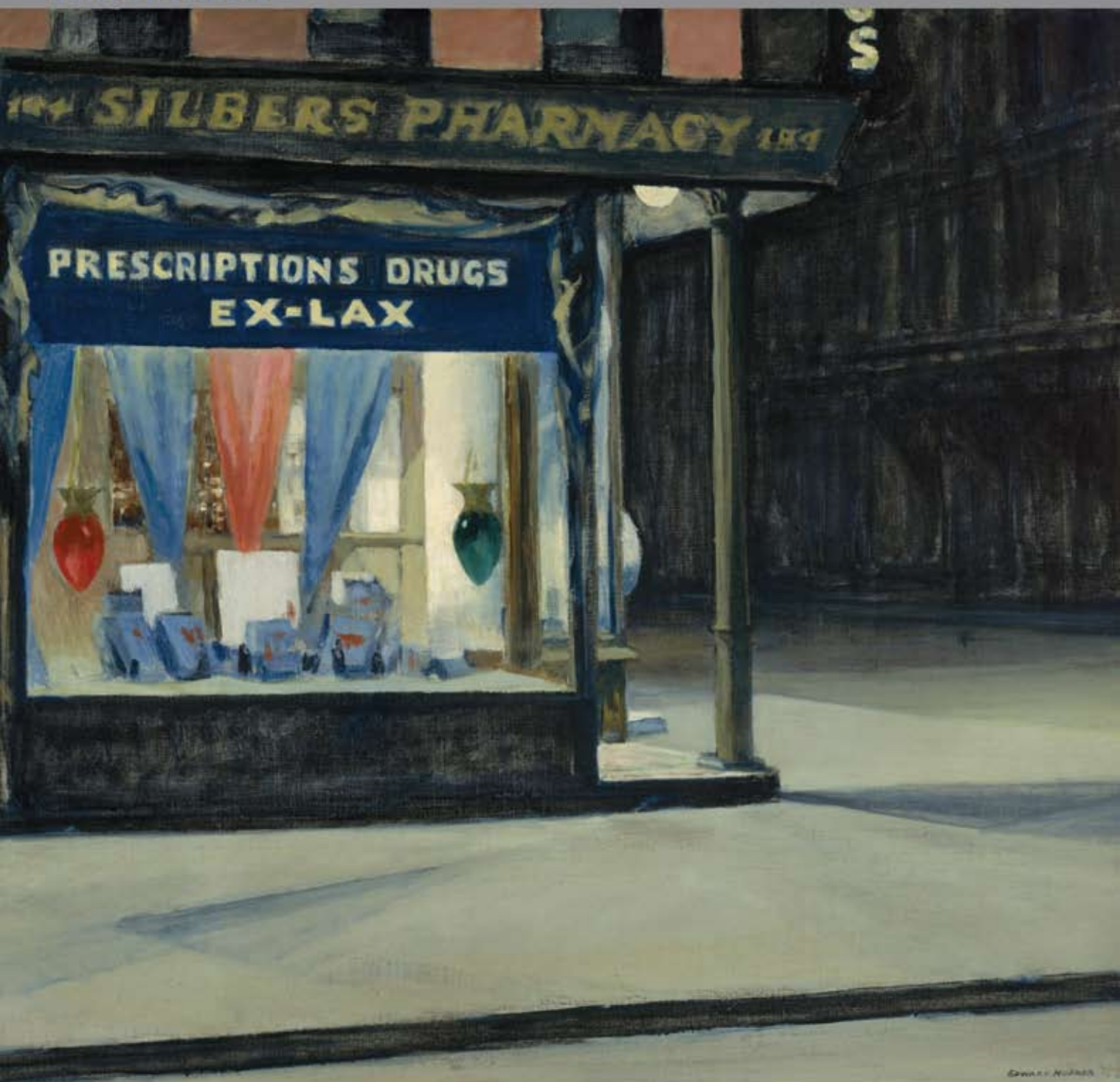
EMERGING INFECTIOUS DISEASES®

April 2010

Enteric Infections



The Museum of Fine Arts, Boston, Massachusetts, USA, Bequest of John T. Spaulding, 48.564

EMERGING
INFECTIOUS DISEASES®

Emerging Infectious Diseases
Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

EMERGING INFECTIOUS DISEASES

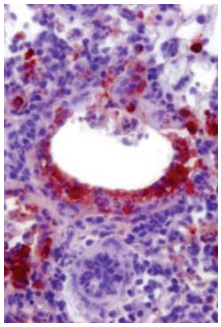
April 2010

On the Cover

Edward Hopper (1882–1967)
Drug Store (1927) (detail)
Oil on canvas (73.6 cm × 101.9 cm)
The Museum of Fine Arts, Boston, Massachusetts, USA
Bequest of John T. Spaulding, 48.564

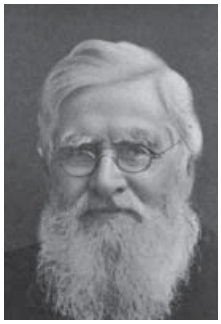
About the Cover p. 751

Research



p. 601

p. 665



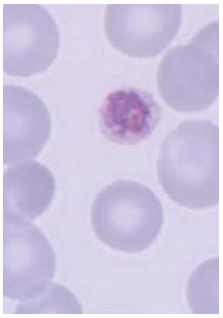
CME Activity

Historical Review

Dispatches

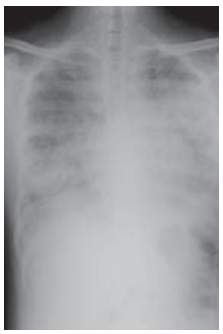
EMERGING INFECTIOUS DISEASES
April 2010

Letters



p. 673

p. 724



Book Reviews

About the Cover

Conference Summaries

Livestock-associated Methicillin-Resistant *Staphylococcus aureus* Sequence Type 398 in Humans, Canada

George R. Golding, Louis Bryden, Paul N. Levett, Ryan R. McDonald, Alice Wong, John Wylie, Morag R. Graham, Shaun Tyler, Gary Van Domselaar, Andrew E. Simor, Denise Gravel, and Michael R. Mulvey

Rates of colonization with livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type 398 have been high for pigs and pig farmers in Canada, but prevalence rates for the general human population are unknown. In this study, 5 LA-MRSA isolates, 4 of which were obtained from skin and soft tissue infections, were identified from 3,687 tested MRSA isolates from persons in Manitoba and Saskatchewan, Canada. Further molecular characterization determined that these isolates all contained staphylococcal cassette chromosome (SCC) *mec*V, were negative for Panton-Valentine leukocidin, and were closely related by macrorestriction analysis with the restriction enzyme *Cfr91*. The complete DNA sequence of the SCC*mec* region from the isolate showed a novel subtype of SCC*mec*V harboring clustered regularly interspaced short palindromic repeats and associated genes. Although prevalence of livestock-associated MRSA seems to be low for the general population in Canada, recent emergence of infections resulting from this strain is of public health concern.

High prevalence of colonization with livestock-associated (LA) methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type (ST) 398 among pigs and pig farmers was first reported in the Netherlands (*1*) and has since been identified in Canada (*2*) and the United States (*3*). In Canada, this LA-MRSA strain was identified in pigs and pig farmers in southwestern Ontario, where prevalence of MRSA colonization was 24.9% (71/285) and 20% (5/25), respectively (*2*). In the United States, nasal samples from 20 production system workers and 299 swine from 2 farms in Illinois and Iowa showed that 45% (9/20) and 49% (147/299), respectively, were colonized with LA-MRSA (*3*). Despite such high prevalence of MRSA colonization on these tested farms, to our knowledge, no human or animal infections resulting from LA-MRSA strains have been reported in North America.

To determine whether LA-MRSA has recently emerged in the general population of Canada, we identified human infections and colonizations associated with the LA-MRSA strain in Canada and molecularly characterized the isolates. We also identified a novel staphylococcal cassette chromosome (SCC) *mec*V subtype harboring clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated genes (*cas*).

Materials and Methods

A convenience sample, totaling 2,358 MRSA isolates from human specimens, was submitted to the National Microbiology Laboratory (NML) for *spa* typing, as described (*4*,*5*). During January 2007–October 2008, the Saskatchewan Disease Control Laboratory submitted 2,008 specimens; during October 2007–August 2008, the Cadham Provincial Laboratory in Manitoba submitted 350 specimens. An additional 1,329 isolates from human specimens were *spa* typed by the Saskatchewan Disease Control Laboratory.

Author affiliations: National Microbiology Laboratory, Winnipeg, Manitoba, Canada (G.R. Golding, L. Bryden, M.R. Graham, S. Tyler, G. Van Domselaar, M.R. Mulvey); Saskatchewan Disease Control Laboratory, Regina, Saskatchewan, Canada (P.N. Levett, R.R. McDonald); Royal University Hospital, Saskatoon, Saskatchewan, Canada (A. Wong); Cadham Provincial Laboratories, Winnipeg (J. Wylie); Sunnybrook Health Sciences Centre, Toronto, Ontario, Canada (A.E. Simor); and Public Health Agency of Canada, Ottawa, Ontario, Canada (D. Gravel)

DOI: 10.3201/eid1604.091435

Given the client base of the Cadham Provincial Laboratory, most of these isolates would have originated from colonized and infected persons living in the community or in personal-care homes or from persons hospitalized in smaller community hospitals, whereas, for surveillance purposes, the Saskatchewan Disease Control Laboratory receives isolates from all colonized and infected persons across the province. Detailed information regarding why cultures were taken (e.g., screening admissions, outbreak investigations) and other clinical and epidemiologic data were limited. Isolates typed in this study represented ≈17% of all MRSA isolates from persons in Manitoba and ≈66% of all MRSA isolates from persons in Saskatchewan within the study period. An additional isolate was sent to the NML from Sunnybrook Health Sciences Centre in Ontario for reference purposes.

Isolates with *spa* types associated with ST398 were confirmed by multilocus sequence typing; tested for Panton-Valentine leukocidin toxin, *mec*A, and *nuc* genes; and typed for SCC*mec* as described (*6*–*9*). Pulsed-field gel electrophoresis (PFGE) of *Sma*I- or *Cfr*91-digested genomic DNA was conducted as described (*10*). Antimicrobial drug susceptibility testing was conducted by using standard broth microdilution panels according to Clinical and Laboratory Standards Institute guidelines (*11*). Breakpoints for fusidic acid and mupirocin resistance, which were not provided in the guidelines, were as described (*12*,*13*).

A fosmid library was constructed by cloning sheared genomic DNA from *S. aureus* isolate 08 BA 02176 into the pCC2FOS vector. The fosmid clones were screened by PCR to identify specific genes *orfX*, *mecA*, and the chromosomal region located downstream of SCC*mec*. Fosmid clone 1G1 was identified and contained the entire SCC*mec* region of the 08 BA 02176 isolate. Colonies were prepared by using the CopyControl Fosmid Library Production Kit (Epicenter Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. Fosmid DNA was column purified by using a QIAGEN Plasmid Mini Kit (QIAGEN, Valencia, CA, USA).

DNA sequencing was performed on the ABI3730xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). Staden (Pregap4) software was used to prepare trace data for sequence assembly (*14*). Sequencing reads were assembled by using the Staden Gap4 program. Gap closure was achieved by primer walking and long-range PCR. Specific primers were designed near the ends of neighboring contigs (contiguous sequence of DNA created by overlapping sequenced fragments of a chromosome), and PCRs were performed with chromosomal template DNA. Regions containing putative frameshifts and point mutations were resequenced to verify the fidelity of the sequence.

Annotation and data mining of the *S. aureus* 08 BA 02176 1G1 fosmid clone sequence were performed by using the GenDB version 2.2 annotation tool (*15*). Putative protein coding sequences were determined according to coding sequence predictions of Glimmer, which is integrated into the GenDB package. Similarity searches were performed by using BLASTN and BLAST2P (www.ncbi.nlm.nih.gov/blast/Blast.cgi) against the nonredundant nucleotide and protein databases, respectively. Additionally, a BLAST2P search was performed against the databases nr (ftp://ftp.ncbi.nlm.nih.gov/blast/db/), SWISS-PROT (www.expasy.ch/sprot/), and KEGG-Genes (ftp://ftp.genome.jp/pub/kegg/genes/); the protein family databases Pfam (http://pfam.sanger.ac.uk/) and TIGRFAM (www.jcvi.org/cms/research/projects/tigrfams/overview/); and predictive signal peptide (Signal P [www.cbs.dtu.dk/services/SignalP/]) and transmembrane helix analysis (TMHMM [www.cbs.dtu.dk/services/TMHMM/]), the nonredundant database on protein level. An automatic functional annotation was followed by a manual annotation of each predicted gene.

Results

LA-MRSA Characterization

A total of 3,687 MRSA isolates were examined; 5 contained ST398-associated *spa* types (4 t034 and 1 t1250). The additional isolate submitted to NML by Sunnybrook Health Sciences Centre in Ontario, isolate T40929, also contained a t034 *spa* type. Further molecular characterization of these 6 isolates determined that they were all ST398, SCC*mec*V, and negative for the Panton-Valentine leukocidin–encoding genes (Table 1). Of the 6 isolates, 5

Table 1. Characteristics of methicillin-resistant *Staphylococcus aureus* sequence type 398 novel staphylococcal cassette chromosome *mec*V subtype isolates, Canada*

Isolate	Collection date	Patient age, y/sex	Region and province	Specimen collection site	*spa* type
07 BA 06477	2007 Feb 27	26/F	Saskatoon, SK	Nasal screen	t034
08 BA 02176	2008 Jan 15	71/F	Sunrise, SK	Leg swab	t034
08 BA 08100	2008 Mar 4	51/M	Five Hills, SK	Left shin open abrasion	t1250
08 BA 13895	2008 Apr 25	79/M	Kelsey Trail, SK	Left hip swab	T034
08 BA 22334	2008 Jul 9	70/M	Prince Albert Parkland, SK	Right leg swab	T034
T40929	2007 Dec 11	59/M	Durham, ON	Nasal and tracheostomy screen	T034

*All isolates were Panton-Valentine leukocidin negative. SK, Saskatchewan; ON, Ontario.

Table 2. Antimicrobial drug susceptibility of the clinical isolates of methicillin-resistant *Staphylococcus aureus* sequence type 398, Canada, 2008*

	Susceptibility, μg/mL					
Drug	07 BA 06477	08 BA 02176	08 BA 08100	08 BA 13895	08 BA 22334	T40929
Clindamycin	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Vancomycin	0.5	0.5	0.5	0.5	0.5	0.5
Erythromycin	0.5	0.5	0.5	0.5	0.5	0.5
SXT	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Synercid	0.5	1	≤0.25	≤0.25	≤0.25	≤0.25
Nitrofurantoin	≤32	≤32	≤32	≤32	≤32	≤32
Tetracycline	>16	>16	>16	≤2	>16	>16
Ciprofloxacin	0.5	0.25	0.5	0.25	0.5	0.5
Rifampin	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25
Fusidic acid	0.25	0.12	0.25	0.12	0.12	0.12
Linezolid	2	2	2	1	0.5	0.5
Gentamicin	1	1	1	1	≤0.5	1
Mupirocin	0.5	0.25	≤0.12	≤0.12	0.25	≤0.12

*SXT, sulfamethoxazole/trimethoprim.

were resistant to tetracycline, but all were susceptible to the other 12 antimicrobial drugs tested (Table 2).

From the surveillance in Manitoba and Saskatchewan, patient information was limited and showed no geographic links (all 5 persons resided in different health regions but were all within the southeastern portion of Saskatchewan) (Figure 1). Of the 5 isolates, 4 were obtained from infected persons (average age 67.8 years, range 51–79 years) (Table 1). The earliest identified LA-MRSA isolate (08 BA 2176) associated with an infection was obtained from a postoperative surgical site. Further follow-up was not possible because of the patient's health problems. This patient is unlikely to have had any recent direct contact with livestock because she had been confined to her home with limited mobility for several years before her hospitalization. Additional nasal swabs from this patient remained positive for this strain for at least 7 months. Additional clinical and epidemiologic information for the remaining 3 patients with skin and soft tissue or wound infections were limited (Table 1).

The isolate submitted to the NML by Sunnybrook Health Sciences Centre, outside the surveillance program, was from a 59-year-old man from Ontario. He had been hospitalized in December 2007 for treatment of metastatic squamous cell carcinoma of the larynx. In the previous year, he had undergone a total laryngectomy, neck node dissection, and tracheostomy. A MRSA isolate was recovered from screening specimens from his nose and the tracheostomy site that had no indication of infection. He was unaware of any animal contact and had no history of exposure to pigs or pig farms. A review of the medical records and standard epidemiologic investigations determined that this was not a nosocomial or healthcare-associated isolate.

The 6 LA-MRSA isolates were nontypeable by PFGE using *Sma*I. However, PFGE using the neoschizomer *Cfr*91 showed that the 6 LA-MRSA isolates were closely related (Figure 2, panel A). Control MRSA strains digested individually with either *Cfr*91 or *Sma*I showed no differences in fingerprint banding patterns when the 2 enzymes were compared (data not shown), which enabled comparisons

Figure 1. Geographic distribution of 5 livestock-associated methicillin-resistant *Staphylococcus aureus* isolates (stars) from humans, Saskatchewan, January 2007–October 2008.

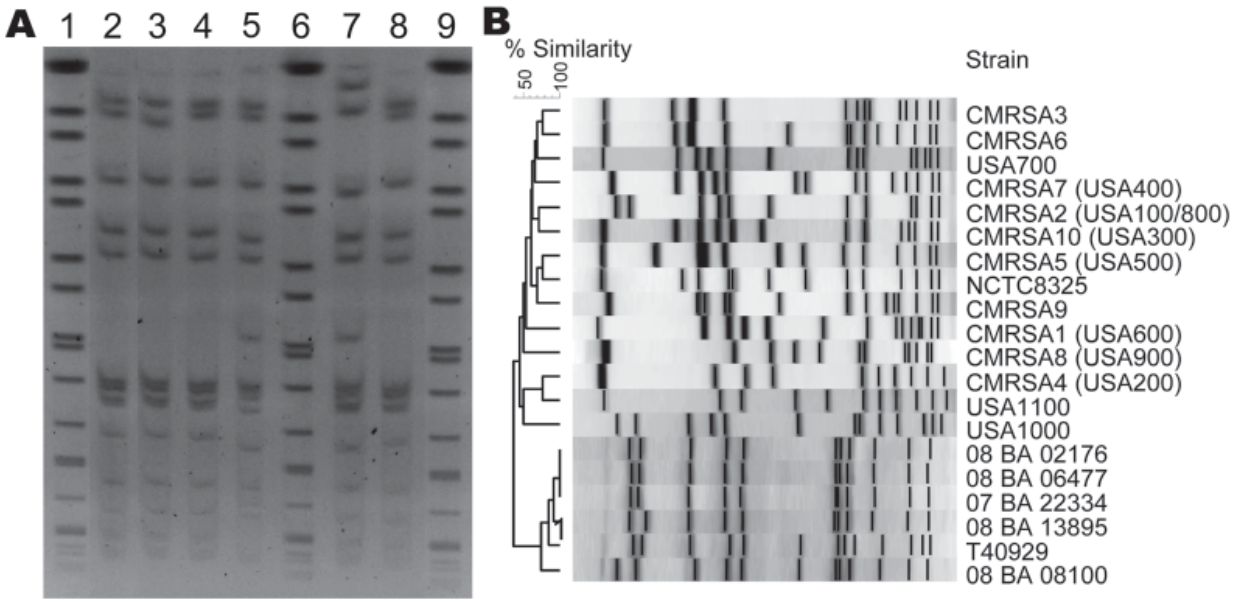


Figure 2. A) Pulsed-field gel electrophoresis (PFGE) of *Cfr*91-digested livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA). Lanes 1, 6, and 9, universal standard *Salmonella* Braenderup H9812; Lane 2, 08 BA 02176; Lane 3, 08 BA 13895; Lane 4, 07 BA 06477; Lane 5, T40929; Lane 7, 08 BA 08100; Lane 8, 07 BA 22334. B) PFGE dendrogram comparing the *Cfr*91 fingerprint patterns of 6 livestock-associated MRSA isolates from humans in Canada with the *Sma*I fingerprints of other human epidemic strains of MRSA circulating in Canada.

of the PFGE patterns obtained for the LA-MRSA isolates with those of other epidemic MRSA strains from hospitals and communities in Canada. No close relatedness was found (≥7 bands difference; <80% similarity) between the LA-MRSA isolates and any other epidemic MRSA strain circulating in Canada (Figure 2, panel B).

SCC*mec* Characterization

DNA sequencing of the entire SCC*mec* element from isolate 08 BA 02176 showed a 32,369-bp element integrated at the 3′ end of *orf*X containing 30 putative open reading frames (ORFs) (Figure 3; Table 3). This element carried a class C2 *mec* complex, which putatively contained a non-

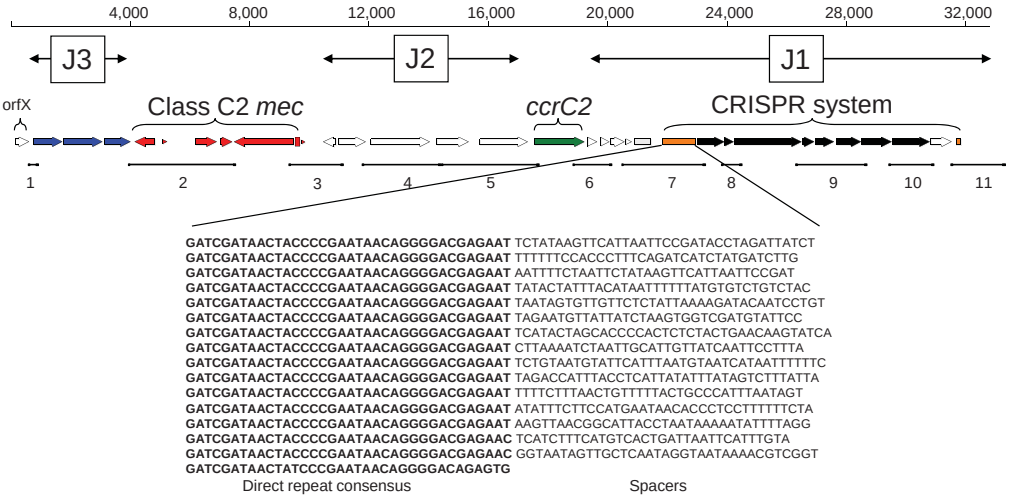


Figure 3. Schematic of the novel staphylococcal cassette chromosome (SCC) *mec*V subtype and DNA sequence of the clustered regularly interspaced short palindromic repeat (CRISPR) array identified in *Staphylococcus aureus* isolate 08 BA 02176. Red and green arrows represent *mec* and *ccr* complexes, respectively. Blue arrows represent 3 open reading frames (ORFs) in the J3 region sharing sequence identity with chromosomal genes of *S. epidermidis* RP62A. Orange boxes indicate confirmed and questionable CRISPRs. Black arrows represent CRISPR-associated genes. Location of primer sets used for coverage of this SCC*mec* element are numbered 1–11 (Table 4) and illustrated as solid lines. Shown below the schematic is the DNA sequence of the confirmed 1,107-bp CRISPR array in the J1 region, which provides the 36-bp direct repeat consensus (**boldface**) and the variable 15 spacer sequences.

functional IS431 transposase and a type 5 *ccr* gene complex (*ccr*C2). Other than *mecA*, no additional antimicrobial drug resistance genes were identified within this element.

The first unique feature of this SCC*mec*V element included 3 ORFs in the J3 region sharing high sequence identity with ORFs from *S. epidermidis* RP62A (GenBank accession no. CP000029), which included an ADP-ribosylglycohydrolase, a permease for cytosine/purines, and a ribokinase (Table 3). A second unique feature was a CRISPR array, identified by using CRISPRFinder (*16*), in the J1 region, which appears to have replaced the type 1 restriction modification system (*hsdR*, *hsdS*, *hsdM*) through recombination. The CRISPR array (1,107 bp) contained a 36-bp direct repeat consensus and 15 spacers of variable sequence and length (33–38 bp) (Figure 3). Downstream of this CRISPR array was a combination of putative CRISPR-associated (*cas*) genes, sharing sequence identity with those previously identified in *S. epidermidis* RP62A. This array was followed by a second questionable CRISPR array (183 bp) containing a 38-bp direct repeat consensus and 2 spacers of variable sequence (Figure 3; Table 3).

Design of primers spanning the entire SCC*mec* element was based on the DNA sequence obtained from 08 BA 02176 (Figure 3; Table 4). PCR of these select regions produced amplicons of expected size for 3 additional LA-MRSA isolates (07 BA 06477, 08 BA 13895, 08 BA 22334) but were negative for some of the J1 and J3 regions in 08 BA 08100 and T40929 (Table 4).

Discussion

The high prevalence of LA-MRSA colonization of pigs and pig farmers in Canada (*2*) and the United States

Table 3. Open reading frames of the novel staphylococcal cassette chromosome *mec*V subtype in methicillin-resistant *Staphylococcus aureus* isolate 08 BA 02176, from woman in Canada, 2008*

ORF	Location, bp†	Predicted gene size, bp	Gene‡	Product description	Amino acid identity, %§	GenBank accession no.
Sk01	1–480	480	*orfX*	Conserved hypothetical protein	100	gb|ACC96139.1|
Sk02	609–1595	987	None	ADP-ribosylglycohydrolase	99	gb|AAW53059.1|
Sk03	1614–2948	1335	None	Permease for cytosine/purines; uracil; thiamine; allantoin	98	gb|AAW53058.1|
Sk04	2999–3883	885	None	Ribokinase	98	gb|AAW53057.1|
Sk05	(4013–4687)	675	*tnp*	Transposase for IS431	100	dbj|BAD24823.1|
Sk06	4945–5112	168	None	HMG-CoA synthase truncation	100	ref|YP_184940.1|
Sk07	6029–6772	744	*ugpQ*	Glycerophosphoryl diester phosphodiesterase	100	ref|NP_370563.1|
Sk08	6869–7297	429	*maoC*	Hypothetical protein	100	ref|YP_184943.1|
Sk09	(7343–9349)	2007	*mecA*	Penicillin-binding protein 2′	100	dbj|BAG06200.1|
Sk10	9449–9559	Unknown	ψ*mecR1*	Truncated signal transducer protein MecR1	100	ref|YP_252007.1|
Sk11	9597–9740	144	ψ*tnp*	Partial transposase for insertion sequence–like element IS431mec	100	dbj|BAH57698.1|
Sk12	(10331–10759)	429	None	Hypothetical protein	100	dbj|BAD24829.1|
Sk13	10840–11769	930	None	Hypothetical protein	100	gb|ACL99839.1|
Sk14	11931–13919	1989	None	Hypothetical protein	100	gb|ACL99840.1|
Sk15	14114–15223	1110	None	Hypothetical protein	100	gb|ACL99841.1|
Sk16	15584–17200	1617	None	Hypothetical protein	100	gb|ACL99843.1|
Sk17	17425–19104	1680	*ccrC*	Cassette chromosome recombinase C	100	gb|ACL99844.1|
Sk18	19193–19531	339	None	Hypothetical protein	100	gb|ACL99845.1|
Sk19	19625–19936	312	None	Hypothetical protein	100	gb|ACL99846.1|
Sk20	19951–20454	504	None	Hypothetical protein	100	gb|ACL99847.1|
Sk21	20469–20690	222	None	Hypothetical protein	100	gb|ACL99848.1|
Sk22	(20853–21256)	403	ψ*hsdR*	Truncated *hsdR*	92	dbj|BAG71456.1|
Sk23	22888–23793	906	*cas1*	CRISPR–associated Cas1 family protein	91	gb|AAW53332.1|
Sk24	23793–24098	306	*cas2*	CRISPR-associated protein Cas2	87	gb|AAW53331.1|
Sk25	24112–26385	2274	*csm1*	CRISPR-associated protein; Csm1 family	92	gb|AAW53330.1|
Sk26	26388–26813	426	*csm2*	CRISPR-system related protein	94	gb|AAW53329.1|
Sk27	26815–27459	645	*csm3*	CRISPR-associated RAMP protein	96	gb|AAW53328.1|
Sk28	27530–28378	849	*csm4*	CRISPR-associated RAMP protein	91	gb|AAW53327.1|
Sk29	28381–29403	1023	*csm5*	CRISPR-associated Csm5 family protein	92	gb|AAW53326.1|
Sk30	29403–30671	1269	*csm6*	CRISPR-associated protein (Cas_Csm6)	73	gb|AAW53325.1|
Sk31	30668–31402	735	*cas6*	CRISPR-associated protein C	86	gb|AAW53324.1|

*ORF, open reading frame; CRISPR, clustered regularly interspaced short palindromic repeats.
†Parentheses indicate complement sequences.
‡None indicates no name given.
§Comparisons of translated query versus protein databases was determined by using BLASTX 2.2.21 (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 4. Primers used for coverage of the novel SCC*mec*V subtype in methicillin-resistant *Staphylococcus aureus* isolates, Canada, 2007–2008*

Primer set	Primer name	Primer 5′ → 3′	Expected amplicon size, bp	Reference position	SCC*mec*V found in isolate					
					08 BA 02176	T 49209	07 BA 06477	08 BA 13895	08 BA 08100	08 BA 22334
1	OrfX	CATTTAAGATTATGCGTGGAG	347	443–789	+	–	+	+	–	+
	Adpr1	CATCTGTAAACTGTCCTTTGG								
2	RibB2	TTGTATATGGGGAAACGAAG	3623	3793–7415	+	–	+	+	–	+
	MecaA1	TGCCAAAATCTCAGGTAAAG								
3	MecB1	CTTCACCATTATCGCTTTTAG	1842	9172–11013	+	+	+	+	+	+
	HypA1	ACCATTTTTCCCTGGATTAC								
4	Hyp3A1	CTTCCACGTATTGGTCTAGC	2671	11636–14306	+	+	+	+	+	+
	Hyp1B1	AAGTGAACGCGAAAGATATAG								
5	Hyp3B1	GCTAGACCAATACGTGGAAG	3301	14287–17587	+	+	+	+	+	+
	CcrCA2	TTTTACCTGAAATGCCTGAG								
6	CcrCB1	ATGAAATGGATAGCGAAATG	1330	18695–20024	+	+	+	+	+	+
	Hyp6A1	TTGAGTAAGTAGCGGTGTTG								
7	Hyp6B1	TGAGCAAGTGATGGAAATG	2835	20331–23165	+	–	+	+	–	+
	Crspr1A1	CTTTGAATCCTTTGAAGACG								
8	Crspr1B1	AAAAAGTGGTGAGGTTACTTG	711	23675–24385	+	–	+	+	–	+
	Crspr3A1	CTCGTCTATCAATACCACTCG								
9	Crspr3B1	AACAGATGAACACGGAAAAG	2417	26166–28582	+	–	+	+	–	+
	Crspr7A1	TTGGTGGGTATCTCAAAAAG								
10	Crspr7B1	GCCTTCTAACGTACCAGTTG	1511	29289–30820	+	–	+	+	–	+
	Hyp11A1	TTGCTTCAATGGACTATAAGC								
11	Hyp11B1	TTAGGCATGGGGAAATATAG	1622	31373–	+	–	+	+	–	+
	Hyp12A1	GTCGCAATGTTTTGAAGTG								

*SCC*mec*, staphylococcal cassette chromosome *mec*V subtype; +, positive; –, negative. Testing by PCR.

(*3*) and this report of human infections suggest that this LA-MRSA strain from Canada poses potential public and occupational health concern in North America. This strain has been associated with various types of infections in pigs (*17*,*18*) and humans (*19*,*20*) and is transmissible from animal patients to veterinary workers (*21*), healthcare workers (*22*), and family members (*1*). Evidence also suggests that this strain might be spreading from animals to the environment, which may facilitate the colonization or infection of persons who are not involved in animal husbandry (*23*). Whereas in 2006 in the Netherlands LA-MRSA accounted for >20% of all MRSA isolated (*24*), carriage of this strain in the general population of 2 provinces in Canada (Manitoba and Saskatchewan) appears rare (0.14%). This difference could be attributed to the substantially higher density of pigs in the Netherlands (1,244 pigs/km^2) than in Manitoba (55 pigs/km^2), Saskatchewan (6 pigs/km^2), and Ontario (91 pigs/km^2) (www.agriculture.gov.sk.ca/Pig_Densities). It is also plausible that the much lower proportions of LA-MRSA in Canada, relative to a country with low MRSA endemicity such as the Netherlands, is attributable to competition with other highly successful human epidemic MRSA clones circulating in Canada, including CMRSA2 (USA200/800), CMRSA7 (USA400), and CMRSA10 (USA300) (*25*,*26*).

The tested LA-MRSA isolates were highly susceptible to most classes of antimicrobial drugs, except β-lactams and tetracyclines, the latter of which has been attributed to its high usage in animal husbandry (*27*). The complete sequence of the SCC*mec* region showed a novel SCC*mec*V subtype sharing sequence identity in its J1 and J3 regions with chromosomal genes in the *S. epidermidis* RP62A chromosome (GenBank accession no. CP000029), including a CRISPR system. CRISPRs and associated *cas* genes are present in many other bacterial (≈40%) and archaeal (≈90%) genomes (*28*,*29*) and have been shown to be involved in sequence-directed immunity against phages (*30*,*31*) and plasmids (*32*). The resistance to plasmids and phages encoded by this system could explain why many of these ST398-MRSA-V strains contain fewer antimicrobial drug resistance genes and phage-encoded virulence factors than do other epidemic MRSA strains (*33*,*34*). The origin of this CRISPR system is unknown, but the propagation of CRISPR loci throughout prokaryote genomes has been proposed to occur through horizontal gene transfer by conjugation of megaplasmids ≥40 kb (*35*). Because the CRISPR system identified in this study is encoded within a putative mobile genetic element, we propose that an additional mechanism of mobilization to other methicillin-susceptible *Staphylococcus* spp. is plausible.

This novel subtype of SCC*mec*V was found in only 4 of the 6 LA-MRSA isolates identified in this study. One isolate not containing this novel SCC*mec* subtype (08 BA 08100) could also be distinguished by a different but closely related *spa* type (t1250) (Table 1) and variant PFGE fingerprint (Figure 2) when compared with the other LA-MRSA

isolates, which suggests that at least 2 epidemiologically different strains of LA-MRSA circulate in Saskatchewan. The other LA-MRSA isolate that did not contain this novel SCC*mec* element was obtained in Ontario. However, this isolate was the same *spa* type (t034) and was closely related, according to PFGE, to the LA-MRSA isolates identified in Saskatchewan. Therefore, in addition to PFGE and *spa* typing, SCC*mec* subtyping could provide a useful epidemiologic tool for surveillance, outbreak investigations, or traceability studies of this emerging strain. For detection of this SCC*mec*V subtype (tentatively designated V.2.1.2; Vb), we propose using primer set 1 (spanning orfX into Sk02 in the J3 region) and primer set 7 (spanning Sk20 into cas1 in the J1 region) (Table 4).

Visual comparison of PFGE fingerprints from this study with those reported from patients from the Dominican Republic and the United States (northern Manhattan, New York, NY) (*36*), showed substantial variations in fingerprint patterns, as well as related but different *spa* types. These variations suggest further molecular and geographic diversity of these LA-MRSA strains on a global scale.

Because cases of LA-MRSA infections have only recently been identified in Canada, additional surveillance efforts are required to monitor the emergence and clinical relevance of this MRSA strain in Canada, including communities, the environment, livestock, farmers, and production facility workers. Whether these strains pose a major threat to human health in light of the low livestock density and continued spread of epidemic hospital and community strains of MRSA in Canada remains unknown.

Acknowledgments

We thank Jennifer Campbell and Dave Spreitzer for performing PFGE; Brynn Kaplen, Claude Ouellette, and Erika Landry for performing amplicon cleanup and DNA sequencing; and Philip Mabon, Kunyan Zhang, and Lisa Louie for helping with analysis of the SCC*mec* element.

Funding was provided by the Federal Genomics Research and Development Initiative.

Dr Golding is a research scientist at the National Microbiology Laboratory, Winnipeg. His primary research interest focuses on antimicrobial drug resistance mechanisms, genomics, typing, and surveillance of *S. aureus*.

References

1. Voss A, Loeffen F, Bakker J, Klaassen C, Wulf M. Methicillin-resistant *Staphylococcus aureus* in pig farming. Emerg Infect Dis. 2005;11:1965–6.
2. Khanna T, Friendship R, Dewey C, Weese JS. Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. Vet Microbiol. 2008;128:298–303. DOI: 10.1016/j.vetmic.2007.10.006
3. Smith TC, Male MJ, Harper AL, Kroeger JS, Tinkler GP, Moritz ED, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. PLoS One. 2009;4:e4258. DOI: 10.1371/journal.pone.0004258
4. Golding GR, Campbell JL, Spreitzer DJ, Veyhl J, Surynicz K, Simor A, et al.; Canadian Nosocomial Infection Surveillance Program. A preliminary guideline for the assignment of methicillin-resistant *Staphylococcus aureus* to a Canadian pulsed-field gel electrophoresis epidemic type using *spa* typing. Can J Infect Dis Med Microbiol. 2008;19:273–81.
5. Harmsen D, Claus H, Witte W, Rothgänger J, Claus H, Turnwald D, et al. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting using a novel software for *spa*-repeat determination and database management. J Clin Microbiol. 2003;41:5442–8. DOI: 10.1128/JCM.41.12.5442-5448.2003
6. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus.* J Clin Microbiol. 2000;38:1008–15.
7. McDonald RR, Antonishyn NA, Hansen T, Snook LA, Nagle E, Mulvey MR, et al. Development of a triplex real-time PCR assay for detection of Panton-Valentine leukocidin toxin genes in clinical isolates of methicillin-resistant *Staphylococcus aureus.* J Clin Microbiol. 2005;43:6147–9. DOI: 10.1128/JCM.43.12.6147-6149.2005
8. Zhang K, McClure JA, Elsayed S, Louie T, Conly JM. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol. 2005;43:5026–33. DOI: 10.1128/JCM.43.10.5026-5033.2005
9. Kondo Y, Ito T, Ma XX, Watanabe S, Kreiswirth BN, Etienne J, et al. Combination of multiplex PCRs for SCC*mec* type assignment: rapid identification system for *mec, ccr*, and major differences in junkyard regions. Antimicrob Agents Chemother. 2007;51:264–74. DOI: 10.1128/AAC.00165-06
10. Mulvey MR, Chui L, Ismail J, Louie L, Murphy C, Chang N, et al.; Canadian Committee for the Standardization of Molecular Methods. Development of a Canadian standardized protocol for subtyping methicillin-resistant *Staphylococcus aureus* using pulsed-field gel electrophoresis. J Clin Microbiol. 2001;39:3481–5. DOI: 10.1128/JCM.39.10.3481-3485.2001
11. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 17th informational supplement. Document M100–S17. Wayne (PA): The Institute; 2007.
12. Skov R, Frimodt-Moller N, Espersen F. Correlation of MIC methods and tentative interpretive criteria for disk diffusion susceptibility testing using NCCLS methodology for fusidic acid. Diagn Microbiol Infect Dis. 2001;40:111–6. DOI: 10.1016/S0732-8893(01)00262-0
13. Walker ES, Levy F, Shorman M, David G, Abdalla J, Sarubbi FA. A decline in mupirocin resistance in methicillin-resistant *Staphylococcus aureus* accompanied administrative control of prescriptions. J Clin Microbiol. 2004;42:2792–5.
14. Staden R, Beal KF, Bonfield JK. The Staden package. In: Misener S, Krawetz SA, editors. Computer methods in molecular biology, bioinformatics methods and protocols. Vol. 132. Totowa (NJ): The Humana Press Inc.; 1998. p. 115–130.
15. Meyer F, Goesmann A, McHardy AC, Bartels D, Bekel T, Clausen J, et al. GenDB—an open source genome annotation system for prokaryote genomes. Nucleic Acids Res. 2003;31:2187–95. DOI: 10.1093/nar/gkg312
16. Grissa I, Vergnaud G, Pourcel C. CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic Acids Res. 2007 Jul;35(Web Server issue):W52–7.
17. Schwarz S, Kalec K, Strommenger B. Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* detected in the Bft-GermVet monitoring programme 2004–2006 in Germany. J Antimicrob Chemother. 2008;61:282–5. DOI: 10.1093/jac/dkm487

18. van Duijkeren E, Jansen MD, Flemming SC, de Neeling H, Wagenaar JA, Schoormans AH, et al. Methicillin-resistant *Staphylococcus aureus* in pigs with exudative epidermitis. Emerg Infect Dis. 2007;13:1408–10.
19. Witte W, Strommenger B, Stanek C, Cuny C. Methicillin-resistant *Staphylococcus aureus* ST398 in humans and animals, Central Europe. Emerg Infect Dis. 2007;13:255–8. DOI: 10.3201/eid1302.060924
20. Krziwanek K, Metz-Gercek S, Mittermayer H. Methicillin-resistant *Staphylococcus aureus* ST398 from human patients, upper Austria. Emerg Infect Dis. 2009;15:766–9. DOI: 10.3201/eid1505.080326
21. Wulf MW, Sørum M, van Nes A, Skov R, Melchers WJ, Klaassen CH, et al. Prevalence of methicillin-resistant *Staphylococcus aureus* among veterinarians: an international study. Clin Microbiol Infect. 2008;14:29–34. DOI: 10.1111/j.1469-0691.2007.01873.x
22. van Rijen MM, Van Keulen PH, Kluytmans JA. Increase in a Dutch hospital of methicillin-resistant *Staphylococcus aureus* related to animal farming. Clin Infect Dis. 2008;46:261–3. DOI: 10.1086/524672
23. Gibbs SG, Green CF, Tarwater PM, Mota LC, Mena KD, Scarpino PV. Isolation of antibiotic-resistant bacteria from the air plume downwind of a swine confined or concentrated animal feeding operation. Environ Health Perspect. 2006;114:1032–7.
24. van Loo I, Huijsdens X, Tiemersma E, de Neeling A, van de Sande-Bruinsma N, Beaujean D, et al. Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. Emerg Infect Dis. 2007;13:1834–9.
25. Simor AE, Ofner-Agostini M, Bryce E, McGeer A, Paton S, Mulvey MR; Canadian Hospital Epidemiology Committee and Canadian Nosocomial Infection Surveillance Program. Laboratory characterization of methicillin-resistant *Staphylococcus aureus* in Canadian hospitals: results of 5 years of national surveillance, 1995–1999. J Infect Dis. 2002;186:652–60. DOI: 10.1086/342292
26. Christianson S, Golding GR, Campbell J; Canadian Nosocomial Infection Surveillance Program, Mulvey MR. Comparative genomics of Canadian epidemic lineages of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol. 2007;45:1904–11.
27. de Neeling AJ, van den Broek MJ, Spalburg EC, van Santen-Verheuvel MG, Dam-Deisz WDC, Boshuizen HC, et al. High prevalence of methicillin resistant *Staphylococcus aureus* in pigs. Vet Microbiol. 2007;122:366–72. DOI: 10.1016/j.vetmic.2007.01.027
28. Kunin V, Sorek R, Hugenholtz P. Evolutionary conservation of sequence and secondary structures in CRISPR repeats. Genome Biol. 2007;8:R61 10.1186/gb-2007-8-4-r61. DOI: 10.1186/gb-2007-8-4-r61
29. Grissa I, Vergnaud G, Pourcel C. The CRISPRdb database and tools to display CRISPRs and to generate dictionaries of spacers and repeats. BMC Bioinformatics. 2007;8:172 DOI: 10.1186/1471-2105-8-172
30. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science. 2008;321:960–4. DOI: 10.1126/science.1159689
31. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science. 2007;315:1709–12. DOI: 10.1126/science.1138140
32. Marraffini LA, Sontheimer EJ. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science. 2008;322:1843–5. DOI: 10.1126/science.1165771
33. Monecke S, Jatzwauk L, Weber S, Slickers P, Ehricht R. DNA microarray-based genotyping of methicillin-resistant *Staphylococcus aureus* strains from eastern Saxony. Clin Microbiol Infect. 2008;14:534–45. DOI: 10.1111/j.1469-0691.2008.01986.x
34. Walther B, Monecke S, Ruscher C, Friedrich AW, Ehricht R, Slickers P, et al. Comparative molecular analysis substantiates zoonotic potential of equine methicillin-resistant *Staphylococcus aureus.* J Clin Microbiol. 2009;47:704–10. DOI: 10.1128/JCM.01626-08
35. Godde JS, Bickerton A. The repetitive DNA elements called CRISPRs and their associated genes: evidence of horizontal transfer among prokaryotes. J Mol Evol. 2006;62:718–29. DOI: 10.1007/s00239-005-0223-z
36. Bhat M, Dumortier C, Taylor BS, Miller M, Vasquez G, Yunen J, et al. *Staphylococcus aureus* ST398, New York City and Dominican Republic. Emerg Infect Dis. 2009;15:285–7. DOI: 10.3201/eid1502.080609

Address for correspondence: Michael R. Mulvey, National Microbiology Laboratory, 1015 Arlington Ave, Winnipeg, Manitoba R3E 3R2, Canada; email: michael_mulvey@phac-aspc.gc.ca

Influenza A Strain-Dependent Pathogenesis in Fatal H1N1 and H5N1 Subtype Infections of Mice

Mutien-Marie Garigliany, Adélite Habyarimana, Bénédicte Lambrecht, Els Van de Paar, Anne Cornet, Thierry van den Berg, and Daniel Desmecht

To determine if fatal infections caused by different highly virulent influenza A viruses share the same pathogenesis, we compared 2 different influenza A virus subtypes, H1N1 and H5N1. The subtypes, which had shown no pathogenicity in laboratory mice, were forced to evolve by serial passaging. Although both adapted viruses evoked diffuse alveolar damage and showed a similar 50% mouse lethal dose and the same peak lung concentration, each had a distinct pathologic signature and caused a different course of acute respiratory distress syndrome. In the absence of any virus labeling, a histologist could readily distinguish infections caused by these 2 viruses. The different histologic features described in this study here refute the hypothesis of a single, universal cytokine storm underlying all fatal influenza diseases. Research is thus crucially needed to identify sets of virulence markers and to examine whether treatment should be tailored to the influenza virus pathotype.

According to the World Health Organization, influenza annually infects 5%–15% of the global population, causing 3–5 million cases of severe illness and ≈500,000 reported deaths. The persistence of influenza A virus (H5N1) in poultry populations over the past 6 years and the ability of those viruses to cause fatal infections in humans, along with the recent pandemic (H1N1) 2009 outbreaks, have raised fears of a renewed catastrophic influenza outbreak comparable to that of 1918, which caused death in 0.2%–8% of those infected in various countries and ≈50 million deaths worldwide (*1*). Standard influenza symptoms include fever, cough, headache, sore throat, and dehydration, with some reports of diarrhea, vomiting, and bleeding from the mouth or throat. In benign cases, not all of these symptoms are exhibited. In severe cases, additional signs typical of either secondary bacterial pneumonia or acute respiratory distress syndrome (ARDS) occur. Notably, these 2 manifestations are those that cause death in patients with influenza, whether seasonal or pandemic or caused by the 1918 subtype H1N1 strain or by recent subtype H5N1 strains.

The catastrophic lethality of the 1918 pandemic makes it paramount that we understand the disease pathogenesis of both severe forms of influenza. Because most secondary bacterial pneumonias can be controlled with antimicrobial agents, prevention and treatment of influenza-associated ARDS are the major medical challenges that must be addressed to reduce the influenza-related death rate. This requires more knowledge about the pathogenesis of ARDS. Alterations in human and mouse lungs have been described for fatal virus infections with pandemic virus strains (subtypes H1N1, H2N2, and H3N2 strains of 1918, 1957, and 1968, respectively) or subtype H5N1 strains. They are all characterized by similar lung dysfunctions and lesions (*2*,*3*). The lung becomes flooded as its alveolocapillary membranes leak, and the alveoli fill with body fluids. Consequently, the exchange of carbon dioxide and oxygen is reduced, and fatal acute lung failure ensues. The histologic findings depend on the stage of the disease. Edema, epithelial necrosis, fibrin, and hyaline membranes are found during the early exudative phase, and fibroblast and type II cell hyperplasia are found during the proliferative phase. This array of morphologic alterations is known as diffuse

Author affiliations: University of Liège, Liège, Belgium (M.-M. Garigliany, E. Van de Paar, A. Cornet, D. Desmecht); and Veterinary Agrochemical Center, Brussels, Belgium (A. Habyarimana, B. Lambrecht, T. van den Berg).

DOI: 10.3201/eid1604.091061

alveolar damage. Moreover, mice infected with the 1918 influenza virus or with a recent subtype H5N1 human isolate also show considerable similarities in overall lung cellularity, composition of lung immune cell subpopulation, and cellular immune temporal dynamics (*4*). On the basis of these mostly retrospective studies, the pathogenesis of influenza-associated ARDS is widely viewed as being the same whatever the infecting strain.

In this study, we closely monitored ARDS in mice, caused by inoculation of identical doses of 2 different influenza strains rendered highly pathogenic toward mice by adaptation. The 2 strains elicited dramatically different disease courses and histopathologic signatures, although both strains caused death in 100% of those infected, evoked the expected diffuse alveolar damage, and led to comparable virus titers in the lungs. The pathogenesis underlying influenza-associated fatal ARDS thus depended on the infecting strain.

Materials and Methods

Animals

Eight-week-old female FVB/J mice weighing 20–25 g were obtained from Charles River Laboratories (L'Arbresle, France). Challenge studies were conducted under BioSafety Level 3 laboratory conditions and in facilities accredited by the Belgian Council for Laboratory Animal Science, under the guidance of the Institutional Animal Care and Use Committees of the Veterinary Agrochemical Research Center and University of Liège. The mice were housed in microisolator cages ventilated under negative pressure with HEPA-filtered air. The light/dark cycle was 12/12 h, and the animals were allowed free access to food and water. Before each inoculation or euthanasia procedure, the animals were anesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (30 mg/kg).

Viruses

Two influenza A virus strain subtypes that had low pathogenicity for laboratory mice were used in this study: a clade 1 avian influenza virus (H5N1) (A/crested_eagle/Belgium/1/2004), and a porcine influenza virus (H1N1) (A/swine/Iowa/4/76). Both viruses were first propagated in the allantoic cavity of 10-day-old embryonating hen eggs and then adapted to the mice by lung-to-lung passaging. At each passage, a set of mice were inoculated intranasally with 50 μL of either allantoic fluid or lung homogenate containing influenza A virus. At 5 days postinoculation (dpi), the mice were killed humanely by an overdose of pentobarbital, followed by exsanguination. The lungs were combined and homogenized in phosphate-buffered saline (PBS)–penicillin-streptomycin, the homogenates were centrifuged at 3,000 g for 10 min, and the supernatant was used for the next passage. The process was stopped when the mice showed a substantial loss of bodyweight on 4 dpi. This occurred after 5 (H5N1) or 31 (H1N1) passages. Lung homogenates from the last passage were homogenized and divided into aliquots for direct use in pathotyping studies, and their titers were determined by standard plaque (subtype H1N1) or median tissue culture infective dose assays (H5N1). Serial dilutions of each adapted virus stock were then injected into FVB/J mice, and the 50% mouse lethal dose (MLD_{50}) was calculated according to the method of Reed and Muench (*5*).

Pathotyping Studies

For assessment of virus-induced pathogenicity, 2 series of mice were inoculated intranasally with 10 MLD_{50} of virus by instillation of 50 μL of diluted stock. Mice were monitored daily for changes in bodyweight to assess virus-induced illness. At selected intervals, 5 (virus titration or histopathology) or 10 (virus titration + dry/wet weight ratio) mice were given an overdose of sodium pentobarbital and exsanguinated by cutting the brachial artery. Lungs and pieces of heart, liver, spleen, pancreas, kidney, brain, and adipose tissue from 5 mice were fixed in 4% neutral-buffered, ice-cold paraformaldehyde, routinely processed, and embedded in paraffin for histopathologic evaluation. Five-micrometer sections were stained with hematoxylin and eosin (HE) or periodic acid–Schiff (PAS) for lesion detection. For virus detection, sections were stained by a streptavidin-biotin complex immunoperoxidase method. An in-house immunoglobulin (Ig) G–purified polyclonal rabbit antiserum raised against recombinant influenza virus nucleoprotein was used as the source of primary antibodies, and horseradish peroxidase (HRP)–conjugated anti–rabbit IgGs (Dako, Glostrup, Denmark) were used as secondary antibodies. Peroxidase was indicated by the bright red precipitate produced in the presence of 3-amino-9-ethyl-carbazole, and sections were counterstained with Mayer hematoxylin. For virus titrations, lungs from 5 mice were weighed, homogenized in 1 mL PBS, and clarified. The supernatants were used for virus titration by plaque or median tissue culture infectious dose assays. Because the appearance of a biphasic expiratory pattern has been shown to announce death within ≈24 h (*6*), this qualitative sign was chosen, for humane reasons, as the endpoint of the experimental disease. On this endpoint day, lungs from 5 mice were sampled and weighed, and homogenates thereof were desiccated for dry weight determination.

Results

Clinical, Gross Pathologic, and Virologic Observations

The influenza A virus strains (subtypes H1N1 and H5N1) used in this study were isolated, respectively, from

a diseased pig in the United States in 1976 and from a crested eagle smuggled from Thailand in 2003 (*7*). Both were nonpathogenic for FVB/J mice (MLD_{50} >10^6 PFU/50% tissue culture infective dose [$TCID_{50}$]). After adaptation, the strains showed a similar pathogenic outcome in FVB/J mice, i.e., close MLD_{50} values: 3.2 PFUs for the subtype H1N1 strain and 6.4 $TCID_{50}$ for the subtype H5N1 strain. These results allowed a relevant comparison of their respective pathologic signatures. Overall, virus-associated illness, bodyweight loss, and gross lesions caused by inoculation of 10 MLD_{50} were similar for both viruses, except that body condition and respiratory function deteriorated far more rapidly after subtype H5N1 inoculation, the endpoint being reached on 4 dpi for subtype H5N1–induced disease and 8 dpi for subtype H1N1–induced disease. The pathologic processes caused no symptoms for the first 2 (H5N1) or 3 (H1N1) days and then gave rise to general signs such as gradually slower, less frequent, and more erratic spontaneous displacements and a ruffled coat. By 3 dpi (H5N1) or 5 dpi (H1N1), all mice became lethargic and abruptly showed clinical signs of respiratory disease, including respiratory distress, labored breathing, and forced expiration. Mice inoculated with subtype H5N1 lost 10% of their bodyweight during the last 48 hours before the endpoint day. In mice that were inoculated with subtype H1N1, weight loss was acute and biphasic: a 10% loss occurred between virus inoculation and the appearance of respiratory symptoms, and an additional 20% was lost during ARDS (Figure 1). Autopsies performed on the endpoint day of subtype H1N1 disease consistently showed dark, purplish, bulky, noncrepitant, liverlike lungs, findings compatible with a diagnosis of massive pulmonary congestion and consolidation. In subtype H5N1–inoculated mice, the lungs at endpoint were bulky, noncrepitant, and diffusely pinkish gray, which suggests a diagnosis of congestion with massive pulmonary edema. Mice inoculated with either virus had a lung wet weight at endpoint approximately double that of controls, but this weight gain was achieved during the last ≈24 hours in mice inoculated with subtype H5N1, whereas mice inoculated with subtype H1N1 showed a progressive lung weight increase over 96 hours, from 4 dpi to the endpoint day (Figure 2). At the endpoint, the dry/wet weight ratio of the lungs was ≈22% lower for subtype H5N1-infected mice (17.6% ± 1.1%) than for subtype H1N1-infected mice (21.4% ± 1.4%). No obvious gross lesions were observed in the heart, liver, spleen, kidney, brain, or perivisceral fat. The lung virus loads measured on 2, 4, 6, and 8 dpi are shown in Figure 3. The time required to reach the peak virus titer was the same for both virus strains. Death occurred at the peak lung virus concentration for subtype H5N1, but subtype H1N1–associated disease did not become fatal until 4 days after this peak, when virus clearance was already substantial (Figure 3).

Histopathologic Observations

An exhaustive list of the histopathologic lesions caused by the 2 viruses is given in the online Appendix Table (www.cdc.gov/EID/content/16/4/595-appT.htm). Some changes in lung morphology were identical for both viruses. First, a clear topographic extension of the lesions was perceptible between the first and the last day of infection, with centrifugal spreading from the terminal bronchioles or the alveoli adjacent to the airways. Qualitatively, all alterations characterizing the exudative phase of the histopathologic condition termed diffuse alveolar damage were identifiable, with intense congestion of the alveolar

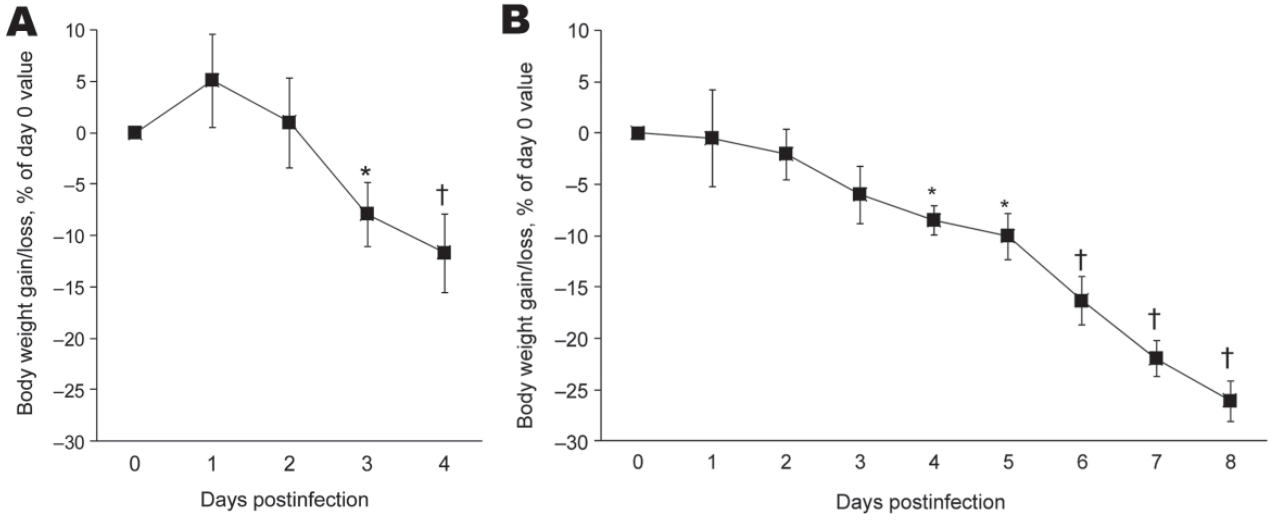


Figure 1. Effect of influenza A virus subtype H5N1 (A) and H1N1 (B) strains on bodyweight gain or loss after intranasal inoculation of 10× the 50% mouse lethal dose on day 0. Relative values are given, as calculated with respect to preinoculation control values (mean ± SD). For each virus strain, means significantly different from baseline are indicated (Student *t* test for paired values). *p<0.05; †p<0.01.

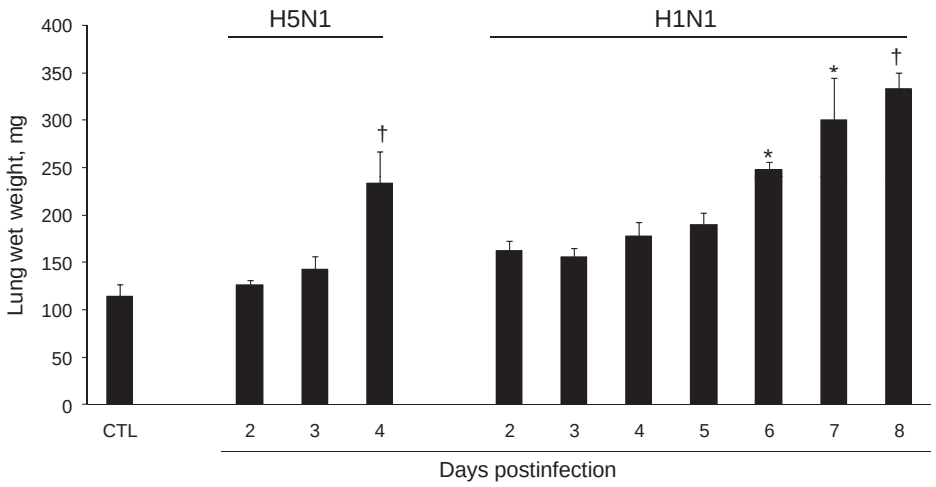


Figure 2. Effect of influenza A virus subtype H5N1 and H1N1 strains on lung weight after intranasal inoculation of 10× the 50% mouse lethal dose on day 0. Absolute values are given as means ± SD for 5 mice at each time point. For each virus strain, means significantly different from those of control (CTL) lungs are indicated (nonparametric Mann-Whitney test). *p<0.05; †p<0.01.

capillaries, marginated intracapillary neutrophils, necrosis of the alveolar epithelium, interstitial and alveolar edema, hyaline membranes, and invasion of the alveoli by (mostly) mononucleate cells. On the other hand, we did not observe cuboidalization of the alveoli (hyperplasia of type II pneumocytes) or hyperplasia or squamous metaplasia of the airway epithelia. These results indicate extremely rapid disease progression, nearly complete elimination of type II pneumocytes, or both. Despite these similarities, when sections of lung tissue samples taken on the last day from infected mice were pooled by subtype, an examiner unaware of which infection he was looking could easily distinguish one from the other (Figures 4, 5). The criteria for attributing lung lesions to the subtype H1N1 strain were the following: 1) earlier and much more extensive degeneration, necrosis, and desquamation of the airway epithelium; 2) a much higher cell density of the peribronchial, peribronchiolar, interstitial, and intra-alveolar infiltrates; 3) the presence of dense cuffs of mononucleate cells around the arterioles; 4) far less extensive alveolar edemas; and 5) the rarity of alveolar hemorrhages. The lesions caused by the subtype H5N1 strain were distinguishable by the late and mild regressive alterations of the airway epithelium, the extent of alveolar edema, the low cell density of the inflammatory infiltrates, the high number of alveolar hemorrhage foci, and the unusual appearance of the pulmonary arterioles (which seemed to have been dissected from the surrounding tissues because of the magnitude of the perivascular edema).

On the other hand, no arteriole showed any cuff of infiltrated mononucleate cells. Some blood-vessel walls also showed hemorrhage inside the muscle layer. No other organ examined was found to carry any histopathologic lesions except, notably, the liver in subtype H5N1–infected mice (Figure 5). These livers displayed multifocal necrosis, with necrotic foci consisting of aggregates of hypereosinophilic, pyknotic, and caryorhectic hepatocytes, admixed with a few neutrophils and lymphocytes. Such foci were

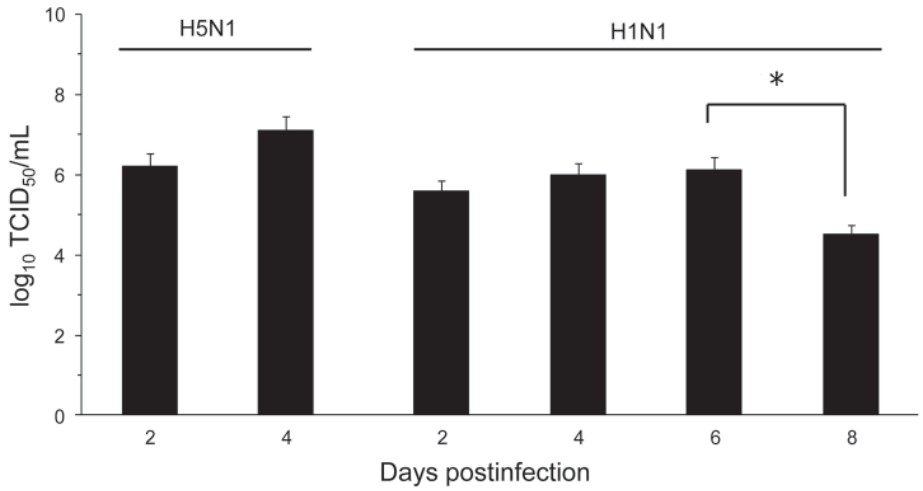


Figure 3. Effect of influenza A virus subtype strains H5N1 and H1N1 on lung virus titers 2–8 days after intranasal inoculation of 10× the 50% mouse lethal dose on day 0. Titers are expressed as the $\log_{10}$ median tissue culture infectious dose ($TCID_{50}$) units per milliliter of lung homogenate. Significantly different titers are indicated (nonparametric Mann-Whitney test). Error bars indicate SD calculated from individual virus titers. *p <0.05.

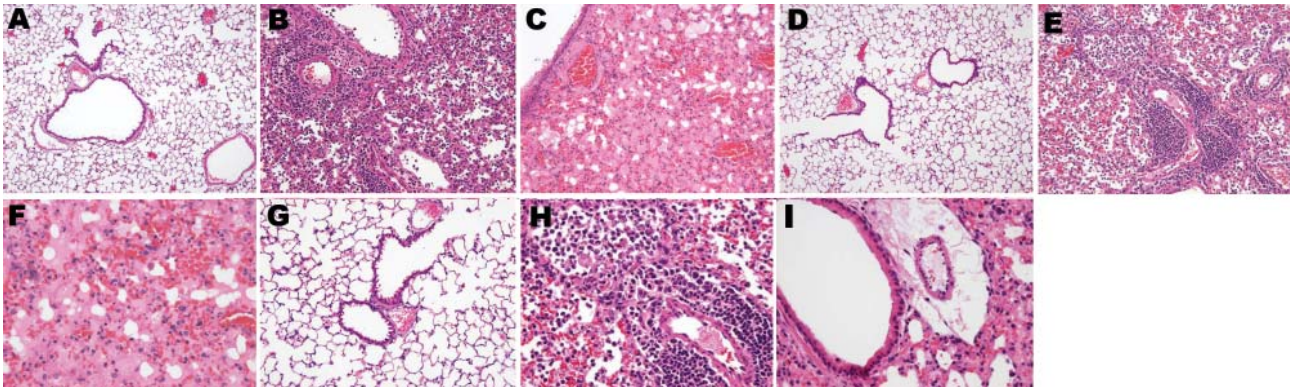


Figure 4. Photomicrographs of the lung sections of influenza A virus (H1N1)– and (H5N1)–infected mice at endpoint (hematoxylin and eosin stain). Dramatically different histopathologic signatures are observed, with either a mostly cellular reaction (H1N1) or a mostly humoral reaction (H5N1). Panels A, D, and G: 3 views of vehicle-infected lungs (original magnification ×100). Panels B and E, subtype H1N1: Dense granulocytic and lymphocytic cell infiltrates in the interstitium and around vessels and airways with focally denuded lamina propria due to epithelial necrosis and desquamation (original magnification ×100). Panel C, subtype H5N1: Airway epithelium is intact; note the striking difference in the number of infiltrated inflammatory cells between subtypes H1N1- and H5N1-infected lungs. Dramatic congestion of the vessels is visible, with extensive interstitial and alveolar edema (original magnification ×100). Panel F, subtype H5N1: Alveoli are completely filled with edema and hemorrhages; cellular infiltrates are conspicuously absent (original magnification ×200). Panel H, subtype H1N1: An airway with a totally denuded lamina propria is shown (top, left), with its lumen filled with granulocytic and lymphocytic exsudate (original magnification ×200). A prominent periarteriolar lymphocytic cuff is visible (bottom right). Panel I, subtype H5N1: Moderate inflammatory cell infiltrate, with no cuffing of any airway or vessel; an airway with a still intact epithelium is shown, located just beside a vessel with dramatic peripheral edema (original magnification ×200).

also seen in the spleen in some animals. Strikingly, numerous PAS-positive islets were detected throughout the livers of subtype H5N1–infected animals, each overlapping with a necrotic focus. Patterns of centrolobular, hydropic, granular (2 dpi), centrolobular (3 dpi), and panlobular (4 dpi) microvesicular fatty degeneration were also observed in the livers of all subtype H5N1-infected animals. Interstitial hemorrhages were seen in the renal medulla.

Detection of Viruses in Tissues

The results of immunohistochemical tests were homogeneous for mice infected with the same strain. Overall, they showed that the subtype H1N1 strain swarmed centrifugally from the bronchioles throughout the lungs over 4–5 days, but remained strictly confined to the lungs. The subtype H5N1 virus, in contrast, conquered the whole lung over 24–48 hours; infected some bronchioles only later; and spread to the liver, pancreas, kidneys, spleen, brain, and perivisceral fat.

Topologic Distribution of Subtype H1N1 Antigens over Time

The virus was first detectable in the epithelium of the bronchi and bronchioles on 3 dpi. By 5 dpi, the stain was more conspicuous and appeared also in the alveolar epithelium of the areas adjacent to the airways. By 7 dpi, the virus was detectable in the epithelia of almost all bronchi and bronchioles and in the alveolar epithelium in extensive areas of the lungs. In the alveolar structures, staining showed the virus in type I and type II pneumocytes and in alveolar macrophages (Figure 6, panels A, C, and E). Nonrespiratory organs sampled on 3, 5, or 7 dpi remained strictly virus negative.

Topologic Distribution of Subtype H5N1 Antigens over Time

The virus was detectable from 2 dpi in some type II pneumocytes in peribronchiolar alveoli, some interstitial/alveolar macrophages, and some endothelial cells in the vicinity of the positive alveoli. In contrast, no nonrespiratory organ examined showed any virus-positive cells. By 3 dpi, staining of the airway epithelium was still discrete and limited, whereas the alveolar epithelium showed more pronounced staining, diffusely distributed throughout the lung. In the liver, multiple nests of positive hepatocytes were detectable, corresponding exactly with the above-mentioned necrotic PAS-positive foci. A few renal tubular epithelial cells were also positive. On 4 dpi, the alveolar epithelium was still diffusely stained, but more intensely than on 3 dpi. For the first time, staining of the bronchiolar epithelium was also visible, but not all bronchioles—far from all, in fact—showed this staining. Type II pneumocytes and alveolar macrophages were more often positive than type I pneumocytes (Figure 6, panels B, D, and F). The appearance of the kidneys and liver was the same as on 3 dpi, with more conspicuous staining. Additionally, virus-

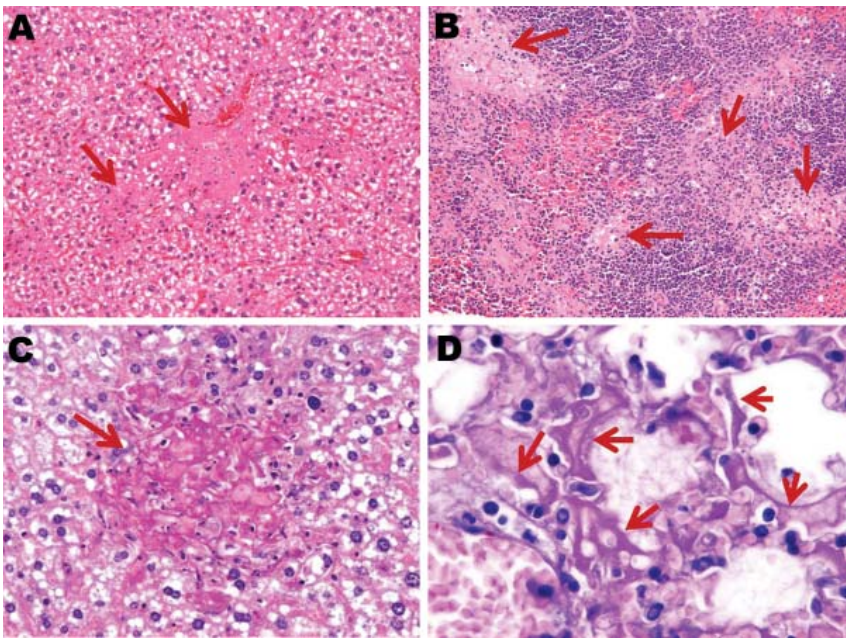


Figure 5. Photomicrographs of liver, spleen, and lung sections from influenza virus A (H5N1)–infected mice at endpoint. Necrotic foci (arrows) scattered throughout the liver (A) (original magnification ×200) and spleen (B) (original magnification ×100) from subtype H5N1–infected mice (hematoxylin and eosin stain); these foci are absent from subtype H1N1–infected mouse livers. C) Necrotic foci in the liver stain periodic acid–Schiff (PAS)–positive (arrow), which suggests focal accumulation of glycogen (original magnification ×400). D) Numerous alveolar walls lined with PAS-stained hyaline membranes (arrows), suggestive of necrosis and desquamation of pneumocytes (original magnification ×1,000).

positive glial cells, splenic macrophages, cardiomyocytes, islets of Langerhans cells, and peritoneal adipocytes were also detected (Figure 7).

Discussion

Two influenza A viruses of different subtypes, derived from different species and showing no pathogenicity toward mice, were forced to evolve by serial passaging in mouse lungs. The 2 adapted viruses obtained showed practically identical virulence levels, with similar MLD_{50} values. On the basis of this criterion, they appear to be more virulent than most other viruses used to date in murine models (*4*,*8*–*16*). Their virulence is of the same order of magnitude as those of the A/Vietnam/1203/2004 (H5N1) and A/Vietnam/1204/2004 (H5N1) viruses, whose respective MLD_{50}s are 0.7 and 2.1 PFUs (*17*). In both cases, inoculation of 10 MLD_{50} causes biphasic weight loss, culminating in death with a loss of ≈10% (H5N1) or ≈25% (H1N1) bodyweight. Viral amplification is maximal for both viruses on 4 dpi, roughly corresponding to the typical inoculation-to-peak lag of natural murine respiratory viruses (*6*,*18*). On the other hand, the 2 viruses adapted in the lungs showed replication kinetics that differed substantially from what is observed with natural viruses, with a quasi-plateau from 2 to 5/6 dpi instead of the classical Gaussian profile. Notably, this peculiar amplification kinetics profile has been described previously for mice infected with mouse-adapted forms of the A/Puerto Rico/8/34 (H1N1) virus (*19*), the A/South Carolina/1/18 (H1N1) virus (*4*), and several human subtype H5N1 strains showing high or low pathogenicity (*4*,*14*). These reports suggest that this profile is typical of influenza virus amplification by the murine respiratory system.

A final common feature of infection with the 2 virus subtypes was diffuse alveolar damage, which dominates both histopathologic profiles; these results corroborate the pathologic data found in the literature. Seasonal human influenza epidemics typically consist of a transient tracheobronchitis caused by preferential attachment of the virus to the laryngeal, tracheal, and bronchial epithelia. In contrast, those influenza viruses which are highly pathogenic toward humans, from the pandemic viruses of 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2) to the subtype H5N1 strains isolated from humans since 2003, additionally colonize the bronchiolar and alveolar epithelia, preferentially or not, and cause diffuse alveolar damage as an additional primary lesion (*20*–*23*). The same lesion has been found in experimental animals injected with a recent subtype H5N1 strain (*14*,*24*–*26*).

Although both viruses share the same pathogenicity, replication kinetics, and concentration peak, and although they both evoke diffuse alveolar damage by the endpoint day, they differ dramatically in terms of the ARDS course and pathologic signature. Flagrant differences make it easy to distinguish infections by the 2 subtypes. In subtype H1N1 infection, the disease becomes fatal at a point when the pulmonary edema is much less intense and leaves a histopathologic picture characterized by much more dense inflammatory cell infiltrates, generating cuffs around the bronchioles and blood vessels. Second, subtype H1N1 colonizes the epithelia of both the upper and lower airways, without any obvious preference, whereas subtype H5N1

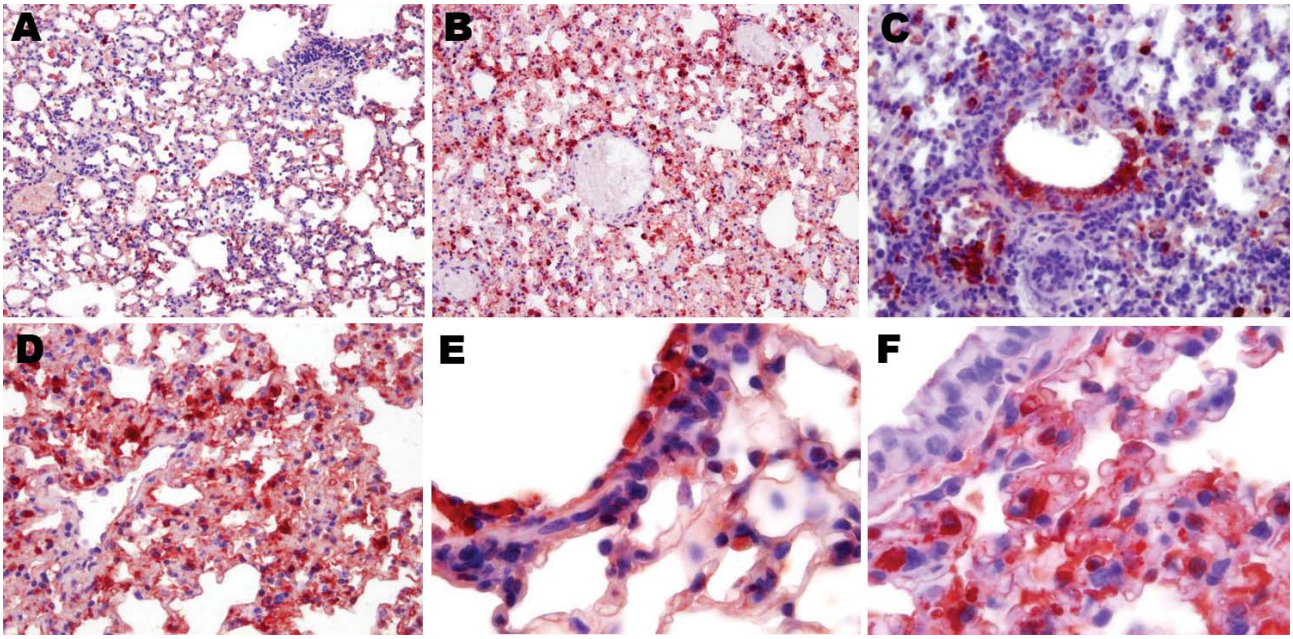


Figure 6. Topologic distribution of influenza antigens in the lungs of mice infected with influenza virus A subtype H1N1 and H5N1 strains at endpoint (antinucleoprotein immunohistochemical staining). A) Subtype H1N1 and B) subtype H5N1, both showing diffusely distributed positive staining of numerous pneumocytes and alveolar macrophages (original magnification ×100). C) Subtype H1N1, showing antigens massively present in the remaining non-desquamated airway epithelial cells (original magnification ×400); viral amplification in type I and type II pneumocytes is far more intense and widespread 4 days after inoculation of the subtype H5N1 virus (D) than 7 days after inoculation of the subtype H1N1 virus (original magnification ×400). E) Desquamated, necrotic, and intensely virus-positive airway epithelial cells in a terminal bronchiole and adjacent alveoli of a mouse infected with subtype H1N1, compared with F) uninfected, intact airway epithelial cells in a terminal bronchiole and adjacent alveoli of a mouse infected with subtype H5N1, illustrating the different pneumotropism of the 2 viruses (original magnification ×1,000). Conversely, the density of virus-positive cells in the lung/alveoli is higher after inoculation of the subtype H5N1 strain (Mayer hematoxylin counterstain).

remains confined essentially to the alveoli and terminal bronchioles. Within the alveoli, unlike the subtype H1N1 strain, the subtype H5N1 strain shows a preferential tropism for type II pneumocytes and alveolar macrophages. Lastly, whereas subtype H1N1 remains strictly confined to the respiratory system, subtype H5N1 spreads to other organs. These differences demonstrate unambiguously that the 2 highly virulent influenza A viruses studied here cause 2 different forms of ARDS. This finding suggests that the physiopathologic data obtained when studying 1 virulent strain should not be extrapolated automatically to other strains. The observed differences also suggest that diverse constellations of critical mutations in the viral genome might lead to the same fatal result.

This work addresses the question of possible differences between 2 fatal diseases caused by influenza A viruses, although some previous evidence that pointed in the same direction has already been reported. For example, the pandemic human strains of 1918, 1957, and 1968, on the one hand, and the recent subtype H5N1 strains, on the other, show different tropisms: panepithelial for the former strains (*20*,*27*,*28*) and limited to the bronchiolar and alveolar epithelia for the latter strains, a result compatible with our own observations on mouse-adapted viruses. Likewise, a panepithelial tropism has been observed for the A/South Carolina/1/18 (H1N1) virus in mice (*29*), whereas a preference for the bronchioles and alveoli has been noted for recent subtype H5N1 strains that have been injected into macaques, mice, ferrets, and cats (*14*,*25*,*30*–*37*). In addition, the observed strict confinement of our subtype H1N1 strain to the respiratory system confirms previously reported data that refute the existence of polysystemic dissemination of non-H5 viruses that are lethal to humans or laboratory animals (*20*,*27*,*29*,*38*). Conversely, our observation that the subtype H5N1 strain spreads beyond the respiratory system confirms similar observations of both humans (*22*,*22*,*39*) and laboratory animals (*14*,*24*,*25*,*30*–*34*).

Although other subtype H5N1 and subtype H1N1 viruses infect other susceptible hosts, they may not show trends similar to those observed here. These results, when integrated with the diverse pieces of evidence reported elsewhere, suggest that fatal infections caused by different highly virulent influenza A viruses do not necessarily share the same pathogenesis. To be convinced, one has only to

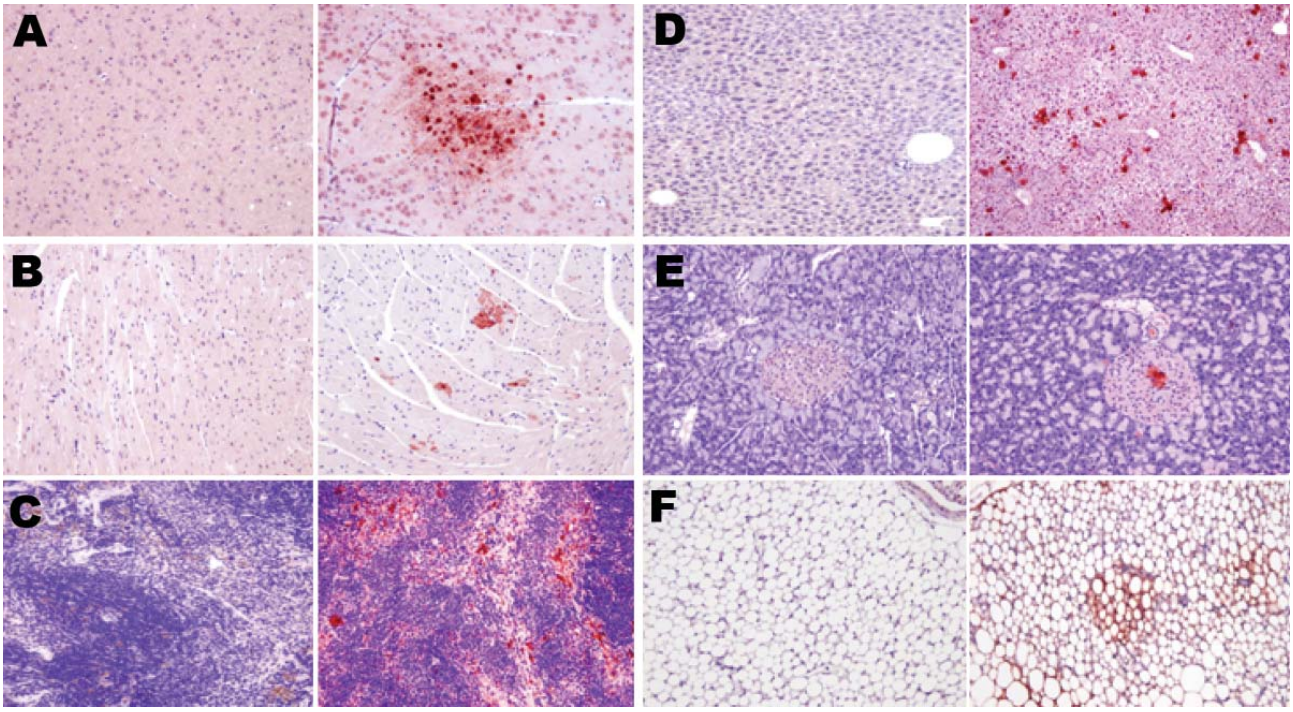


Figure 7. Topologic distribution of antigens in mice infected with influenza A virus subtype H1N1 at day 7 postinfection (left columns) and subtype H5N1 at day 4 postinfection (right columns) in various nonrespiratory organs. A) Glial cells (mostly oligodendrocytes); B) cardiomyocytes; C) spleen macrophages; D) hepatocytes; E) islets of Langerhans cells in the pancreas; and F) adipocytes. Bright virus-positive staining can be seen in subtype H5N1–infected mice (antinucleoprotein immunohistochemical staining), while absence of any staining can be seen in subtype H1N1–infected mice (Mayer hematoxylin counterstain). Original magnification ×100.

note the ease of distinguishing, in the absence of any virus labeling, the histopathologic sections typical of the 2 strains used here (Figure 4). These different histopathologic signatures and different pathogeneses probably reflect the presence of specific sets of virulence markers that will have to be decrypted to anticipate the emergence of a pandemic. In this respect, sequence analysis of both strains will lead to insight on specific residues that are relevant for the adaptation and virulence of an influenza strain in a new host.

Furthermore, the differences between these 2 strains suggest that >1 universal cytokine storm underlies fatal influenza diseases. Thus, it might be advantageous to tailor the therapeutic approach to the influenza virus pathotype.

Acknowledgments

We thank Michaël Sarlet and François Cornet for logistical and technical support.

This work was supported by the Interuniversity Attraction Poles, phase VI, project P6/14 (GPCRS, to A.C.) and by the European Union–funded European Animal Disease Genomics Network of Excellence.

Dr Garigliany is a research fellow of the Fonds National de la Recherche Scientifique, Brussels, Belgium. His research interests include influenza virus biology and host–virus interactions.

References

1. Murray CJ, Lopez AD, Chin B, Feehan D, Hill KH. Estimation of potential global pandemic influenza mortality on the basis of vital registry data from the 1918–20 pandemic: a quantitative analysis. Lancet. 2006;368:2211–8. DOI: 10.1016/S0140-6736(06)69895-4
2. Korteweg C, Gu J. Pathology, molecular biology, and pathogenesis of avian influenza A (H5N1) infection in humans. Am J Pathol. 2008;172:1155–70. DOI: 10.2353/ajpath.2008.070791
3. Kuiken T, Taubenberger JK. Pathology of human influenza revisited. Vaccine. 2008;26:D59–66. DOI: 10.1016/j.vaccine.2008.07.025
4. Perrone LA, Plowden JK, García-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog. 2008;4:e1000115. DOI: 10.1371/journal.ppat.1000115
5. Reed LJ, Muench HA. A simple method of estimating fifty per cent endpoints. Am J Hyg. 1938;27:493–7.
6. Anh BD, Faisca P, Desmecht DJ. Differential resistance/susceptibility patterns to pneumovirus infection among inbred mouse strains. Am J Physiol Lung Cell Mol Physiol. 2006;291:L426–35. DOI: 10.1152/ajplung.00483.2005
7. Van Borm S, Thomas I, Hanquet G, Lambrecht B, Boschmans M, Dupont G, et al. Highly pathogenic H5N1 influenza virus in smuggled Thai eagles, Belgium. Emerg Infect Dis. 2005;11:702–5.
8. Brown EG, Liu H, Kit LC, Baird S, Nesrallah M. Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: identification of functional themes. Proc Natl Acad Sci U S A. 2001;98:6883–8. DOI: 10.1073/pnas.111165798
9. Evseenko VA, Bukin EK, Zaykovskaya AV, Sharshov KA, Ternovoi VA, Ignatyev GM, et al. Experimental infection of H5N1 HPAI in

BALB/c mice. Virol J. 2007;4:77–82. DOI: 10.1186/1743-422X-4-77
10. Isobe H, Alt F, Bona CA, Schulman J. Intact antiinfluenza virus immune response in targeted kappa-deficient mice. Viral Immunol. 1994;7:25–30. DOI: 10.1089/vim.1994.7.25
11. Kawaoka Y. Equine H7N7 influenza A viruses are highly pathogenic in mice without adaptation: potential use as an animal model. J Virol. 1991;65:3891–4.
12. Lipatov AS, Andreansky S, Webby RJ, Hulse DJ, Rehg JE, Krauss S, et al. Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses. J Gen Virol. 2005;86:1121–30. DOI: 10.1099/vir.0.80663-0
13. Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol. 1999;73:5903–11.
14. Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, et al. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J Virol. 2005;79:11788–800. DOI: 10.1128/JVI.79.18.11788-11800.2005
15. Tumpey TM, García-Sastre A, Taubenberger JK, Palese P, Swayne DE, Basler CF. Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. Proc Natl Acad Sci U S A. 2004;101:3166–71. DOI: 10.1073/pnas.0308391100
16. Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solórzano A, Swayne DE, et al. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science. 2005;310:77–80. DOI: 10.1126/science.1119392
17. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, et al. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog. 2007;3:1374–9. DOI: 10.1371/journal.ppat.0030133
18. Faisca P, Anh DB, Desmecht DJ. Sendai virus-induced alterations in lung structure/function correlate with viral loads and reveal a wide resistance/susceptibility spectrum among mouse strains. Am J Physiol Lung Cell Mol Physiol. 2005;289:L777–87. DOI: 10.1152/ajplung.00240.2005
19. Hennet T, Ziltener HJ, Frei K, Peterhans E. A kinetic study of immune mediators in the lungs of mice infected with influenza A virus. J Immunol. 1992;149:932–9.
20. Winternitz MC, Wason IM, McNamara FP. The pathology of influenza. New Haven (CT): Yale University Press; 1920. p. 20–45.
21. To KF, Chan PK, Chan KF, Lee WK, Lam WY, Wong KF, et al. Pathology of fatal human infection associated with avian influenza A H5N1 virus. J Med Virol. 2001;63:242–6. DOI: 10.1002/1096-9071(200103)63:3<242::AID-JMV1007>3.0.CO;2-N
22. Gu J, Xie Z, Gao Z, Liu J, Korteweg C, Ye J, et al. H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet. 2007;370:1137–45. DOI: 10.1016/S0140-6736(07)61515-3
23. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, et al. Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol. 2007;171:1215–23. DOI: 10.2353/ajpath.2007.070248
24. Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, Ortin J, et al. Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci U S A. 2006;103:12121–6. DOI: 10.1073/pnas.0605134103
25. Nishimura H, Itamura S, Iwasaki T, Kurata T, Tashiro M. Characterization of human influenza A (H5N1) virus infection in mice: neuro-, pneumo- and adipotropic infection. J Gen Virol. 2000;81:2503–10.
26. Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, Hulse-Post DJ, et al. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med. 2006;203:689–97. DOI: 10.1084/jem.20051938
27. Opie EL, Blake FG, Small JC, Rivers TM. Respiratory disease. London (UK): H. Kimpton;1921. p. 402–20.
28. Herfs JF, Mulder J. Broad aspects of the pathology and pathogenesis of human influenza. Am Rev Respir Dis. 1961;83:84–97.
29. Kash JC, Tumpey TM, Proll SC, Carter V, Perwitasari O, Thomas MJ, et al. Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. Nature. 2006;443:578–81.
30. Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA, et al. Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol. 2006;168:176–83. DOI: 10.2353/ajpath.2006.050466
31. Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, et al. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol. 2005;79:2191–8. DOI: 10.1128/JVI.79.4.2191-2198.2005
32. Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J Virol. 2002;76:4420–9. DOI: 10.1128/JVI.76.9.4420-4429.2002
33. Yen HL, Lipatov AS, Ilyushina NA, Govorkova EA, Franks J, Yilmaz N, et al. Inefficient transmission of H5N1 influenza viruses in a ferret contact model. J Virol. 2007;81:6890–8. DOI: 10.1128/JVI.00170-07
34. Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD. Pathogenesis of influenza A (H5N1) virus infection in a primate model. J Virol. 2001;75:6687–91. DOI: 10.1128/JVI.75.14.6687-6691.2001
35. Xu T, Qiao J, Zhao L, Wang G, He G, Li K, et al. Acute respiratory distress syndrome induced by avian influenza A (H5N1) virus in mice. Am J Respir Crit Care Med. 2006;174:1011–7. DOI: 10.1164/rccm.200511-1751OC
36. Katz JM, Lu X, Frace AM, Morken T, Zaki SR, Tumpey TM. Pathogenesis of and immunity to avian influenza A H5 viruses. Biomed Pharmacother. 2000;54:178–87. DOI: 10.1016/S0753-3322(00)89024-1
37. Kuiken T, Rimmelzwaan GF, Van Amerongen G, Osterhaus AD. Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (*Macaca fascicularis*). Vet Pathol. 2003;40:304–10. DOI: 10.1354/vp.40-3-304
38. Lowen AC, Mubareka S, Tumpey TM, García-Sastre A, Palese P. The guinea pig as a transmission model for human influenza viruses. Proc Natl Acad Sci U S A. 2006;103:9988–92. DOI: 10.1073/pnas.0604157103
39. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med. 2006;12:1203–7. DOI: 10.1038/nm1477

Address for correspondence: Daniel Desmecht, University of Liège, Department of Pathology, Faculty of Veterinary Medicine, Sart Tilman B43, Liège 4000, Belgium; email: daniel.desmecht@ulg.ac.be

Clostridium difficile Infections among Hospitalized Children, United States, 1997–2006

Marya D. Zilberberg, Glenn S. Tillotson, and L. Clifford McDonald

We evaluated the annual rate (cases/10,000 hospitalizations) of pediatric hospitalizations with *Clostridium difficile* infection (CDI; International Classification of Diseases, 9th revision, clinical modification code 008.45) in the United States. We performed a time-series analysis of data from the Kids' Inpatient Database within the Health Care Cost and Utilization Project during 1997–2006 and a cross-sectional analysis within the National Hospital Discharge Survey during 2006. The rate of pediatric CDI-related hospitalizations increased from 7.24 to 12.80 from 1997 through 2006; the lowest rate was for children <1 year of age. Although incidence was lowest for newborns (0.5), incidence for children <1 year of age who were not newborns (32.01) was similar to that for children 5–9 years of age (35.27), which in turn was second only to incidence for children 1–4 years of age (44.87). Pediatric CDI-related hospitalizations are increasing. A better understanding of the epidemiology and outcomes of CDI is urgently needed.

The epidemiology of *Clostridium difficile* infection (CDI) has been shifting over the past decade. Since 2000, the molecular evolution of the hypervirulent toxigenic bacterial strain BI/NAP1/027, which causes severe disease in massive outbreak settings, has been well documented (*1*–*4*). Furthermore, the increasing detection of this strain in the United States and other countries coincides with reports of increasing hospitalizations either resulting from or complicated by CDI and associated with increased case-fatality rates (*5*–*7*). Although in the past it was not thought to affect pediatric populations substantially, CDI has more recently been implicated as an increasingly prevalent diarrheal pathogen in children (*8*–*10*). Moreover, evidence suggests that a large proportion of pediatric CDI cases are community-acquired infections and that many of these infections lack the traditional risk factor of exposure to antimicrobial drugs (*11*–*13*). These changes in the epidemiology of pediatric CDI, although not definitively caused by the BI/NAP1/027 strain, are likely related to this strain because at least 2 reports suggest a high prevalence (10%–38%) of this strain in pediatric CDI populations and a 4× increase in complication rates associated with this strain compared with other strains (*14*,*15*).

Current age-specific epidemiology of CDI among children remains poorly studied. Literature predating the emergence of the epidemic strain suggests that although up to 67% of all neonates (i.e., <1 month of age) become colonized with *C. difficile* in the perinatal period, they do not appear to be at risk for the development of CDI-associated symptoms (*16*). Conversely, children 1 month–2 years of age, although less likely to become colonized with this bacterium, are more likely to have attendant disease (*16*). Finally, children 3–18 years of age have been reported to have similar risk for CDI as that seen in adults (*16*). Because the epidemiology of CDI is changing rapidly in children and adults, we examined age-specific trends in CDI-related hospitalizations in the US population <18 years of age.

Materials and Methods

To characterize the epidemiology of CDI-related hospitalizations among US children, we performed 2 analyses using 2 databases. These databases are based on administrative coding, and consistency in results obtained from >1 data source potentially indicates a higher chance of

Author affiliations: University of Massachusetts, Amherst, Massachusetts, USA (M.D. Zilberberg); Evi*Med* Research Group, LLC, Goshen, Massachusetts, USA (M.D. Zilberberg); ViroPharma Inc., Exton, Pennsylvania, USA (G.S. Tillotson); and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (L.C. McDonald)

DOI: 10.3201/eid1604.090680

accuracy. In addition, the format in which we analyzed 1 of the databases (Kids' Inpatient Database [KID]) did not enable separating newborn discharges (defined as those hospitalizations during which the child was born) from those of other children <1 year of age. Because newborns represent a unique population prone to colonization but not overt disease, we chose a second database that enables separate analysis of newborns (National Hospital Discharge Survey [NHDS]).

The first analysis was a time-series analysis of all CDI-related hospitalizations among US children between 1997 and 2006 based on the data from KID within the Healthcare Cost and Utilization Project (HCUP) administered by the Agency for Healthcare Research and Quality (Rockville, MD, USA). This type of longitudinal analysis is useful for tracking disease patterns over long periods. KID was specifically designed to identify, track, and analyze national trends in healthcare use, access, charges, quality, and outcomes; in 2006, it included data from 3,739 hospitals from 38 states in the United States (*17*). Complex survey methods exist to develop national and regional estimates for conditions addressed in the database. The Agency for Healthcare and Research Quality assesses completeness and data quality, and documentation is provided with the dataset. Data quality checks are limited to logical issues (e.g., birth date precedes age at hospital admission, excessively low total charges or long length of stay, age <10 years or >55 years on a maternal record, and mixed neonatal and maternal records), i.e., no chart reviews are undertaken by the agency.

For the current study, all data were derived in aggregate from the publicly available HCUPNet website (*18*). Because the years for which data were available were 1997, 2000, 2003, and 2006, our observations were limited to these periods. We examined the annual incidence of CDI-related hospitalizations on the basis of the International Classification of Diseases, 9th revision, clinical modification (ICD-9-CM), code 008.45 (intestinal infection with *C. difficile*) as a proportion of all hospitalizations. We additionally determined the time trends for CDI as the principal discharge diagnosis in this population. Finally, to understand better the context of increasing CDI-related hospitalizations, we examined trends in hospitalizations related to other diarrheal diseases, specifically *Salmonella* (ICD-9-CM 00.30), rotavirus (ICD-9-CM 008.61), viral enteritis (ICD-9-CM 008.8), and other infectious enteritides (ICD-9-CM codes 009.0–009.3, 487.8).

The second analysis was a cross-sectional characterization of all CDI hospitalizations for patients <18 years of age in 2006 reported in the NHDS collected by the Centers for Disease Control and Prevention (Atlanta, GA, USA) and available as a public utility file from the National Center for Health Statistics (NCHS) (Hyattsville, MD, USA) (*19*). NHDS covers discharges from ≈500 noninstitutional, nonfederal, short-stay hospitals in the United States. The 3-stage survey design enables balanced geographic representation; data are abstracted either manually or electronically for an ≈1% representative sample of all US hospitalizations. To ensure quality and completeness of the data, NCHS at the Centers for Disease Control and Prevention conducted studies in the late 1970s. These surveys indicated that the NHDS data met the general standards for quality set by NCHS (*20*).

Results

According to data from KID, the national number of pediatric CDI-related hospitalizations increased from 4,626 in 1997 to 8,417 in 2006 (Table 1). This change corresponded to an increased rate from 7.24/10,000 hospitalizations in 1997 to 12.80/10,000 hospitalizations in 2006 and represented a crude 9.0% per year increase. Although the group <1 year of age consistently accounted for the largest proportion of all pediatric CDI-related hospitalizations (Table 1), this group accounted for the lowest rate of CDI hospitalizations across the entire group of children; the highest incidence in all 4 years was detected in the group 1–4 years of age (Figure 1). The proportion of all CDI-related hospitalizations that had CDI listed as the principal discharge diagnosis remained essentially stable over the period examined (Table 1). Among other infectious

Table 1. *Clostridium difficile* infection–related hospitalizations, by year and age group, HCUP and KID, United States*

Characteristic	1997	2000	2003	2006
Age group, y				
<1	1,269	1,444	1,586	2,269
1–4	1,480	1,453	1,880	2,587
5–9	699	673	934	1,255
10–14	602	815	920	1,197
15–17	576	574	716	1,110
All	4,625	4,960	6,035	8,417
% CDIs as principal diagnosis	0.31	0.29	0.27	0.29

*HCUP, Health Care Utilization Project; KID, Kids' Inpatient Database; CDIs, *C. difficile* infections. The sums of individual age groups may not add up to the total cases because numbers represent rounded values of weighted estimates.

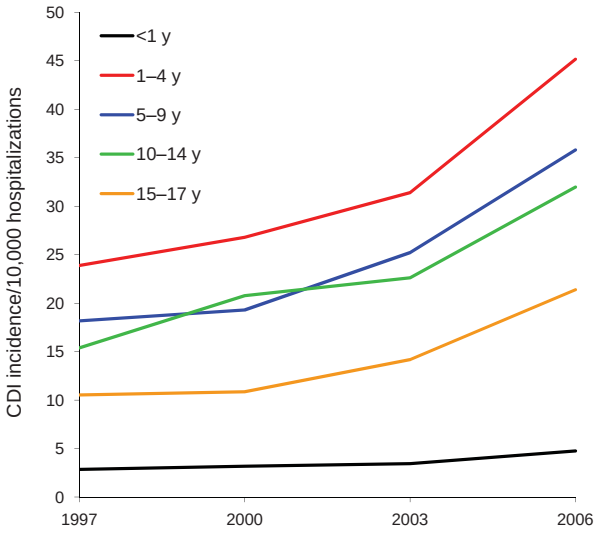


Figure 1. Age-specific incidence of patients with *Clostridium difficile* infection (CDI) per 10,000 hospitalizations, Health Care Utilization Project Kids' and Inpatient Database, United States, 1997–2006.

gastroenteritis-related hospitalizations, only rotavirus had a similar upward trajectory during this period (Figure 2).

Characteristics of CDI-related and nonrelated hospitalizations in the NHDS are shown in Table 2. The overall rate of pediatric CDI hospitalizations was 14.03/10,000 hospitalizations, and the age-specific rate did not differ substantially from that seen in the KID 2006 database (Figure 3). Although the newborn group, defined as infants whose hospitalizations originated at their birth, in NHDS represented 57.4% of all children hospitalized in 2006 and 84.2% of all children <1 year of age hospitalized, it had the lowest CDI rate of all pediatric age groups; the annual rate was 0.5/10,000 hospitalizations (Figure 3). Although the groups 1–4 and 5–9 years of age had the first (44.87/10,000) and second (35.27/10,000) highest rates of CDI hospitalizations, the third highest rate was seen in the non-newborn, <1 year of age group (32.01/10,000). The groups 10–14 and 15–17 years of age had the lowest rates of CDI hospitalizations in the pediatric cohort (Figure 3). There were no observed meaningful age-specific or sex-specific differences between the populations represented in the 2 data sources.

Discussion

We found that CDI-related hospitalizations as a proportion of all hospitalizations among US children increased dramatically between 1997 and 2006, from 7.24 to 12.80/10,000 hospitalizations. Most of this increase occurred between 2000 and 2006, which possibly reflects spread of the new *C. difficile* strain into medical institutions. Consistent with finding from previous studies, children 1–4 years of age were as a group most likely to have a hospitalization that was CDI related, and newborns were the least likely. Such a low rate in newborns is consistent with long-standing recommendations against routine testing of children <1 year of age (*21*). This rate, rather than representing a truly low risk for CDI in this age group, may be the result of an inflated denominator, given that most births in the United States occur in a hospital setting. In contrast, non-newborn infants (i.e., those <1 year of age and not meeting the newborn definition) had the second highest rate of CDI-related hospitalizations. In addition to the overall increase in pediatric CDI-related hospitalizations, there was a coincident increase in hospitalizations either resulting from or complicated by rotavirus infection.

Several of these findings are consistent with other recent epidemiologic and microbiology-based investigations. For example, Klein et al. examined billing records for the testing of diarrheal stool specimens from children who came to the emergency department at a children's hospital between 1998 and 2001 and identified *C. difficile* toxin in 6.7% (*8*). However, viral pathogens were isolated from 33% of the samples. A more recent study tracked changes between 2001 and 2006 in the epidemiology of *C. difficile* toxin testing performed on children at 1 academic medical center (*11*). The proportion of children <2 years of age who were positive increased from 46% to 64%, and there was a substantial increase in the incidence of community-onset infections and a concomitant decrease in hospital-onset infections. Interestingly, 43% of all patients had no recent

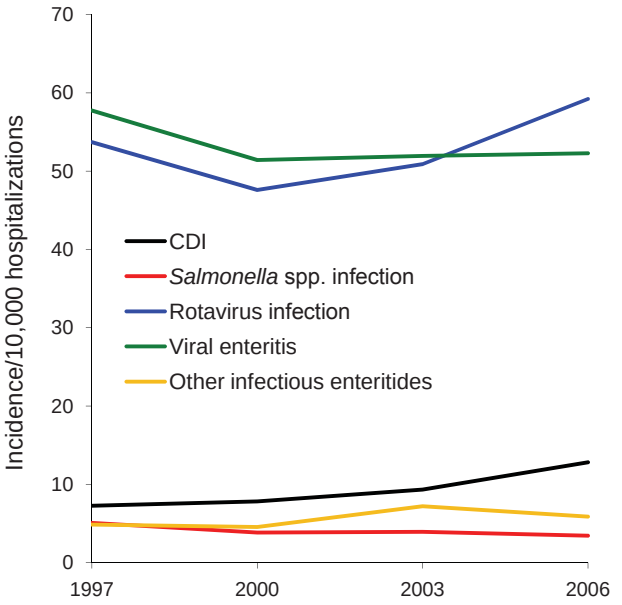


Figure 2. Incidence of infectious diarrhea hospitalizations per 10,000 all-cause hospitalizations, Health Care Utilization Project and Kids' Inpatient Database, United States, 1997–2006. CDI, *Clostridium difficile* infection.

Table 2. Demographic characteristics for CDIs, National Hospital Discharge Survey, United States, 2006*

Characteristic	No. (%) CDIs	No. (%) non-CDIs
All pediatric hospitalizations	98	69,651
Age, y		
Newborn	2	40,022
<1 but not newborn	24	7,474
1–4	32	7,099
5–9	16	4,521
10–14	13	4,943
15–17	11	5,690
Sex		
M	48 (49.0)	35,941 (51.5)
F	50 (51.0)	33,808 (48.5)
Race		
Caucasian	59 (60.2)	39,457 (52.3)
African American	12 (12.2)	9,602 (13.8)
Other	7 (7.1)	5,589 (8.0)
Not stated	20 (20.4)	18,069 (26.0)

*CDIs, *Clostridium difficile* infections.

history of exposure to antimicrobial drugs (*11*). Kim et al. estimated the rate of CDI in 22 US children's hospitals and also found a steady increase from 4.4 cases/10,000 patient-days in 2001 to 6.5 cases/10,000 patient-days in 2006 (*9*).

The role of *C. difficile* in the pathogenesis of disease among non-newborn children <1 year of age remains perplexing. Because of historically low rates of pseudomembranous colitis (the characteristic pathologic lesion caused by toxins A and B) among infants and high rates of asymptomatic *C. difficile* carriage in neonates, it has been recommended that laboratory testing for CDI not be routinely performed for children <1 year of age (*21*). However, in the study by Kim et al., in which tests for *C. difficile* laboratory assays were combined with ICD-9-CM discharge diagnoses, 26% of all CDI cases were identified in infants and 5% in neonates (*9*). Although rates increased from 2001 through 2006 for children 1–5 years of age (from 0.7 to 1.3 cases/1,000 hospitalizations; p = 0.04) and those 5–17 years of age (from 1.2 to 1.8/1,000 hospitalizations; p = 0.03), these rates did not change for the group <1 year of age (from 3.1 to 3.0/1,000 hospitalizations).

Our data, which are more broadly representative of all pediatric admissions in the United States, have similar trends between 2000 and 2006 for children 1–4 years of age (from 2.68 to 4.52/1,000 hospitalizations) and those 5–17 years of age (from 1.62 to 2.86/1,000 hospitalizations). In contrast, the <1 year age group rates from our time series were an order of magnitude lower in 2000 and 2006. However, the rate for non-newborn children <1 year of age in our 2006 cross-sectional study (3.20/1,000 hospitalizations) was comparable with that observed by Kim et al. (*9*). The lower overall rate for children <1 year of age from our data likely reflects that healthy newborns have an exceedingly low risk for CDI and that although it is unlikely for these children to end up at a children's hospital unless peripartum problems are encountered, neonates account for >80% of all hospitalized children <1 year of age in the HCUP and KID databases.

We could not determine whether the relatively high rate of CDI-related hospitalizations among non-newborn infants represents predominantly true disease or colonization. Although more specific than recovery of a toxin-producing strain from culture, even the detection of free toxins A, B, or both in the stool of a symptomatic infant does not ensure a pathogenic role for *C. difficile*, especially if another cause for diarrhea can be identified. Rates of hospitalizations for rotavirus infections have exhibited a similar increase as those with CDI between 1997 and 2006. Although 2 recent analyses of discharge data for adults suggest that non-CDI causes of diarrhea are not likely leading to a reporting bias as the explanation for the observed increase in CDI rates (*22*,*23*), the situation may be different for children in whom rotavirus is a serious pathogen and related hospitalizations are clearly increasing. Although Kim et al. did not report an increase in the frequency of testing for *C. difficile* in their study, our findings implicate this finding as a distinct possibility that needs to be investigated further (*9*).

Our study has several limitations. First, case identification was based on administrative coding, thus predisposing to misclassification. However, the degree of misclassification may not be substantial because multiple studies have shown the ICD-9-CM code 008.45 to be a relatively accurate way to identify CDI (*24–26*). Second, because we had no clinical data available, we could not distinguish stool colonization from CDI infection. Third, we were unable to distinguish community-acquired from healthcare-associated disease.

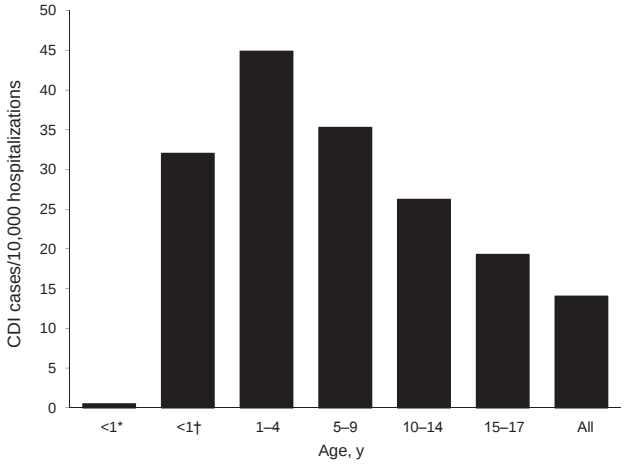


Figure 3. Age-specific incidence of *Clostridium difficile* infection (CDI) hospitalizations, National Hospital Discharge Survey, United States, 2006. *Newborn (i.e., during hospitalization for birth); †not newborn (i.e., during subsequent hospitalization).

However, our study has several strengths. Because we explored 2 databases and discovered results that are highly consistent not only with each other but with those of previous recent investigations, we have augmented the accuracy of estimates of pediatric CDI incidence (*9*). In addition, our data are generalizable to most US-based institutions that care for the pediatric populations. This generalizability sets our results apart from those reported previously because they were limited to the highly specialized setting of children's hospitals (*8*,*9*,*11*).

In summary, the incidence of CDI in the pediatric population appears to be increasing in US hospitals. A reporting bias for diarrheal diseases may play a role in this trend given the concomitant increase in rotavirus-related hospitalizations we identified. Future data may clarify this finding because widespread immunization with available rotavirus vaccines may soon lead to reduced incidence of related hospitalizations. The low incidence of CDI-related hospitalizations among newborns reflects current recommendations against routine testing and may support the concept that *C. difficile* does not cause disease among neonates. In contrast, the relatively high rate of CDI-related hospitalizations among non-newborn infants indicates an urgent need for studies to determine how often *C. difficile* causes true disease in this population.

M.D.Z. was supported by a grant from ViroPharma Inc.

Dr Zilberberg is a health services researcher at the Evi*Med* Research Group, LLC, Goshen, Massachusetts, and the University of Massachusetts, Amherst. Her research interests include reducing complications and optimizing the efficiency of healthcare delivery in the hospital setting.

References

1. McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, SambolSP, et al. An epidemic, toxin gene-variant strain of *Clostridium difficile.* N Engl J Med. 2005;353:2433–41. DOI: 10.1056/NEJMoa051590
2. Muto CA, Pokrywka M, Shutt K, Mendelsohn MB, Nouri K, Posey K, et al. A large outbreak of *Clostridium difficile*–associated disease with an unexpected proportion of deaths and colectomies at a teaching hospital following increased fluoroquinolone use. Infect Control Hosp Epidemiol. 2005;26:273–80. DOI: 10.1086/502539
3. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, et al. A predominantly clonal multi-institutional outbreak of *Clostridium difficile*–associated diarrhea with high morbidity and mortality. N Engl J Med. 2005;353:2442–9. DOI: 10.1056/NEJMoa051639
4. Pépin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, et al. *Clostridium difficile*–associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ. 2004;171:466–72. DOI: 10.1503/cmaj.1041104
5. Centers for Disease Control and Prevention. Data and statistics about *Clostridium difficile* infections [cited 2009 Jan 15]. http://www.cdc.gov/ncidod/dhqp/id_Cdiff_data.html
6. Redelings MD, Sorvillo F, Mascola L. Increase in *Clostridium difficile*–related mortality rates, United States, 1999–2004. Emerg Infect Dis. 2007;13:1417–9.
7. Zilberberg MD, Shorr AF, Kollef MH. Increase in adult *Clostridium difficile*–related hospitalizations and case fatality in the US, 2000–2005. Emerg Infect Dis. 2008;14:929–31. DOI: 10.3201/eid1406.071447
8. Klein EJ, Boster DR, Stapp JR, Wells JG, Qin X, Clausen CR, et al. Diarrhea etiology in a children's hospital emergency department: a prospective cohort study. Clin Infect Dis. 2006;43:807–13. DOI: 10.1086/507335
9. Kim J, Smathers SA, Prasad P, Leckerman KH, Coffin S, Zaoutis T. Epidemiological features of *Clostridium difficile*–associated disease among inpatients in the United States, 2001–2006. Pediatrics. 2008;122:1266–70. DOI: 10.1542/peds.2008-0469
10. Zilberberg MD, Shorr AF, Kollef MH. Increase in *Clostridium difficile*–related hospitalizations among infants in the United States, 2000–2005. Pediatr Infect Dis J. 2008;27:1111–3. DOI: 10.1097/INF.0b013e31817eef13
11. Benson L, Song X, Campos J, Singh N. Changing epidemiology of *Clostridium difficile*–associated disease in children. Infect Control Hosp Epidemiol. 2007;28:1233–5. DOI: 10.1086/520732
12. Centers for Disease Control and Prevention. Severe *Clostridium difficile*–associated disease in populations previously at low risk—four states, 2005. MMWR Morb Mortal Wkly Rep. 2005;54:1201–5.
13. Centers for Disease Control and Prevention. Surveillance for community-associated *Clostridium difficile*—Connecticut, 2006. MMWR Morb Mortal Wkly Rep. 2008;57:340–3.
14. Toltzis P, Kim J, Dul M, Zoltanski J, Smathers S, Zaoutis T. Presence of the epidemic North American pulsed field type 1 *Clostridium difficile* strain in hospitalized children. J Pediatr. 2009;154:607–8. DOI: 10.1016/j.jpeds.2008.10.016
15. Suh KN, Gravel D, Mulvey MR, Moore DL, Miller M, Simor AE, et al. *Clostridium difficile*–associated infections in children admitted to acute care hospitals participating in the Canadian Nosocomial Infections Surveillance Program (CNISP), 2004–2005 [abstract 306]. In: Program of the 18th Annual Scientific Meeting of the Society of Healthcare Epidemiology of America; 2008 Apr 5–8; Orlando, FL. Arlington (VA): The Society.
16. McFarland LV, Brandmarker SA, Guandaline S. Pediatric *Clostridium difficile*: a phantom menace or clinical reality? J Pediatr Gastroenterol Nutr. 2000;31:220–31. DOI: 10.1097/00005176-200009000-00004
17. Kids' Inpatient Database (KID) in the Healthcare Cost and Utilization Project (HCUP) [cited 2009 Jan 15]. http://www.hcup-us.ahrq.gov/kidoverview.jsp
18. HCUPnet. Healthcare Cost and Utilization Project (HCUP). Rockville (MD): Agency for Healthcare Research and Quality [cited 2009 Jan 15]. http://hcupnet.ahrq.gov
19. Centers for Disease Control and Prevention. National hospital discharge survey public utility files. [cited 2008 Oct 13]. http://www.cdc.gov/nchs
20. Vital Health Statistics. Design and operation of the National Hospital Discharge Survey: 1988 redesign. Series 1: programs and collection procedures no. 39. Hyattesville (MD): National Center for Health Statistics. December 2000. DHHS publication no. (PHS) 2001–1315 [cited 2009 Oct 8]. http://www.cdc.gov/nchs/data/series/sr_01/sr01_039.pdf
21. Gerding DN, Johnson S, Peterson LR, Mulligan ME, Silva J Jr. *Clostridium difficile*–associated diarrhea and colitis. Infect Control Hosp Epidemiol. 1995;16:459–77. DOI: 10.1086/648363
22. Jagai J, Naumova E. *Clostridium difficile*–associated disease in the elderly, United States. Emerg Infect Dis. 2009;15:343–4. DOI: 10.3201/eid1502.080785

23. Zilberberg MD. Assessment of reporting bias for *Clostridium difficile* hospitalizations, United States. Emerg Infect Dis. 2008;14:1334. DOI: 10.3201/eid1408.080446
24. Dubberke ER, Reske KA, McDonald LC, Fraser VJ. ICD-9 codes and surveillance for *Clostridium difficile*–associated disease. Emerg Infect Dis. 2006;12:1576–9.
25. Scheurer DB, Hicks LS, Cook EF, Schnipper JL. Accuracy of ICD-9 coding for *Clostridium difficile* infections: a retrospective cohort. Epidemiol Infect. 2007;135:1010–3. DOI: 10.1017/S0950268806007655
26. Schmiedeskamp M, Harpe S, Polk R, Oinonen M, Pakyz A. Use of International Classification of Disease, ninth revision, clinical modification codes and medication use data to identify nosocomial *Clostridium difficile* infection. Infect Control Hosp Epidemiol. 2009;30:1070–6. DOI: 10.1086/606164

Address for correspondence: Marya D. Zilberberg, Evi*Med* Research Group LLC, PO Box 303, Goshen, MA 01032, USA; email: marya@evimedgroup.org

Phylogenetic Analysis of Enterohemorrhagic *Escherichia coli* O157, Germany, 1987–2008

Christian Jenke, Dag Harmsen, Thomas Weniger, Jörg Rothgänger, Eija Hyytiä-Trees, Martina Bielaszewska, Helge Karch, and Alexander Mellmann

Multilocus variable number tandem repeat analysis (MLVA) is a subtyping technique for characterizing human pathogenic bacteria such as enterohemorrhagic *Escherichia coli* (EHEC) O157. We determined the phylogeny of 202 epidemiologically unrelated EHEC O157:H7/H$^-$ clinical isolates through 8 MLVA loci obtained in Germany during 1987–2008. Biodiversity in the loci ranged from 0.66 to 0.90. Four of 8 loci showed null alleles and a frequency ≤44.1%. These loci were distributed among 48.5% of all strains. Overall, 141 MLVA profiles were identified. Phylogenetic analysis assigned 67.3% of the strains to 19 MLVA clusters. Specific MLVA profiles with an evolutionary persistence were identified, particularly within sorbitol-fermenting EHEC O157:H$^-$.These pathogens belonged to the same MLVA cluster. Our findings indicate successful persistence of this clone.

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infections have substantial medical, public health, and economic effects (*1*,*2*). Most symptomatically infected patients have painful bloody diarrhea (*2*,*3*). Hemolytic uremic syndrome (HUS) develops in ≈15% of infected children ≈1 week after the first loose stool. HUS is a thrombotic microangiopathy and consists of nonimmune hemolytic anemia, thrombocytopenia, and renal failure (*1*). Currently, HUS is the main cause of acute renal failure in children (*4*). In Germany, *E. coli* O157:H7, which is the most frequent EHEC serotype implicated in HUS, is not the only relevant EHEC O157 involved. Sorbitol-fermenting (SF) *E. coli* O157:H$^-$ (nonmotile) strains cause ≈20% of all cases of HUS (*5*). Unlike *E. coli* O157:H7, organisms within this clone can ferment sorbitol after overnight incubation on sorbitol MacConkey agar. Although EHEC O157:H7 causes a zoonotic disease mainly associated with cattle, efforts to determine the animal reservoir of SF EHEC O157:H$^-$ have been unsuccessful (*5*).

To identify reservoirs of EHEC O157:H7 infections and of other foodborne pathogens and to elucidate the molecular epidemiology of these pathogens in the United States, PulseNet was established in 1996 (*6*). This US national molecular subtyping network for foodborne disease surveillance facilitates subtyping of bacterial foodborne pathogens for epidemiologic purposes. This network is based on characterization of whole bacterial genomes by using macrorestriction digestion patterns that are separated by pulsed-field gel electrophoresis (PFGE), a technique that has emerged as a common standard for subtyping EHEC O157 isolates (*6*). Despite its high discriminatory power, PFGE can be problematic because it requires great efforts to ensure intralaboratory and interlaboratory reproducibility (*7*–*10*). Furthermore, its application is labor-intensive and difficult to automate. Thus, this technique can be biased by subjective interpretation of band patterns (*7*,*8*). In addition, band patterns can be altered by the presence of mobile genetic elements.

To overcome these drawbacks, other molecular methods were developed, among them multilocus variable number tandem repeat (VNTR) analysis (MLVA). MLVA is based on the characterization of different VNTR regions throughout the bacterial genome. Repeat regions are am-

Author affiliations: Institute for Hygiene, Münster, Germany (C. Jenke, M. Bielaszewska, H. Karch, A. Mellmann); Department of Periodontology, Münster (D. Harmsen, T. Weniger); Ridom GmbH, Würzburg, Germany (J. Rothgänger); and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (E. Hyytiä-Trees)

DOI: 10.3201/eid1604.091361

plified by using PCRs, and resulting fragments are sized to determine the number of repeats. The combination of numbers of repeats of different VNTR loci results in an allelic profile known as the typing result. First developed in 1995 for *Mycobacterium tuberculosis* (*11*), MLVA is now a common typing method for an increasing number of pathogens (*12*,*13*). For EHEC O157, different MLVA schemes with some overlaps of VNTR regions have been published and have demonstrated a capability to detect outbreaks and differentiate closely related EHEC O157 isolates not discriminated by PFGE (*8*,*14*,*15*). These findings qualify MLVA as the second-generation subtyping method for PulseNet (*8*).

In addition to its use in infectious disease surveillance, MLVA also can be used to study phylogeny of pathogens, especially recently evolved clonal pathogens such as *M. tuberculosis* (*16*,*17*) or *Bacillus anthracis* (*18*). However, because of limited diversity in their housekeeping genes, which are the genomic targets for phylogenetic investigations based on multilocus sequence typing (MLST), the common technique for phylogenetic studies (*19*,*20*), certain monomorphic organisms could not be sufficiently differentiated by MLST (*16*,*18*). Similarly, EHEC O157 lacks diversity in its housekeeping genes (*21*,*22*), which hampers phylogenetic analysis of EHEC O157 by MLST.

We investigated the phylogeny of EHEC O157:H7 and SF EHEC O157:H$^-$ strains isolated during 1987–2008 in Germany by applying the current PulseNet MLVA protocol for *E. coli* O157 (*23*). The purpose of our study was to gain a deeper insight into the evolution and spread of this pathogen since 1987, when the first cases of EHEC O157 infections were detected (*24*,*25*).

Materials and Methods

Clinical Isolates

Up to 17 epidemiologically unrelated EHEC O157:H7/H$^-$ isolates per year obtained during 1987–2008 were randomly selected from the strain collection of the Institute of Hygiene and the National Consulting Laboratory on HUS, University Hospital Münster, Germany. All 202 O157 strains (61 of which were SF EHEC O157:H$^-$) were isolated from humans, including patients with HUS (145), bloody diarrhea (12), or diarrhea without visible blood (40), and asymptomatic carriers (5) during epidemiologic investigations. Isolates were obtained from areas throughout Germany. Procedures used for detecting and isolating EHEC O157 from stool samples were described (*26*,*27*). Isolates were confirmed as *E. coli* by the API 20 E test (bioMérieux, Marcy l'Etoile, France) and serotyped by using antisera against *E. coli* O antigens 1–181 and H antigens 1–56 (*28*). Subtyping of *fliC* genes in nonmotile isolates by using *Hha*I restriction fragment length polymorphism of amplicons obtained with primers FSa1 and rFSa1 (*29*,*30*) confirmed the presence of *fliC*H7 in all isolates. EHEC O157:H7 strain EDL933 (*31*,*32*) was used as a reference strain in all analyses.

MLVA of EHEC O157

Strains were grown overnight on Columbia blood agar (Heipha; Eppelheim, Germany) at 37°C. A loop of a fresh culture was suspended in 100 μL of Chelex-100 solution (Bio-Rad, Hercules, CA, USA) and vortexed briefly. After boiling and thorough mixing, samples were centrifuged and DNA-containing supernatants were stored at –20°C until use. To calibrate sequencer-specific variation of fragment length, the exact number of repeats of reference strain O157:H7 EDL933 was initially determined in silico on the basis of its genome sequence (reference sequences NC_002655 [chromosome] and NC_007414 [plasmid]; National Center for Biotechnology Information [NCBI], Bethesda, MD, USA) by using Tandem Repeats Finder software (*33*). Subsequently, the length of the in silico–determined repeats was subtracted from the fragment length of each respective VNTR locus generated in 8 independent capillary electrophoresis runs of strain EDL933 to determine the offset (primer plus VNTR-flanking regions). This locus-specific offset was then used to calculate the correct number of repeats of unknown isolates. Fragments for MLVA typing were generated in 2 multiplex PCRs comprising either VNTR loci 3, 9, 25, and 34 (multiplex 1) or VNTR loci 17, 19, 36, and 37 (multiplex 2) (online Appendix Table, www.cdc.gov/EID/content/16/4/610-appT.htm), according to the current PulseNet MLVA protocol for *E. coli* O157 (*23*).

PCR amplification was performed in a reaction mixture of 10 μL containing 5 μL of Type-it Multiplex Master Mix (QIAGEN, Hilden, Germany), ≈30 ng of DNA template, and VNTR-specific primers for each of the 4 VNTR loci. Concentration, primer sequences, and respective dyes used are shown in the online Appendix Table. PCRs were performed and prepared for subsequent analysis on sequencers in accordance with the manufacturer's instructions (online Appendix Table). PCR products were diluted 1:10 with water purified by high performance liquid chromatography, and 1.0 μL of diluted DNA was mixed with 13.7 μL of HiDi formamide (Applied Biosystems, Foster City, CA, USA) and 0.3 μL of GeneScan-600 LIZ Size Standard (Applied Biosystems) as internal lane size standard. Before fragment sizing in the ABI Prism 3130xl Genetic Analyzer System (Applied Biosystems), samples were incubated for 5 min at 95°C and immediately frozen at –20°C for ≥3 min to denature the DNA.

If a VNTR locus was not detected during fragment analysis, reactions were repeated by using singleplex reactions with minor modifications to amplify the specific

locus. In that particular instance, the primer concentration was increased to 0.2 μmol/L, annealing temperatures were reduced to 55°C, and the extension time was tripled to amplify larger fragments because of possible insertion sequence element transposition or other genetic events. Subsequently, fragments were characterized by using standard agarose gel electrophoresis. If the fragment was larger than the usual range of fragment sizes of the corresponding VNTR, the PCR product was sequenced.

Data Analysis

After fragment analysis, corresponding peak data were examined by using GeneMapper 4.0 software (Applied Biosystems) to calculate the repeat number for each VNTR locus on the basis of fragment length. Partial repeats were rounded to the closest repeat number in accordance with the current Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) MLVA O157 protocol (*23*). If >1 amplicon for a specific VNTR locus was detected and the size difference matched ≥1 repeat lengths (so-called stutter peaks), the one with the highest fluorescence level was used to calculate the repeat number. A null allele was assigned if either no amplicon was detected or agarose gel electrophoresis data showed an amplicon of a size that was beyond the usual range of fragment size of the specific VNTR locus. Corresponding alleles were designated as –2. In the hypothetical situation in which an amplicon without the repeat region was detected, it was designated as –1 (*8*). Null alleles were also included in the overall number of alleles in a specific VNTR locus.

Index of diversity (ID) (*34*) and typeability were calculated by using EpiCompare 1.0 software (Ridom GmbH, Würzburg, Germany). A minimum spanning tree (MST) was generated by using SeqSphere software 0.9 β (Ridom GmbH). All MLVA profiles that differed at ≤2 alleles were grouped as an MLVA cluster. To determine the cluster-defining profile of clusters containing >2 MLVA profiles, the MST priority rule (that the profile with the highest number of single locus variants is chosen) was applied.

Significance of associations of MLVA profiles or clusters comprising ≥4 strains with clinical outcome (HUS vs. non-HUS) were calculated by using a χ^2 test with Yates correction (EpiInfo 6 software; CDC) when appropriate. p values <0.05 were considered significant.

Results

EHEC O157 Strains and VNTR Loci Characterization

The 202 EHEC O157 strains showed 141 MLVA profiles. Of these profiles, MLVA profile 4/8/-2/2/3/9/-2/5 was the most common and was present in 30 isolates. In contrast, 122 profiles were detected only once. Detailed characteristics of the different VNTR loci identified in this study are shown in Table 1. The number of alleles for the VNTRs ranged from 6 (VNTR-25) to 22 (VNTR-3). Calculation of the ID resulted in values from 0.66 (VNTR-34) to 0.9 (VNTR-9). Half of the VNTR loci (VNTR-3, VNTR-9, VNTR-36, and VNTR-37) showed null alleles with different frequencies ranging from 2.0% (VNTR-3 and VNTR-37) to 44.1% (VNTR-36).

To determine whether amplification failure caused by mutations in primer-binding regions or by complete deletions of the VNTR region were the reason for these null alleles or the insertion of fragments such as mobile genetic elements resulted in larger (and therefore by capillary electrophoresis) undetectable fragments, the respective fragments were analyzed by using standard gel electrophoresis. In some cases, large fragments (>1.3 kb) were detected. Sequence analysis and an NCBI nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of randomly selected samples indicated the presence of insertion sequence elements of the IS3 family. The typeability of different VNTR loci ranged from 55.9% to 100% (Table 1). Null alleles were present in 48.5% of the strains (98/202). The overall ID of all MLVA profiles was 0.98.

Table 1. VNTR characteristics of enterohemorrhagic *Escherichia coli*, Germany, 1987–2008*

VNTR locus	Alternative name†	Repeat length, bp	Inside ORF (no.)‡	No. repeats		No. alleles§	Null alleles	Null allele frequency, %	ID	Typeability, %¶
				Minimum	Maximum					
3	Vhec3, TR5	6	+ (Z0268)	3	23	22	+	2.0	0.86	98.0
34	Vhec2, TR6	18	+ (Z5865)	4	12	8	–	–	0.66	100.0
9	Vhec4, TR1	6	+ (Z3935/Z3936)	6	23	15	+	31.7	0.90	68.3
25	TR4	6	–	2	15	6	–	–	0.74	100.0
17	TR3	6	+ (Z5935)	2	19	10	–	–	0.80	100.0
19	TR7	6	+ (Z3274)	3	11	9	–	–	0.76	100.0
36#	Vhec7	7	–	3	15	13	+	44.1	0.87	55.9
37#	–	6	+ (L7083)	3	17	11	+	2.0	0.82	98.0

*VNTR, variable number tandem repeat; ORF, open reading frame; ID, index of diversity without null alleles.
†Vhec loci as explained by Lindstedt et al. (*15*). TR loci are from Noller et al. (*14*).
‡Number is based on current EDL933 genome data. ORF encoding VNTR loci encoded either hypothetical proteins or proteins with unknown function.
§Including null alleles.
¶Typeability determines the proportion of all alleles without null alleles.
#Located on plasmid pO157 of reference strain EDL933.

MLVA Clustering and Phylogeny

Comparison of MLVA profiles assembled 136 of the 202 strains (67.3%) into clusters sharing ≥6 of the 8 VNTR loci. This grouping resulted in 19 MLVA clusters consisting of 2–61 isolates comprising 81 MLVA profiles. The remaining 66 strains (32.7%), which had 60 MLVA profiles, could not be associated with any cluster (Table 2). Strains within clusters consisting of ≥4 MLVA profiles were isolated over a period ≥7 years. The 61 strains of cluster 1 were isolated during 1988–2008. Among these strains, the cluster-defining MLVA profile 4/8/-2/2/3/9/-2/5 was the most common profile. It included 30 (14.9%) of 202 strains isolated during 1988–2008. The profile of the same cluster (cluster 1) with the second highest number of strains differed only in VNTR-37 with an additional repeat and included 5.0% of the strains (10/202). The corresponding strains were isolated during 1995–2008 (Table 2, Figure).

All 61 strains in cluster 1 were SF EHEC O157:H$^-$. The remaining 141 strains did not ferment sorbitol (Table 2, Figure). The phylogenetic relationship of 202 EHEC O157 strains based on the 141 MLVA profiles is shown in an MST in the Figure. The reference strain EDL933, which is also included in the MST, shares its MLVA profile with 4 isolates from Germany obtained during 2007–2008.

Association of MLVA Profiles with HUS

To determine whether there was an association between MLVA profiles or clusters and the ability of these strains to cause HUS, we performed a significance test. The 2 most common MLVA profiles were significantly associated with HUS ($p = 0.023$). Testing for specific clusters resulted in a significant association of HUS with cluster 1 ($p = 0.009$) (Figure).

Discussion

Using 8 VNTR loci of the current PulseNet MLVA O157 protocol (*23*), we analyzed a large collection of 202 EHEC O157:H7/H$^-$ strains isolated over >2 decades in Germany to determine their molecular epidemiology. Of the 141 MLVA profiles detected, 81 were clustered into 19 groups of related profiles that differed at ≥2 loci. The remaining 60 profiles were not clustered. Our data demonstrate a great diversity of EHEC O157:H7 associated with human diseases in Germany over the past 2 decades. The wide distribution of strains within the MST based on MLVA typing reflects frequent occurrence of genetic events outside the EHEC O157 core genome (Table 2, Figure). The 19 MLVA clusters included 67.3% (136/202) of the analyzed strains. Further analysis of the clusters including >4 MLVA profiles did not show any specific clustering of strains in time. Most of the larger clusters (clusters 1, 2, 4, 5, and 6; Table 2) contained strains widespread in the period of 10–20 years. Only cluster 3 is defined by profiles starting from 2001, which indicates a later appearance than clusters 1, 2, 4, 5, and 6 (Table 2).

The 2 most frequently identified MLVA profiles are parts of cluster 1, which indicates a consensus profile among SF EHEC O157:H$^-$ isolates over time within this

Table 2. MLVA cluster profile of enterohemorrhagic *Escherichia coli* O157, Germany, 1987–2008*

MLVA cluster	Cluster-defining MLVA profile	No. strains (% sorbitol-fermenting)	No. MLVA profiles	Years of strain isolation
1	4/8/-2/2/3/9/-2/5	61 (100)	16	1988, 1989, 1993, 1995–2008
2	7/7/13/4/5/7/-2/7	11 (0)	7	1996–1998, 2000, 2002, 2005, 2006
3	11/8/10/3/5/6/8/3	9 (0)	8	2001, 2004, 2005, 2007, 2008
4	18/7/8/3/5/7/-2/7	8 (0)	8	1996–1999, 2001, 2005, 2006
5	5/7/13/5/6/6/6/6	7 (0)	5	1991, 1999, 2007
6	12/7/16/3/5/6/5/7	5 (0)	5	1990, 1992, 1999, 2001, 2004
7	18/7/10/4/4/9/4/9	5 (0)	4	1996, 1998, 2001–2003
8	9/7/9/4/6/7/9/8	3 (0)	3	2007, 2008
9	13/7/11/4/4/8/4/8	3 (0)	3	1992, 1993, 2002
10	6/6/16/3/6/6/6/6	3 (0)	3	2005, 2006
11	11/9/10/5/7/6/11/7	3 (0)	3	1996, 2000, 2007
12	8/10/13/6/7/7/5/6	3 (0)	2	2002
13	9/7/13/3/6/6/10/7	3 (0)	2	1997, 2002
14	NA	2 (0)	2	2000
15	NA	2 (0)	2	1998, 2000
16	NA	2 (0)	2	1991
17	NA	2 (0)	2	1995, 1997
18	NA	2 (0)	2	1998, 2000
19	NA	2 (0)	2	2001, 2003
Singletons	–	66 (0)	60	All years except 1990, 1995, and 2004

*MLVA, multilocus variable number tandem repeat analysis; NA, not applicable because cluster comprises <3 strains. Singletons, MLVA profiles that are not included in a certain cluster. MLVA profiles are defined by repeat numbers after the following variable number tandem repeat (VNTR) locus order: 3/34/9/25/17/19/36/37 with EDL933 as standard with the following MLVA profile: 9/10/11/5/6/6/8/7 (based on genome sequence analysis). Strains belonged to 1 cluster if they differed at ≤2 VNTR loci.

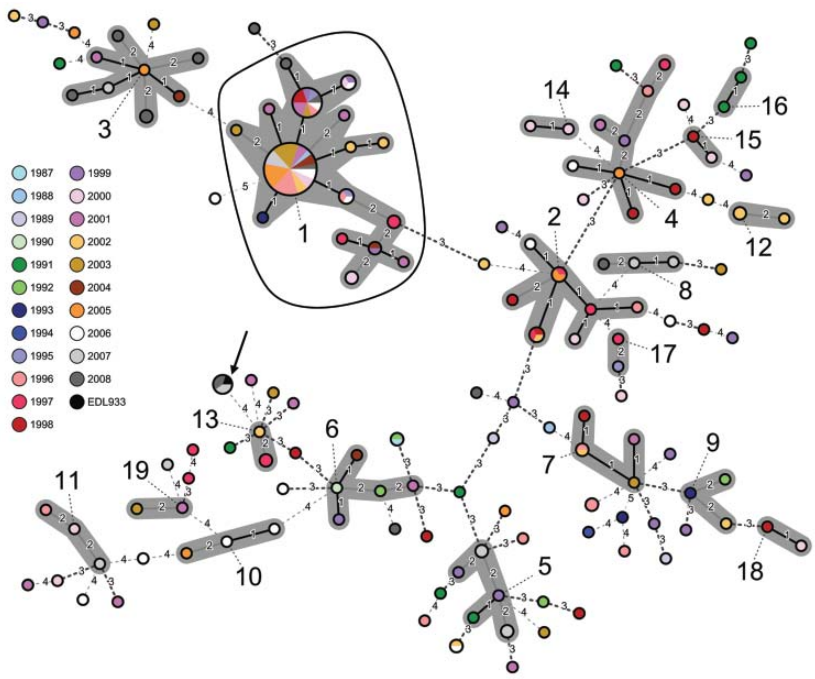


Figure. Minimum spanning tree based on multilocus variable number tandem repeat analysis (MLVA) allelic profiles, showing the phylogenetic relationship between 202 epidemiologically unrelated enterohemorrhagic *Escherichia coli* (EHEC) O157:H7/H$^-$ strains, Germany, 1987–2008. Each node represents a unique MLVA profile. Size of the nodes is proportional to the number of isolates per MLVA profile. Small numbers and specific dashed lines between nodes represent distance between the nodes, i.e., number of different alleles. Clusters are indicated by numbers according to their size (Table 2) and are highlighted in gray. All strains within the enclosed area are sorbitol-fermenting EHEC O157:H$^-$. The arrangement of colors within each node reflects the proportion of strains isolated from different years per node and corresponding MLVA profile. Arrow indicates reference strain *E. coli* O157:H7 EDL933.

cluster. The corresponding strains include strain 493/89, which was isolated during the first documented outbreak caused by SF EHEC O157:H$^-$ (*25*). All other isolates that exhibited the 2 most common MLVA profiles also fermented sorbitol, which identified strain 493/89 as a prototype of these strains. This finding corroborates the assumption of an epidemic bacterial population structure within a background population comprising a network between different genotypes, and that superimposed strains emerge from highly adaptive, ancestral genotypes and may be persistent for decades (*35*). Nodes from cluster 1, which represent the 2 most prevalent MLVA profiles, include strains from 1988–2008 and 1995–2008. This finding indicates a persistence of these successful clones, which supports this hypothesis. Moreover, evolutionary success and uniqueness of this SF clone was recently supported by whole genome single nucleotide polymorphism analysis, in which distinct branching of these clones was determined during evolution of the O157 serotype (*22*).

Statistical analysis demonstrated that the 2 most common profiles and the entire cluster 1 are associated with HUS, which indicates that specific MLVA profiles are associated with severe disease. Cluster 1 comprised 61 of the strains and was distributed over more than a decade. Although not statistically significant, 10 of 11 isolates in cluster 2 were also associated with HUS. Despite these similarities, they exhibited different MLVA profiles (Table 2; Figure). Extensive heterogeneity of EHEC O157:H7, in contrast to conservation of SF EHEC O157:H$^-$, could be related to observed differences in the nature of the reservoirs and vehicles for transmission. In addition, the epidemiology of SF EHEC O157:H$^-$ infections differs markedly because these infections occur predominantly during cold (winter) months and in children <3 years of age (*5*). Moreover, although EHEC O157:H7 infections have zoonotic origins, SF EHEC O157:H$^-$ are rarely found in animals (*36*). Humans are plausibly the main reservoirs, as is the case with classical enteropathogenic *E. coli* and enteroinvasive *E. coli*. This relatively stable niche may lead to the conserved genome structure and high pathogenicity for the host (*37*).

Four strains isolated in 2007 and 2008 exhibited the same MLVA profile as the reference strain EDL933 isolated in 1982 in the United States (*38*) (Table 2, Figure). Among the 3,200 entries in the CDC MLVA database, the EDL933 MLVA profile was detected only during an outbreak in 1982 (E. Hyytiä-Trees, pers. comm.). There are 2 possible explanations for this phenomenon. This finding is coincidental because of genetic changes in the O157 genome or EDL933 shares a common MLVA profile with other strains. The presence of such common profiles is known, especially in foodborne pathogens and other monomorphic species (*39*) and frequently seen by using other typing techniques, such as PFGE.

Analysis of the number of alleles of different VNTRs produced results similar to those of a previous study (*8*). Whereas the ID was high (0.74–0.90; Table 1) in VNTR loci consisting of 6–7-bp repeats (all VNTR loci except VNTR-34), the ID was low (0.66) for the 18-bp repeat (VNTR-34). Whether a VNTR locus is located within

an open reading frame did not influence the ID (Table 1). However, the frequency of null alleles differed markedly. A total of 98 (48.5%) of 202 strains exhibited null alleles in 4 of the 8 VNTR loci. Especially in VNTR-9 and VNTR-36, the frequency of null alleles was high (31.7% and 44.1%). Although null alleles were reported in other MLVA O157 studies (*8*,*40*), this high frequency of null alleles determined in our study might indicate a specific feature of EHEC O157 strains from central Europe or Germany. An explanation for the frequent occurrence might be that VNTR-36 and VNTR-9 are located in noncoding or hypothetical protein encoding regions of the EHEC genome (Table 1). Nevertheless, all strains had a high ID regarding the complete MLVA profile.

Our study had some limitations. Because of the limited number of isolates obtained during 1987–1995, clustering might be biased and a more year-specific clustering might be observable. However, cluster 1 represents 30.2% (61/202) of strains widespread during 1988–2008, which contradicts this thesis, and infers a certain genetic stability of such clusters over time. In contrast to phylogenetic studies based on whole genome sequencing data (*22*), we report a phylogeny based on 8 genetic loci that might be biased by larger recombinational events. However, all VNTR loci are ≥50 kb from the *rfb-gnd* segment, which was determined to be the only genomic region in EHEC O157 with a higher mutation rate (*22*).

Strains (66/202, 32.7%) that were not classifiable into any MLVA cluster complement the assumption of the highly dynamic EHEC O157 genome. This finding likely indicates that genetic changes in *E. coli* lead to adaptation to a host-specific environment (in this case human), especially during pathogenesis and host-specific immune responses.

Applying MLVA to this highly diverse strain collection resulted in new insights into the phylogeny of EHEC O157 in Germany since their first description in 1987. In addition to its already demonstrated ability to differentiate outbreak and sporadic case strains, MLVA of O157 emerged as a major typing tool that can further characterize EHEC O157 subpopulations and associated strains. This tool can be used for studying phylogeny coherences and identifying successful clones.

Acknowledgment

We thank Phillip I. Tarr for extensive discussions regarding the manuscript.

This study was supported by a grant from the German Federal Ministry of Education and Research (0315219A).

Mr Jenke is a PhD candidate at the Institute of Hygiene at the University Hospital Münster. His research interests include the molecular epidemiology and phylogeny of Shiga toxin–producing *E. coli*.

References

1. Tarr PI, Gordon CA, Chandler WL. Shiga toxin–producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet. 2005;365:1073–86.
2. Holtz LR, Neill MA, Tarr PI. Acute bloody diarrhea: a medical emergency for patients of all ages. Gastroenterology. 2009;136:1887–98. DOI: 10.1053/j.gastro.2009.02.059
3. Levine MM. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J Infect Dis. 1987;155:377–89.
4. Williams DM, Sreedhar SS, Mickell JJ, Chan JCM. Acute kidney failure: a pediatric experience over 20 years. Arch Pediatr Adolesc Med. 2002;156:893–900.
5. Karch H, Tarr PI, Bielaszewska M. Enterohaemorrhagic *Escherichia coli* in human medicine. Int J Med Microbiol. 2005;295:405–18. DOI: 10.1016/j.ijmm.2005.06.009
6. Swaminathan B, Barrett TJ, Hunter SB, Tauxe RV. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg Infect Dis. 2001;7:382–9.
7. Lindstedt BA. Multiple-locus variable number tandem repeats analysis for genetic fingerprinting of pathogenic bacteria. Electrophoresis. 2005;26:2567–82. DOI: 10.1002/elps.200500096
8. Hyytiä-Trees E, Smole SC, Fields PA, Swaminathan B, Ribot EM. Second generation subtyping: a proposed PulseNet protocol for multiple-locus variable-number tandem repeat analysis of Shiga toxin–producing *Escherichia coli* O157 (STEC O157). Foodborne Pathog Dis. 2006;3:118–31. DOI: 10.1089/fpd.2006.3.118
9. Murchan S, Kaufmann ME, Deplano A, de Ryck R, Struelens M, Zinn CE, et al. Harmonization of pulsed-field gel electrophoresis protocols for epidemiological typing of strains of methicillin-resistant *Staphylococcus aureus:* a single approach developed by consensus in 10 European laboratories and its application for tracing the spread of related strains. J Clin Microbiol. 2003;41:1574–85. DOI: 10.1128/JCM.41.4.1574-1585.2003
10. Tenover FC, Arbeit R, Archer G, Biddle J, Byrne S, Goering R, et al. Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus.* J Clin Microbiol. 1994;32:407–15.
11. Frothingham R. Differentiation of strains in *Mycobacterium tuberculosis* complex by DNA sequence polymorphisms, including rapid identification of *M. bovis* BCG. J Clin Microbiol. 1995;33:840–4.
12. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis.* J Clin Microbiol. 2006;44:4498–510. DOI: 10.1128/JCM.01392-06
13. van Belkum A. Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA). FEMS Immunol Med Microbiol. 2007;49:22–7. DOI: 10.1111/j.1574-695-X.2006.00173.x
14. Noller AC, McEllistrem MC, Pacheco AGF, Boxrud DJ, Harrison LH. Multilocus variable-number tandem repeat analysis distinguishes outbreak and sporadic *Escherichia coli* O157:H7 isolates. J Clin Microbiol. 2003;41:5389–97. DOI: 10.1128/JCM.41.12.5389-5397.2003
15. Lindstedt BA, Heir E, Gjernes E, Vardund T, Kapperud G. DNA fingerprinting of Shiga-toxin producing *Escherichia coli* O157 based on multiple-locus variable-number tandem-repeats analysis (MLVA). Ann Clin Microbiol Antimicrob. 2003;2:12. DOI: 10.1186/1476-0711-2-12
16. Supply P, Warren RM, Banuls A, Lesjean S, Van Der Spuy GD, Lewis L, et al. Linkage disequilibrium between minisatellite loci supports clonal evolution of *Mycobacterium tuberculosis* in a high tuberculosis incidence area. Mol Microbiol. 2003;47:529–38. DOI: 10.1046/j.1365-2958.2003.03315.x

17. Wirth T, Hildebrand F, Allix-Beguec C, Wolbeling F, Kubica T, Kremer K, et al. Origin, spread and demography of the *Mycobacterium tuberculosis* complex. PLoS Pathog. 2008;4:e1000160. DOI: 10.1371/journal.ppat.1000160
18. Helgason E, Tourasse NJ, Meisal R, Caugant DA, Kolsto A. Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. Appl Environ Microbiol. 2004;70:191–201. DOI: 10.1128/AEM.70.1.191-201.2004
19. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc Natl Acad Sci U S A. 1998;95:3140–5. DOI: 10.1073/pnas.95.6.3140
20. Feil EJ. Small change: keeping pace with microevolution. Nat Rev Microbiol. 2004;2:483–95. DOI: 10.1038/nrmicro904
21. Noller AC, McEllistrem MC, Stine OC, Morris JGJ, Boxrud DJ, Dixon B, et al. Multilocus sequence typing reveals a lack of diversity among *Escherichia coli* O157:H7 isolates that are distinct by pulsed-field gel electrophoresis. J Clin Microbiol. 2003;41:675–9. DOI: 10.1128/JCM.41.2.675-679.2003
22. Leopold SR, Magrini V, Holt NJ, Shaikh N, Mardis ER, Cagno J, et al. A precise reconstruction of the emergence and constrained radiations of *Escherichia coli* O157 portrayed by backbone concatenomic analysis. Proc Natl Acad Sci U S A. 2009;106:8713–8.
23. Hyytiä-Trees E, Lafon P, Vauterin P, Ribot E. Multilaboratory validation study of standardized multiple-locus variable-number tandem repeat analysis protocol for Shiga toxin–producing *Escherichia coli* O157: a novel approach to normalize fragment size data between capillary electrophoresis platform. Foodborne Pathog Dis. 2009 Sep; [Epub ahead of print].
24. Karch H, Heesemann J, Laufs R, O'Brien AD, Tacket CO, Levine MM. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. Infect Immun. 1987;55:455–61.
25. Karch H, Wiss R, Gloning H, Emmrich P, Aleksic S, Bockemuhl J. Hemolytic-uremic syndrome in infants due to verotoxin-producing *Escherichia coli* [in German]. Dtsch Med Wochenschr. 1990;115:489–95. DOI: 10.1055/s-2008-1065036
26. Friedrich AW, Bielaszewska M, Zhang W, Pulz M, Kuczius T, Ammon A, et al. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J Infect Dis. 2002;185:74–84. DOI: 10.1086/338115
27. Mellmann A, Bielaszewska M, Zimmerhackl LB, Prager R, Harmsen D, Tschäpe H, et al. Enterohemorrhagic *Escherichia coli* in human infection: in vivo evolution of a bacterial pathogen. Clin Infect Dis. 2005;41:785–92. DOI: 10.1086/432722
28. Prager R, Strutz U, Fruth A, Tschäpe H. Subtyping of pathogenic *Escherichia coli* strains using flagellar (H)-antigens: serotyping versus *fliC* polymorphisms. Int J Med Microbiol. 2003;292:477–86. DOI: 10.1078/1438-4221-00226
29. Sonntag AK, Prager R, Bielaszewska M, Zhang W, Fruth A, Tschäpe H, et al. Phenotypic and genotypic analyses of enterohemorrhagic *Escherichia coli* O145 strains from patients in Germany. J Clin Microbiol. 2004;42:954–62. DOI: 10.1128/JCM.42.3.954-962.2004
30. Zhang Y, Laing C, Steele M, Ziebell K, Johnson R, Benson AK, et al. Genome evolution in major *Escherichia coli* O157:H7 lineages. BMC Genomics. 2007;8:121. DOI: 10.1186/1471-2164-8-121
31. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med. 1983;308:681–5.
32. Perna NT, Plunkett G III, Burland V, Mau B, Glasner JD, Rose DJ, et al. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. Nature. 2001;409:529–33. DOI: 10.1038/35054089
33. Benson G. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res. 1999;27:573–80. DOI: 10.1093/nar/27.2.573
34. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol. 1988;26:2465–6.
35. Smith JM, Feil EJ, Smith NH. Population structure and evolutionary dynamics of pathogenic bacteria. Bioessays. 2000;22:1115–22. DOI: 10.1002/1521-1878(200012)22:12<1115::AID-BIES9>3.0.CO;2-R
36. Karch H, Bielaszewska M. Sorbitol-fermenting Shiga toxin–producing *Escherichia coli* O157:H$^-$ strains: epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. J Clin Microbiol. 2001;39:2043–9. DOI: 10.1128/JCM.39.6.2043-2049.2001
37. Holt KE, Parkhill J, Mazzoni CJ, Roumagnac P, Weill F, Goodhead I, et al. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. Nat Genet. 2008;40:987–93. DOI: 10.1038/ng.195
38. Wells JG, Davis BR, Wachsmuth IK, Riley LW, Remis RS, Sokolow R, et al. Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare *Escherichia coli* serotype. J Clin Microbiol. 1983;18:512–20.
39. Achtman M. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu Rev Microbiol. 2008;62:53–70. DOI: 10.1146/annurev.micro.62.081307.162832
40. Kawamori F, Hiroi M, Harada T, Ohata K, Sugiyama K, Masuda T, et al. Molecular typing of Japanese *Escherichia coli* O157:H7 isolates from clinical specimens by multilocus variable-number tandem repeat analysis and PFGE. J Med Microbiol. 2008;57:58–63. DOI: 10.1099/jmm.0.47213-0

Address for correspondence: Alexander Mellmann, Institut für Hygiene, Universitätsklinikum Münster, Robert-Koch-Strasse 41, 48149 Münster, Germany; email: mellmann@uni-muenster.de

Use of Norovirus Genotype Profiles to Differentiate Origins of Foodborne Outbreaks

Linda Verhoef, Harry Vennema, Wilfrid van Pelt, David Lees, Hendriek Boshuizen, Kathleen Henshilwood, and Marion Koopmans, on behalf of the Food-Borne Viruses in Europe Network[1]

Because secondary transmission masks the connection between sources and outbreaks, estimating the proportion of foodborne norovirus infections is difficult. We studied whether norovirus genotype frequency distributions (genotype profiles) can enhance detection of the sources of foodborne outbreaks. Control measures differ substantially; therefore, differentiating this transmission mode from person-borne or food handler–borne outbreaks is of public health interest. Comparison of bivalve mollusks collected during monitoring (n = 295) and outbreak surveillance strains (n = 2,858) showed 2 distinguishable genotype profiles in 1) human feces and 2) source-contaminated food and bivalve mollusks; genotypes I.2 and I.4 were more frequently detected in foodborne outbreaks. Overall, ≈21% of all outbreaks were foodborne; further analysis showed that 25% of the outbreaks reported as food handler–associated were probably caused by source contamination of the food.

Noroviruses are members of the family *Caliciviridae* and recognized as major pathogens in outbreaks of gastroenteritis worldwide. Because these viruses have environmental stability (*1*), ability to use different transmission routes, and low infective doses (*2*), their source may be difficult to determine during an outbreak. Transmission can occur through contact with shedding persons; food contaminated during processing, preparation or serving; sewage-contaminated water used for consumption, cultivation or irrigation of food; contaminated aerosols resulting from vomiting; and environmental contamination (*3*,*4*). Five genogroups have been described (GI–V), subdivided into at least 40 genetic clusters (*5*,*6*).

To implement effective measures for prevention, recognition of the transmission routes is necessary. Consequently, the relative importance of different transmission routes in the total number of outbreaks is of interest for estimation of cost-effectiveness of reducing the number and size of norovirus outbreaks, particularly for geographically disseminated foodborne outbreaks. Such outbreaks are difficult to detect when the primary introduction of viruses through food occurs simultaneously in several countries or continents (*7*–*9*). Globalization of the food industry with consequential international distribution of products increases the risk for such outbreaks. For example, the first reported GII.b outbreak occurred in August 2000 during a large waterborne outbreak in southern France (*10*). After this outbreak, in December and January, 4 multipathogen and oyster-related outbreaks with this newly emerging genotype were reported from France. In the same period, Denmark, Finland, and the Netherlands reported norovirus cases resulting from oysters originating from a French batch that probably was sold in these countries, as well as in Sweden, Italy, and Belgium (*6*). All these outbreaks seemed to involve closely related and newly detected GII.b strains. After active case identification, further linked cases

Author affiliations: National Institute for Public Health and the Environment, Bilthoven, the Netherlands (L. Verhoef, H. Vennema, W. van Pelt, H. Boshuizen, M. Koopmans); and Centre for Environment, Fisheries and Aquaculture Science, Weymouth, UK (D. Lees, K. Henshilwood)

DOI: 10.3201/eid1604.090723

[1]Additional members of the Food-Borne Viruses in Europe Network are listed at the end of this article.

were detected in Germany, the United Kingdom, Spain, Slovenia, and Sweden (*11*,*12*). Another example of a geographically disseminated outbreak was several seemingly independent norovirus outbreaks in Denmark that were traced back to consumption of raspberries from Poland. Although raspberries from this contaminated batch were exported to other European countries, an alert in the Rapid Alert System for Food and Feed did not result in further linked outbreak reports (*7*). Thus, geographically disseminated outbreaks are sometimes identified but only after the joint and exhaustive efforts of different organizations, such as laboratory networks, food safety authorities, and public health institutions. Knowledge of the proportion of geographically disseminated foodborne outbreaks to all norovirus outbreaks will therefore provide insight into the cost-effectiveness of such efforts.

We studied whether the genotype frequency distributions (genotype profiles) of strains can be used to differentiate foodborne outbreaks related to contamination early in the food chain (i.e., during primary production) from those related to contamination later in the food chain (i.e., during preparation or serving). If so, detection of food origins likely to cause geographically disseminated outbreaks will be enhanced. We considered methods for attribution to multiple sources commonly applied to *Salmonella* infections (*13*) because different transmission routes involved in norovirus infections can disguise the foodborne origin. However, such methods require strain collections representative of noroviruses in the potential sources that are as yet unavailable because of difficulties in the direct detection of viruses in food (*14*–*16*). Therefore, we compared 2 strain collections: noroviruses identified through filter-feeding bivalve mollusk monitoring representing source contamination of food and noroviruses collected through systematic surveillance of illness in the population. The first was collected by the European Community Reference Laboratory for Monitoring Bacteriological and Viral Contamination of Bivalve Mollusks during 1995–2004 (*17*) and the second by the Food-Borne Viruses in Europe (FBVE) network, which has conducted surveillance for norovirus outbreaks in Europe since 1999. Prior investigation of the FBVE database of systematically collected epidemiologic and microbiological norovirus surveillance data (*6*) showed that the epidemiology of norovirus outbreaks in Europe varies between genogroups. An analysis of the properties of reported outbreaks indicated a clear difference between GII.4 strains and other noroviruses; non-GII.4 strains were found more frequently in outbreaks with a foodborne mode of transmission, and GII.4 strains were found more frequently in healthcare settings with person-to-person transmission (*18*,*19*). Here we demonstrate that further specification into genotypes shows additional differences in the epidemiology of norovirus outbreaks.

Methods

Data Sources

We used 2 broad databases reflecting norovirus prevalence within the European countries under surveillance. These databases provided us the opportunity to compare genotype proportions as detected in outbreaks, i.e., human surveillance data, with those detected in source-contaminated food products, i.e., bivalve mollusks monitoring data.

Human Surveillance Data

From January 1999 through December 2004, FBVE collected molecular information on 2,727 norovirus outbreaks and sporadic cases in Denmark, Finland, France, Germany, England and Wales, Hungary, Ireland, Italy, the Netherlands, Norway, Sweden, Slovenia, and Spain (*20*,*21*). Although the name FBVE suggested a foodborne focus, the network actually investigated outbreaks from all modes of transmission to obtain a comprehensive overview of viral activity in the community (strengths and limitations of the FBVE data collection were described by Kroneman et al. [*20*]; to compare newly detected strains with the FBVE database and find potential linked outbreaks, we used a comparison tool [www.rivm.nl/bnwww]). Data were reported to FBVE at outbreak level; therefore, no informed consent was needed. Outbreaks were categorized as follows on the basis of the cause of infection as reported in the surveillance system:

- Foodborne-food (FB-food) when an outbreak was reported to be caused by food and the outbreak strain was detected in food;
- Foodborne-feces (FB-feces) when an outbreak was reported to be caused by food and the outbreak strain was detected in human feces only;
- Foodborne (FB) when an outbreak was classified as FB-food or FB-feces;
- Food handler–borne (FHB) when an outbreak was reported to be caused by an infected food handler contaminating the food and the outbreak strain was detected in human feces;
- Person-borne (PB) when an outbreak was reported to be caused by person-to-person transmission and the outbreak strain was detected in human feces;
- Unknown (UN) when the mode of transmission was not reported or was reported to be unknown and the outbreak strain was detected in human feces.

When the mode of transmission was not reported but information was given in text data fields, this information was used to categorize the outbreak. Because we were interested in the origin of the virus, we categorized outbreaks involving PB transmission but starting with food as FB-food, FB-feces, or FHB, depending on available information. Strains detected in sporadic cases were clustered into outbreaks if information was available. The remaining strains detected in sporadic cases were considered of interest with respect to the genotypes causing human illness and representative of potential unreported outbreaks. When we detected multiple genotypes during an outbreak or in sporadic cases, we recorded each genotype.

Bivalve Mollusk Monitoring Data

The European Community Reference Laboratory for Monitoring Bacteriological and Viral Contamination of Bivalve Mollusks systematically collected sequence data on norovirus strains routinely detected in bivalve mollusks in Europe. During January 1999–December 2004, the laboratory systematically collected 295 strain sequences with region A sequence lengths varying from 76 to 78 nt. These strain sequences were detected as part of production area monitoring studies or outbreak investigations of gastroenteritis in Denmark, England and Wales, Ireland, Scotland, and Spain. All samples were first routinely tested with GI and GII PCR methods; then all positive samples were cloned (*22*), resulting in a representative reflection of norovirus presence in bivalve shellfish. If we detected multiple genotypes in 1 sample, we recorded each genotype.

Assignment of Genotypes

Strains were genotyped by using a previously described method for sequence analysis of a fragment of the RNA-dependent RNA polymerase gene regions B, C, and D (*23*) because these regions were used in the FBVE network. From the start, the network used sequence-based genotyping of the then most commonly used diagnostic PCR fragment, targeting the RNA-dependent RNA polymerase gene. Since then, however, it has become clear that recombination is common and mainly occurs in the area between the overlap between the polymerase and the capsid gene. Therefore, capsid-based and polymerase-based typing may be discordant. Genotype assignment was therefore performed only after clustering of query strains against all relevant available sequences in the FBVE database (M. Koopmans et al., unpub. data). This process resulted in the genotyping of all but 68 (2%) strains. Genotypes were classified on the basis of their similarity to reference strains representing known genotypes by using the norovirus typing library (www.noronet.nl/nov_quicktyping). If the (clustered) genotypes occurred <5 times in our 5-year covering data selection, the frequencies were considered too low to be ascribed a separate genotype and excluded. This was the situation for GII.18 and 6 clusters of nonassigned GII strains (n = 25, 1%).

Data Analysis

First, we compared the genotype frequency distributions detected in outbreak categories reported as FB-food, FB-feces, FHB, PB, and UN and in routinely tested bivalve mollusks. To evaluate the correlation and measures of association of these 6 proportional profiles, Pearson correlation coefficient ρ was calculated on the basis of frequencies (ρ_1) and logarithm (ρ_2) of the frequencies of 22 genotypes, as well as Cramer V and simulated p values by using 20,000 replications with the exact variant of the χ^2 test. The exploratory technique correspondence analysis allows for examining the structure of categorical variables in a multiway table and was used to visualize the measure of correspondence in the 6 genotype profiles. p values <0.05 were considered significant.

Second, to differentiate the remaining genotype profiles detected in outbreaks, we used the genotype profiles of the 2 main transmission modes to be distinguished during an outbreak investigation. For each genotype in the human surveillance collection, the fraction of outbreaks of known origin being FB (i.e., FB-food and FB-feces) or PB was estimated on the basis of the proportion of FB outbreaks of all FB + PB outbreaks in each genotype. We used the estimated proportion of FB outbreaks of all FB + PB outbreaks in each genotype to estimate the probability that an FHB or UN outbreak was foodborne. We calculated 95% confidence intervals (CIs) using Monte Carlo simulation with 10,000 random draws from the β distributions, which are the posterior probabilities of the proportions (*24*).

Results

Of 3,022 detected noroviruses, 25 (1%) were excluded because of low frequencies; for 68 (2%), assignment of a genotype was not possible because of short sequences or inability of the method applied to type the detected norovirus beyond its genogroup. Of the remaining 2,929 strains, 71 (2%) could not be linked to epidemiologic data, and therefore their origin remained unknown, leaving 2,858 (95%) strains for analysis: 922 originating from PB outbreaks, 24 from FB-food outbreaks, 151 FB-feces outbreaks, 20 FHB outbreaks, 1,446 UN outbreaks, and all 295 bivalve mollusk monitoring strains. Among the outbreaks of known origin, 175 (16%) of 1,117 were reported to be FB (i.e., FB-food and FB-feces).

The proportion of genogroup I was significantly higher in bivalve mollusks (137/295, 46%) than in infected humans (313/2,539, 12%) (Table 1). All genotypes detected in bivalve mollusks were also detected in humans; however, 9 genotypes causing human illness were not detected in bi-

Table 1. Number of norovirus strains detected in samples from humans, bivalve mollusks, and food, 1999–2004*

Genotypes		Human surveillance, no. strains					Bivalve monitoring, no. strains	Total no. strains
Pol-based	Cap-based	FB-food	FB-feces	FHB	PB	UN		
Genogroups								
I.1	I.1	1	8	0	5	18	0	32
I.2		0	6	0	1	32	8	47
I.3	I.3	0	8	3	16	80	13	120
I.4	I.4	9	8	1	8	46	86	158
I.5		0	0	0	1	5	3	9
I.6	I.6	2	3	1	21	17	25	69
I.7		0	1	0	0	7	2	10
NA I.a	NA I.a	0	1	0	0	4	0	5
II.1		0	5	2	12	94	7	120
II.2	II.2	0	13	1	27	66	0	107
II.3		0	1	0	1	38	11	51
II.3R	II.3	0	1	0	1	41	2	45
II.4	II.4	5	47	9	681	584	63	1,389
II.5		0	3	0	6	12	0	21
II.8		1	0	1	1	13	0	16
NA II.a		0	0	0	2	7	0	9
NA II.c		0	2	0	8	31	1	42
NA II.d		0	1	0	3	8	0	12
IV.1		0	2	0	1	8	0	11
Recombinants								
NA II.b	II.1, II.2, II.3	4	23	1	100	200	63	391
II.1	II.10	0	0	0	8	19	11	38
II.7	II.6, II.7	2	18	1	19	116	0	156
Total		24	151	20	922	1,446	295	2,858

*Pol, polymerase; Cap, capsid; FB-food, foodborne-food, i.e., an outbreak was reported to be caused by food and the outbreak strain was detected in food; FB-feces, foodborne-feces, i.e., an outbreak was reported to be caused by food and the outbreak strain was detected in human feces only; FHB, food handler–borne, i.e., an outbreak was reported to be caused by an infected food handler contaminating the food and the outbreak strain was detected in human feces; PB, person-borne, i.e., an outbreak was reported to be caused by person-to-person transmission and the outbreak strain was detected in human feces; UN, unknown, i.e., the mode of transmission was not reported or was reported to be unknown and the outbreak strain was detected in human feces.

valve mollusks. Overall, the II.4 genotype was responsible for most of the human outbreaks (1,326/2,539, 52%), followed by II.b (328/2,539, 13%) and II.7 (156/2,539, 6%).

We visualized genotype frequency distributions as profiles for the observed categories of outbreaks and sorted them for their relevance in UN outbreaks, presented with different scales allowing for proportional comparison (online Appendix Figure 1, www.cdc.gov/EID/content/16/4/617-appF1.htm). The genotype profiles vary between these groups. The correlation coefficients based on frequencies, ρ_1, showed that 2 genotype profiles were distinguishable (Table 2): 1 profile typically seen in human feces (FB-feces, FHB, or PB), and another profile typically detected in sources other than human feces, i.e., in food (FB-food) or bivalve mollusks. The ρ_1 reflects some genotypes frequently and others rarely seen in FB-food and bivalve mollusks. Because FB-food strains include oyster-related outbreaks as well, we assumed that the correlation between FB-food and bivalve mollusks can be explained partly by these oyster-related outbreaks. We therefore calculated an additional correlation coefficient using the 14 strains detected in food items other than bivalve mollusks. Despite low numbers, this calculation resulted in a high, significant correlation coefficient ($\rho = 0.81$, $p < 0.001$). The logarithm of the frequencies, ρ_2 (Table 2), is less sensitive to peak frequencies of genotypes and therefore capable of differentiating profiles with respect to the rare genotypes and approaching the Cramer V. Cramer V and ρ_2 show less clear association of profiles, with diverging results for the FHB and UN profiles.

Table 2 shows the quantification of association; the associated genotype profiles illustrated by correspondence analysis is shown in the Figure. The values of the 6 columns in Table 1 can be considered coordinates in a 6-dimensional space, and the distances are computed. These distances summarize information about the similarity between the rows in Table 1. Dimension 1 may be considered to differentiate transmission modes explaining 59.12% of the correspondence, confirming that the profiles found in bivalve mollusks and FB-food are similar with regard to the pattern of relative frequencies in genotypes (rows in Table 1) and differ from those in PB. It also shows that the FHB, UN, and FB-feces profiles are mutually similar, with their distance somewhere between the PB and FB-food/bivalve mollusk profiles. Dimension 2 may represent dual origin, explaining an additional 31.40%, showing that FB-feces,

FHB, and UN mutually correspond and differ from FB-food, bivalve mollusks, and PB that mutually correspond.

When we compared the proportions of genotypes detected in FB outbreaks with those in PB outbreaks, we detected genotypes I.2 and I.4 significantly more frequently in FB outbreaks (online Appendix Figure 2, www.cdc.gov/EID/content/16/4/617-appF2.htm). On the other hand, genotypes I.6, II.1W, II.2, II.4, II.b, II.c, and II.d were detected significantly more frequently in PB outbreaks. Using these proportional FB and PB genotype profiles and their confidence intervals to distinguish between FB and PB transmission among 20 FHB outbreaks, we could ascribe 5 (95% CI 4–6) to FB and 15 (95% CI 14–16) to PB transmission. Ascribing 1,446 unexplained human norovirus outbreaks to either FB or PB transmission resulted in ≈367 (95% CI 327–417) FB outbreaks and ≈1,079 (95% CI 1,026–1,120) PB outbreaks. Overall, use of the genotype patterns increases the estimated number of FB proportion of outbreaks to 21% (547/2,563; range 20%–23%) compared with the 16% previously mentioned among the outbreaks of known origin.

Discussion

Our combined epidemiologic and virologic analysis demonstrated that norovirus genotype profiles, derived from long-term norovirus strain collections, can be used to differentiate foodborne outbreaks caused by food contamination early in the food chain from those caused by food handlers contaminating food. Our study is one step in deriving practical applicable information from the existing record and possible only through the availability of continuously updated databases containing detailed epidemiologic data and virus characterization. We confirmed a significant difference in the GI:GII ratio; GI strains were more prevalent in bivalve mollusks. On the basis of the 5-year strain collections, some genotypes (I.2 and I.4) suggest FB instead of PB preference, and others (II.2 and II.6/II.7) are commonly seen in outbreaks but not detected in bivalve mollusks (and FB-food). Strains detected in food that caused outbreaks (FB-food) showed a genotype profile similar to those in bivalve mollusk monitoring and dissimilar to the profile detected in human feces (i.e., FB-feces, FHB, PB, UN) with respect to the frequently seen genotypes. This finding may reflect the ability of these genotypes to survive outside humans or their diminished ability to spread or replicate within the human population. Genotype profiles of FHB and UN resulted in diverging association outcomes, which may reflect their potential dual origin.

Although consumption of contaminated food causes both types of outbreaks, outbreaks resulting from infected food handlers clearly necessitate different measures than do outbreaks resulting from food contaminated early in the

Table 2. $\rho 1$, $\rho 2$, and Cramer V results with simulated p values (20,000 replications) of norovirus 6 genotype patterns as detected in routinely tested bivalve shellfish and during norovirus outbreaks, 1999—2004*

Source	FB-food (p value)	FB-feces (p value)	FHB (p value)	PB (p value)	UN (p value)	Bivalve mollusk (p value)
FB-food						
ρ_1	1.00	0.48 (0.02)	0.40 (0.07)	0.43 (0.04)	0.48 (0.02)	0.91 (<0.01)
ρ_2		0.46 (0.03)	–0.15 (0.51)	0.34 (0.12)	0.24 (0.26)	0.47 (0.03)
Cramer V		0.47 (0.01)	0.62 (0.04)	0.48 (<0.01)	0.26 (<0.01)	0.41 (0.02)
FB-feces						
ρ_1		1.00	0.93 (<0.01)	0.92 (<0.01)	0.96 (<0.01)	0.53 (0.01)
ρ_2			0.40 (0.06)	0.55 (<0.01)	0.66 (<0.01)	0.69 (<0.01)
Cramer V			0.34 (0.41)	0.43 (<0.01)	0.19 (<0.01)	0.57 (<0.01)
FHB						
ρ_1			1.00	0.97 (<0.01)	0.93 (<0.01)	0.43 (<0.05)
ρ_2				0.22 (0.32)	0.39 (0.07)	0.47 (0.03)
Cramer V				0.25 (<0.01)	0.09 (0.75)	0.46 (<0.01)
PB						
ρ_1				1.00	0.96 (<0.01)	0.51 (0.01)
ρ_2					0.61 (<0.01)	0.53 (0.01)
Cramer V					0.42 (<0.01)	0.63 (0.01)
UN						
ρ_1					1.00	0.59 (<0.01)
ρ_2						0.65 (<0.01)
Cramer V						0.48 (<0.01)
Bivalve mollusk						1.00

*ρ_1 = based on frequencies; ρ_2 = based on logarithm of frequencies; Cramer V, χ^2 test with simulated p values; FB-food, foodborne-food, i.e., an outbreak was reported to be caused by food and the outbreak strain was detected in food; FB-feces, foodborne-feces, i.e., an outbreak was reported to be caused by food and the outbreak strain was detected in human feces only; FHB, food handler–borne, i.e., an outbreak was reported to be caused by an infected food handler contaminating the food and the outbreak strain was detected in human feces; PB, person-borne, i.e., an outbreak was reported to be caused by person-to-person transmission and the outbreak; strain was detected in human feces; UN, unknown, i.e., the mode of transmission was not reported or was reported to be unknown and the outbreak strain was detected in human feces.

food chain. Consequently, differentiation of these modes of transmission is of interest to food safety authorities and public health institutions. Food handler–borne outbreaks are end-of-chain outbreaks easily recognized as such, as numerous outbreak reports illustrate (*25*–*29*). Such outbreaks can be prevented or limited by exclusion of infected or shedding food handlers from work until 48–72 hours after recovery (*25*,*27*,*29*,*30*), education of food handlers (*26*), and standard testing of food handlers during outbreaks (*28*). A common source of contamination early in the food chain, however, may be more difficult to detect. Such contamination may result from sewage influx containing multiple viruses (*8*,*9*,*31*), making a link difficult to identify (*31*). Moreover, sewage most likely contains noroviruses from person-to-person outbreaks, which can contaminate the food and thereby dilute the genotype profiling effect. Use of genotype profiles is a first step toward recognizing outbreaks resulting from contamination early in the food chain because it allows estimation of the incidence in surveillance data retrospectively and objectively minimizes misclassification of outbreaks. However, genotyping data need to be interpreted with care, and continuous updating of the database remains necessary.

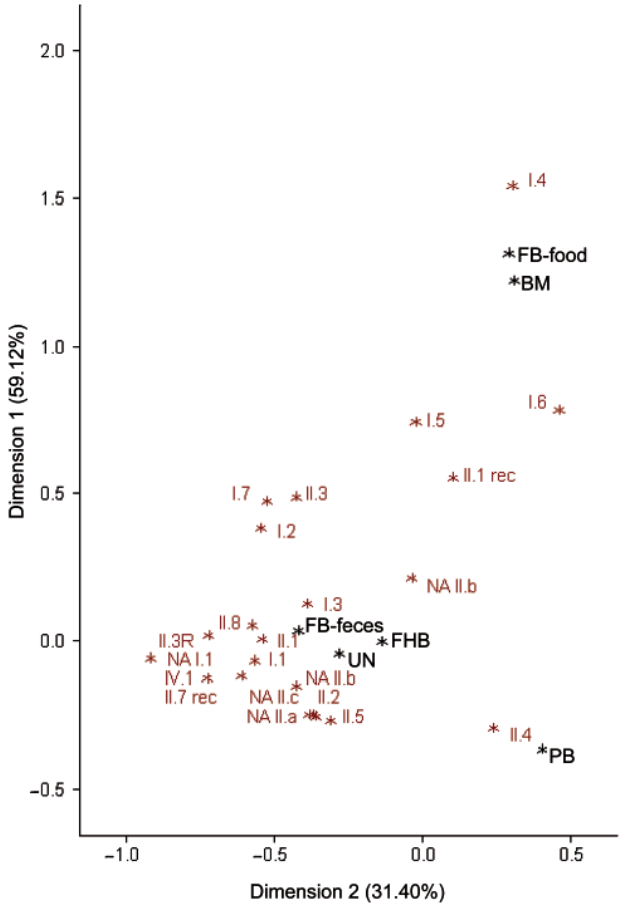


Figure. Two-dimensional display of the correspondence analysis of 6 norovirus genotype profiles based on nucleotide sequences in which points close to each other are similar with regard to the pattern of relative frequencies across genotypes. Dimension 1 explains 59.12% and dimension 2 an additional 31.40%. In dimension 1, foodborne-feces (FB-feces; i.e., outbreak reported to be caused by food with the outbreak strain detected in human feces only) and bivalve mollusk (BM) genotype profiles are mutually similar and differ from other profiles; the most distinct profile is person-borne (PB; i.e., an outbreak reported to be caused by person-to-person transmission with the outbreak strain detected in human feces). In dimension 2, food handler–borne (FHB; i.e., outbreak reported to be caused by an infected food handler contaminating the food with the outbreak strain detected in human feces), FB-feces, and unknown (UN; i.e., mode of transmission was not reported or was reported to be unknown with the outbreak strain detected in human feces) mutually correspond and differ from the mutually corresponding foodborne-food (FB-food; i.e., outbreak reported to be caused by food with the outbreak strain detected in food), BM, and PB.

Our study has some limitations. First, our measures of association could not detect differences between genotype profiles with respect to the rare genotypes. Even so, the rare outbreak or sporadic strains are of interest because they may represent potential emerging or zoonotic genotypes with consequences for public health. Types that were initially rare may remain in human surveillance, as seen with the emergence of GII.b after a large waterborne outbreak (*10*) followed by, among others, foodborne distribution throughout Europe. Since then, GII.b strains have caused 13% of all outbreaks (Table 1), now mainly PB, suggesting good adaptation. On the other hand, if the rare types are unable to adapt for persistence in the human population, they may be repeatedly reintroduced, causing only sporadic cases but not outbreaks. This repeated introduction of sporadic cases would remain undetected at present because routine surveillance for sporadic cases is rare (*32*) and is not the current practice of FBVE. To identify the origin of newly emerging and rare strains, systematic monitoring of additional potential sources, such as cattle and swine (*33*) as well as sporadic human cases, is necessary.

Second, in our analysis, the transmission route was reported as unknown for 57% of outbreak strains. Incompleteness of surveillance data is a common problem (*34*) and has been recognized in surveillance of foodborne viral infections (*35*), including in the FBVE database (*19*,*20*). Incomplete data may have resulted in underestimation of the number of foodborne outbreaks because they may be complicated to identify. Food safety authorities routinely confirm FB clusters by detecting pathogens in food, but such confirmation is difficult for viruses because viruses, unlike bacteria, do not replicate in food, resulting in a low viral load for extraction and concentration. In addition, the matrix involved may complicate these procedures, and successful detection methods are available primarily for fresh produce with surface contamination and virus-accumulating shellfish (*36*,*37*). However, knowledge of

the prevalence of strains in the environment, foods, and humans is necessary for the interpretation of matching. Such knowledge requires monitoring, which is limited to shellfish and norovirus outbreaks (*38*). For monitoring of foods other than shellfish, methods sensitive enough to detect viruses in naturally contaminated (and not spiked) food are required. The technical advisory group (TAG 4) of the Viruses in Food workgroup (WG 6) in the Technical Committee of Horizontal Methods for Food Analysis (TC 275) of the European Committee for Standardization (CEN) is validating standard methods for norovirus detection in bivalve mollusks, soft fruit, leafy vegetables, and bottled water (*39*). Until such methods are available and provide knowledge about the prevalence of viral presence in foods, the use of genetic profiles retrospectively derived from outbreak surveillance data is likely to improve foodborne viral surveillance. Because the norovirus strain population is continuously evolving, our analysis needs to be repeated periodically to ensure that retrospective findings remain predictive.

Third, international comparison of norovirus strains is complicated because of their genetic diversity and the involvement of several laboratories in diagnosis; consequential different assays result in sequences with diverging lengths and from diverging genomic regions. However, this limitation is not likely to have influenced our results because it affects mostly the comparison of sequence clusters and not genotypes. Moreover, within FBVE, standardization of diagnostic methods occurs by having participating laboratories regularly test a representative panel of fecal samples (*40*).

We showed that norovirus genotype profiles can be used to estimate the foodborne proportion of norovirus outbreaks while excluding those of the food handler as a source. Distinction at genogroup level had already indicated epidemiologic differences (*19*), and we have now demonstrated that genotype profiles can be used to differentiate transmission modes. The profiles and proportions are likely to be helpful for estimating the number of outbreaks with potential of causing geographically disseminated outbreaks. Because identification and investigation of such outbreaks provides insight into effective prevention measures during the production process, detection should enable containment of viral foodborne infection and thus prevent further spread and the consequent potential for large numbers of human infections.

Members of the Food-Borne Viruses in Europe Network: B. Böttiger, K. Mølbak, C. Johnsen (Denmark); K.-H. von Bonsdorff, L. Maunula, M. Kuusi (Finland); P. Pothier, K. Balay, J. Kaplon, G. Belliot, S. Le Guyader (France); E. Schreier, K. Stark, J. Koch, M. Höhne (Germany); G. Szücs, G. Reuter, K. Krisztalovics (Hungary); Ireland: M. Lynch, B. Foley, P. McKeown, S. Coughlan (Ireland); E. Duizer, A. Kroneman, Y. van Duynhoven (the Netherlands); K. Vainio, K. Nygard, G. Kapperud (Norway); M. Poljsak-Prijatelj, D. Barlic-Maganja, A. Hocevar Grom (Slovenia); F. Ruggeri, I. Di Bartolo (Italy); A Bosch, A. Dominguez, J. Buesa, A. Sanchez Fauquier, G. Hernández-Pezzi (Spain); K.-O. Hedlund, Y. Andersson, M. Thorhagen, M. Lysén, M. Hjertqvist (Sweden); D. Brown, B. Adak, J. Gray, J. Harris, M. Iturriza (United Kingdom).

Acknowledgments

We thank Annelies Kroneman, Soizick Le Guyader, and Leena Maunula for critically reviewing the manuscript and Amal Chatterjee for editorial assistance.

This work was supported by the Dutch Food and Consumer Product Safety Authority, the European Commission Directorate General Research Quality of Life Program, 6th Framework (EVENT, SP22-CT-2004-502571) and Secretariat General SANCO (DIVINE-net, 2003213).

Ms Verhoef is an epidemiologist in the Virology Division of the Diagnostic Laboratory for Infectious Diseases in the National Institute for Public Health and the Environment. Her work focuses on the epidemiology and surveillance of infectious diseases, particularly noroviruses.

References

1. Cannon JL, Papafragkou E, Park GW, Osborne J, Jaykus LA, Vinje J. Surrogates for the study of norovirus stability and inactivation in the environment: a comparison of murine norovirus and feline calicivirus. J Food Prot. 2006;69:2761–5.
2. Dolin R, Blacklow NR, DuPont H, Formal S, Buscho RF, Kasel JA, et al. Transmission of acute infectious nonbacterial gastroenteritis to volunteers by oral administration of stool filtrates. J Infect Dis. 1971;123:307–12.
3. Duizer E, Koopmans M. Tracking emerging pathogens: the case of noroviruses. In: Motarjemi Y, Adams M, editors. Emerging foodborne pathogens. Boca Raton (FL): Woodhead Publishing; 2006. p. 77–110.
4. Food and Agriculture Organization of the United Nations/World Health Organization. Viruses in food: scientific advice to support risk management activities. Microbiological Risk Assessment series no. 13. Rome: The Organization; 2008.
5. Greening G. Human and animal viruses in food (including taxonomy of enteric viruses). In: Goyal SM, editor. Viruses in foods. New York (NY): Springer; 2006. p. 5–42.
6. Koopmans M, Vennema H, Heersma H, van Strien E, van Duynhoven Y, Brown D, et al. Early identification of common-source foodborne virus outbreaks in Europe. Emerg Infect Dis. 2003;9:1136–42.
7. Falkenhorst G, Krusell L, Lisby M, Madsen SB, Bottiger B, Molbak K. Imported frozen raspberries cause a series of norovirus outbreaks in Denmark, 2005. Euro Surveill. 2005;10:pii2795 [cited 2005 Sep 22]. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2795
8. Le Guyader FS, Bon F, DeMedici D, Parnaudeau S, Bertone A, Crudeli S, et al. Detection of multiple noroviruses associated with an international gastroenteritis outbreak linked to oyster consumption. J Clin Microbiol. 2006;44:3878–82. DOI: 10.1128/JCM.01327-06

9. Webby RJ, Carville KS, Kirk MD, Greening G, Ratcliff RM, Crerar SK, et al. Internationally distributed frozen oyster meat causing multiple outbreaks of norovirus infection in Australia. Clin Infect Dis. 2007;44:1026–31. DOI: 10.1086/512807
10. Gallay A, De Valk H, Cournot M, Ladeuil B, Hemery C, Castor C, et al. A large multi-pathogen waterborne community outbreak linked to faecal contamination of a groundwater system, France, 2000. Clin Microbiol Infect. 2006;12:561–70. DOI: 10.1111/j.1469-0691.2006.01441.x
11. Buesa J, Collado B, Lopez-Andujar P, Abu-Mallouh R, Rodriguez Diaz J, Garcia Diaz A, et al. Molecular epidemiology of caliciviruses causing outbreaks and sporadic cases of acute gastroenteritis in Spain. J Clin Microbiol. 2002;40:2854–9. DOI: 10.1128/JCM.40.8.2854-2859.2002
12. Koopmans M, Lopman B, Vennema H, Reacher M, Carrique-Mas J, Van Duijnhoven Y, et al. Foodborne viruses in Europe: web-based technologies for investigation of transnational outbreaks of viral gastroenteritis. Proceedings of the International Conference on Emerging Infectious Diseases. 2002 Mar 24–27. Atlanta, GA, USA.
13. Hald T, Vose D, Wegener HC, Koupeev T. A Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. Risk Anal. 2004;24:255–69. DOI: 10.1111/j.0272-4332.2004.00427.x
14. Boxman IL, Tilburg JJ, te Loeke NA, Vennema H, de Boer E, Koopmans M. An efficient and rapid method for recovery of norovirus from food associated with outbreaks of gastroenteritis. J Food Prot. 2007;70:504–8.
15. Le Guyader FS, Mittelholzer C, Haugarreau L, Hedlund KO, Alsterlund R, Pommepuy M, et al. Detection of noroviruses in raspberries associated with a gastroenteritis outbreak. Int J Food Microbiol. 2004;97:179–86. DOI: 10.1016/j.ijfoodmicro.2004.04.018
16. Rutjes SA, Lodder Verschoor F, van der Poel WH, van Duijnhoven YT, de Roda Husman AM. Detection of noroviruses in foods: a study on virus extraction procedures in foods implicated in outbreaks of human gastroenteritis. J Food Prot. 2006;69:1949–56.
17. Green J, Henshilwood K, Gallimore CI, Brown DW, Lees DN. A nested reverse transcriptase PCR assay for detection of small round-structured viruses in environmentally contaminated molluscan shellfish. Appl Environ Microbiol. 1998;64:858–63.
18. Kroneman A, Verhoef L, Harris J, Vennema H, Duizer E, van Duynhoven Y, et al. Analysis of integrated virological and epidemiological reports of norovirus outbreaks collected within the foodborne viruses in Europe Network from 1 July 2001 to 30 June 2006. J Clin Microbiol. 2008;46:2959–65. DOI: 10.1128/JCM.00499-08
19. Verhoef LP, Kroneman A, Van Duijnhoven Y, Boshuizen H, van Pelt W, Koopmans M. Selection tool for foodborne norovirus outbreaks. Emerg Infect Dis. 2009;15:31–8. DOI: 10.3201/eid1501.080673
20. Kroneman A, Harris J, Vennema H, Duizer E, van Duynhoven Y, Gray J, et al. Data quality of 5 years of central norovirus outbreak reporting in the European Network for food-borne viruses. J Public Health (Oxf). 2008;30:82–90. DOI: 10.1093/pubmed/fdm080
21. Lopman BA, Reacher MH, Van Duijnhoven Y, Hanon FX, Brown D, Koopmans M. Viral gastroenteritis outbreaks in Europe, 1995–2000. Emerg Infect Dis. 2003;9:90–6.
22. Henshilwood K, Green J, Lees DN. Monitoring the marine environment for small round structured viruses (SRSVs): a new approach to combating the transmission of these viruses by mulluscan shellfish. Water Sci Technol. 1998;38(12):51–6. DOI: 10.1016/S0273-1223(98)00799-9
23. Vinje J, Koopmans MP. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. J Infect Dis. 1996;174:610–5.
24. Brown LD, Cai TT, DasGupta A. Interval estimation for a binomial proportion. Stat Sci. 2001;16:101–33.
25. Centers for Disease Control and Prevention. Multisite outbreak of norovirus associated with a franchise restaurant—Kent County, Michigan, May 2005. MMWR Morb Mortal Wkly Rep. 2006;55:395–7.
26. Centers for Disease Control and Prevention. Norovirus outbreak associated with ill food-service workers—Michigan, January–February 2006. MMWR Morb Mortal Wkly Rep. 2007;56:1212–6.
27. Friedman DS, Heisey Grove D, Argyros F, Berl E, Nsubuga J, Stiles T, et al. An outbreak of norovirus gastroenteritis associated with wedding cakes. Epidemiol Infect. 2005;133:1057–63. DOI: 10.1017/S0950268805004760
28. Godoy P, Izcara J, Bartolome R, Bach P, Escobar A, Pal M, et al. Outbreak of food-borne norovirus associated with the consumption of sandwiches [in Spanish]. Med Clin (Barc). 2005;124:161–4. DOI: 10.1157/13071479
29. Schmid D, Stuger HP, Lederer I, Pichler AM, Kainz Arnfelser G, Schreier E, et al. A foodborne norovirus outbreak due to manually prepared salad, Austria 2006. Infection. 2007;35:232–9. DOI: 10.1007/s15010-007-6327-1
30. Rooney RM, Cramer EH, Mantha S, Nichols G, Bartram JK, Farber JM, et al. A review of outbreaks of foodborne disease associated with passenger ships: evidence for risk management. Public Health Rep. 2004;119:427–34. DOI: 10.1016/j.phr.2004.05.007
31. Gallimore CI, Pipkin C, Shrimpton H, Green AD, Pickford Y, McCartney C, et al. Detection of multiple enteric virus strains within a foodborne outbreak of gastroenteritis: an indication of the source of contamination. Epidemiol Infect. 2005;133:41–7. DOI: 10.1017/S0950268804003218
32. Zintz C, Bok K, Parada E, Barnes-Eley M, Berke T, Staat MA, et al. Prevalence and genetic characterization of caliciviruses among children hospitalized for acute gastroenteritis in the United States. Infect Genet Evol. 2005;5:281–90. DOI: 10.1016/j.meegid.2004.06.010
33. Mattison K, Shukla A, Cook A, Pollari F, Friendship R, Kelton D, et al. Human noroviruses in swine and cattle. Emerg Infect Dis. 2007;13:1184–8.
34. Doyle TJ, Glynn MK, Groseclose SL. Completeness of notifiable infectious disease reporting in the United States: an analytical literature review. Am J Epidemiol. 2002;155:866–74. DOI: 10.1093/aje/155.9.866
35. O'Brien S. The challenge of estimating the burden of an underreported disease. In: Koopmans M, Cliver DO, Bosch A, editors. Foodborne viruses. Washington (DC): ASM Press; 2008. p. 87–116.
36. Atmar RL. Molecular methods of virus detection in food. In: Goyal SM, editor. Viruses in foods. New York: Springer 2006. p. 121–43.
37. Goyal SM. Conventional methods of virus detection in foods. In: Goyal SM, editor. Viruses in foods. New York: Springer; 2006. p. 101–20.
38. Verhoef L, Boxman I, Koopmans M. Viruses transmitted through the food-chain: a review of the latest developments. CAB Reviews. 2008;3:1–15 .
39. Costafreda MI, Bosch A, Pinto RM. Development, evaluation, and standardization of a real-time TaqMan reverse transcription–PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. Appl Environ Microbiol. 2006;72:3846–55. DOI: 10.1128/AEM.02660-05
40. Vinje J, Vennema H, Maunula L, von Bonsdorff CH, Hoehne M, Schreier E, et al. International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses. J Clin Microbiol. 2003;41:1423–33. DOI: 10.1128/JCM.41.4.1423-1433.2003

Address for correspondence: Linda Verhoef, National Institute for Public Health and the Environment (RIVM), Postbak 22, PO Box 1, 3720 BA Bilthoven, the Netherlands; email: linda.verhoef@rivm.nl

Reassortment of Human Rotavirus Gene Segments into G11 Rotavirus Strains

Jelle Matthijnssens, Mustafizur Rahman, Max Ciarlet, Mark Zeller, Elisabeth Heylen, Toyoko Nakagomi, Ryuichi Uchida,[1] Zahid Hassan, Tasnim Azim, Osamu Nakagomi, and Marc Van Ranst

G11 rotaviruses are believed to be of porcine origin. However, a limited number of G11 rotaviruses have been recently isolated from humans in combination with P[25], P[8], P[6], and P[4]. To investigate the evolutionary relationships of these strains, we analyzed the complete genomes of 2 human G11P[25] strains, 2 human G11P[8] strains, and 3 porcine reference strains. Most of the 11 gene segments of these 7 strains belonged to genotype 1 (Wa-like). However, phylogenetic clustering patterns suggested that an unknown G11P[25] strain with a new I12 VP6 genotype was transmitted to the human population, in which it acquired human genotype 1 gene segments through reassortment, resulting in a human G11P[8] rotavirus strain with an entire human Wa-genogroup backbone. This Wa-like backbone is believed to have caused the worldwide spread of human G9 and G12 rotaviruses. G11 human rotavirus strains should be monitored because they may also become major human pathogens.

Group A rotaviruses are the most frequently detected viral cause of diarrhea in children worldwide and cause ≈600,000 deaths in children <5 years of age annually, mainly in developing countries (*1*). Rotaviruses have a genome composed of 11 segments of double-stranded RNA that encodes 6 structural (VP) and 5 or 6 nonstructural (NSP) proteins (*2*). The 2 outer capsid proteins VP7 and VP4 are the basis for a widely used dual classification system defining G-types and P-types, respectively. Currently 23 G-genotypes and 31 P-genotypes have been described, of which 12 of each type have been found in human rotavirus isolates (*3*–*9*). However, only a limited number of G/P-genotype combinations are found frequently in humans, such as G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8], and, more recently, the G12 genotype in combination with P[8] or P[6] (*10*). It has been hypothesized that the G9 and G12 genotypes have been able to successfully infect, spread, and persist in humans because of reassortment events with human Wa-like rotavirus strains, which has resulted in the G9 and G12 rotaviruses combining with P[8] and the 9 remaining gene segments belonging to genotype 1 (*10*).

A nucleotide sequence–based classification and nomenclature system encompassing the 11 rotavirus genome segments has been introduced recently, and it defines genotypes for each of the 11 gene segments (*11*). This system has been useful in investigating reassortment events and interspecies transmission of rotaviruses and their interhost relationships (*5*,*12*–*20*). In this system, the VP1–VP3, VP6, and NSP1–NSP5 genotypes comprising Wa-like strains have been designated as genotype 1 (R1, C1, M1, I1, A1, N1, T1, E1, and H1, respectively), and DS-1 and AU-1-like strains have been designated as genotypes 2 and 3, respectively (*11*). A Rotavirus Classification Working Group (RCWG) was formed to maintain and update this system and to assign successive genotype numbers to newly discovered rotavirus genotypes (*21*).

G11 rotaviruses are believed to be circulating in pigs, albeit in low numbers. Only 2 G11P[7] porcine strains have been isolated, strain YM in Mexico in 1983 and strain A253 in Venezuela in 1989 (*22*,*23*). Each of these porcine strains was identified as a single isolate in large strain col-

Author affiliations: Rega Institute for Medical Research, Leuven, Belgium (J. Matthijnssens, M. Zeller, E. Heylen, M. Van Ranst); International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh (M. Rahman, Z. Hassan, T. Azim); Merck and Company, Inc., North Wales, Pennsylvania, USA (M. Ciarlet); and Nagasaki University, Nagasaki, Japan (T. Nakagomi, R. Uchida, O. Nakagomi)

DOI: 10.3201/eid1604.091591

[1]Current affiliation: Osaka University, Osaka, Japan.

lections obtained during epidemiologic surveys. In subsequent years, no additional G11 strains were detected in the same or nearby pig farms. However, more than a decade after the detection of these 2 porcine G11 strains, several reports have described the isolation of G11 rotavirus strains from humans. Three G11 rotaviruses, Dhaka6, KTM368, and CRI 10795, have been found in combination with the rare human genotype P[25] in India (*24*), Bangladesh (*25*), and Nepal (*26*). Furthermore, G11 human rotaviruses have been found in combination with P[8] (Matlab36–02 and Matlab22–01), with P[6] in Ecuador (EC2184) (*27*) and Bangladesh (Dhaka13–06) (*28*) and with P[4] (CUK-1) in South Korea (*29*).

It was recently suggested that human rotavirus strains belonging to the Wa-like genogroup and porcine rotaviruses have a common origin (*11*). This report prompted us to investigate the level of genetic relatedness, and possibly evolutionary origins, between these unusual human G11 rotavirus strains and porcine rotavirus strains by complete genome analyses of 2 human G11P[25] strains (KTM368 and Dhaka6), 2 human G11P[8] strains (Matlab36–02 and Dhaka22–01), and 3 cell culture–adapted porcine strains YM (G11P[7]), Gottfried (G4P[6]), and OSU (G5P[7]).

Methods

Strain Collection

Strain KTM368 (G11P[25]) was isolated in Nepal in 2004 (*26*), and rotavirus strains Dhaka6 (G11P[25]), Dhaka22–01 (G11P[8]), and Matlab36–02 (G11P[8]) were isolated in Bangladesh in 2001, 2001, and 2002, respectively (*25*,*28*). Double-stranded RNA of tissue culture–adapted reference porcine strains YM (G11P[7]), isolated in Mexico in 1983 (*23*), and OSU (G5P[7]) and Gottfried (G4P[6]) isolated in the United States in 1976 (*30*), was used in our study.

RNA Extraction and Reverse Transcription–PCR

Virus RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN, Leusden, the Netherlands) according to the manufacturer's instructions. Extracted RNA was denatured at 97°C for 5 min, and reverse transcription–PCR was performed by using the OneStep RT-PCR Kit (QIAGEN). Forward and reverse primers used for amplification of different gene segments were synthesized on the basis of alignments of known 5′ and 3′ sequences of respective gene segments found in GenBank (primers are available upon request from J.M.). PCRs were performed by using an initial reverse transcription step at 50°C for 30 min, followed by PCR activation at 95°C for 15 min, 40 cycles of amplification, and a final extension at 72°C for 10 min in a BiometraT3000 Thermocycler (Biometra, Westburg, the Netherlands). Cycle conditions for the amplification of VP1, VP2, VP3, and VP4 were 30 s at 94°C, 30 s at 50°C, and 6 min at 70°C. For other gene segments, conditions were 30 s at 94°C, 30 s at 45°C, and 3 min at 72°C.

Nucleotide Sequencing

PCR products were purified with the MSB Spin PCRapace Kit (Invitek, Berlin, Germany) and sequenced by using the dideoxy-nucleotide chain termination method with the ABI PRISM BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 3100 automated sequencer (Applied Biosystems). Complete 5′ and 3′ terminal nucleotide sequences of the 11 gene segments were determined by using a modified rapid amplification of cDNA ends technique as described (*15*).

Nucleotide and Protein Sequence Analysis

Chromatogram sequencing files were analyzed by using Chromas 2.3 (Technelysium, Helensvale, Queensland, Australia), and contigs were prepared by using SeqMan II (DNASTAR, Madison, WI, USA). Multiple sequence alignments were constructed in ClustalX 2 (*31*) and subsequently edited in MEGA4 (*32*).

Phylogenetic Analysis

Phylogenetic and molecular evolutionary analyses were conducted by using MEGA4 (*32*). Genetic distances were calculated by using the Kimura-2 correction parameter at the nucleotide level, and phylogenetic trees were constructed by using the neighbor-joining method with 500 bootstrap replicates.

Assignment of Newly Identified Genotypes

Genotypes of each of the 11 genome segments for all the rotavirus strains under investigation were determined according to the genotyping recommendations of the RCWG by using the RotaC rotavirus genotyping tool (*21*,*33*). Because the sequences of the VP6 gene segment of strain KTM368 did not belong to any of the established VP6 I genotypes, it was submitted to the RCWG for appropriate genotype assignment. GenBank accession numbers for each of the gene segments of strains Dhaka6, KTM368, Dhaka22–01, Matlab36–02, YM, OSU, and Gottfried are shown in online Technical Appendix 1 (www.cdc.gov/EID/content/16/4/625-Techapp1.pdf).

Results

Complete genome sequences of 3 human rotavirus strains (Dhaka6, KTM368, and Matlab36–02) were determined. For strain Dhaka22–01, only short nucleotide sequences ranging from 199 to 962 nt per segment could be determined because of insufficient sample. For porcine rotavirus strains YM, OSU, and Gottfried, variable amounts

of gene sequences have been reported. We sequenced the remaining porcine rotavirus gene segments (online Technical Appendix 1).

Genotyping

According to guidelines of the RCWG, all gene segments belonged to established genotypes, except for the VP6 gene segment of KTM368 (*21*). This sequence was submitted to the RCWG, accepted as a new VP6 genotype, and designated I12. Complete genotype assignments of the 6 strains fully sequenced and reference strain Wa are shown in Table 1. All 3 human G11 strains have gene segments belonging to genotype 1 for the gene segments encoding VP1–VP3 and NSP1–NSP5. The VP4-encoding gene segment of human strains KTM368 and Dhaka6 had the unusual P[25] genotype, and human strains Matlab36–02 and Dhaka22–01 had the typical human P[8] genotype. With regard to VP6-encoding gene segments, only the human strain KTM368 from Nepal had the I12 genotype, and other human rotavirus strains isolated in Bangladesh had the typical human Wa-like VP6 genotype I1. The 3 porcine rotavirus strains YM, Gottfried, and OSU all had genotype 1 gene segments for VP1–VP3 and NSP2–NSP5 and the expected different G (G11, G4, and G5) and P (P[7] and P[6]) genotypes. The VP6 and NSP1 genotypes were either I1 or I5 and A1 or A8, respectively (Table 1).

For several gene segments of the partially sequenced strains Dhaka22–01, insufficient sequence data were available for a definitive classification according to the guidelines of the RCWG (*21*). However, when available sequence data of strain Dhaka22–01 were compared pairwise with those of the other human G11 strains, sequences of VP7, VP6, and VP4 of strain Dhaka22–01 were nearly identical with those of the strain Matlab36–02 (99.8%, 99.0% and 98.4% identity at the nucleotide level, respectively) (online Technical Appendix 1). In addition, for the VP1–VP2 and NSP1–NSP5 gene segments of strain Dhaka22–01, high identities (range 97.9%–100%) at the nucleotide level were found between strain Dhaka22–01 and the other human Wa-like G11 strains. For the VP3 gene segment, identities between strain Dhaka22–01 and strains KTM368, Dhaka6, and Matlab36–02 were low (range 86.3%–86.7%), and the VP3 gene segment of strain Dhaka22–01 was closely related to strains in the human M1 subcluster (99.6% identity with strain Dhaka12–03). These findings suggest a typical human Wa-like origin for Dhaka22-01 (online Technical Appendix 1).

Phylogenetic and Pairwise Identity Analyses

To study the relationships between human G11 and porcine rotavirus strains in greater detail, we constructed phylogenetic trees by using entire open reading frame nucleotide sequences for the 11 gene segments (online Technical Appendix 2, www.cdc.gov/EID/content16/4/625-Techapp2.pdf). For VP7, strains KTM368, Dhaka6, and Matlab36–02 cluster closely within the G11 genotype, together with the G11P[8] strain CUK1 from South Korea; the porcine G11 rotavirus strains YM and A253 are more distantly related (online Technical Appendix 2). For VP6, strain KTM368 (genotype I12) was only distantly related to strains belonging to genotype I1. Human strains Dhaka6 and Matlab36–02 cluster in a large I1 subcluster, which contains mainly human and a few porcine strains isolated in Bangladesh, Belgium, the United States, Thailand, India, Australia, and Japan (online Technical Appendix 2). For VP4, strains KTM368 and Dhaka6 cluster closely in the rare P[25] genotype, whereas strain Matlab36–02 is closely related to recently isolated P[8] human strains from Bangladesh, Belgium, South Korea, and the Democratic Republic of the Congo (online Technical Appendix 2).

In the phylogenetic trees of the remaining 8 gene segments (VP1–VP3 and NSP1–NSP5), at least 1 major human monophyletic subcluster could be distinguished within genotype 1. We also observed 1 major porcine genotype 1 subcluster, a finding that is consistent with the assumption that Wa-like human rotavirus strains and porcine rotaviruses have a common ancestor (online Technical Appendix 2) (*11*). Several gene segments of human G11 strains cluster closely in human genotype 1 subcluster, but a few of them did not cluster closely with any known human or porcine rotavirus strains and formed a distinct (nonhuman, nonporcine) branch or subcluster within genotype 1.

For the VP2 and NSP1 gene segments, human strains KTM368, Dhaka6, and Matlab36–02 clustered closely

Table 1. Genomic constellation of 3 human rotavirus strains, 3 porcine strains, and human reference strain Wa*

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Hu/Wa	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Hu/KTM368	G11	P[25]	I12	R1	C1	M1	A1	N1	T1	E1	H1
Hu/Dhaka6	G11	P[25]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Hu/Matlab36–02	G11	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Po/YM	G11	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1
Po/Gottfried	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1
Po/OSU	G5	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1

*VP, structural protein; NSP, nonstructural protein; Hu, human; Po, porcine. Wa genogroup genotypes are indicated in green, G11 and the rare P[25] and I12 genotypes are indicated in orange, and typical porcine genotypes are indicated in blue.

within the genotype 1 (C1 and A1, respectively) human subcluster (online Technical Appendix 2). Regarding the VP1, NSP2, NSP3, NSP4, and NSP5 gene segments, human rotavirus strains Dhaka6 and Matlab36–02 are localized within the human subcluster, whereas strain KTM368 formed a distinct branch inside genotype 1 and did not cluster with any known human or porcine rotavirus strain (online Technical Appendix 2). For VP3, all 3 human G11 strains (KTM368, Dhaka6, and Matlab36–02) belonged to a distinct subcluster inside the VP3 M1 genotype (online Technical Appendix 2).

Acquisition of Human Rotavirus Genes

The distinct genotype constellations (Table 2) differed in their degree of relatedness to typical human Wa genogroup rotavirus strains. With the exception of the G11 genotype, human strain Dhaka22–01 is nearly indistinguishable from other locally or more distantly circulating human Wa-genogroup rotavirus strains, such as strains Dhaka16–03 (G1P[8]) and Dhaka12–03 (G12P[6]) from Bangladesh or strain B4633–03 (G12P[8]) from Belgium, all isolated in 2003 (online Technical Appendix 1) (*19*). Human strain Matlab36–02 is closely related to strain Dhaka22–01, except for the VP3 gene (online Technical Appendix 2), for which human strains KTM368, Dhaka6, and Matlab36–02 form a distinct subcluster inside the M1 genotype. This finding suggests a recent reassortment event. Strain Dhaka6 is closely related to strain Matlab36–02 and only differs in the VP4 genotype (P[25] versus P[8]), respectively (online Technical Appendix 2). This finding also suggests a recent reassortment event. Strain KTM368 from Nepal has VP2 and NSP1 gene segments that belong to the typical human subcluster within the C1 and A1 genotypes, respectively. All other gene segments belong to non–Wa-like genotypes (VP7: G11, VP4: P[25], and VP6: I12) or to a distinct subcluster or branch inside genotype 1 with an unknown origin.

There are 2 possible hypotheses for the observed acquisition of human rotavirus genes by G11 strains. The first hypothesis is that different human G11 virus strains described in this study may have originated from several unrelated interspecies transmission events of animal G11 strains to humans, followed by reassortment events that involved Wa-like human strains. The second hypothesis is that a gradual acquisition of human rotavirus genes occurred after 1 interspecies transmission event, followed by multiple successive reassortment events. The second hypothesis is that a currently unknown ancestral rotavirus, of probable porcine origin and having the G11-P[25]-I12 genotypes in a nonhuman Wa genogroup background, might have undergone multiple reassortment events with co-circulating human rotavirus strains, resulting in the different natural reassortant rotavirus strains described in this study. These reassortments resulted in a human G11P[8] rotavirus composed entirely of typical human genotype 1 (Wa-like) RNA segments.

Discussion

G11 rotaviruses are considered porcine rotaviruses because they were first isolated from pigs in Venezuela and Mexico in the 1980s (*22*,*23*,*34*). Although G11 porcine rotaviruses have been detected infrequently on pig farms (*22*,*23*,*34*), these viruses have been recently detected in humans in several locations (India, Bangladesh, Nepal, South Korea, and Ecuador) (*24*–*29*). Our data show that multiple reassortment events have occurred between porcine or human G11 rotaviruses and co-circulating human Wa-like rotavirus strains (all human G11 strains were isolated during 2001–2006). In addition to G11 strains described in this study, another G11P[25] human rotavirus strain (CRI 10795) has been isolated in India, but only partial VP7, VP4, VP6, and NSP4 gene sequences of this strain are available (*24*). The CRI 10795 strain is yet another G11P[25] human rotavirus variant with a VP6 gene of the human I1 genotype and an NSP4 gene of the nonhuman subcluster of the E1 genotype. This finding suggests that additional reassortments have occurred between G11 and Wa-like strains.

Because the few human G11 strains investigated most likely represent only a small part of a complex set of events, our primary hypothesis of a linear stepwise acquisition of human rotavirus genes through successive reassortment events, which result in a human G11 rotavirus with an entire human Wa-like genomic background, may be oversimplified. An

Table 2. Subcluster-based genomic constellation of 3 human rotavirus strains, 3 porcine strains, and human reference strain Wa*

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Hu/Wa	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Hu/KTM368	G11	P[25]	I12	R1	C1	M1	A1	N1	T1	E1	H1
Hu/Dhaka6	G11	P[25]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Hu/Matlab36–02	G11	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1
Po/YM	G11	P[7]	I5	R1	C1	M1	A8	N1	T1	E1	H1
Po/Gottfried	G4	P[6]	I1	R1	C1	M1	A8	N1	T1	E1	H1
Po/OSU	G5	P[7]	I5	R1	C1	M1	A1	N1	T1	E1	H1

*VP, structural protein; NSP, nonstructural protein; Hu, human; Po, porcine. Genotypes belonging to the Wa genogroup indicated in green in Table 1 are further subdivided into subclusters shown in green, purple, and light blue, based on phylogenetic trees shown in online Technical Appendix 2 (www.cdc.gov/EID/content/16/4/625-Techapp2.pdf), to distinguish additional patterns. G11 and the rare P[25] and I12 genotypes are indicated in orange, and typical porcine genotypes are indicated in dark blue.

alternative hypothesis is that rare (porcine?) G11-P[25]-I12 progenitor strains are more widely spread and that genes of such viruses were introduced into the human population by interspecies transmissions, followed by multiple independent reassortment events with human rotavirus strains (G1P[8], G3P[8], G4P[8], G9P[8]). These reassortments could have resulted in different numbers of porcine gene segments being transferred to human Wa-like rotaviruses, as described for the G11 strains used in this study. Reassortment of individual or small numbers of porcine rotavirus genes into human rotaviruses, which are well adapted to propagation in the human host, can result in a virus with a genetic makeup that is optimal for replication in the human host and spread in the human population. However, close phylogenetic clustering of most genes of different G11 strains (online Technical Appendix 2) suggests that these strains have a recent common ancestor and that gradual acquisition of human rotavirus genes is a plausible hypothesis.

The 2 hypotheses are not mutually exclusive, and a combination of both cannot be ruled out or proven at this time. In addition to G11P[25] and G11P[8] human rotaviruses, G11 rotaviruses in combination with P[4] and P[6] genotypes have been isolated in Bangladesh (*28*) and South Korea (*29*). However, additional sequence data are not available for these strains, which are suggestive for additional reassortment events with P[4] (DS-1 like) and P[6] genotype rotaviruses. A recent report describes another human G11P[6] strain in Ecuador, which was found to be the likely result of reassortment between a typical porcine rotavirus and a human Wa-like rotavirus (*27*).

Early detection in ongoing surveillance programs and detailed analyses of G11 strains might provide unique insights into adaptation mechanisms of nonhuman rotaviruses to the human host through reassortment. G11 rotaviruses appear to be acquiring genotype 1 genome segments through multiple reassortment events (or Wa-like strains are acquiring genes of G11 rotavirus strains) in a short period. It will be useful to monitor whether new G11P[8] human rotavirus strains, which carry mainly human Wa-like genes, will be as successful as G9 and G12 rotaviruses in finding a niche in the human population, and whether the currently licensed rotavirus vaccines will afford protection against rotavirus disease caused by G11P[8] human rotavirus strains. Given that available rotavirus vaccines contain the virus P[8] component, it is more likely that they will also protect humans against G9, G11, and G12 strains with the VP4 genotype P[8].

Acknowledgments

We thank Koki Taniguchi for providing cell culture–adapted YM, OSU, and Gottfried porcine rotavirus strains, and Ulrich Desselberger for valuable comments and discussions regarding the manuscript.

J.M. was supported by a postdoctoral fellowship from Fonds voor Wetenschappelijk Onderzoek.

Dr Matthijnssens is a postdoctoral fellow at the Rega Institute for Medical Research in Leuven, Belgium. His research interests are the molecular epidemiology, genetic diversity, and evolution of rotaviruses.

References

1. Parashar UD, Gibson CJ, Bresse JS, Glass RI. Rotavirus and severe childhood diarrhea. Emerg Infect Dis. 2006;12:304–6.
2. Estes M, Kapikian A. 2007. Rotaviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, et al., editors. Fields virology, 5th ed. Philadelphia: Kluwer Health/Lippincott, Williams and Wilkins; 2007. p. 1919–74.
3. Abe M, Ito N, Morikawa S, Takasu M, Murase T, Kawashima T, et al. Molecular epidemiology of rotaviruses among healthy calves in Japan: Isolation of a novel bovine rotavirus bearing new P and G genotypes. Virus Res. 2009;144:250–7. DOI: 10.1016/j.virusres.2009.05.005
4. Matthijnssens J, Bilcke J, Ciarlet M, Martella V, Banyai K, Rahman M, et al. Rotavirus disease and vaccination: impact on genotype diversity. Future Microbiol. 2009;4:1303–16. DOI: 10.2217/fmb.09.96
5. Matthijnssens J, Potgieter CA, Ciarlet M, Parreno V, Martella V, Banyai K, et al. Are human P[14] rotavirus strains the result of interspecies transmissions from sheep or other ungulates belonging to the mammalian order of Artiodactyla? J Virol. 2009;83:2917–29. DOI: 10.1128/JVI.02246-08
6. Schumann T, Hotzel H, Otto P, Johne R. Evidence of interspecies transmission and reassortment among avian group A rotaviruses. Virology. 2009;386:334–43. DOI: 10.1016/j.virol.2009.01.040
7. Solberg OD, Hasing ME, Trueba G, Eisenberg JN. Characterization of novel VP7, VP4, and VP6 genotypes of a previously untypeable group A rotavirus. Virology. 2009;385:58–67. DOI: 10.1016/j.virol.2008.11.026
8. Trojnar E, Otto P, Johne R. The first complete genome sequence of a chicken group A rotavirus indicates independent evolution of mammalian and avian strains. Virology. 2009;386:325–33. DOI: 10.1016/j.virol.2009.01.034
9. Ursu K, Kisfali P, Rigo D, Ivanics E, Erdelyi K, Dan A, et al. Molecular analysis of the VP7 gene of pheasant rotaviruses identifies a new genotype, designated G23. Arch Virol. 2009;154:1365–9. DOI: 10.1007/s00705-009-0439-0
10. Matthijnssens J, Rahman M, Ciarlet M, Van Ranst M. 2008. Emerging human rotavirus genotypes. In: Palombo EA, Kirkwood CD, editors. Viruses in the environment. Trivandrum (India): Research Signpost; 2008. p. 171–219.
11. Matthijnssens J, Ciarlet M, Heiman E, Arijs I, Delbeke T, McDonald SM, et al. Full genome-based classification of rotaviruses reveals a common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J Virol. 2008;82:3204–19. DOI: 10.1128/JVI.02257-07
12. Banyai K, Martella V, Molnar P, Mihaly I, Van Ranst M, Matthijnssens J. Genetic heterogeneity in human G6P[14] rotavirus strains detected in Hungary suggests independent zoonotic origin. J Infect. 2009;59:213–5. DOI: 10.1016/j.jinf.2009.06.009
13. Ciarlet M, Hoffmann C, Lorusso E, Baselga R, Cafiero MA, Bányai K, et al. Genomic characterization of a novel group A lamb rotavirus isolated in Zaragoza, Spain. Virus Genes. 2008;37:250–65. DOI: 10.1007/s11262-008-0257-6

14. Martella V, Banyai K, Matthijnssens J, Buonavoglia C, Ciarlet M. Zoonotic aspects of rotaviruses. Vet Microbiol. 2009 Aug 28; [Epub ahead of print].
15. Matthijnssens J, Rahman M, Martella V, Xuelei Y, De Vos S, De Leener K, et al. Full genomic analysis of human rotavirus strain B4106 and lapine rotavirus strain 30/96 provides evidence for interspecies transmission. J Virol. 2006;80:3801–10. DOI: 10.1128/JVI.80.8.3801-3810.2006
16. Matthijnssens J, Rahman M, Van Ranst M. Two out of the 11 genes of an unusual human G6P[6] rotavirus isolate are of bovine origin. J Gen Virol. 2008;89:2630–5. DOI: 10.1099/vir.0.2008/003780-0
17. Matthijnssens J, Rahman M, Yang X, Delbeke T, Arijs I, Kabue JP, et al. G8 rotavirus strains isolated in the Democratic Republic of Congo belong to the DS-1-like genogroup. J Clin Microbiol. 2006;44:1801–9. DOI: 10.1128/JCM.44.5.1801-1809.2006
18. McDonald SM, Matthijnssens J, McAllen JK, Hine E, Overton L, Wang S, et al. Evolutionary dynamics of human rotaviruses: balancing reassortment with preferred genome constellations. PLoS Pathog. 2009;5:e1000634. DOI: 10.1371/journal.ppat.1000634
19. Rahman M, Matthijnssens J, Yang X, Delbeke T, Arijs I, Taniguchi K, et al. Evolutionary history and global spread of the emerging G12 human rotaviruses. J Virol. 2007;81:2382–90. DOI: 10.1128/JVI.01622-06
20. Tsugawa T, Hoshino Y. Whole genome sequence and phylogenetic analyses reveal human rotavirus G3P[3] strains Ro1845 and HCR3A are examples of direct virion transmission of canine/feline rotaviruses to humans. Virology. 2008;380:344–53. DOI: 10.1016/j.virol.2008.07.041
21. Matthijnssens J, Ciarlet M, Rahman M, Attoui H, Banyai K, Estes MK, et al. Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol. 2008;153:1621–9. DOI: 10.1007/s00705-008-0155-1
22. Ciarlet M, Hidalgo M, Gorziglia M, Liprandi F. Characterization of neutralization epitopes on the VP7 surface protein of serotype G11 porcine rotaviruses. J Gen Virol. 1994;75:1867–73. DOI: 10.1099/0022-1317-75-8-1867
23. Ruiz AM, Lopez IV, Lopez S, Espejo RT, Arias CF. Molecular and antigenic characterization of porcine rotavirus YM, a possible new rotavirus serotype. J Virol. 1988;62:4331–6.
24. Banerjee I, Iturriza-Gomara M, Rajendran P, Primrose B, Ramani S, Gray JJ, et al. Molecular characterization of G11P[25] and G3P[3] human rotavirus strains associated with asymptomatic infection in South India. J Med Virol. 2007;79:1768–74. DOI: 10.1002/jmv.20988
25. Rahman M, Matthijnssens J, Nahar S, Podder G, Sack DA, Azim T, et al. Characterization of a novel P[25],G11 human group a rotavirus. J Clin Microbiol. 2005;43:3208–12. DOI: 10.1128/JCM.43.7.3208-3212.2005
26. Uchida R, Pandey BD, Sherchand JB, Ahmed K, Yokoo M, Nakagomi T, et al. Molecular epidemiology of rotavirus diarrhea among children and adults in Nepal: detection of G12 strains with P[6] or P[8] and a G11P[25] strain. J Clin Microbiol. 2006;44:3499–505. DOI: 10.1128/JCM.01089-06
27. Banyai K, Esona MD, Kerin TK, Hull JJ, Mijatovic S, Vasconez N, et al. Molecular characterization of a rare, human-porcine reassortant rotavirus strain, G11P[6], from Ecuador. Arch Virol. 2009;154:1823–9. DOI: 10.1007/s00705-009-0499-1
28. Rahman M, Sultana R, Ahmed G, Nahar S, Hassan ZM, Saiada F, et al. Prevalence of G2P[4] and G12P[6] rotavirus, Bangladesh. Emerg Infect Dis. 2007;13:18–24. DOI: 10.3201/eid1301.060910
29. Hong SK, Lee SG, Lee SA, Kang JH, Lee JH, Kim JH, et al. Characterization of a G11,P[4] strain of human rotavirus isolated in South Korea. J Clin Microbiol. 2007;45:3759–61. DOI: 10.1128/JCM.01505-07
30. Bohl EH, Theil KW, Saif LJ. Isolation and serotyping of porcine rotaviruses and antigenic comparison with other rotaviruses. J Clin Microbiol. 1984;19:105–11.
31. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947–8. DOI: 10.1093/bioinformatics/btm404
32. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
33. Maes P, Matthijnssens J, Rahman M, Van Ranst M, Rota C. A web-based tool for the complete genome classification of group A rotaviruses. BMC Microbiol. 2009;9:238. DOI: 10.1186/1471-2180-9-238
34. Rosen BI, Parwani AV, Lopez S, Flores J, Saif LJ. Serotypic differentiation of rotaviruses in field samples from diarrheic pigs by using nucleic acid probes specific for porcine VP4 and human and porcine VP7 genes. J Clin Microbiol. 1994;32:311–7.

Address for correspondence: Jelle Matthijnssens, Laboratory of Clinical and Epidemiological Virology, Department of Microbiology and Immunology, Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium; email: jelle.matthijnssens@uz.kuleuven.be

Household Transmission of Pandemic (H1N1) 2009, San Antonio, Texas, USA, April–May 2009

Oliver W. Morgan, Sharyn Parks, Trudi Shim, Patricia A. Blevins, Pauline M. Lucas, Roger Sanchez, Nancy Walea, Fleetwood Loustalot, Mark R. Duffy, Matthew J. Shim, Sandra Guerra, Fernando Guerra, Gwen Mills, Jennifer Verani, Bryan Alsip, Stephen Lindstrom, Bo Shu, Shannon Emery, Adam L. Cohen, Manoj Menon, Alicia M. Fry, Fatimah Dawood, Vincent P. Fonseca, and Sonja J. Olsen

To assess household transmission of pandemic (H1N1) 2009 in San Antonio, Texas, USA, during April 15–May 8, 2009, we investigated 77 households. The index case-patient was defined as the household member with the earliest onset date of symptoms of acute respiratory infection (ARI), influenza-like illness (ILI), or laboratory-confirmed pandemic (H1N1) 2009. Median interval between illness onset in index and secondary case-patients was 4 days (range 1–9 days); the index case-patient was likely to be ≤18 years of age (p = 0.034). The secondary attack rate was 4% for pandemic (H1N1) 2009, 9% for ILI, and 13% for ARI. The secondary attack rate was highest for children <5 years of age (8%–19%) and lowest for adults ≥50 years of age (4%–12%). Early in the outbreak, household transmission primarily occurred from children to other household members and was lower than the transmission rate for seasonal influenza.

On April 15 and 17, 2009, the first 2 cases of pandemic (H1N1) 2009 in the United States were identified among children in California; within 10 weeks, the strain was identified in 99 countries or territories (*1*). Texas was the second US state to confirm human transmission of pandemic (H1N1) 2009. On April 24, 2009, the Texas Department of State Health Services reported 2 patients with laboratory-confirmed pandemic (H1N1) 2009 infection in Guadalupe County. The strain was similar to that isolated previously from patients in Mexico and California (*2*). On June 11, 2009, the World Health Organization raised the pandemic alert to phase 6, indicating that a global pandemic was under way (*3*).

Characterizing transmission dynamics in various settings, such as households, schools, and the community, is critical to the development of appropriate guidance and public health interventions. Household contacts of persons with seasonal influenza are at increased risk for infection (*4–7*), but the household transmission characteristics of pandemic (H1N1) 2009 have yet to be fully characterized. This study reports household secondary attack rates and serial time intervals between illness onset in the index case-patient to illness onset in a household contact. We investigated persons with laboratory-confirmed pandemic (H1N1) 2009 and their household contacts in 1 health service region of Texas.

Methods

Population

The Texas Department of State Health Services consists of 11 health service regions. We conducted our investigation in Health Service Region 8, which includes 28 counties in south-central Texas, bordered on the south by Mexico (Figure). Approximately 2.4 million persons live in the region; 1.5 million live in the city of San Antonio (*8*).

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (O.W. Morgan, S. Parks, F. Loustalot, J. Verani, S. Lindstrom, B. Shu, S. Emery, A.L. Cohen, M. Menon, A.M. Fry, F. Dawood, S.J. Olsen); Department of State Health Services, Austin, Texas, USA (S. Parks, T. Shim, N. Walea, S. Guerra, V.P. Fonseca); United States Air Force School of Aerospace Medicine, Brooks City-Base, Texas, USA (P.M. Lucas, M.R. Duffy, M.J. Shim); San Antonio Metropolitan Health District, San Antonio, Texas, USA (P.A. Blevins, R. Sanchez, F. Guerra, B. Alsip); and Comal County Health Department, New Braunfels, Texas, USA (G. Mills)

DOI: 10.3201/eid1604.091658

Figure. Texas Department of State Health Services Health Service Region 8 (gray shading), Texas, USA.

The Texas Department of State Health Services provides public health services in counties without local health departments. Within Health Service Region 8, public health services for Bexar County are provided by the San Antonio Metropolitan Health District and for Comal County by the Comal County Health Department.

Case Definitions

We defined a laboratory-confirmed case-patient as a resident of Health Service Region 8 who had a positive respiratory specimen showing nucleic acid sequences unique to pandemic (H1N1) 2009; a real-time reverse transcription–PCR (rRT-PCR) assay was used to detect the virus (*9*). For persons with no laboratory test performed, we assessed whether they had influenza-like illness (ILI), defined as fever (measured or unmeasured) with either cough or sore throat; or acute respiratory infection (ARI), defined as ≥2 of the following signs or symptoms: fever, cough, sore throat, and rhinorrhea. The index case-patient was defined as the household member with the earliest symptom onset date of ARI, ILI, or laboratory-confirmed pandemic (H1N1) 2009. A secondary case-patient was defined as a household member with ARI, ILI, or laboratory-confirmed pandemic (H1N1) 2009 and symptom onset 1–9 days after symptom onset in the index case-patient. We chose the maximum interval of 9 days because shedding of seasonal influenza virus uncommonly lasts >8 days (*10*) and the median incubation period for seasonal influenza is ≈1.4 days (*11*). Household members were defined as persons who lived at the same address as a case-patient who had laboratory-confirmed pandemic (H1N1) 2009 infection.

Case Finding

During April 10–May 8, 2009, we identified laboratory-confirmed cases of pandemic (H1N1) 2009 by reviewing 1,167 laboratory records of influenza specimens submitted by healthcare providers for rRT-PCR testing by the regional public health laboratory in San Antonio. We also reviewed 1,251 laboratory records of all specimens submitted by military medical treatment facilities in San Antonio. These specimens were tested for influenza by rRT-PCR at the Epidemiology Laboratory Service of the Department of Defense Global Influenza Surveillance Program at the United States Air Force School of Aerospace Medicine in San Antonio. In addition, we conducted telephone interviews with 540 (67%) of 802 high school students who were reported as absent by their school administrators during April 9–28 in the Texas counties where the first 2 identified case-patients attended school. Respiratory samples were collected from students who reported an acute respiratory illness at the time of interview. Additional case-patients were identified by collecting respiratory samples from nonhousehold contacts of laboratory-confirmed case-patients (i.e., those who had been within 6 feet of someone with ARI for at least 1 hour during the period 1 day before through 7 days after onset of illness in the contact).

Household Investigations

We interviewed case-patients with laboratory-confirmed infection and all household members about the occurrence of illness, receipt of influenza vaccination in the previous 12 months, and medical history. We asked all persons about their use of antiviral medication and reviewed health department pharmacy records where appropriate to ascertain the type, dosage, and timing of antiviral medication and to define whether antiviral medications were prescribed for treatment or prophylaxis. Respiratory samples were collected from household contacts who had an acute respiratory illness at the time of interview; respiratory samples were collected from all members of 9 households identified early in the investigation, regardless of respiratory symptoms.

Sample Collection and Laboratory Testing

Nasal wash samples were collected from military servicemen and their household family members, and nasopharyngeal swabs were collected from all others. Nasal wash samples were sent to the Epidemiology Laboratory Service at the United States Air Force School of Aerospace Medicine; nasopharyngeal swabs were sent to the regional public health laboratory in San Antonio. We used rRT-PCR to test all respiratory samples for seasonal influenza (A/H1 and A/H3 influenza viruses). Specimens positive for influenza A but negative for seasonal influenza by rRT-PCR were sent to the Centers for Disease Control and Preven-

tion (CDC) for confirmatory testing for pandemic (H1N1) 2009 (*12*).

Statistical Analysis

We calculated the serial interval as the number of days from the onset date of illness in the index case-patient to onset date of illness in the secondary case-patient. Secondary household attack rates were calculated by dividing the number of secondary case-patients (excluding the index case-patient) by the total number of household members (excluding the index case-patient). Secondary case-patients for ILI and ARI attack rates also included laboratory-confirmed case-patients. We compared characteristics between groups by using the χ^2 test or Fisher exact test for categorical data and the Wilcoxon signed-rank test for continuous variables (*13*).

Ethics

The collection of information about cases of pandemic (H1N1) 2009 was part of the emergency public health practice response and was not deemed to be research in accordance with the federal human subjects protection regulations (45 Code of Federal Regulations 46.101c and 46.102d) and CDC's Guidelines for Defining Public Health Research and Public Health Non-Research. All protocols pertaining to the pandemic were reviewed for protection concerns and the necessity of Institutional Review Board review by the CDC's National Center for Immunization and Respiratory Diseases (NCIRD) Human Subjects Contact and the NCIRD Associate Director of Science.

Results

We identified 110 persons with laboratory-confirmed pandemic (H1N1) 2009 infection. We were unable to contact 23 (21%) of these persons, and 3 (3%) did not agree to provide further information. Of 84 persons with laboratory-confirmed pandemic (H1N1) 2009 infection who provided information, 77 (92%) lived with ≥1 persons. These 77 households comprised 349 persons; the median household size was 4 persons (range 2–9 persons), including the index case-patient. Seventy five percent of household interviews were conducted ≥8 days (range 0–24 days) after the onset of infection in the index case-patient.

From household interviews, we identified an additional 47 persons who reported respiratory symptoms or had laboratory evidence of pandemic (H1N1) 2009 infection: 13 persons with laboratory-confirmed pandemic (H1N1) 2009 infection, 24 persons with ILI, and 10 persons whose illness met the case definition for ARI only. We did not classify 15 of these persons as secondary case-patients: 8 persons had the same date of symptom onset as the index case-patient; we could not establish the date of symptom onset for 3 persons; and 4 persons reported illness onset 10–15 days after the index case-patient. In 1 household where 2 persons had ILI, 1 had a nasopharyngeal swab that was positive for pandemic (H1N1) 2009; the other was positive for influenza A, but the subtype could not be determined, possibly because of the quality of the sample or because 9 days had elapsed between illness and sample collection, thus decreasing viral load. We considered this person to have laboratory-confirmed pandemic (H1N1) 2009 on the basis of an epidemiologic link to another laboratory-confirmed case. In 2 households where secondary case-pateints were identified, nasal swab samples were obtained from members of all 7 households; 1 person, 14 years of age, who did not report any respiratory symptoms, was positive for pandemic (H1N1) 2009 infection.

Among the 97 symptomatic laboratory-confirmed case-patients (84 identified through case finding and 13 through household investigation), illness onset dates ranged from April 11 through May 8, 2009. Eleven (11%) were ≤4 years of age, 61 (63%) 5–18 years of age, 22 (23%) 19–49 years of age, and 3 (3%) ≥50 years of age. Forty-six (47%) were male. The most common signs and symptoms were fever (93%), cough (91%), rhinnorhea (70%), headache (67%), and sore throat (58%). Vomiting was reported by 26% and diarrhea by 25%. Ninety-two percent of laboratory-confirmed case-patients met the definition for ARI, and 85% met the definition for ILI. One laboratory-confirmed case-patient was hospitalized: a child who was admitted to the hospital with pneumonia in early April. No deaths occurred. Compared with household contacts who did not have laboratory-confirmed pandemic (H1N1) 2009 or did not report respiratory illness, laboratory-confirmed case-patients (index and secondary) were significantly younger (median age 17 vs. 24 years; $p<0.001$).

Secondary case-patients were found in 24 (31%) of 77 households; 5 had 2 secondary case-patients, and 1 had 3 case-patients (Table 1). Secondary infections appeared most likely to be transmitted between children (12/32, 38%) or children to adults (10/32, 31%) than from adults to children (6/32, 19%) or adults to adults (4/32, 13%) ($p = 0.034$). The median serial interval for ARI, ILI, and laboratory-confirmed pandemic (H1N1) 2009 combined was 4 days (range 1–9 days) (Table 1; online Appendix Figure, www.cdc.gov/EID/content/16/4/631-appF.htm). Antiviral treatment was given to the index case-patient of 23 (72%) of 32 secondary case-patients; in these households, the serial interval was 3 days, compared with 5 days when the index case-patient was not given treatment ($p = 0.17$). Inclusion of 5 household contacts with illness that occurred 10–15 days after symptom onset of the index case-patient did not alter the median serial interval estimate. The median serial interval also remained unchanged when only members of households interviewed ≥9 days after the onset of symptoms in the household index case-patient were

included. Limiting the estimate of median serial interval to include only persons with ILI or laboratory-confirmed case-patients reduced the median serial interval to 3 days (range 1–8 days).

The secondary household attack rate was 13% for ARI, 9% for ILI, and 4% for laboratory-confirmed pandemic (H1N1) 2009 (Table 2). Secondary attack rates were highest in children <5 years of age and were higher in children 5–18 years of age than in adults 19–49 and ≥50 years of age (Table 2). By household size, secondary attack rates for ARI, ILI, and laboratory-confirmed pandemic (H1N1) 2009 were highest in households with 2–3 persons (ARI 23%, ILI 23%, laboratory-confirmed pandemic [H1N1] 2009 6%) and were lowest in households with 7–9 persons (ARI 9%, ILI 9%, laboratory-confirmed pandemic [H1N1] 2009 2%) (online Appendix Table 1, www.cdc.gov/EID/content/16/4/631-appT1.htm). The secondary household attack rate did not differ by receipt of seasonal influenza vaccination in the previous 12 months (online Appendix Table 2, www.cdc.gov/EID/content/16/4/631-appT2.htm).

Treatment with antiviral medication was given to 77% of index case-patients (57/74 of persons with ARI, ILI, and laboratory-confirmed pandemic [H1N1] 2009 combined for whom data were available) and 72% of secondary cases (23/32 of ARI, ILI, and laboratory-confirmed pandemic [H1N1] 2009 combined); 90% took oseltamivir; 7% took zanamivir; and 3% took an unknown type of antiviral medication. Neither the age of the index case-patient, household

Table 1. Index and secondary household case-patients with ARI, ILI, or laboratory-confirmed pandemic (H1N1) 2009, Region 8, Texas, April–May, 2009*

	Index case-patients			Secondary case-patients			Serial
Household	Date of onset	Age, y	Case definition	Date of onset	Age, y	Case definition	interval, d†
A	Apr 18	14‡	A, no subtype	Apr 25	21	Pandemic (H1N1) 2009	7
B	Apr 19	5	Pandemic (H1N1) 2009	Apr 21	9	Pandemic (H1N1) 2009	2
C	Apr 22	18	Pandemic (H1N1) 2009	Apr 25	<1	Pandemic (H1N1) 2009	3
D	Apr 26	1	Pandemic (H1N1) 2009	May 4	27	Pandemic (H1N1) 2009	2
E	Apr 26	16	Pandemic (H1N1) 2009	Apr 27	51	Pandemic (H1N1) 2009	1
				Apr 27	8	ILI	1
F	Apr 27	<1	Pandemic (H1N1) 2009	Apr 29	22	Pandemic (H1N1) 2009	2
				May 6	47	ARI	9
G	Apr 27	16	Pandemic (H1N1) 2009	May 1	16	Pandemic (H1N1) 2009	4
				May 1	14	Pandemic (H1N1) 2009	4
H	Apr 29	6	Pandemic (H1N1) 2009	Apr 3	<1	Pandemic (H1N1) 2009	1
I	May 3	33	Pandemic (H1N1) 2009	May 7	15	Pandemic (H1N1) 2009	4
				May 8	14	Pandemic (H1N1) 2009	5
Subtotal no. case-patients			9			13	3 (1–9)
J	Apr 20	17	Pandemic (H1N1) 2009	Apr 26	14	ARI	6
K	Apr 24	71	Pandemic (H1N1) 2009	Apr 27	65	ILI	3
L	Apr 25	16	Pandemic (H1N1) 2009	Apr 27	16	ILI	2
M	Apr 25	12	Pandemic (H1N1) 2009	Apr 28	30	ARI	3
				Apr 28	33	ARI	3
				Apr 30	6	ARI	5
N	Apr 26	30	Pandemic (H1N1) 2009	May 1	28	ARI	5
O	Apr 27	33	Pandemic (H1N1) 2009	May 5	53	ARI	8
P	Apr 28	25	Pandemic (H1N1) 2009	May 4	14	ILI	6
Q	Apr 29	1	Pandemic (H1N1) 2009	May 1	21	ILI	2
				May 2	2	ILI	3
R	Apr 29	8	Pandemic (H1N1) 2009	May 3	44	ARI	4
S	May 1	6	Pandemic (H1N1) 2009	May 3	45	ILI	2
Subtotal no. case-patients			10			13	3 (1–8)
T	Apr 17	11	ILI	Apr 21	18	Pandemic (H1N1) 2009	4
U	Apr 18	48	ILI	Apr 26	10	Pandemic (H1N1) 2009	8
V	Apr 23	53	ILI	Apr 26	42	Pandemic (H1N1) 2009	3
W	Apr 24	5	ILI	Apr 29	<1	Pandemic (H1N1) 2009	5
X	Apr 28	26	ILI	May 2	7	Pandemic (H1N1) 2009	4
				May 3	4	ILI	5
Subtotal no. case-patients			5			6	4.5 (3–8)
Total no. case-patients			24			32	4 (1–9)

*ARI, acute respiratory infection; ILI, influenza-like illness (fever measured or subjective and cough or sore throat).
†Median (range) number of days between symptom onset of the index and secondary case-patients.
‡The influenza virus from this person could not be subtyped, possibly because of the quality of the sample or the length of time from symptom onset to sample collection. We considered this case-patient to have been infected with pandemic (H1N1) 2009.

Table 2. Household secondary attack rates for ARI, ILI, and laboratory-confirmed pandemic (H1N1) 2009, by age group, Region 8, Texas, April–May 2009*

Illness type by age group, y	No. index case-patients	Household contacts			Household members not included	Secondary attack rate (A/A + B), %
		Secondary case-patients, A	Not ill, B	Total household contacts, A + B		
ARI						
<5	7	5	23	28	1	18
5–18	50	13	83	96	3	14
19–49	17	11	96	107	3	10
≥50	3	3	22	25	1	12
All ages	77	32	224	256	8	13
ILI						
<5	6	5	23	28	2	18
5–18	50	11	86	97	2	11
19–49	18	6	102	108	1	6
≥50	3	2	23	25	1	8
All ages	77	24	234	258	6	9
Laboratory-confirmed pandemic (H1N1) 2009						
<5	8	2	26	28	0	7
5–18	51	5	92	97	1	5
19–49	16	3	108	111	0	3
≥50	2	1	26	27	0	4
All ages	77	11	252	263	1	4

*ARI, acute respiratory infection; ILI, influenza-like illness (fever measured or subjective and cough or sore throat). Ill household members were not included in the calculation of the secondary attack rate if they had the same symptom onset as the index case or if symptom onset was not known.

size, nor diagnosis of the index patient (with ARI, ILI, or laboratory-confirmed pandemic [H1N1] 2009) were predictive of treatment with antiviral medication. The secondary household attack rates for ARI, ILI, and laboratory-confirmed pandemic (H1N1) 2009 combined in households where the index case-patient was given antiviral treatment was 12% compared with 16% in other households (p = 0.64) (Table 3). Antiviral prophylaxis was given to 39% of household contacts (92/235 with data available) (Table 3), and the secondary attack rate of ARI, ILI, and laboratory-confirmed pandemic (H1N1) 2009 combined was 14% (12/83) in households where the index patient took treatment, compared with 66% (6/9) (p = 0.003) in households where the index patient did not take treatment (Table 3).

Discussion

During an outbreak of pandemic (H1N1) 2009 in the San Antonio, Texas, area, we identified 97 persons with laboratory-confirmed infection in 77 households. The epidemiologic and clinical features were similar to summary reports from the United States (*14*,*15*) and other countries (*15*,*16*). Nearly one third of households had secondary case-patients who also had respiratory illness, with a median of 4 days between onset of illness in the index case-patient and household members, a finding similar to that for seasonal influenza (*17*).

The secondary attack rate was 4% for laboratory-confirmed pandemic (H1N1) 2009, 9% for ILI, and 13% for ARI. In general, these rates are lower than for seasonal influenza and lower than anticipated for a pandemic strain, although rates vary from 13% to 30%, depending on influenza subtype and year and pandemic period (*4*,*5*,*18*–*21*). The highest proportion of laboratory-confirmed pandemic (H1N1) 2009 and secondary attack rates occurred in children, a finding consistent with the epidemiology of seasonal and pandemic influenza, where we know children experience higher rates of illness (*4*,*5*,*7*) and higher secondary attack rates (*19*). Adults may have some cross-protection against pandemic (H1N1) 2009 from antibodies developed during infections with seasonal influenza A virus (H1N1) (*22*–*24*).

Four randomized controlled trials of zanamivir and oseltamivir for seasonal influenza have shown that these antiviral medications reduce but do not eliminate viral shedding

Table 3. Household secondary attack rates for ARI, ILI, and laboratory-confirmed pandemic (H1N1) 2009, by antiviral medication treatment and prophylaxis, Region 8, Texas, April–May 2009*

Type of contact	No. contacts	Index case-patient received antiviral treatment (attack rate, %)		
		Yes	No	p value†
All contacts of index case-patients	235	22/185 (12)	8/50 (16)	0.64
Contacts who took antiviral prophylaxis	92	12/83 (14)	6/9 (67)	0.003

*ARI, acute respiratory infection; ILI, influenza-like illness (fever measured or subjective and cough or sore throat).
†Fisher exact test comparing the secondary attack rate for any treatment to no antiviral treatment. Data about antiviral medication were missing for 2 index case-patients and 15 contacts.

and are effective in preventing disease among household contacts, especially if taken within 48 hours of illness onset in the index case-patient (*19*,*20*,*25*,*26*). We found that secondary attack rates for all households were lower when the index case-patient received treatment, although this difference was not significant. The role of prophylaxis in the absence of treatment of the index case-patient was difficult to determine; our investigation included only a small number of such persons. Nevertheless, because most index and secondary case-patients received antiviral treatment, household secondary attack rates may have been reduced.

Our investigation has several limitations. Because early case finding was most intensive among high school children associated with school outbreaks, our cohort may have been biased in favor of households where the index case-patients were children; however, this would not explain a lower secondary attack rate among adult household contacts. We did not assess the role of mild or asymptomatic pandemic (H1N1) 2009 infection because we collected respiratory samples only; serologic assays to detect influenza antibodies are the most sensitive method for detecting asymptomatic infection, but virus assays for pandemic (H1N1) 2009 were not available at the time of the investigation. Volunteer challenge studies with seasonal influenza viruses have found that up to 30% of infected persons may be asymptomatic and could be identified through serologic testing (*10*). Because 25% of household interviews were conducted <8 days after onset of illness of the index case-patient, we may have underestimated the secondary attack rate if these households had secondary case-patients with long serial intervals. However, when we restricted our analysis to persons interviewed ≥8 days after onset of symptoms in the index case-patient, we found no difference in the median serial interval or distribution of attack rates by age. Conversely, household members interviewed ≥8 days after onset of illness in the index case-patient may have had incomplete recall of acute respiratory infections. Finally, some of the secondary illnesses may have been acquired in the community, leading to overestimate of household secondary attack rates.

We found that pandemic (H1N1) 2009 disproportionately affected children, who in turn posed a risk for secondary household transmission, especially to their caregivers and siblings. The Advisory Committee on Immunization Practices (2009) recommends that children 6–18 years of age and caregivers of infants be included as initial target groups for the new pandemic (H1N1) 2009 vaccine (*27*), which may reduce household transmission. As pandemic (H1N1) 2009 continues to spread internationally, ongoing investigations are needed to shed further light on transmission dynamics, to monitor epidemiologic changes over time, and to assess the effectiveness of public health interventions.

Acknowledgments

We thank the study participants and their household members and are grateful for the contribution of a large number of colleagues at the Texas Department of State Health Services, the San Antonio Metropolitan Health District, the Comal County Health Department, the United States Air Force School of Aerospace Medicine, and the Centers for Disease Control and Prevention in Atlanta for their assistance with the study.

Dr Morgan is an epidemiologist with the International Emerging Infections Program, National Center for Preparedness Detection and Control of Infections Disease, Centers for Disease Control and Prevention, Atlanta, Georgia, USA. His primary research interest is the global epidemiology of respiratory diseases.

References

1. World Health Organization. Influenza A (H1N1)—update 52. Laboratory-confirmed cases of new influenza A (H1N1) as officially reported to WHO by States Parties to the International Health Regulations (2005) [cited 2009 Jun 22]. http://www.who.int/csr/don/2009_06_22/en/index.html
2. Centers for Disease Control and Prevention. Update: swine influenza A (H1N1) infections—California and Texas, April 2009. MMWR Morb Mortal Wkly Rep. 2009;58:435–7.
3. World Health Organization. Transcript of statement by Margaret Chan, Director-General of the World Health Organization. 11 June 2009 [cited 2009 Jul 7]. http://www.who.int/mediacentre/influenzaAH1N1_presstranscript_20090611.pdf
4. Fox JP, Cooney MK, Hall CE, Foy HM. Influenza virus infections in Seattle families, 1975–1979. II. Pattern of infection in invaded households and relation of age and prior antibody to occurrence of infection and related illness. Am J Epidemiol. 1982;116:228–42.
5. Fox JP, Hall CE, Cooney MK, Foy HM. Influenzavirus infections in Seattle families, 1975–1979. I. Study design, methods and the occurrence of infections by time and age. Am J Epidemiol. 1982;116:212–27.
6. Foy HM, Cooney MK, Allan I. Longitudinal studies of types A and B influenza among Seattle schoolchildren and families, 1968–74. J Infect Dis. 1976;134:362–9.
7. Taber LH, Paredes A, Glezen WP, Couch RB. Infection with influenza A/Victoria virus in Houston families, 1976. J Hyg (Lond). 1981;86:303–13.
8. Texas Department of State Health Services. Health Service Region 8. 2009 [cited 2009 Jun 22]. http://www.dshs.state.tx.us/region8/default.shtm
9. Centers for Disease Control and Prevention. Interim guidance on case definitions to be used for investigations of novel influenza A (H1N1) cases. 2009 [cited 2009 Jun 27]. http://www.cdc.gov/H1N1flu/guidance/
10. Carrat F, Vergu E, Ferguson NM, Lemaitre M, Cauchemez S, Leach S, et al. Time lines of infection and disease in human influenza: a review of volunteer challenge studies. Am J Epidemiol. 2008;167:775–85. DOI: 10.1093/aje/kwm375
11. Lessler J, Reich NG, Brookmeyer R, Perl TM, Nelson KE, Cummings DA. Incubation periods of acute respiratory viral infections: a systematic review. Lancet Infect Dis. 2009;9:291–300. DOI: 10.1016/S1473-3099(09)70069-6
12. Centers for Disease Control and Prevention. CDC protocol of real-time RT-PCR for influenza A (H1N1) 2009 [cited 2009 Jun 23]. http://www.who.int/csr/resources/publications/swineflu/realtimeptpcr/en/index.html

13. Altman DG. Practical statistics for medical research. London: Chapman and Hall/CRC Press; 1999.
14. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009;360:2605–15. DOI: 10.1056/NEJMoa0903810
15. Centers for Disease Control and Prevention. Update: novel influenza A (H1N1) virus infection—Mexico, March–May, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:585–9.
16. Health Protection Agency; Health Protection Scotland; National Public Health Service for Wales; HPA Northern Ireland Swine influenza investigation teams. Epidemiology of new influenza A (H1N1) virus infection, United Kingdom, April–June 2009. Euro Surveill. 2009;14. pii: 19232.
17. Cowling BJ, Fang VJ, Riley S, Malik Peiris JS, Leung GM. Estimation of the serial interval of influenza. Epidemiology. 2009;20:344–7. DOI: 10.1097/EDE.0b013e31819d1092
18. Woodall J, Rowson KE, Mc DJ. Age and Asian influenza, 1957. BMJ. 1958;2:1316–8. DOI: 10.1136/bmj.2.5108.1316
19. Hayden FG, Belshe R, Villanueva C, Lanno R, Hughes C, Small I, et al. Management of influenza in households: a prospective, randomized comparison of oseltamivir treatment with or without postexposure prophylaxis. J Infect Dis. 2004;189:440–9. DOI: 10.1086/381128
20. Welliver R, Monto AS, Carewicz O, Schatteman E, Hassman M, Hedrick J, et al. Effectiveness of oseltamivir in preventing influenza in household contacts: a randomized controlled trial. JAMA. 2001;285:748–54. DOI: 10.1001/jama.285.6.748
21. Blumenfeld HL, Kilbourne ED, Louria DB, Rogers DE. Studies on influenza in the pandemic of 1957–1958. I. An epidemiologic, clinical and serologic investigation of an intrahospital epidemic, with a note on vaccination efficacy. J Clin Invest. 1959;38:199–212. DOI: 10.1172/JCI103789
22. Centers for Disease Control and Prevention. Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine. MMWR Morb Mortal Wkly Rep. 2009;58:521–4.
23. Cate TR, Kasel JA, Couch RB, Six HR, Knight V. Clinical trials of bivalent influenza A/New Jersey/76-A/Victoria/75 vaccines in the elderly. J Infect Dis. 1977;136(Suppl):S518–25.
24. Dolin R, Wise TG, Mazur MH, Tuazon CU, Ennis FA. Immunogenicity and reactogenicity of influenza A/New Jersey/76 virus vaccines in normal adults. J Infect Dis. 1977;136(Suppl):S435–42.
25. Hayden FG, Gubareva LV, Monto AS, Klein TC, Elliot MJ, Hammond JM, et al. Inhaled zanamivir for the prevention of influenza in families. Zanamivir Family Study Group. N Engl J Med. 2000;343:1282–9. DOI: 10.1056/NEJM200011023431801
26. Monto AS, Pichichero ME, Blanckenberg SJ, Ruuskanen O, Cooper C, Fleming DM, et al. Zanamivir prophylaxis: an effective strategy for the prevention of influenza types A and B within households. J Infect Dis. 2002;186:1582–8. DOI: 10.1086/345722
27. Centers for Disease Control and Prevention. Use of influenza A (H1N1) 2009 monovalent vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. National Center for Immunization and Respiratory Diseases. MMWR Recomm Rep. 2009;58(RR-10):1–8.

Address for correspondence: Oliver W. Morgan, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop C12, Atlanta, GA 30333, USA; email: omorgan@cdc.gov

Escherichia albertii in Wild and Domestic Birds

J. Lindsay Oaks, Thomas E. Besser, Seth T. Walk, David M. Gordon, Kimberlee B. Beckmen, Kathy A. Burek, Gary J. Haldorson, Dan S. Bradway, Lindsey Ouellette, Fred R. Rurangirwa, Margaret A. Davis, Greg Dobbin, and Thomas S. Whittam[1]

Escherichia albertii has been associated with diarrhea in humans but not with disease or infection in animals. However, in December 2004, *E. albertii* was found, by biochemical and genetic methods, to be the probable cause of death for redpoll finches (*Carduelis flammea*) in Alaska. Subsequent investigation found this organism in dead and subclinically infected birds of other species from North America and Australia. Isolates from dead finches in Scotland, previously identified as *Escherichia coli* O86:K61, also were shown to be *E. albertii*. Similar to the isolates from humans, *E. albertii* isolates from birds possessed intimin (*eae*) and cytolethal distending toxin (*cdtB*) genes but lacked Shiga toxin (s*tx*) genes. Genetic analysis of *eae* and *cdtB* sequences, multilocus sequence typing, and pulsed-field gel electrophoresis patterns showed that the *E. albertii* strains from birds are heterogeneous but similar to isolates that cause disease in humans.

In late December 2004, deaths of common redpoll finches (*Carduelis flammea*) were reported around the city of Fairbanks, Alaska, USA, coincident with a prolonged period of extreme cold (below –40°F). The final reported death occurred on February 24. At the beginning of the outbreak, the local at-risk population was estimated to be ≈8,000 redpoll finches, a historic high for the area. Although ≈100 deaths were documented, the actual number is assumed to be considerably higher.

Outbreaks of disease in wild finches (family Fringillidae) have been associated with *Salmonella enterica* subsp. *enterica* serotype Typhimurium, *Mycoplasma gallisepticum*, poxvirus, and *Escherichia coli* (*1*–*6*). Diagnostic investigation into the Alaska outbreak identified *Escherichia albertii* as the probable cause of death and as a new pathogen for birds. *E. albertii* had been identified as an enteric pathogen of humans in Asia (*7*) and, more recently, in Africa and North America (T.S. Whittam and H. Steinsland, unpub. data), but to our knowledge, until this outbreak its presence in animals had not been observed.

We describe the identification and characterization of *E. albertii* from birds in North America, Europe, and Australia. We show that bacterial isolates from dead finches in Scotland, previously identified as *E. coli* O86:K61, were actually *E. albertii*. The genetic diversity of 2 virulence loci (intimin and cytolethal distending toxin) for the bird isolates was compared with characterized human pathotypes. We also determined genetic relatedness among isolates from birds and humans by multilocus sequence typing (MLST) and clonality of multiple isolates from dead or clinically healthy birds by pulsed-field gel electrophoresis (PFGE).

Methods

Bird Isolate Collection

In the United States during 2004–2005, dead redpoll finches from Alaska were submitted to the Alaska Department of Fish and Game. Three clinically healthy redpoll finches were trapped near the outbreak site. Standard necropsies included gross examination and collection of tissues into 10% neutral buffered formalin for histopatho-

Author affiliations: Washington State University, Pullman, Washington, USA (J.L. Oaks, T.E. Besser, G.J. Haldorson, D.S. Bradway, F.R. Rurangirwa, M.A. Davis); University of Michigan Health System, Ann Arbor, Michigan, USA (S.T. Walk); The Australian National University, Canberra, Australian Capital Territory, Australia (D.M. Gordon); Alaska Department of Fish and Game, Fairbanks, Alaska, USA (K.B. Beckmen); Alaska Veterinary Pathology Services, Eagle River, Alaska, USA (K.A. Burek); Michigan State University, East Lansing, Michigan, USA (L. Ouellette, T.S. Whittam); and University of Prince Edward Island, Charlottetown, Prince Edward Island, Canada (G. Dobbin)

DOI: 10.3201/eid1604.090695

[1]Deceased.

logic examination. Fresh tissues were frozen for microbiologic assays. Two other dead birds, a captive adult gyrfalcon (*Falco rusticulos*) from Idaho and a chicken (*Gallus gallus*) from Washington, were submitted for diagnosis to the Washington Animal Disease Diagnostic Laboratory in Pullman, Washington, USA.

In Canada in 2005, isolates were obtained from feces of clinically healthy redpolls and pine siskins (*Carduelis pinus*) trapped on Prince Edward Island. A total of 158 finches were sampled and included redpolls, pine siskins, and purple finches (*Carpodacus purpureus*).

In Australia in 2001–2002, isolates from birds were obtained from feces of clinically healthy domestic and trapped wild birds (*8*). Domestic birds included 9 chickens (*G. gallus*), 4 geese (*Anser anser domesticus*), 3 ducks (*Anas platyrhynchos domesticus*), and 1 guinea fowl (family Numididae). Wild birds totaled 634 birds representing 112 species.

In Scotland, isolates obtained during 1998–2000 from dead birds—Eurasian siskins (*Carduelis spinus*), greenfinches (*Carduelis chloris*), and chaffinches (*Fringilla coelebs*)—previously identified as *E. coli* O86:K61 (*4*), were obtained from M.J. Woodward (Veterinary Laboratories Agency Weybridge, Inverness, UK). Information about all isolates is summarized in Table 1.

Isolation and Identification of Bacteria

To detect Enterobacteriaceae, we inoculated tissues, intestinal contents, or feces onto MacConkey agar and incubated the plates at 35°C. Isolated colonies were char-

Table 1. *Escherichia albertii* isolates and host information

Isolate	Origin (state)*	Host species of origin	Clinical status	Year of isolation	Reference
1568-05-27C†	USA (AK)	Redpoll finch (*Carduelis flammea*)	Dead	2005	This study
1568-05-27D†	USA (AK)	Redpoll finch (*C. flammea*)	Dead	2005	This study
1615-05-A†	USA (AK)	Redpoll finch (*C. flammea*)	Dead	2005	This study
1615-05-B†	USA (AK)	Redpoll finch (*C. flammea*)	Dead	2005	This study
1297-05-19†‡§¶	USA (AK)	Redpoll finch (*C. flammea*)	Dead	2005	This study
7991-07†‡§¶	USA (WA)	Chicken (*Gallus gallus*)	Dead	2007	This study
12055-07†‡§¶	USA (ID)	Gyrfalcon (*Falco rusticulos*)	Dead	2007	This study
5419-05-R†‡§¶	Canada	Redpoll finch (*C. flammea*)	Healthy	2005	This study
5419-05-S†‡	Canada	Pine siskin (*Carduelis pinus*)	Healthy	2005	This study
EC370-98†‡§¶	Scotland	Finch spp. (*Carduelis* spp.)	Dead	1998	(*9*)
EC558-00†	Scotland	Eurasian siskin (*Carduelis spinus*)	Dead	2000	(*9*)
EC744-99†	Scotland	Greenfinch (*Carduelis chloris*)	Dead	1999	(*9*)
EC746-99‡	Scotland	Eurasian siskin (*C. spinus*)	Dead	1999	(*9*)
EC748-99†	Scotland	Greenfinch (*C. chloris*)	Dead	1999	(*9*)
B090†‡¶	Australia	Magpie (*Gymnorhina tibicen*)	Healthy	2001	This study
B101†‡§¶	Australia	Magpie(*G. tibicen*)	Healthy	2001	This study
B156†‡§¶	Australia	Magpie (*G. tibicen*)	Healthy	2001	This study
B249†‡¶	Australia	Magpie (*G. tibicen*)	Healthy	2001	This study
B198†‡§¶	Australia	Honeyeater (*Melithreptus brevirostris*)	Healthy	2002	This study
B992†‡§¶	Australia	Wren (*Malurus cyaneus*)	Healthy	2001	This study
B1086†‡§¶	Australia	Fantail (*Rhipidura fulginosa*)	Healthy	2002	This study
B1068†‡§¶	Australia	Chicken (*G. gallus*)	Healthy	2002	This study
B1074†‡§¶	Australia	Chicken (*G. gallus*)	Healthy	2002	This study
616‡	No data	Human	Diarrhea	No data	(*10*)
3103-99‡	USA (IL)	Human	Diarrhea	No data	(*10*)
C-425‡§	No data	Human	Diarrhea	No data	(*10*)
106A5‡§	Guinea-Bissau	Human	Healthy	1997	(*11*)
9194†‡	Bangladesh	Human	Diarrhea	1990	(*10*)
19982‡§¶	Bangladesh	Human	Diarrhea	1990	(*10*)
79D4†‡	Guinea-Bissau	Human	Healthy	1997	(*11*)
97F8‡	Guinea-Bissau	Human	Healthy	1997	(*11*)
M2005000616 #8‡	USA (MN)	Human	Diarrhea	No data	T.S. Whittam, unpub data
I2005002880 #36‡	USA (MN)	Human	Diarrhea	No data	T.S. Whittam, unpub data
K-694¶	Bangladesh	Human	Diarrhea	No data	(*10*)
K-1‡§¶	Bangladesh	Human	Diarrhea	No data	(*10*)

*AK, Alaska; WA, Washington; ID, Idaho; IL, Illinois; MN, Minnesota.
†Included in pulsed-field gel electrophoresis analysis.
‡Included in multilocus sequence typing analysis.
§Included in *eae* allele analysis.
¶Included in *cdtB* allele analysis.

acterized by fermentation of lactose and glucose, production of oxidase, and production of indole from tryptophan. Additional biochemical characterization was performed by using a commercial kit (API 20E; bioMérieux, Hazelwood, MO, USA). *E. coli* serotyping was performed by the Gastroenteric Disease Center (Wiley Laboratory, Pennsylvania State University, University Park, PA, USA).

Genetic identification was based on 16S rRNA gene sequencing and/or PCR to detect housekeeping gene polymorphisms unique for the *E. albertii*/*Shigella boydii* lineage. 16S rRNA analysis was performed on 1 isolate from Alaska by sequencing >1,400 nt of the 16S rRNA gene (*12*). The amplicon was cloned into the pCR2.1 sequencing vector (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA, USA) and sequenced bidirectionally by automated dideoxy DNA methods. Partial 16S rRNA sequences of ≈500 bp of the 5′ end, including the V1, V2, and V3 variable regions (*13*), were determined for other isolates with the same primers, after which direct dideoxy sequencing was performed. A sequence similarity search was performed by searching the GenBank database with BLASTN.2.2.3 (*14*), and sequences were aligned with ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html). PCR was used to detect *E. albertii* lineage–specific genetic polymorphisms in the housekeeping genes *lysP* and *mdh* (*10*). As a positive control, PCR for the gene *clpX*, which is conserved in *E. coli*, *Shigella*, and the *E. albertii*/*S. boydii* lineage, was performed as described (*10*), except a corrected primer sequence for *clpX*_28 (5′-TGG CGT CGA GTT GGG CA-3′) (T.S. Whittam, unpub. data) was used. The negative control for the *lysP* and *mdh* PCR was *E. coli* strain DH10b.

Virulence Gene PCR and Sequence Analysis

PCR was used to test isolates for virulence genes found in Enterobacteriaceae—the central conserved region of intimin (*eae*, the attaching-and-effacing ligand), heat-stable enterotoxin (*sta*), and Shiga toxins (*stx1* and *stx2*)—as described (*15*). Positive controls were *E. coli* strains S2 (for *sta*) and S14 (for *stx1*, *stx2*, and *eae*) from the Pennsylvania State University *E. coli* Reference Center. A multiplex PCR protocol that amplified the consensus portion of the B subunit of the cytolethal distending toxin gene (*cdtB*) as described by Toth (*16*) was modified by using each of the primer pairs (s1/as1 and s2/as2) individually to screen for *cdtB* in all isolates.

For sequencing *eae*, PCR primers that amplified ≈800 nt of the variable 3′ end of the *eae* gene were used as described (*9*). Because these primers did not work for all bird isolates, additional primer sequences were either taken from other studies or designed for this study (Tables 2 and 3). Sequence analysis was based on ≈726 nt in the 3′ variable region of *eae*, which corresponded to amino acids 33–275 within the C-terminal 280 aa of intimin (Int280). Nucleotide sequences were determined for each of the *cdtB* products obtained by using the s1/as1 (403 bp) and s2/as2 (411 bp) primer pairs (*16*). Predicted amino acid sequences for *eae* and *cdtB* were aligned with reference alleles by using the ClustalW method and MegAlign software (DNASTAR, Madison, WI, USA). Neighbor-joining dendrograms were constructed by using MEGA version 4 (*18*) with the p-distance metric and pairwise gap deletion.

MLST

MLST was performed on 26 isolates of *E. albertii* (Table 1) as described (*10*), with slight modification. Briefly, partial gene sequences for 6 conserved housekeeping loci (*aspC*, *clpX*, *fadD*, *icdA*, *lysP*, and *mdh*) were obtained by PCR and direct sequenced by automated dideoxy sequencing. Raw sequences were aligned by using Seqman Pro software (DNASTAR). Sequences for 11 *E. albertii* isolates from humans and 6 common *E. coli* pathotypes were obtained from www.shigatox.net. *E. coli* was used as an outgroup; strains used were enterohemorrhagic *E. coli* strain EDL933, *Shigella flexneri* strain 2747-71, enteroaggregative *E. coli* strain 042, enteropathogenic *E. coli* strain e2348/69, uropathogenic *E. coli* strain CFT073, and *E. coli* K-12. A neighbor-joining dendrogram was based on the concatenated nucleotide sequence and the maximum composite likelihood model by using MEGA version 4 (*18*). Details of the MLST procedure, including allelic typing and sequence type assignment methods, can be found at www.shigatox.net.

Table 2. Primers used for amplification and sequencing of *eae* gene of *Escherichia albertii*

Primer	Sequence, 5′ → 3′	Position (GenBank accession no.)	Reference
Intimin γ F	CGTTGAAGTCGAGTACGCCA	1867–1887 (AF081185)	(*9*)
Intimin γ R	TTCTACACAAACCGCATAGA	2782–2803 (AF081185)	(*9*)
EaeA-F	CAAACCAAGGCCAGCATTAC	1963–1982 (AF081185)	This study
EaeA-R outer	CCCCAAGAGAGAGGGTTCTT	2743–2763 (AF081185)	This study
EaeA-R inner	ACTTGATACCCCAGACCTTCA	2703–2725 (AF081185)	This study
EscD-R1	GTATCAACATCTCCCGCCA	27918–27937 (AF022236)	(*17*)
Intimin-R2	CAGAATATTAAACAAGCGCAGTTG	3103–3126 (FJ609833)	This study
EaeA F06s	GTAACGGACTTTACGGCTGATA	1803–1824 (FJ609833)	(*10*)
Intimin B101 int R	TGACCATATTGCAACCA	2460–2476 (FJ609833)	This study
Intimin B156 int R	TGACCATATCGCAACCA	2459–2475 (FJ609822)	This study

Table 3. Primer sets used for amplification and sequencing of *eae* gene of *Escherichia albertii* in specific isolates

Isolate	PCR amplification	Sequencing
1297–05–19	Intimin γ F, intimin γ R	Intimin γ F, intimin γ R
7991–07	EaeA-F, EaeA-R outer	EaeA-F, EaeA-R inner
12055–07	Intimin γ F, intimin γ R	Intimin γ F, intimin γ R
5419–05-R	Intimin γ F, intimin γ R	Intimin γ F, intimin γ R
EC370–98	Intimin γ F, intimin γ R	Intimin γ F, intimin γ R
EC746–99	Intimin γ F, intimin γ R	Intimin γ F, intimin γ R
B101	Intimin γ F, intimin γ R	Intimin γ F, intimin γ R
B156	EaeA-F, Intimin-R2	EaeA-F, intimin-R2, intimin B156 int R
B198	EaeA-F, intimin-R2	EaeA-F, intimin-R2, intimin B156 int R
B992	EaeA F06s, EscD-R1	EaeA F06s, EscD-R1, EaeA-F, intimin B101 int R
B1086	EaeA-F, intimin γ R	EaeA-F, intimin γ R, EaeA-R outer
B1068	EaeA F06s, EscD-R1	EaeA F06s, EscD-R1, EaeA-F
B1074	EaeA F06s, EscD-R1	EaeA F06s, EscD-R1, EaeA-F

An overall phylogenetic representation of the genus *Escherichia* was generated by combining nucleotide sequence data from GenBank for *Escherichia fergusonii* with outgroup strains *Salmonella bongori*, *S. enterica* subsp. *enterica* serotype Typhi, and *S. enterica* subsp. *enterica* serotype Typhimurium. A neighbor-net network analysis was generated by using SplitsTree 4 software (*19*) (Figure 1, inset).

PFGE

E. albertii isolates were compared by using a standard PFGE method (*20*) with minor modifications. Briefly, fragments of *Xba*I-digested bacterial DNA were separated in 1% agarose gel by using a CHEF-DR III PFGE apparatus (Bio-Rad, Hercules, CA, USA); pulse times were ramped from 2.2 to 54.2 seconds over 19 hours. Digital gel images were analyzed with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium) by using the unweighted pair group method with arithmetic mean algorithm for cluster analysis of Dice similarity coefficients with a position tolerance of 2%.

Nucleotide Sequence Accession Numbers

Nucleotide sequences from this study were deposited in GenBank. Their accession numbers are EU926632–EU926649 and GQ140242–GQ140261.

Results

Pathologic Findings

Redpoll finches from the Alaska outbreak were typically found dead without obvious signs of disease. All those evaluated had adequate pectoral muscle mass, suggestive of acute death. Some had green fecal material pasted around their cloacae, suggestive of diarrhea. Gross lesions were inconsistent, but a few birds had darkened intestines distended with excessive yellow to green digesta. Histologic lesions were also inconsistent, but when present they were consistent with acute, severe, fibrinous, and necrotizing proventriculitis; multifocal heterophilic enteritis; and small-crypt abscessation. For some, gram-negative bacilli in large numbers were observed within the intestinal lumens. Attachment of bacteria to intestinal epithelial cells was not observed, although autolysis precluded assessment of the epithelium and ultrastructural studies to detect attaching-and-effacing lesions. No lesions consistent with septicemia were observed in any affected redpolls.

The affected gyrfalcon had appeared clinically healthy until found dead. Histologic examination failed to demonstrate enteric lesions, although evidence of septicemia was found. The chicken from Washington died after

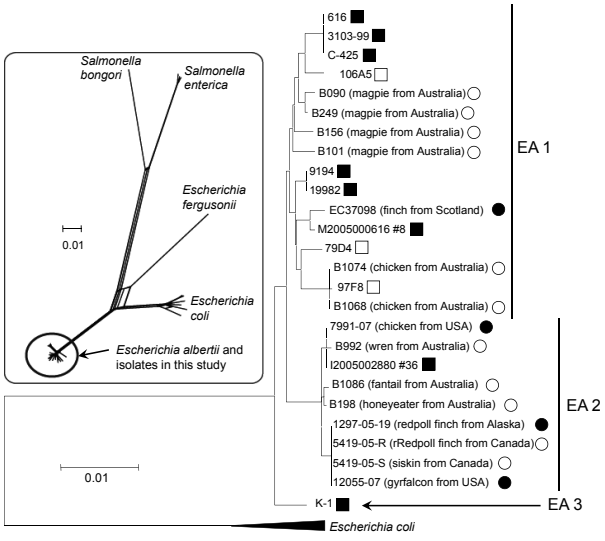


Figure 1. Neighbor-joining dendrogram of bird and human isolates, based on nucleotide variation at 6 conserved housekeeping loci in the *Escherichia albertii* genome (multilocus sequence typing), supporting identification of all isolates as *E. albertii*. Three distinct clades are designated EA 1–EA 3. No clustering of isolates is apparent on the basis of their host type, geographic origin, or association with disease. Inset is a SplitsTree phylogenetic network of the genus *Escherichia*, showing the *E. albertii*/*Shigella boydii* serotype 7/13 lineage with respect to the other named species. *S. bongori*, *Salmonella bongori*; *S. enterica*, *Salmonella enterica*. Scale bars indicate genetic distance.

≈1 week of illness, during which it appeared depressed and anorexic. Histopathologic examination showed severe, diffuse, necrotizing typhlitis; mild to moderate enterocolitis; and septicemia.

Bacteria Isolated

Bacterial cultures were performed for 8 dead redpolls from Alaska and 3 healthy redpolls trapped in the same area. Large numbers of non–lactose-fermenting gram-negative rods were isolated from the intestines and tissues of 5 of the dead redpolls but from none of the 3 healthy redpolls. Similar organisms were also isolated in large numbers from the tissues of the gyrfalcon and intestines of the chicken. In the healthy birds trapped on Prince Edward Island, non–lactose-fermenting bacteria were isolated from the feces of 11 (12%) of 95 siskins and 4 (12%) of 33 redpolls but from none of 30 samples from purple finches. From the healthy birds trapped in Australia, non–lactose-fermenting bacteria were isolated from 4 (18%) of 22 magpies (*Gymnorhina tibicen*), 1 (10%) of 10 honeyeaters (*Melithreptus brevirostris*), 1 (3%) of 38 wrens (*Malurus cyaneus*), 1 (7%) of 15 fantails (*Rhipidura fulginosa*), and 2 (22%) of 9 chickens.

The isolates were oxidase negative; fermented glucose but not lactose, sucrose, or xylose; produced indole from tryptophan; and were nonmotile at 35°C. Further biochemical characterization with the API 20E panel indicated that the isolates produced lysine decarboxylase and ornithine decarboxylase and fermented D-glucose, D-mannitol, and L-arabinose. The isolates did not utilize citrate; did not produce arginine decarboxylase, hydrogen sulfide, urease, tryptophan deaminase, acetoin, or gelatinase; and did not ferment inositol, L-rhamnose, D-sucrose, D-melibiose, or amygdalin. All isolates except that from the fantail from Australia (B1086) used β-galactosidase. Fermentation of D-sorbitol varied; it was not fermented by the isolates from finches from Alaska, Canada, and Scotland or the gyrfalcon or by 3 of the 9 isolates from birds in Australia (B1068, B1074, and B1086). API 20E testing identified the isolates that used β-galactosidase and fermented D-sorbitol as code 5144102 and weakly (43%) identified them as *E. coli*. Identical API 20E profiles were reported for the isolates identified as *E. coli* from the dead finches from Scotland (*4*). Although the isolates from Scotland were serotyped as O86:K61, an isolate from Alaska (1297-05-019) did not react with any of the 175 O *E. coli* antiserum samples, including O86. Variable use of β-galactosidase and fermentation of D-sorbitol resulted in API 20E codes of 5144502 or 4144102 and more robust identifications as *E. coli* (84% and 90%, respectively).

The nearly full-length 16S rRNA sequence of isolate 1297-05-19 from the redpoll in Alaska was most similar (1,470 [99.7%] of 1,475 nt) to sequences of *E. albertii* (AY696669) and *S. boydii* (1,467 [99.5%] of 1,475 nt, AY696670) isolated from humans. The 495-nt sequence of the 5′ end of 16S rRNA was determined for redpoll 5419-05-R from Canada, finch EC37098 from Scotland, and gyrfalcon 12055-07 and chicken 7991-07 from the United States. In all, 9 nucleotide polymorphisms were observed among these 5 isolates, including 5 clustered in the V1 region (98.2% overall identity). These sequences were 99.2%–99.6% identical to the sequence of an *eae*-positive strain of *Hafnia alvei* (Z83203), *E. albertii* from humans (AJ508775, AY696662–AY696664, AY696669), and *S. boydii* serotypes 7 and 13 (AY696670–AY696680). PCRs were positive for the *E. albertii*–specific alleles of *lysP* and *mdh* (*10*) in all isolates from birds. Collectively, these data tentatively identified these isolates as *E. albertii*.

Virulence Genes

All isolates from birds were positive for *eae* and *cdtB* but negative for *stx1*, *stx2*, and *sta*, the same repertoire of virulence genes reported for *E. albertii* isolates from humans (*10*). Alignment of the 3′ portion of the *eae* gene showed that the bird isolates possessed a variety of *eae* alleles, some novel and some similar to previously reported alleles (Figure 2, panel A). There was no clustering of bird *eae* alleles related to geographic origin, bird versus human origin, or isolation from diseased versus clinically healthy birds or humans. The largest cluster of bird alleles was found in representative isolates (Figure 2, panel A) from the redpolls from Alaska and Canada, the gyrfalcon and chicken from the United States, and the fantail from Australia, which were all nearly identical (1 nonsynonymous nt change each in the isolate from the redpoll and fantail from Alaska). This allele was distinct from other reference *eae* alleles and thus novel, but it was most similar to γ intimin. Alleles from the isolates from finch E37098 and siskin EC74699 from Scotland and magpie B101 from Australia were identical to each other but were also novel alleles most closely related to the μ allele in *E. coli* (*17*). The alleles from isolates from other wild birds and chickens from Australia were similar to previously reported allelic subtypes, including ε, α, and ν (*17*). Only the isolates from chickens B1068 and B1074 in Australia had an allelic subtype, ν 1.1, previously reported for an *E. albertii* isolate from a human (*10*,*17*).

All isolates tested (Figure 2, panel B) were PCR positive for *cdtB* with the s1/as1 primer pair. With the exception of the chicken from the United States and 5 isolates from birds in Australia (2 chickens [B1068 and B1074], 1 fantail, 1 honeyeater, and 1 wren), all were also positive for *cdtB* with the s2/as2 primer pair. Because these 2 primer pairs are specific for different types of cytolethal distending toxin (*16*), at least some isolates from birds appeared to carry multiple *cdtB* genes. Sequencing and alignment of the s1/as1 PCR products showed these to have ≈91% nt and 92% aa identity. On the basis of amino acid polymor-

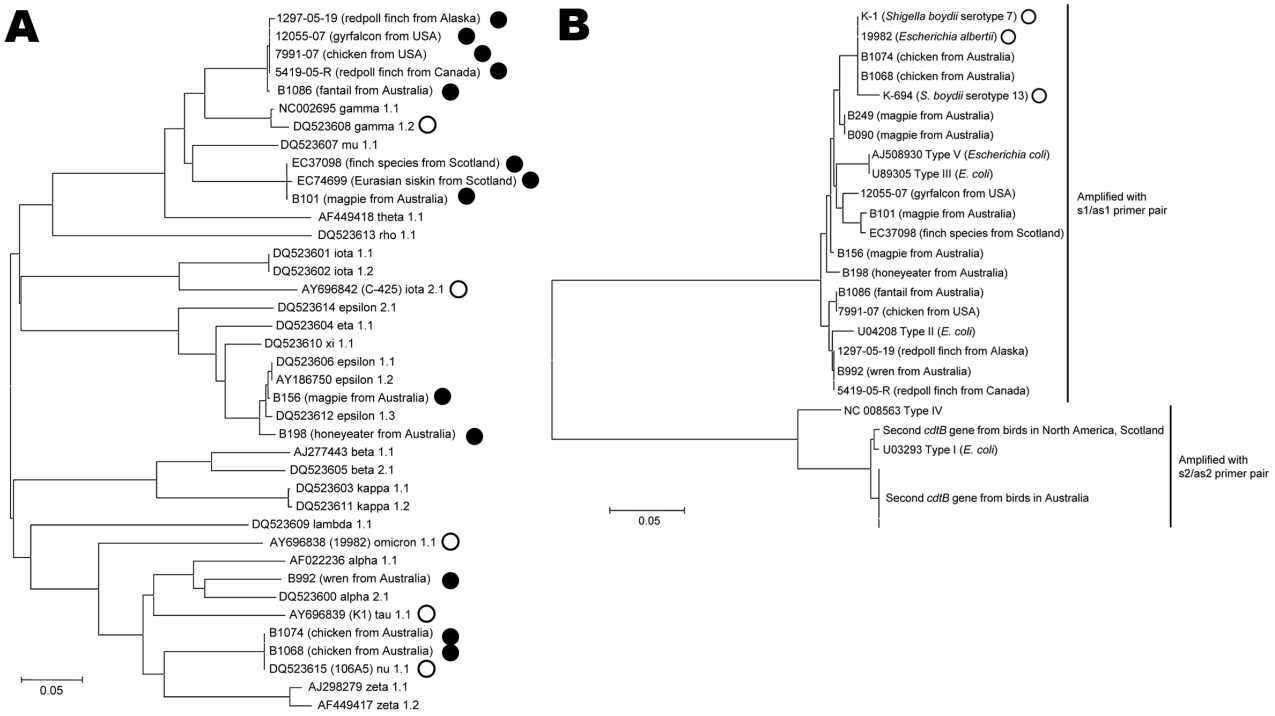


Figure 2. Neighbor-joining dendrogram based on the predicted amino acid sequences of the intimin (*eae*) and cytolethal distending toxin (*cdtB*) loci of *Escherichia* spp. Isolates analyzed in this study are designated by their isolate identification number; reference alleles are designated by their GenBank accession numbers. All *Escherichia albertii* isolates are indicated by circles or squares; all other isolates are *E. coli*. Scale bars indicate genetic distance. A) *eae* alleles carried by *E. albertii* isolates from birds (filled circles) represent diverse allelic subtypes and do not form separate clusters from allelic subtypes carried by isolates from humans (open circles). The *eae* alleles from North America and 1 from Australia (B1086) are novel but most similar to γ intimins. The *eae* alleles from Scotland and 1 from Australia (B101) are also novel but most similar to μ intimins. B) The *E. albertii cdtB* alleles from bird isolates, amplified by the s1/as1 primers, are most similar to *cdtB* types II, III, and V. *E. albertii* alleles from human isolates are marked with open circles. The bird *E. albertii cdtB* alleles amplified by the s2/as2 primers are distantly related to the other bird and human *E. albertii* alleles and are most similar to *cdtB* type I in *E. coli*. Because all the avian alleles amplified by the s2/as2 primers in each group are identical, only 1 sequence for each is shown.

phisms (Figure 2, panel B), avian *cdtB* alleles amplified by the s1/as1 primers were most similar to type II (birds from North America and Australia) and types III and V (the gyrfalcon, finches from Scotland, other birds from Australia) reference alleles (*21*–*23*). Sequencing and alignment of the s2/as2 PCR product showed that the sequences from the isolates from Australia were identical to each other, that the sequences from the isolates from North America and Scotland were identical to each other, and that these 2 groups of sequences were similar to each other with ≈99% identity at both the nucleotide and amino acid levels. These sequences were similar (1- or 2-aa differences) to the type I *cdtB* reference allele (*24*). The presence of a type I *cdtB* is consistent with the previous finding of a type I *cdtB* in the isolates from the finches from Scotland, identified by type-specific PCRs (*16*).

MLST Findings

MLST of nucleotide variation at 6 loci (a total of 3,165 bp) in the genomes of isolates (Table 1) showed 3 main clades of *E. albertii* (EA 1, EA 2, and EA 3 in Figure 1). Isolates did not appear to cluster on the basis of host disease status (healthy, with diarrhea, or dead) or host type. All isolates from birds from North America were closely related and clustered in clade EA 2, along with 3 isolates from birds from Australia (honeyeater, wren, and fantail) and an isolate from a human with diarrhea (I2005002880 #36). The isolate from chicken 7991-07 was slightly divergent from the rest of the isolates from North America (5 synonymous and 1 nonsynonymous nt changes) and was indistinguishable from isolate I2005002880 #36 from the human. Isolates from the dead redpolls from Alaska, healthy finches from Canada, and the gyrfalcon were identical. The isolate from finch EC370-98 from Scotland was distantly related to other bird isolates and clustered with an isolate from a human with diarrhea (M2005000616 #8) in clade EA 1.

MLST strongly supported the biochemical and other molecular data indicating that the bird isolates in this study were *E. albertii*. Collectively, these isolates represent a dis-

tant relative of *E. coli*, a divergent lineage in the genus *Escherichia*, and novel diversity within the *E. albertii* species (Figure 1, inset).

PFGE Findings

PFGE showed that isolates from the 2 bird death epornithics (in Alaska and Scotland) each formed a clonal group (Figure 3), which suggests that these events were associated with expansion of a single clone from either a common source or bird-to-bird transmission. Overall, the PFGE banding patterns and dendrogram indicate that the isolates from birds and humans constitute a heterogeneous group, consistent with the heterogeneity identified in *eae* and *cdtB* and by MLST.

Discussion

E. albertii is a recently described member of the Enterobacteriaceae and has been associated with diarrheal illness in humans (*25*–*27*). Until now, however, it has not been associated with disease or infection in animals. *E. albertii* was originally described as an unusual strain of *H. alvei* with virulence genes that included *eae* and the *cdtABC* operon (*26*,*28*). Subsequent characterization of these *H. alvei* strains demonstrated that they were members of the genus *Escherichia* (*7*,*10*,*27*) and constituted a new taxon for which the name *E. albertii* was proposed (*7*). The *E. albertii* lineage diverged before the radiation of *E. coli* and *Shigella* spp. and includes the atypical *S. boydii* serotypes 7 and 13 (*10*). The prevalence, epidemiology, and clinical relevance of *E. albertii* are poorly defined, in part because *E. albertii* is likely to either remain unidentified or be misidentified by current commercial biochemical identification methods as *E. coli*, *H. alvei*, *S. boydii*, or *Yersinia ruckeri* (*7*,*29*,*30*).

Our phenotypic, biochemical, 16S rRNA sequence, and MLST analyses are in strong agreement that the bird isolates in this study, including the previously identified O86:K61 *E. coli* isolates from Scotland, are correctly classified as *E. albertii*. In addition, all bird isolates carried genes for 2 characteristic *E. albertii* virulence factors (intimin and cytolethal distending toxin). Our findings indicate that *E. albertii* is likely pathogenic to birds and can be associated with epornithics and sporadic disease. The primary pathologic lesion in birds was consistent with enteritis, but the classic attaching-and-effacing lesions typically associated with *eae*-positive pathogens were not detected. The postmortem condition of the dead finches may have prevented such detection, but experimental work with chicks and the isolates from Scotland suggests that other disease mechanisms need to be considered (*9*).

We also conclude that *E. albertii* is able to subclinically colonize various species of wild birds globally. The determinants of pathogenicity of *E. albertii* in birds remain to be clarified, but its isolation from diseased and healthy birds suggests that its epizoology in songbirds may resemble that of *S. enterica* subsp. *enterica* serotype Typhimurium, which is maintained by subclinical carriers and causes outbreaks of disease under conditions of increased stress or high bacterial doses (*5*,*6*,*31*).

The *E. albertii* isolates from birds in this study differed from those from humans in several notable ways, although the lack of phylogenetic clustering based on host of origin suggests that it would be premature to conclude that these differences are truly host related. First, all bird isolates produced indole from tryptophan, resulting in weak (43% level of confidence) identification as *E. coli* in contrast to indole-negative isolates from humans, which are identified as *H. alvei* (45% level of confidence) according to API

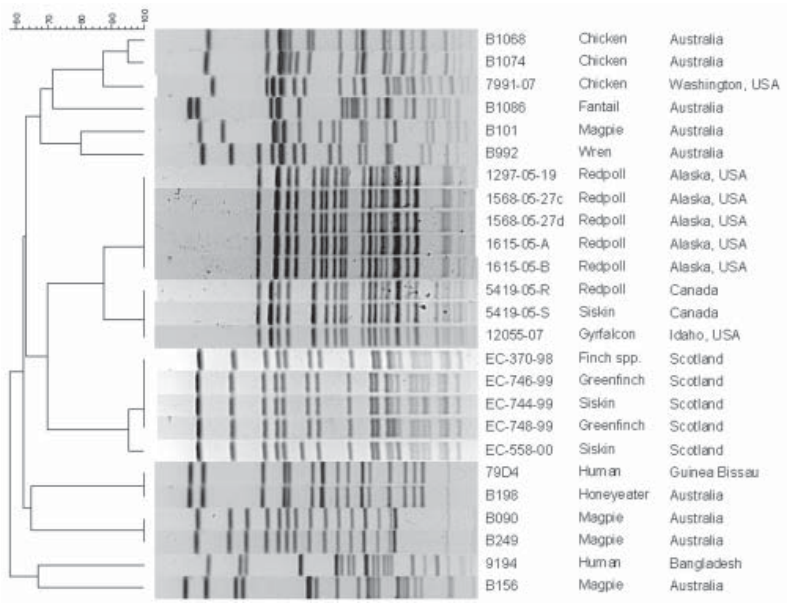


Figure 3. Dendrogram (unweighted pair group method with arithmetic mean) of *Escherichia albertii* isolates from birds, based on pulsed-field gel electrophoresis band profiles. Isolates from disease outbreaks in Alaska and Scotland form clonal groups, indicating that these outbreaks are associated with expansion of a single clone. The profiles for the other isolates indicate that the bird and human *E. albertii* isolates are heterogeneous and do not segregate on the basis of host, geographic origin, or disease status. Scale bar indicates percent similarity.

20E databases. However, when the positive indole result is combined with the positive reaction for D-sorbitol found for some bird isolates, the identification as *E. coli* is more robust (84%) and thus more likely to lead to misidentification. Second, we demonstrated the presence of 2 different *cdtB* genes. Whether the presence of multiple *cdtB* genes in the human or other bird *E. albertii* isolates was missed for technical reasons, or whether these other isolates contain a single gene, requires further investigation.

In conclusion, *E. albertii* appears to be a pathogen of animals and humans and may be carried subclinically by some birds. *E. albertii* is a member of a more heterogeneous group than was previously appreciated, and additional variation will likely become apparent as additional isolates from other animal hosts and geographic regions are characterized. Whether *E. albertii* can be transmitted from animals to humans is unknown, although the *eae*, *cdtB*, MLST, and PFGE data indicating that the bird isolates cluster among isolates from humans suggest that zoonoses or anthroponoses are possible. Regardless, identification of *E. albertii* in the clinical laboratory remains a challenge, and it is likely that this pathogen is often unidentified or misidentified in human and veterinary medicine.

Acknowledgments

This work is dedicated to the memory of Tom Whittam, who passed away before the study was completed.

We thank the following for providing case materials: Robert Gerlach, Susan Sharbaugh, Bernard Jilly, Tricia L. Franklin, the Alaska Department of Fish and Game staff in Fairbanks and Delta Junction, and the Alaskan Bird Observatory. Excellent technical assistance was provided by Katherine N.K. Baker, Lusha Evans, Heather Matthews, Charlene Teitzel, Joyce Wisinger, Yubei Zhang, and Julia Christenson.

Financial support was provided by the Washington Animal Disease Diagnostic Laboratory; by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Department of Health and Human Services contract no. N01-AI-30055; and by NIH research contract N01-AI30058.

Dr Oaks is a veterinarian and microbiologist at Washington State University. His primary interests are diagnostic microbiology in domestic and wild animals, identification of new and emerging pathogens through conventional and molecular means, establishment of the role of these new agents in disease, and mechanisms of persistence and host–virus interactions with lentiviruses and herpesviruses.

References

1. Daoust PY, Busby DG, Ferns L, Goltz J, McBurney S, Poppe C, et al. Salmonellosis in songbirds in the Canadian Atlantic provinces during winter–summer 1997–1998. Can Vet J. 2000;41:54–9.
2. Dhondt AA, Tessaglia DL, Slothower RL. Epidemic mycoplasmal conjunctivitis in house finches from eastern North America. J Wildl Dis. 1998;34:265–80.
3. Docherty DE, Long RI. Isolation of a poxvirus from a house finch, *Carpodacus mexicanus* (Muller). J Wildl Dis. 1986;22:420–2.
4. Foster G, Ross HM, Pennycott TW, Hopkins GF, McLaren IM. Isolation of *Escherichia coli* O86:K61 producing cyto-lethal distending toxin from wild birds of the finch family. Lett Appl Microbiol. 1998;26:395–8. DOI: 10.1046/j.1472-765X.1998.00359.x
5. Pennycott TW, Ross HM, McLaren IM, Park A, Hopkins GF, Foster G. Causes of death of wild birds of the family Fringillidae in Britain. Vet Rec. 1998;143:155–6.
6. Refsum T, Vikoren T, Handeland K, Kapperud G, Holstad G. Epidemiologic and pathologic aspects of *Salmonella* Typhimurium infection in passerine birds in Norway. J Wildl Dis. 2003;39:64–72.
7. Huys G, Cnockaert M, Janda JM, Swings J. *Escherichia albertii* sp. nov., a diarrhoeagenic species isolated from stool specimens of Bangladeshi children. Int J Syst Evol Microbiol. 2003;53:807–10. DOI: 10.1099/ijs.0.02475-0
8. Gordon DM, Cowling A. The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. Microbiology. 2003;149:3575–86. DOI: 10.1099/mic.0.26486-0
9. La Ragione RM, McLaren IM, Foster G, Cooley WA, Woodward MJ. Phenotypic and genotypic characterization of avian *Escherichia coli* O86:K61 isolates possessing a gamma-like intimin. Appl Environ Microbiol. 2002;68:4932–42. DOI: 10.1128/AEM.68.10.4932-4942.2002
10. Hyma KE, Lacher DW, Nelson AM, Bumbaugh AC, Janda JM, Strockbine NA, et al. Evolutionary genetics of a new pathogenic *Escherichia* species: *Escherichia albertii* and related *Shigella boydii* strains. J Bacteriol. 2005;187:619–28. DOI: 10.1128/JB.187.2.619-628.2005
11. Valentiner-Branth P, Steinsland H, Fischer TK, Perch M, Scheutz F, Dias F, et al. Cohort study of Guinean children: incidence, pathogenecity, conferred protection, and attributable risk for enteropathogens during the first 2 years of life. J Clin Microbiol. 2003;41:4238–45. DOI: 10.1128/JCM.41.9.4238-4245.2003
12. Pettersson B, Leitner T, Ronaghi M, Bolske G, Uhlen M, Johansson K. Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. J Bacteriol. 1996;178:4131–42.
13. Baker GC, Smith JJ, Cowan DA. Review and re-analysis of domain-specific 16S primers. J Microbiol Methods. 2003;55:541–55. DOI: 10.1016/j.mimet.2003.08.009
14. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997;25:3389–402. DOI: 10.1093/nar/25.17.3389
15. Franck SM, Bosworth BT, Moon HW. Multiplex PCR for enterotoxigenic, attaching and effacing, and Shiga toxin–producing *Escherichia coli* strains from calves. J Clin Microbiol. 1998;36:1795–7.
16. Toth I, Herault F, Beutin L, Oswald E. Production of cytolethal distending toxins by pathogenic *Escherichia coli* strains isolated from human and animal sources: establishment of the existence of a new *cdt* variant (type IV). J Clin Microbiol. 2003;41:4285–91. DOI: 10.1128/JCM.41.9.4285-4291.2003
17. Lacher DW, Steinsland H, Whittam TS. Allelic subtyping of the intimin locus (*eae*) of pathogenic *Escherichia coli* by fluorescent RFLP. FEMS Microbiol Lett. 2006;261:80–7. DOI: 10.1111/j.1574-6968.2006.00328.x
18. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
19. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006;23:254–67. DOI: 10.1093/molbev/msj030

20. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis. 2006;3:59–67. DOI: 10.1089/fpd.2006.3.59
21. Janka A, Bielaszewska M, Dobrindt U, Greune L, Schmidt MA, Karch H. Cytolethal distending toxin gene cluster in enterohemorrhagic *Escherichia coli* O157:H- and O157:H7: characterization and evolutionary considerations. Infect Immun. 2003;71:3634–8. DOI: 10.1128/IAI.71.6.3634-3638.2003
22. Peres SY, Marches O, Daigle F, Nougayrede JP, Herault F, Tasca C, et al. A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G2/M phase. Mol Microbiol. 1997;24:1095–107. DOI: 10.1046/j.1365-2958.1997.4181785.x
23. Pickett CL, Cottle DL, Pesci EC, Bikah G. Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. Infect Immun. 1994;62:1046–51.
24. Scott DA, Kaper JB. Cloning and sequencing of the genes encoding *Escherichia coli* cytolethal distending toxin. Infect Immun. 1994;62:244–51.
25. Albert MJ, Alam K, Islam M, Montanaro J, Rahaman AS, Haider K, et al. *Hafnia alvei*, a probable cause of diarrhea in humans. Infect Immun. 1991;59:1507–13.
26. Albert MJ, Faruque SM, Ansaruzzman M, Islam MM, Haider K, Alam K, et al. Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei.* J Med Microbiol. 1992;37:310–4. DOI: 10.1099/00222615-37-5-310
27. Janda JM, Abbott SL, Albert MJ. Prototypical diarrheagenic strains of *Hafnia alvei* are actually members of the genus *Escherichia.* J Clin Microbiol. 1999;37:2399–401.
28. Pickett CL, Lee RB, Eyigor A, Elitzur B, Fox EM, Strockbine NA. Patterns of variations in *Escherichia coli* strains that produce cytolethal distending toxin. Infect Immun. 2004;72:684–90. DOI: 10.1128/IAI.72.2.684-690.2004
29. Abbott SL, O'Connor J, Robin T, Zimmer BL, Janda JM. Biochemical properties of a newly described *Escherichia* species, *Escherichia albertii.* J Clin Microbiol. 2003;41:4852–4. DOI: 10.1128/JCM.41.10.4852-4854.2003
30. Stock I, Rahman M, Sherwood KJ, Wiedemann B. Natural antimicrobial susceptibility patterns and biochemical identification of *Escherichia albertii* and *Hafnia alvei* strains. Diagn Microbiol Infect Dis. 2005;51:151–63. DOI: 10.1016/j.diagmicrobio.2004.10.008
31. Daoust PY, Prescott JF. Salmonellosis. In: Thomas NJ, Hunter DB, Atkinson CT, editors. Infectious diseases of wild birds. Hoboken (NJ): Blackwell Publishing Company; 2007. p. 270–88.

Address for correspondence: J. Lindsay Oaks, Washington Animal Disease Diagnostic Laboratory, Washington State University, PO Box 647034, Pullman, WA 99164-7034, USA; email: loaks@vetmed.wsu.edu

Community-associated Methicillin-Resistant *Staphylococcus aureus* Strains in Pediatric Intensive Care Unit[1]

Aaron M. Milstone, Karen C. Carroll, Tracy Ross, K. Alexander Shangraw, and Trish M. Perl

CME ACTIVITY

Medscape, LLC is pleased to provide online continuing medical education (CME) for this journal article, allowing clinicians the opportunity to earn CME credit. Medscape, LLC is accredited by the Accreditation Council for Continuing Medical Education (ACCME) to provide CME for physicians. Medscape, LLC designates this educational activity for a maximum of 0.5 *AMA PRA Category 1 Credits*™. Physicians should only claim credit commensurate with the extent of their participation in the activity. All other clinicians completing this activity will be issued a certificate of participation. To participate in this journal CME activity: (1) review the learning objectives and author disclosures; (2) study the education content; (3) take the post-test and/or complete the evaluation at **http://www.medscape.com/cme/eid**; (4) view/print certificate.

Learning Objectives

Upon completion of this activity, participants will be able to:

- Identify risk factors among children for being a community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) carrier.
- Recognize the benefits of screening for MRSA colonization in children being admitted to the hospital.
- Predict a consequence of undetected CA-MRSA carriers admitted to a hospital setting.

Editor

Carol Snarey, Copyeditor, Emerging Infectious Diseases. *Disclosure: Carol Snarey has disclosed no relevant financial relationships.*

CME Author

Charles P. Vega, MD, Associate Professor; Residency Director, Department of Family Medicine, University of California, Irvine, CA, USA. *Disclosure: Charles P. Vega, MD, has disclosed no relevant financial relationships.*

Authors

Disclosures: ***Aaron M. Milstone, MD, MHS****, has disclosed the following relevant financial relationship: received grants for clinical research from Sage Products, Inc.* ***Karen C. Carroll, MD,*** *has disclosed the following relevant relationships: served as an advisor or consultant for Quidel Diagnostics; OpGen, Inc.; Boehringer Ingelheim Pharmaceuticals, Inc.; received grants for clinical research from BD GeneOhm; Ibis Biosciences, Inc.; MicroPhage, Inc.* ***Tracy Ross, BS,*** *and* ***K. Alexander Shangraw, MSPH,*** *have disclosed no relevant financial relationships.* ***Trish M. Perl, MD, MSc,*** *has disclosed the following relevant financial relationships: served as an advisor or consultant for Cadence Pharmaceuticals; 3M; TheraDoc Inc.; received grants for clinical research from US Centers for Disease Control and Prevention; Merck & Co., Inc.; Sage Products, Inc.; US Department of Veterans Affairs.*

Virulent community-associated methicillin-resistant *Staphylococcus-aureus* (CA-MRSA) strains have spread rapidly in the United States. To characterize the degree to which CA-MRSA strains are imported into and transmitted in pediatric intensive care units (PICU), we performed a retrospective study of children admitted to The Johns Hopkins Hospital PICU, March 1, 2007–May 31, 2008. We found that 72 (6%) of 1,674 PICU patients were colonized with MRSA. MRSA-colonized patients were more likely to be younger (median age 3 years vs. 5 years; p = 0.02) and African American (p<0.001) and to have been hospitalized within 12 months (p<0.001) than were noncolonized patients. MRSA isolates from 66 (92%) colonized patients were fingerprinted; 40 (61%) were genotypically CA-MRSA strains. CA-MRSA strains were isolated from 50% of patients who became colonized with MRSA and caused the only hospital-acquired MRSA catheter-associated bloodstream infection in the cohort. Epidemic CA-MRSA strains are becoming endemic to PICUs, can be transmitted to hospitalized children, and can cause invasive hospital-acquired infections. Further appraisal of MRSA control is needed.

Author affiliation: The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

DOI: 10.3201/eid1604.090107

[1]These data were presented in part at the Annual Scientific Meeting of the Society of Healthcare Epidemiology of America, Orlando, Florida, USA, April 2008.

Methicillin-resistant *Staphylococcus aureus* (MRSA) frequently infects children. Traditionally, MRSA infections were confined to those who frequented healthcare facilities or had predisposing healthcare-associated risk factors. In the 1990s, reports emerged of MRSA infections in healthy children in the community who had no predisposing risk factors (*1*). Community-onset MRSA infections were caused by MRSA strains belonging to the genotypes USA300 and USA400 (identified by pulsed-field gel electrophoresis [PFGE]), also referred to as the community-associated MRSA (CA-MRSA) strains (*2*,*3*). These CA-MRSA strains are associated with increased production of toxins and are less resistant to antimicrobial drugs than are traditional hospital-acquired MRSA (HA-MRSA) strains (*4*,*5*). Although CA-MRSA strains usually cause mild skin and soft tissue infections, they can also cause severe and fatal disease (*6*–*8*).

As the community prevalence of MRSA has risen (*9*), more children colonized or infected with MRSA have been admitted to hospitals (*10*–*12*), especially with phenotypic CA-MRSA strains. Notably, CA-MRSA strains can cause outbreaks in hospitals (*13*) and have become a frequent cause of hospital-onset infections (*14*,*15*). Aside from ways to manage outbreaks (*16*) and a report that clinical cultures underestimate MRSA prevalence (*17*), little is known about the prevalence of MRSA colonization of hospitalized children. The degree to which CA-MRSA strains are imported into and transmitted in high-risk settings such as pediatric intensive care units (PICUs) has not been determined. Understanding the effects of MRSA in hospitalized children is essential to guide, assess, and plan MRSA prevention and control programs among hospitalized children. Our objectives were to measure the prevalence of MRSA colonization at the time of admission to the PICU and to determine the effects of CA-MRSA strains on MRSA colonization, transmission, and hospital-acquired infections in the PICU.

Materials and Methods

Setting and Design

The Johns Hopkins Hospital is a 920-bed tertiary care academic medical center with an embedded 175-bed children's hospital. The institution serves Baltimore, Maryland, USA, and the surrounding area. The 26-bed PICU admits ≈1,700 medical and surgical patients each year, including patients needing hematopoietic stem cell transplants and organ transplants, as well as cardiac, orthopedic, and neurosurgical patients. Beginning March 1, 2007, as part of a hospital MRSA prevention and control program, the Department of Hospital Epidemiology and Infection Control initiated screening of patients for MRSA colonization at the time of admission to the PICU and weekly thereafter. Nares swab specimens were obtained by PICU nurses and cultured for MRSA as described later. Newly identified patients or those known to be colonized or infected with MRSA were isolated in cohort groups. Compliance with admission screening was reported back to the unit monthly. Hospital policy required strict hand hygiene, use of standard precautions, and contact isolation for all patients colonized or infected with MRSA.

During March 1, 2007–May 31, 2008, we performed a retrospective cohort study to identify all MRSA-colonized patients in the Johns Hopkins Hospital PICU, including those colonized at the time of admission and those who became colonized while in the PICU. If patients were admitted to the PICU multiple times, only the first admission was included. The institutional review board approved this study and waived informed consent to review retrospective data collected during hospital care.

Definitions

MRSA colonization at the time of PICU admission was defined as having a nasal surveillance culture obtained at the time of admission that grew MRSA or any clinical culture that grew MRSA within 3 days of PICU admission (*18*,*19*). Known MRSA carriers were defined as any patients with an institutional history of MRSA colonization or infection before PICU admission. Newly identified MRSA patients (incident cases) had no institutional history of MRSA colonization or infection (either had negative cultures on prior admissions or clinic visits or had not previously been tested). Case-patients with incident MRSA became colonized or infected in the PICU and met the following criteria: 1) had a positive screening or clinical culture obtained >3 days after admission to the PICU (*19*), 2) had no institutional history of MRSA, and 3) had a previous negative culture from the same site during the current PICU admission. Incidence density was calculated as the number of incident MRSA cases per 1,000 patient-days at risk for MRSA acquisition (i.e., patient days during which patients were not known to be colonized or infected with MRSA). Healthcare-associated MRSA (HA-MRSA) infections met the criteria established by the National Healthcare Safety Network's surveillance definition for healthcare-associated infections (*20*). CA-MRSA strains included those belonging to the PFGE genotypes USA300 and USA400 (*3*). HA-MRSA strains included those belonging to other PFGE genotypes.

Data Collection and Case Identification

We searched a computerized surveillance support system (Theradoc Inc., Salt Lake City, UT, USA) to identify all patients with microbiology cultures of samples from any body site that grew MRSA from March 1, 2007, through May 31, 2008, and to determine compliance in performing screening cultures at the time of PICU admission. Adminis-

trative databases were searched to obtain patient characteristics. Medical records were reviewed to determine whether MRSA infections met the National Healthcare Safety Network's surveillance definition for healthcare-associated infections.

Laboratory Methods

Nasal surveillance swabs were plated on BBL CHROMagar MRSA plates (BD Diagnostics, Sparks, MD, USA), a selective and differential medium to detect MRSA. Mauve-colored colonies present after 24 or 48 hours of incubation were confirmed as *S. aureus* by Gram stain and slide coagulase testing (*21*). We performed PFGE on available stored isolates. DNA was extracted and digested by using *Sma*1 (*22*,*23*). We used *S. aureus* subspecies NCTC 8325 as a control strain, and all USA PFGE strain types were included for comparison (*3*). The USA type strains were obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* program (supported under National Institute of Allergy and Infectious Diseases/National Institutes of Health contract no. HHSN272200700055C). We performed PFGE on the CHEF-DR III (BioRad Laboratories, Hercules, CA, USA). Gels were stained and scanned by using a molecular analysis fingerprinting software (Fingerprinting II Version 3.0; BioRad Laboratories). We considered isolates to be related if their patterns had ≤3 band differences (*3*) and to be unrelated if they had >3 band differences.

Statistical Analysis

Data were maintained in Microsoft Access 2003 (Microsoft Corp., Redmond, WA, USA) and analyzed by using Stata version 10.0 (StataCorp., College Station, TX, USA). Means, medians, and interquartile ranges (IQRs) were calculated for select demographic variables. Categorical variables were expressed as numbers and percentages. Comparisons were made by using the Pearson χ^2 or Fisher exact test. For continuous variables, the Student *t* test or the Wilcoxon rank-sum test was used to compare groups, depending on the distribution of the data. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) and to evaluate the strength of associations. A pairwise correlation coefficient was calculated to assess an association between incident cases and monthly admission colonization prevalence. A 2-tailed p value <0.05 was considered significant for all statistical tests.

Results

From March 1, 2007, through May 31, 2008, 1,674 children were admitted to the Johns Hopkins Hospital PICU. The median age was 5 years (IQR 1–12 years), and 55% of patients were male. Only 53 patients (3.2%) had an institutional history of MRSA colonization or infection. Screening cultures were performed on nasal swabs from 1,210 children (72%) obtained at the time of PICU admission. When patients that were screened were compared with those that were not screened, screened patients were more likely to have been hospitalized in the previous 12 months (29% vs. 22%, p<0.01). No other significant differences in demographic or clinical characteristics were found between those patients screened for MRSA colonization and those not screened (Table 1).

At the time of admission to PICU, 72 (6.0%) patients were colonized with MRSA: 68 patients (94%) identified by results of nasal screening cultures and 4 patients whose clinical culture grew MRSA within 3 days of PICU admission. Characteristics of patients colonized with MRSA at the time of admission (group 1, currently colonized) were compared with those of patients not colonized with MRSA and never known to be colonized (group 2, never colonized) and patients not colonized with MRSA at admission but who had an institutional history of prior colonization or infection (group 3, previously colonized) (Table 2). Compared with never-colonized patients, MRSA-colonized patients tended to be younger (median age 3 years vs. 5 years,

Table 1. Characteristics of patients screened for and not screened for MRSA colonization at the time of PICU admission, The Johns Hopkins University Hospital, Baltimore, MD, USA, March 2007–May 2008*

Characteristic	Patients screened for MRSA, n = 1,210	Patients not screened for MRSA, n = 464	p value
Demographic			
Median age, y (IQR)	5 (0–12)	6 (1–12)	0.28
Male sex	667 (55)	255 (56)	0.78
Race			
White	676 (56)	234 (51)	Referent†
African American	403 (33)	173 (37)	0.07
Other	131 (11)	57 (12)	0.19
Clinical			
Known MRSA carrier‡	41 (3)	12 (3)	0.40
Hospitalized in previous 12 mo	355 (29)	102 (22)	<0.01

*MRSA, methicillin-resistant *Staphylococcus aureus*; PICU, pediatric intensive care unit; IQR, interquartile range. Values reported as no. (%) unless otherwise specified.
†Obtained from univariate logistic regression analysis.
‡Patients with institutional history of MRSA colonization or infection.

Table 2. Characteristics of patients with and without MRSA colonization at the time of PICU admission, The Johns Hopkins University Hospital, Baltimore, MD, USA, March 2007–May 2008*

Characteristic	Group 1,† n = 72	Group 2,‡ n = 1,117	Group 3, n = 24§	p value: Group 2 vs. group 1	p value: Group 3 vs. group 1
Demographic					
Median age, y (IQR)	3 (0–7.5)	5 (1–12)	10.5 (3–13)	0.02	<0.01
Male sex	38 (53)	616 (55)	13 (54)	0.69	0.91
Race					
White	28 (39)	640 (57)	10 (42)	Referent¶	Referent¶
African American	39 (54)	352 (32)	13 (54)	<0.001	0.89
Other	5 (7)	126 (11)	1 (4)	0.86	0.62
Clinical					
Known MRSA carrier#	18 (25)	0 (0)	24 (100)		
Hospitalization in previous 12 mo	42 (58)	308 (28)	14 (58)	<0.001	0.41
Outcomes					
PICU length of stay,** median (IQR)	3 (1–7)	2 (1–4)	2.5 (1–9)	<0.001	0.96
Hospital length of stay,** median (IQR)	8 (3.5–15.5)	5 (3–10)	6 (3/5–14.5)	<0.01	0.70

*MRSA, methicillin-resistant *Staphylococcus aureus*; PICU, pediatric intensive care unit; IQR, interquartile range. Values reported as no. (%) unless otherwise specified.
†MRSA colonized: patients who had MRSA grow in an admission nasal surveillance culture or in any clinical culture within 3 days of PICU admission.
‡Not MRSA colonized/no institutional history of MRSA colonization.
§Not MRSA colonized/institutional history of MRSA colonization.
¶Obtained from univariate logistic regression analysis.
#Patients with institutional history of MRSA colonization or infection.
**Data were log transformed before regression analysis to account for skewing.

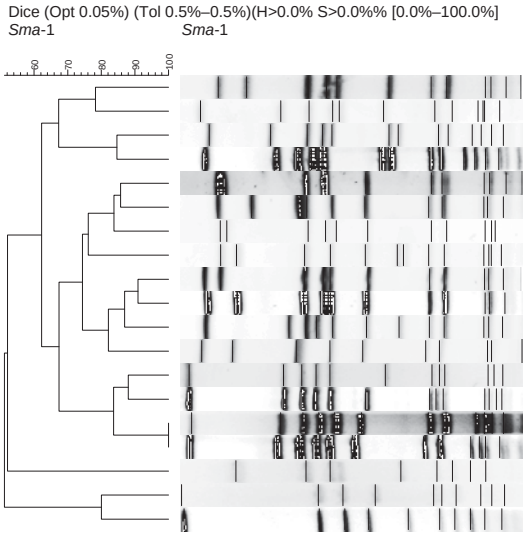
p = 0.02), to be African American (54% vs. 32%; p<0.001), and to have been hospitalized in the previous 12 months (p<0.001). MRSA-colonized patients had a longer stay in the PICU (3 days vs. 2 days, p<0.001) and a longer stay in the hospital (8 days vs. 5 days; p<0.01). MRSA-colonized patients tended to be younger than previously colonized patients (median age 3 years vs. 10.5 years; p<0.01), but these 2 groups were otherwise similar.

Of the 72 MRSA colonized patients, 54 (75%) were newly identified MRSA carriers (51 by screening cultures and 3 by clinical cultures). Of the 51 patients newly identified by screening cultures, 8 (16%) had a subsequent clinical culture grow MRSA during their PICU stay. Therefore, 43 (60%) of 72 MRSA-colonized patients would have gone undetected had admission screening cultures not been performed. Most MRSA-colonized patients were <6 years of age (71%) and African American (54%). Eighteen percent were admitted from an in-patient unit, and in the previous 12 months, 58% had been hospitalized and 43% had been admitted to the PICU.

MRSA isolates from 66 (92%) of 72 patients colonized with MRSA at the time of admission were analyzed by PFGE; 14 distinct strains were identified (Figure). Forty (61%) isolates were CA-MRSA strains, including those identical to or related to PFGE types USA300 (n = 39) and USA400 (n = 1). Twenty-six isolates (39%) were HA-MRSA strains related to USA100 (n = 11), USA200 (n = 1), USA700 (n = 1), and 8 other strains had unique PFGE fingerprints (n = 13). Many isolates did not have identical band patterns, but they did have ≤3 band differences and were considered related strains (*3*).

Patients colonized with CA-MRSA were compared with those colonized with HA-MRSA (Table 3). Patients colonized with CA-MRSA strains were less likely to have been admitted to the PICU within the previous 12 months (OR 0.31; 95% CI 0.11–0.84). No significant differences were found between the 2 groups in other demographic, clinical, or outcome characteristics, including median age, sex, number of newly identified MRSA carriers, length of stay in the hospital before PICU admission, admission from home or patient care unit, admission to a medical or surgical service, PICU length of stay, or hospital length of stay.

To identify patients who became colonized with MRSA while in the PICU, MRSA screening cultures from nares specimens were sent weekly, and clinical cultures from patients in the PICU for >3 days were monitored. During the study period, 8 incident MRSA cases were identified (Table 4), an incidence density of 1.01 cases per 1,000 patient days at risk. No correlation was shown between monthly colonization prevalence at time of admission and incident MRSA cases (p = 0.09). Six (75%) of 8 incident cases were identified by a screening culture. Of these 6 patients, 2 had subsequent clinical cultures grow MRSA. If weekly MRSA screening cultures were not performed, only 4 (50%) of 8 incident MRSA cases would have been identified. Patients with incident MRSA cases were in the PICU for a median of 6 days (range 5–24 days) before acquiring MRSA and had a median age of 6 years (range 1–11 years). Seven (88%) of 8 MRSA isolates were available for PFGE analysis. Four (57%) of 7 isolates were identical to or related to PFGE-type USA300, documenting healthcare-associated transmission of CA-MRSA strains. Six (75%) of 8 patients



PFGE strain type	Isolate
B	1
G	1
USA400-related	1
USA400*	–
H	1
C	5
J	1
I	1
USA100-related	11
USA100*	–
E	1
F	1
USA700-related	1
USA700*	–
USA300 or related	39
USA300*	–
D	1
USA200-related	1
USA200*	–

Figure. Dendrogram of methicillin-resistant *Staphylococcus aureus* strains that colonized children admitted to the pediatric intensive care unit, The Johns Hopkins Hospital, Baltimore, MD, USA, 2007–2008. Isolates were characterized by pulsed-field gel electrophoresis (PFGE). Not all strains within a PFGE type had identical patterns, but strains were considered related with ≤3 band differences; 66 isolates were analyzed. The number of isolates related to each PFGE type is listed. *Reference strains.

with incident cases were admitted to a surgical service. Of the patients who became colonized with MRSA, 3 (38%) acquired a subsequent MRSA infection during their stay in the PICU (1 central-line–associated bloodstream infection, 1 case of ventilator-associated pneumonia, 1 case of ventilator-associated tracheitis). Both respiratory infections were caused by PFGE strain A, a strain that was not associated with colonization of any patients at the time of PICU admission. The only HA-MRSA bloodstream infection was caused by a USA300-related strain.

Discussion

These data describe the prevalence of MRSA colonization in patients admitted to the PICU and suggest that CA-MRSA strains may be becoming endemic in hospitalized children. We found that 6.0% of patients screened at the time of admission to the PICU were colonized with MRSA. Most (60%) MRSA-colonized patients would not have been recognized if admission screening cultures had not been performed. Sixty-one percent of MRSA colonized patients harbored CA-MRSA strains, mostly USA300. Our data show that epidemic CA-MRSA strains are endemic to the PICU. These strains can be transmitted to children in the hospital and can cause invasive hospital-acquired infections, including bacteremia.

Aside from how to manage an MRSA outbreak, little research has attempted to characterize the epidemiology of MRSA colonization and transmission in the PICU. The lack of research in PICU patients is surprising, given the abundance of published articles that have characterized the epidemiology of MRSA in adult ICU patients (*24*,*25*). Our findings agree with those of studies of adult patients: screening cultures detect a large reservoir of MRSA-colonized patients, cases that would otherwise go undetected (*18*,*24*). Most (75%) patients colonized at the time of PICU admission had no institutional history of MRSA colonization or infection. MRSA-colonized patients serve as a reservoir for contamination of healthcare workers' hands and subsequent MRSA transmission to patients. Active detection and isolation of MRSA carriers can reduce MRSA transmission in hospitals (*26*,*27*). Given the risk to patients for MRSA acquisition and subsequent infection (*28*), many hospitals screen high-risk populations to identify MRSA carriers. In an attempt to curb the spread of MRSA in healthcare facilities, some states have passed legislation mandating MRSA screening.

As the community prevalence of MRSA has risen (*9*), more children infected with MRSA have been admitted to hospitals (*10*,*11*). Our data suggest that these patients represent a small fraction of the patients colonized with CA-MRSA strains who enter the hospital. We found that 61% of children colonized with MRSA at the time of PICU admission harbored a CA-MRSA strain. Findings from a previous cohort showed a lower percentage of colonization with CA-MRSA strains (*29*). Among patients >13 years of age who were admitted to an urban hospital in Atlanta, 7.3% were colonized with MRSA, and 30% of those were colonized with CA-MRSA strains (*29*).

Several factors may explain the high prevalence of colonization with CA-MRSA strains in our cohort. First, the community prevalence of MRSA colonization in children is increasing nationwide and in Baltimore (*30*–*32*), largely the result of spread of CA-MRSA strains. Second, children generally have less exposure to healthcare settings where they would be exposed to traditional HA-MRSA strains. Third, children frequently are in settings of close personal contact where opportunities for hygiene may be limited, such as day care, schools, and sports teams, settings postulated as high-risk environments for MRSA transmission.

Notably, children in this study who were colonized with MRSA at the time of admission to PICU were more

Table 3. Characteristics of patients colonized with different MRSA strain types at the time of PICU admission, The Johns Hopkins Hospital, Baltimore, MD, USA, March 2007–May 2008*

Characteristic	Patients colonized with CA-MRSA strain, n = 40	Patients colonized with HA-MRSA strain, n = 26	OR (95% CI)†
Demographic			
Median age, y (IQR)	3.8 (1.0–5.9)	4.0 (1.0–9.5)	0.98 (0.90–1.07)
Male sex	22 (55)	14 (54)	1.05 (0.39–2.82)
Race			
White	15 (35)	9 (38)	Referent
African American	23 (58)	13 (50)	1.1 (0.36–3.10)
Other	2 (5)	4 (15)	0.3 (0.05–1.98)
Clinical			
Newly identified MRSA carrier	32 (80)	20 (77)	1.2 (0.36–3.97)
Hospitalized in previous 12 mo	20 (50)	19 (73)	0.37 (0.13–1.07)
ICU admission in previous 12 mo	13 (33)	26 (62)	0.31 (0.11–0.84)
Length of stay in hospital before PICU admission, median (range)	0 (0–28)	0 (0–14)	1.02 (0.92–1.15)
Primary service			
Medical	24 (60)	12 (46)	Referent
Surgical	16 (40)	14 (54)	0.57 (0.21–1.55)
Admitted to PICU from inpatient unit	6 (19)	5 (15)	0.74 (0.20–2.73)
Outcomes			
PICU length of stay,‡ median (IQR)	3 (1–7.5)	3 (2–7)	1.05 (0.79–1.40)
Hospital length of stay,‡ median (IQR)	8 (4.5–28.5)	8.5 (3–15)	1.04 (0.97–2.04)

*MRSA, methicillin-resistant *Staphylococcus aureus*; PICU, pediatric intensive care unit; CA-MRSA, community-associated MRSA; HA-MRSA, hospital-associated MRSA; OR, odds ratio; CI, confidence interval; IQR, interquartile range. Values reported as no. (%) unless otherwise specified.
†Obtained from univariate logistic regression analysis.
‡Data were log transformed before regression analysis to account for skewing.

likely to be younger and African American. Our finding of younger age is consistent with data from the 2003–2004 Centers for Disease Control and Prevention National Health and Nutrition Examination Survey Nasal Swab survey, which found that children 1–5 years of age had the second highest MRSA colonization rates, behind adults >60 years of age (R. Gorwitz, pers. comm.). Similarly, we found that children with previous, but not current, MRSA colonization tended to be older. Why colonization prevalence is different in various pediatric age groups remains unknown. Previous studies have found racial disparities in rates of invasive MRSA disease (*6*,*33*). In a 2007 report by Klevens et al., incidence rates of invasive MRSA disease were more than twice as high for African Americans than for whites, and mortality rates were 80% higher for African Americans (*7*). The reason for these racial disparities is unknown. MRSA colonization is a known risk factor for invasive MRSA, so higher colonization rates in African Americans may in part explain higher rates of invasive disease.

CA-MRSA strains are changing the landscape of MRSA infection prevention and control in hospitals. We found that CA-MRSA strain USA300 was the most commonly acquired MRSA strain identified in the PICU. All patients who acquired MRSA had negative nares swab cultures at the time of PICU admission, and all subsequently exhibited MRSA nasal colonization. Of 8 patients, 3 (38%) had subsequent MRSA infection during their PICU stay. We were unable to monitor these patients after PICU discharge and likely have underestimated their long-term risk for subsequent MRSA infection. CA-MRSA strains, with the potential to spread rapidly and cause severe disease, have now been shown to cause hospital-acquired infections and hospital outbreaks (*7*,*13*–*15*). Hospital outbreaks confirm that CA-MRSA strains can be transmitted and acquired in the healthcare setting. The role of CA-MRSA strains in endemic MRSA transmission has not been elucidated. The extent to which endemic CA-MRSA strains will affect the epidemiology of HA-MRSA transmission and infections remains unknown. Our data suggest that this topic requires further study.

As CA-MRSA strains become more prevalent in hospitals, the importance of distinguishing between MRSA strains remains unclear. However, CA-MRSA strains appear to be highly transmissible and may have increased virulence (*33*–*35*). When attempting to distinguish between patients colonized with CA-MRSA strains and those colonized with non–CA-MRSA strains, we found that patients colonized with HA-MRSA strains were 3× more likely to have been admitted to an ICU within the previous 12 months. Other demographic characteristics did not differ between the groups. Overall, our findings agree with those of other studies that have shown that demographic data and risk factors may not reliably distinguish between patients colonized or those infected with various MRSA strains (*14*,*36*).

Our study has several limitations. First, only nares cultures were performed to identify asymptomatic MRSA

Table 4. Characteristics of patients who acquired MRSA colonization in the PICU, The Johns Hopkins University Hospital, Baltimore, MD, USA, March 2007–2008*

Patient no.	Age, y	Culture type	Days in PICU before MRSA acquisition	Strain type	Clinical service
1	7.0	Clin	19	A	Surg
2	4.5	Surv	7	A	Med
3	11.2	Surv	5	B	Surg
4	2.5	Surv	9	USA300	Surg
5	1.2	Surv	5	USA300	Surg
6	9.9	Surv	24	USA300	Surg
7	3.8	Clin	5	USA300	Surg
8	7.5	Surv	5	Unknown	Med

*MRSA, methicillin-resistant *Staphylococcus aureus*; PICU, pediatric intensive care unit; Clin, clinical; Surg, surgical; Surv, surveillance; Med, medical.

carriers at the time of admission to the PICU. Recent studies have shown that screening extranasal sites or subtances, such as throat, axilla, perineum, or stool can increase the detection of MRSA carriers (*37*,*38*), especially those colonized with CA-MRSA strains. However, the best sites to detect MRSA, in combination with nasal culture, remain unclear (*38*). Nares cultures, if poorly carried out, can have false-negative results. Therefore, if we misclassified MRSA carriers with negative nasal screening cultures, we may have underestimated the MRSA prevalence. Sensitive, yet cost-effective, methods of screening for MRSA colonization are still needed.

The second limitation is that admission screening cultures were instituted in March 2007, and compliance with screening was only 72%. Our PICU did not have admission order sets, and in the absence of a physician's written order, cultures were not always performed. Compliance improved over time with initiation of a patient order entry system, a visual reminder to perform screening cultures on the front of the patient's chart, and frequent compliance auditing by the nurse manager. However, given the similarities between screened and unscreened patients, we expect that our measured prevalence was representative of the entire cohort. Third, our PICU has low MRSA incidence rates and may have a low prevalence of MRSA colonization at the time of admission compared with other PICUs. These conditions may limit how our findings can be generalized to other institutions.

Overall, we found that epidemic CA-MRSA strains are likely endemic to PICUs. These virulent and transmissible strains are entering the PICU through infected or colonized patients, they are being transmitted to children, and they are responsible for hospital-onset MRSA infections. CA-MRSA strains often colonize children without healthcare-associated risk factors. Traditional infection-control strategies, in which risk factors are used to target high-risk patients for screening and intervention, may prove insufficient for MRSA. Future studies to determine optimal approaches to control MRSA transmission in hospitalized children are needed. As CA-MRSA strains enter the hospital environment, the increased frequency of methicillin resistance and the coexistence of multiple strain types may lead to the selection of novel MRSA strains with enhanced capacity for transmission and infection. These conditions would be especially concerning with regard to children, for whom a more restricted antimicrobial drug arsenal is available. Sound epidemiologic investigation and feasible interventions are needed to control MRSA and protect hospitalized children.

Acknowedgments

We thank Kathleen Speck; Claire Beers; and Johns Hopkins Hospital microbiology laboratory staff, PICU nursing staff, and Epidemiology and Infection Control Group for their support of this study.

A.M. was supported by Johns Hopkins Clinical Research Career Development Award, National Institutes of Health/National Center for Research Resources 1KL2RR025006-01, NIH/National Institute for Allergy and Infectious Diseases 1 K23 AI081752-01, and the Thomas Wilson Sanitarium for Children of Baltimore City (Baltimore, MD, USA). T.P. was supported by Centers for Disease Control and Prevention Grant UR8/CCU315092.

Dr Milstone is an assistant professor of pediatric infectious diseases at The Johns Hopkins University School of Medicine. His research interests include the prevention of hospital-acquired infections in children; he studies the prevalence and transmission of multidrug-resistant bacteria in hospitalized children and tests interventions to prevent their spread and reduce hospital-acquired infections.

References

1. Herold BC, Immergluck LC, Maranan MC, Lauderdale DS, Gaskin RE, Boyle-Vavra S, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. JAMA. 1998;279:593–8. DOI: 10.1001/jama.279.8.593
2. Daum RS, Ito T, Hiramatsu K, Hussain F, Mongkolrattanothai K, Jamklang M, et al. A novel methicillin-resistance cassette in community-acquired methicillin-resistant *Staphylococcus aureus* isolates of diverse genetic backgrounds. J Infect Dis. 2002;186:1344–7. DOI: 10.1086/344326
3. McDougal LK, Steward CD, Killgore GE, Chaitram JM, McAllister SK, Tenover FC. Pulsed-field gel electrophoresis typing of oxacillin-resistant *Staphylococcus aureus* isolates from the United States: establishing a national database. J Clin Microbiol. 2003;41:5113–20. DOI: 10.1128/JCM.41.11.5113-5120.2003
4. Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, Etienne J, et al. Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection. JAMA. 2003;290:2976–84. DOI: 10.1001/jama.290.22.2976
5. Vandenesch F, Naimi T, Enright MC, Lina G, Nimmo GR, Heffernan H, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence. Emerg Infect Dis. 2003;9:978–84.

6. Fridkin SK, Hageman JC, Morrison M, Sanza LT, Como-Sabetti K, Jernigan JA, et al. Methicillin-resistant *Staphylococcus aureus* disease in three communities. N Engl J Med. 2005;352:1436–44. DOI: 10.1056/NEJMoa043252
7. Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, et al. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. JAMA. 2007;298:1763–71. DOI: 10.1001/jama.298.15.1763
8. Agwu A, Brady KM, Ross T, Carroll KC, Halsey NA. Cholera-like diarrhea and shock associated with community-acquired methicillin-resistant *Staphylococcus aureus* (USA400 clone) pneumonia. Pediatr Infect Dis J. 2007;26:271–3. DOI: 10.1097/01.inf.0000256766.25247.d4
9. Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, Fosheim GE, et al. Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001–2004. J Infect Dis. 2008;197:1226–34. DOI: 10.1086/533494
10. Kaplan SL, Hulten KG, Gonzalez BE, Hammerman WA, Lamberth L, Versalovic J, et al. Three-year surveillance of community-acquired *Staphylococcus aureus* infections in children. Clin Infect Dis. 2005;40:1785–91. DOI: 10.1086/430312
11. Alfaro C, Mascher-Denen M, Fergie J, Purcell K. Prevalence of methicillin-resistant *Staphylococcus aureus* nasal carriage in patients admitted to Driscoll Children's Hospital. Pediatr Infect Dis J. 2006;25:459–61. DOI: 10.1097/01.inf.0000215228.25382.bd
12. Zaoutis TE, Toltzis P, Chu J, Abrams T, Dul M, Kim J, et al. Clinical and molecular epidemiology of community-acquired methicillin-resistant *Staphylococcus aureus* infections among children with risk factors for health care–associated infection: 2001–2003. Pediatr Infect Dis J. 2006;25:343–8. DOI: 10.1097/01.inf.0000207403.67197.cc
13. Saiman L, O'Keefe M, Graham PL III, Wu F, Said-Salim B, Kreiswirth B, et al. Hospital transmission of community-acquired methicillin-resistant *Staphylococcus aureus* among postpartum women. Clin Infect Dis. 2003;37:1313–9. DOI: 10.1086/379022
14. Popovich KJ, Weinstein RA, Hota B. Are community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) strains replacing traditional nosocomial MRSA strains? Clin Infect Dis. 2008;46:787–94.
15. Seybold U, Kourbatova EV, Johnson JG, Halvosa SJ, Wang YF, King MD, et al. Emergence of community-associated methicillin-resistant *Staphylococcus aureus* USA300 genotype as a major cause of health care–associated blood stream infections. Clin Infect Dis. 2006;42:647–56. DOI: 10.1086/499815
16. Lin YC, Lauderdale TL, Lin HM, Chen PC, Cheng MF, Hsieh KS, et al. An outbreak of methicillin-resistant *Staphylococcus aureus* infection in patients of a pediatric intensive care unit and high carriage rate among health care workers. J Microbiol Immunol Infect. 2007;40:325–34.
17. Milstone AM, Song X, Beers C, Berkowitz I, Carroll KC, Perl TM. Unrecognized burden of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* carriage in the pediatric intensive care unit. Infect Control Hosp Epidemiol. 2008;29:1174–6. DOI: 10.1086/592093
18. Warren DK, Guth RM, Coopersmith CM, Merz LR, Zack JE, Fraser VJ. Epidemiology of methicillin-resistant *Staphylococcus aureus* colonization in a surgical intensive care unit. Infect Control Hosp Epidemiol. 2006;27:1032–40. DOI: 10.1086/507919
19. Cohen AL, Calfee D, Fridkin SK, Huang SS, Jernigan JA, Lautenbach E, et al. Recommendations for metrics for multidrug-resistant organisms in healthcare settings: SHEA/HICPAC Position paper. Infect Control Hosp Epidemiol. 2008;29:901–13. DOI: 10.1086/591741
20. Horan TC, Andrus M, Dudeck MA. CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting. Am J Infect Control. 2008;36:309–32. DOI: 10.1016/j.ajic.2008.03.002
21. Farley JE, Stamper PD, Ross T, Cai M, Speser S, Carroll KC. Comparison of the BD GeneOhm methicillin-resistant *Staphylococcus aureus* (MRSA) PCR assay to culture by use of BBL CHROMagar MRSA for detection of MRSA in nasal surveillance cultures from an at-risk community population. J Clin Microbiol. 2008;46:743–6. DOI: 10.1128/JCM.02071-07
22. Murray BE, Singh KV, Heath JD, Sharma BR, Weinstock GM. Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. J Clin Microbiol. 1990;28:2059–63.
23. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233–9.
24. Lucet JC, Chevret S, Durand-Zaleski I, Chastang C, Regnier B. Prevalence and risk factors for carriage of methicillin-resistant *Staphylococcus aureus* at admission to the intensive care unit: results of a multicenter study. Arch Intern Med. 2003;163:181–8. DOI: 10.1001/archinte.163.2.181
25. Davis KA, Stewart JJ, Crouch HK, Florez CE, Hospenthal DR. Methicillin-resistant *Staphylococcus aureus* (MRSA) nares colonization at hospital admission and its effect on subsequent MRSA infection. Clin Infect Dis. 2004;39:776–82. DOI: 10.1086/422997
26. Jernigan JA, Titus MG, Groschel DH, Getchell-White S, Farr BM. Effectiveness of contact isolation during a hospital outbreak of methicillin-resistant *Staphylococcus aureus*. Am J Epidemiol. 1996;143:496–504.
27. Khoury J, Jones M, Grim A, Dunne WM Jr, Fraser V. Eradication of methicillin-resistant *Staphylococcus aureus* from a neonatal intensive care unit by active surveillance and aggressive infection control measures. Infect Control Hosp Epidemiol. 2005;26:616–21. DOI: 10.1086/502590
28. Huang SS, Platt R. Risk of methicillin-resistant *Staphylococcus aureus* infection after previous infection or colonization. Clin Infect Dis. 2003;36:281–5. DOI: 10.1086/345955
29. Hidron AI, Kourbatova EV, Halvosa JS, Terrell BJ, McDougal LK, Tenover FC, et al. Risk factors for colonization with methicillin-resistant *Staphylococcus aureus* (MRSA) in patients admitted to an urban hospital: emergence of community-associated MRSA nasal carriage. Clin Infect Dis. 2005;41:159–66. DOI: 10.1086/430910
30. Creech CB II, Kernodle DS, Alsentzer A, Wilson C, Edwards KM. Increasing rates of nasal carriage of methicillin-resistant *Staphylococcus aureus* in healthy children. Pediatr Infect Dis J. 2005;24:617–21. DOI: 10.1097/01.inf.0000168746.62226.a4
31. Chen AE, Goldstein M, Carroll K, Song X, Perl TM, Siberry GK. Evolving epidemiology of pediatric *Staphylococcus aureus* cutaneous infections in a Baltimore hospital. Pediatr Emerg Care. 2006;22:717–23. DOI: 10.1097/01.pec.0000236832.23947.a0
32. Szczesiul JM, Shermock KM, Murtaza UI, Siberry GK. No decrease in clindamycin susceptibility despite increased use of clindamycin for pediatric community-associated methicillin-resistant *Staphylococcus aureus* skin infections. Pediatr Infect Dis J. 2007;26:852–4. DOI: 10.1097/INF.0b013e318124aa5c
33. Moran GJ, Krishnadasan A, Gorwitz RJ, Fosheim GE, McDougal LK, Carey RB, et al. Methicillin-resistant *S. aureus* infections among patients in the emergency department. N Engl J Med. 2006;355:666–74. DOI: 10.1056/NEJMoa055356
34. Gonzalez BE, Martinez-Aguilar G, Hulten KG, Hammerman WA, Coss-Bu J, Avalos-Mishaan A, et al. Severe staphylococcal sepsis in adolescents in the era of community-acquired methicillin-resistant *Staphylococcus aureus*. Pediatrics. 2005;115:642–8. DOI: 10.1542/peds.2004-2300
35. Adem PV, Montgomery CP, Husain AN, Koogler TK, Arangelovich V, Humilier M, et al. *Staphylococcus aureus* sepsis and the Waterhouse-Friderichsen syndrome in children. N Engl J Med. 2005;353:1245–51. DOI: 10.1056/NEJMoa044194

36. Miller LG, Perdreau-Remington F, Bayer AS, Diep B, Tan N, Bharadwa K, et al. Clinical and epidemiologic characteristics cannot distinguish community-associated methicillin-resistant *Staphylococcus aureus* infection from methicillin-susceptible *S. aureus* infection: a prospective investigation. Clin Infect Dis. 2007;44:471–82. DOI: 10.1086/511033
37. Rohr U, Mueller C, Wilhelm M, Muhr G, Gatermann S. Methicillin-resistant *Staphylococcus aureus* whole-body decolonization among hospitalized patients with variable site colonization by using mupirocin in combination with octenidine dihydrochloride. J Hosp Infect. 2003;54:305–9. DOI: 10.1016/S0195-6701(03)00140-3
38. Ringberg H, Cathrine Petersson A, Walder M, Hugo Johansson PJ. The throat: an important site for MRSA colonization. Scand J Infect Dis. 2006;38:888–93. DOI: 10.1080/00365540600740546

Address for correspondence: Aaron M. Milstone, Departments of Pediatric Infectious Diseases and Hospital Epidemiology and Infection Control, The Johns Hopkins University School of Medicine, 200 N Wolfe St, Rubenstein 3141, Baltimore, MD 21287, USA; email: amilsto1@jhmi.edu

Contribution of *Streptococcus anginosus* to Infections Caused by Groups C and G Streptococci, Southern India

Silvana Reißmann, Claudia Friedrichs, Reena Rajkumari, Andreas Itzek, Marcus Fulde, Arne C. Rodloff, Kootallur N. Brahmadathan, Gursharan S. Chhatwal, and D. Patric Nitsche-Schmitz

Vellore, a region in southern India, has a high incidence of severe human infections with β-hemolytic group C and G streptococci (GCGS). To determine the causative species in these infections, we conducted 16S rRNA gene sequencing: *Streptococcus dysgalactiae* subsp. *equisimilis* (81%) and *S. anginosus* (19%) were the causative organisms in the 2-year study period (2006–2007). We used PCR to detect the virulence-related *emm* gene; results showed that it was restricted to *S. dysgalactieae* subsp. *equisimilis* isolates of 99.2% tested positive. Due to a novel marker, *S. anginosus* and *S. constellatus* can be quickly and accurately distinguished from other members of the genus. The notable contribution of the anginosus group to human infections suggests that this group of obligate pathogens deserves more attention in healthcare and research.

Group C and group G streptococci (together GCGS) were first recognized as human pathogens in 1935 by Lancefield and Hare (*1*). Since then, awareness about their importance has greatly increased, especially within recent years (*2*–*6*). Similar to infections with *Streptococcus pyogenes,* the prime example of a pyogenic streptococcal pathogen, infections with GCGS can develop into life-threatening necrotizing fasciitis, sepsis, and streptococcal toxic shock–like syndrome. Lancefield groups C and G comprise a variety of species; one of those species, *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE), frequently causes human infections. This species can cause the whole spectrum of infections caused by *S. pyogenes* (*4*,*5*).

SDSE likely owes its virulence in humans to homologs of prominent *S. pyogenes* virulence genes (*7*,*8*). Most SDSE strains isolated from human infections possess *emm* genes (*9*,*10*), which code for the potent virulence factor called M protein (*11*). This surface localized protein contributes substantially toward the virulence of both *S. pyogenes* and SDSE in human hosts because it acts as an adhesin, invasin, and antiphagocytic factor (*11*). Strain-to-strain variability in the N terminus of M proteins, driven by the adaptive immune response of the host, has led to a vast *emm* type diversity. More than 100 genetically distinct M proteins exist within the GCGS group and form the basis for *emm* genotyping (*12*). However, SDSE is not the only species that causes severe diseases in humans. The variety of GCGS includes the typical animal pathogens *S. equi* subsp. *zooepidemicus* (group C) and *S. canis* (group G), which have the potential to cause zoonotic infections (*13*,*14*). Other streptococcal species that are pathogenic in humans and that occasionally expose groups C and G carbohydrates are gathered under the umbrella term anginosus group (*15*).

In the literature, the designation *S. milleri* (*16*) has often been used for streptococci of this group, although it has never been an officially approved name (*15*). Streptococci of the anginosus group can reside commensally in the human oral cavity but have a certain propensity to cause pharyngitis, bacteremia, and serious purulent infections in the deep neck and soft tissue and in internal organs such as the brain, lung, and liver (*17*–*25*). The bacteria cause severe infections after surgical treatments and infect im-

Author affiliations: Helmholtz Centre for Infection Research, Braunschweig, Germany (S. Reißmann, A. Itzek, M. Fulde, G.S. Chhatwal, D.P. Nitsche-Schmitz); University Hospital Leipzig, Leipzig, Germany (C. Friedrichs, A.C. Rodloff); and Christian Medical College, Vellore, Tamil Nadu, India (R. Rajkumari, K.N. Brahmadathan)

DOI: 10.3201/eid1604.090448

planted material, thereby posing a problem of substantial clinical relevance (*20*,*26*,*27*). The species diversity within the GCGS highlights the limits of Lancefield grouping by agglutination assays, the typically applied method in the diagnosis of streptococcal infections. The genetically distinct GCGS species differ in pathogenesis, virulence mechanisms, and antimicrobial drug susceptibility. Thus, finding the optimal treatment regimen can be facilitated by species determination. Diagnosis of anginosus group infections is particularly difficult. The group comprises the species *S. anginosus*, *S. intermedius*, and *S. constellatus* of which the 2 subspecies, *S. constellatus* subsp. *constellatus* and *S. constellatus* subsp. *pharyngis*, are further distinguished. Identification of the anginosus group is complicated by wide phenotypic and antigenic diversity, even within 1 species. Although most anginosus group isolates belong to the non-β-hemolytic oral streptococci, β-hemolytic strains are found in all 3 species. Some anginosus group strains carry a typeable Lancefield group antigen, which belongs to group F, C, G, or A (*28*).

Routine microbiologic diagnosis of streptococcal infections is often restricted to determination of the type of hemolysis and of the Lancefield group. Identification of streptococci to the species level is rarely carried out. This leaves a considerable risk for misidentification of causative pathogens, which can lead to an inappropriate treatment of the infection (*29*–*31*). As a further consequence of the complications associated with species determination, insight into the epidemiology of infections with certain streptococci remains imprecise, and the epidemiology of the anginosus group, in particular, remains widely elusive. Comprehensive insights are missing that could enable clinicians to reevaluate respective diagnostic and therapeutic routines. Moreover, such insights may stimulate research that aims at clarifying the pathogenesis of these streptococcal species and the development of specialized treatments and prevention strategies. These goals have motivated our cross-species study of human pathogenic GCGS from Vellore, India, a region with a high incidence of such infections. Examination of epidemiologic contributions of the different streptococcal species was combined with a cross-species screening for *emm* genes, which identified a novel gene in *S. anginosus* and *S. constellatus*. The use of this gene as a marker for fast detection of infections caused by these 2 streptococcal species was investigated.

Methods

Bacterial Strains, Lancefield Typing, and Genomic DNA

Clinical isolates of group G and group C streptococci were collected at the Department of Clinical Microbiology, Christian Medical College, Vellore, India, from 2006 through 2007. The collection comprised isolates from patients with pharyngitis (throat swabs), patients with respiratory (sputum) and urinary tract infections (urine), and from other suppurative foci (pus) and blood. Streptococci were collected from 2004 through 2006 at the University Hospital in Leipzig, Germany. The isolates were recovered from blood cultures, wound swabs, aspirates of peritonsillar abscesses, abscesses in the inner body, and catheter tips. The collection was typed as described previously (*32*). Bacterial strains were subcultured on Columbia agar with 5% sheep blood (Becton-Dickinson, Franklin Lakes, NJ, USA). Cultures from single colonies were grown overnight (37°C in 5% CO_2) in Todd-Hewitt broth (Becton-Dickinson) supplemented with 0.5% yeast extract. The Lancefield group was determined by using the Slidex streptococcal grouping kit (Oxoid, Basingstoke, UK). Genomic DNA was isolated by using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol with a minor variation: the incubation with proteinase K was carried out at 70°C for 30 min.

Sequencing of *emm* and 16S rRNA Genes

Amplification of *emm* genes from streptococcal genomic DNA samples was performed by using the primers 1 and 2 recommended by the Centers for Disease Control and Prevention (*12*) (*emm*-PCR). To amplify the 16S rRNA gene, PCR was performed with a pair of generic primers: 16S rDNA fwd and 16S rDNA rev (Table 1), for gram-positive bacteria as described (*33*). PCR experiments were analyzed by agarose (1%) gel electrophoresis. PCR products were purified by using the QIAGEN PCR purification Kit (QIAGEN, Hilden, Germany) and sequenced by using primer 16S rDNA fwd (Table 1), the Big Dye Terminator reaction, and an ABI Prism 377 system (Applied Biosystems, Foster City, CA, USA).

Inverse PCR and Sequencing of a Marker of *S. anginosus* and *S. constellatus*

Application of *emm*-PCR on the genomic DNA of *S. anginosus* strain SV52 produced a 1.1-kb amplicon, subsequently identified as a fragment of a marker of *S. anginosus* and *S. constellatus* (*moac*). The fragment was cloned into the pCR2.1-TOPO vector by using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and then sequenced by using primers M13 rev and M13 fwd (Table 1). Inverse PCR was used to amplify the genomic DNA segments flanking the 1.1-kb fragment of *moac*. Genomic DNA (1 μg) was digested separately with 1–3 of the following enzymes: *Ase*I, *Avr*II, *Bam*HI, *Bgl*II, *Bsa*I, *Bse*YI, *Eco*RI, *Hind*III, *Nde*I, *Nsi*I, *Pst*I, *Sac*I, *Sal*I, *Spe*I, *Xba*I, *Xho*I (New England Biolabs, Ipswich, MA, USA). Digestion was carried out for 16 h under conditions recommended by the manufacturer. Digested genomic DNA

Table 1. Sequences of primers used in study of groups C and G streptococci, Vellore, India, and Leipzig, Germany*

Name	Sequence (5′ → 3′)	Application
16S rDNA fwd	AGAGTTTGATCCTGGCTC	16S rDNA amplification
16S rDNA rev	GGTTACCTTGTTACGACTT	
Primer 1	TATTCGCTTAGAAAATTAA	*emm* amplification
Primer 2	GCAAGTTCTTCAGCTTGTTT	
M13 rev	CAATTTCACACAGGAAACAGCTATGAC	Sequencing of 1.1-kb fragment of *moac*
M13 fwd	GTAAAACGACGGCCAGTGAATTG	
*moac*1	CAAGGAATTGATTCAGCAACAGTGC	Inverse PCR and sequencing of *moac*
*moac*3	CTTCTCAACAAGCATTGGCAGATGC	
*moac*6	GTGTGTATACACGTCGGACATTTCC	
*moac*7	GGTACAGTAATGGGAAGTTTGTTAGG	
*moac*8	GCGGATTGACTTCATTTGGCGTCG	
*moac*9	GGTTTGGGGATGTCTTCTTCCATGG	
*moac*10	GCATCTCAAATCAGACGAGCAAGC	
*moac*11	CTTGAACTTGTCTTCGCATGGAGC	
*moac*12	GACTATTATCAAACGGTATTTGCTCG	
*moac*2	CCAATTCACTTGAATTGACGAATCC	
*moac*4	GCCCAACCTGAAGACAGTTGAGC	
*moac*5	CTGACGAAAAGAGAGCCAGATATCC	
*moac*13	CTGATACCATAATCTGACATCACTGC	
*moac*14	GAAGTTGAACTATCTCCAATCACCG	
moac-SP	ATGAAAAAATCCATTCTAAATAAGGATATC	Screening for 3,272-bp fragment of *moac*
moac-TMH7	AAGACTGGCACAAGATATAC	
moac-BamH1	GCGGATCCGGTCATTTTCCAAGCAAGG	Screening for 962-bp fragment of *moac*
moac-Sal1	GCTGTCGACTTATTAAATTCAGCCTGCTTTTTCTCC	

**moac*, marker of *Streptococcus anginosus* and *S. constellatus.*

was diluted both 100-fold and 10-fold before self-ligation with 160 U ligase (16 h, 16°C). Afterwards the ligations were used as a template in PCR experiments with different combinations of the primers moac1 to moac14 (Table 1). PCR was performed in a thermocycler (Biometra GmbH, Goettingen, Germany) with an initial denaturation step (4 min at 96°C), followed by 30 cycles of denaturation (40 s at 94°C), annealing (30 s at 56°C), and extension (1 min, 30 s, up to 3 min, 72°C). A final extension was carried out for 5 min at 72°C. PCR products were analyzed, purified, and sequenced as described above.

Screening for *moac*

Isolated genomic DNA was tested by PCR for the presence of the *moac* gene. For this purpose, 2 primer pairs (Table 1) were used to amplify a 3,272-bp fragment (primers: *moac*-SP, *moac*-TMH7) and a 962-bp internal fragment (primers: *moac*-*Bam*HI, *moac*-*Sal*I) of *moac*, respectively. For amplification of the 962-bp fragment, initial denaturation (4 min at 96°C) was followed by 25 cycles of denaturation (40 s at 94°C), annealing (30 s at 53°C), and extension (1 min 30 s at 72°C), and then by a final extension step for 5 min at 72°C. The 3,272-bp fragment was amplified with 30 cycles by using an annealing temperature of 50°C and an extension time of 3 min, 20 s. The PCR products were analyzed, purified, and sequenced as described above.

Sequence Analysis

Sequence data were processed and analyzed by using the software BioEdit version 7.0.1 (Isis Pharmaceuticals, Carlsbad, CA, USA). Coiled coil structures and transmembrane helixes were predicted with the Web-based programs Coils (*34*) and TMHMM Server v. 2.0 (*35*), respectively.

Results

Typing of GCGS Isolates and Screening for *emm* (-like) Genes

The Vellore region in India has a high incidence of group C and G streptococcal infections (*36*). For characterization of local pathogenic GCGS strains, 313 isolates were collected from patients with suppurative or invasive infections at the Christian Medical College. The study was designed to be cross-species; therefore, the only preselection criteria applied were type of hemolysis and Lancefield type. Sequencing showed that 254 of the strains showed highest homology with the 16S rRNA gene from SDSE and that the sequences of the remaining 59 strains were homologous to *S. anginosus* (Table 2). Notably, all *S. anginosus* strains belonged to Lancefield group G. M proteins have a fundamental role in streptococcal infections. Therefore, and because of a potential power to discriminate between SDSE and anginosus group strains, the collection was examined by *emm*-PCR (*12*) (Table 2). Specific PCR products were obtained for 252 of the 313 stains. Two of the

Table 2. Typing of groups C and G streptococci collection, Vellore, India

Species (N = 313)	16S rRNA gene	*emm*-PCR positive/negative	*moac*-PCR* positive/negative	Lancefield group G	Lancefield group C
S. dysgalactiae subsp. *equisimilis*	254	252/2	0/254	208	46
S. anginosus	59	0/59	59/0	59	0

*Tested with 2 primer pairs (Figure). *moac*, marker of *Streptococcus anginosus* and *S. constellatus*.

SDSE strains and the 59 anginosus group strains were not *emm*-typeable. The SDSE strains comprised 44 different *emm* types, of which 2, stG120 and stG351, have not been previously described (Table 3).

Characteristics of a Newly Discovered Open Reading Frame of *S. anginosus*

Our recent survey at the University Hospital in Leipzig, comprising 127 cases of severe infections with oral streptococci, found that a large number of infections were caused by strains that belonged to the anginosus group (26%). We included the anginosus group strains from the Leipzig collection in the *emm*-typing experiments described above to increase the number of isolates and the phenotypic diversity of the collection in terms of Lancefield antigen and type of hemolysis. Like the *S. anginosus* strains from Vellore, none of the 33 strains from Leipzig was *emm*-typeable. Under less stringent conditions, the PCR produced a low concentration amplicon of 1.1 kb (Figure).

Sequencing of the of 1.1-kb PCR product did not show considerable similarities with *emm*-genes, however, the lack of stop codons in 1 frame of translation motivated further investigations on that PCR product. Inverted PCR experiments on *S. anginosus* strain SV52 identified an open reading frame (ORF) of 3,363-bp (GenBank accession no. GQ456155). Computational analysis predicts a 124-kDa membrane protein with 7 transmembrane sequences and a signal peptide for secretion (Figure). The predicted protein further consists of 2 larger extracellular regions, one of 23 kDa located between the fourth and fifth transmembrane sequence and one of 60 kDa at its N terminal end. The central part of the N terminal extracellular region contains a stretch of heptad-repeats (aa 204 to 520 of the mature protein), which may enable coiled-coil oligomerization. Other than this, the protein has no obvious or significant features of an M- or M-related protein. Prediction of 7 transmembrane sequences suggests a receptor function or a function in transport processes. The latter assumption is corroborated by a high similarity of the protein sequence to numerous bacterial permease components of ATP-binding cassette transporters.

Distinguishing of *S. anginosus* and *S. constellatus* from Other Oral Streptococci by a Newly Discovered ORF

The distribution of the newly discovered ORF in the Leipzig collection of oral streptococci was examined by PCR with 2 different primer pairs (Table 1). Both primer combinations gave identical results. The collection consists of 127 clinical isolates of which 33 belong to the anginosus group (17 *S. anginosus*, 8 *S. constellatus* subsp. *constellatus*, 4 *S. constellatus* subsp. *pharyngis*, 4 *S. intermedius*); 78 belong to species of the mitis group (*S. mitis*, *S. oralis*, *S. sanguinis*, *S. parasanguinis*). Fourteen strains have been typed as *S. salivarius* and 2 as *S. gallolyticus*. Specific PCR products were obtained exclusively within the anginosus group. All *S. anginosus* and *S. constellatus* isolates tested positive. Negative PCR distinguished *S. intermedius* from the other 2 species. The results were confirmed in experi-

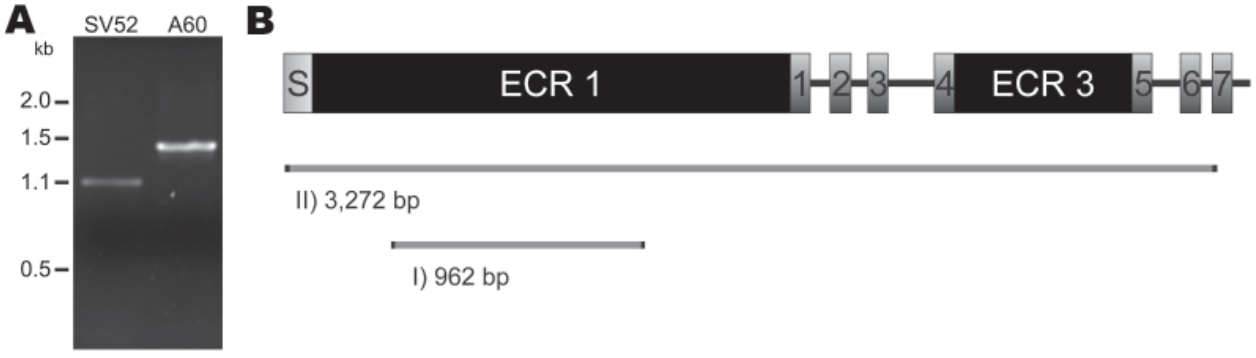


Figure. Amplification and characterization of a newly discovered open reading frame (ORF) of *Streptococcus anginosus*. *A*) Gel electrophoresis after *emm*-PCR on *S. anginosus* isolate SV52 (*SV52*) and *S. pyogenes* strain A60 (*A60*). The latter isolate was used as a control that possesses an *emm*3 gene. The *S. anginosus* strain generated a low concentration 1.1-kb amplicon, as compared with the 1.4-kb product of the *S. pyogenes* strain. Inverse PCR based on the 1.1-kb sequence of SV52 showed an ORF of 3,363 bp. Its sequence is predicted to code for the membrane protein that is schematically depicted in panel B. It comprises an N terminal signal peptide (S) followed by a large extracellular region of 60 kDa (ECR1), 7 transmembrane helixes (1–7), and another large extracellular region of 23 kDa situated between the fourth and the fifth transmembrane helix (ECR3). Positions of the 2 alternative marker of *Streptococcus anginosus* and *S. constellatus* PCR products (I and II) relative to the full-length sequence and their length are indicated in basepairs.

ments with reference strains from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (Table 4). Taken together, the results demonstrate that the newly discovered gene is a marker that discriminates *S. anginosus* and *S. constellatus* from other oral streptococci. The gene was therefore designated *moac*.

Moac as a Marker for *S. anginosus* within GCGS

The data that were obtained with the Leipzig collection of oral streptococci suggested that *moac* could also be exploited as a marker for β-hemolytic strains of the anginosus group. To further test the quality of *moac* as a marker, the Vellore GCGS collection was subjected to *moac*-specific PCR (*moac*-PCR). The results are summarized in Table 2. All 254 strains that were specified as SDSE on the basis of their 16S rRNA gene sequence were negative in *moac*-PCR. The 59 remaining strains, which could be assigned to the species *S. anginosus* by 16S rRNA gene sequencing, were positive for *moac*. Moreover, strains of the species *S. canis* (*2*), *S. equi* subsp. *zooepidemicus* (*3*), *S. equi* subsp. *equi* (*2*), and *S. dysgalactiae* subsp. *dysgalactiae* (*2*) were negative in the *moac*-PCR, proving that it is a reliable method for identifying anginosus group strains in collections of GCGS. The examination showed that *S. anginosus* isolates constitute 19% of the collection of β-hemolytic GCGS isolates from clinical suppurative infections in Vellore; thus, these pathogens play a considerable epidemiologic role in acute streptococcal infections that occur in this region.

Discussion

Lancefield groups C and G comprise a variety of distinct species; of these species, *S. dysgalactiae* subsp. *equisimilis*, *S. equi* subsp. *zooepidemicus*, *S. canis*, and streptococci of the anginosus group cause severe infections in humans (*2–5*, *17–27*). Examination of clinical isolates from patients with purulent infections of the upper respiratory tract, the urinary tract, and invasive infections showed that SDSE is dominant in GCGS infections in Vellore, accounting for 81% of the cases. SDSE was responsible for all 7 cases of invasive infections included in this study. *S. anginosus* that possess group G antigens accounted for the remaining 19% of the suppurative infections, which indicates a considerable epidemiologic role for this species in the Vellore region. In contrast, β-hemolytic group C streptococci of the pharyngitis-associated species *S. constellatus* subsp. *pharyngis* (*37*) were not detected. Infections with the typically zoonotic β-hemolytic GCGS *S. equi* subsp. *zooepidemicus* (group C) and *S. canis* (group G) were also not diagnosed, which suggests a comparatively low incidence of such infections in Vellore. Groups C and G antigens are rare in *S. constellatus* subsp. *constellatus* and in *S. intermedius* (*28*), if they are present at all in these species. Their absence in the Vellore GCGS collection, therefore, cannot be considered as indicative that these species have less clinical relevance.

The high phenotypic and antigenic diversity within the anginosus group, and the circumstance that non–β-hemolytic strains remain inconspicuous in samples that contain commensal flora, make anginosus group infections difficult to

Table 3. *emm* typing of pathogenic *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from Vellore, India*

emm type	No. strains
stG245	32
stG6792	18
stG643	17
stG6	14
stG653	14
stC1400	12
stGL265	12
stG652	11
stG866	10
stC5345	9
stG485	9
stC74a	9
stC6979	8
stC839	7
stGLP1	7
stGrobn	5
stC922	4
stG211	4
stC2sk	3
stG2574	3
stG4222	3
stG480	3
stG4831	3
stG5420	3
stG7882	3
stG120*	3
*emm*31	2
stC36	2
stCK401	2
stG166b	2
stG2078	2
stG840	2
stG97	2
stG10	2
stG351*	1
*emm*23	1
stC1741	1
stC3852	1
stC46	1
stC5344	1
stC6746	1
stC-NSRT2	1
stGM220	1
stMD216	1

*The 5′ sequences of newly identified *emm* genes are deposited in GenBank (accession nos. FJ036933, FJ036936, and FJ036937) and in the *emm*-gene database of the Centers for Disease Control and Prevention, Atlanta, GA, USA.

Table 4. Distribution of *moac* within a collection of oral streptococci, Vellore, India, and Leipzig, Germany*

Group/species	No. strains	*moac*-PCR
Anginosus group	33	
Streptococcus anginosus	17	+
S. constellatus subsp. *constellatus*	8	+
S. constellatus subsp. *pharyngis*	4	+
S. intermedius	4	–
Bovis group	2	
S. gallolyticus	2	–
Mitis group	78	
S. gordonii	5	–
S. mitis/S. oralis	12	–
S. mitis	12	–
S. oralis	24	–
S. parasanguinis	18	–
S. sanguinis	7	–
Salivarius group	14	
S. salivarius	14	–
Reference strains (DSMZ)		
Anginosus group	4	
S. anginosus	1	+
S. constellatus subsp. *constellatus*	1	+
S. constellatus subsp. *pharyngis*	1	+
S. intermedius	1	–
Mutans group	1	
S. mutans	1	–

**moac*, marker of *Streptococcus anginosus* and *S. constellatus;* DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen.

diagnose. Therefore, contribution of the anginosus group as a whole to the epidemiology of streptococcal infections is poorly understood and needs further investigation, not only in Vellore but also in other regions with suspected high incidence. The required surveys will become feasible with the identification of potent markers for detection of anginosus strains such as the *moac* marker. The isolates from Vellore and Leipzig together represent a highly diverse collection of anginosus strains that differ not only in their geographic origin, but greatly in Lancefield type. The collection comprises strains with groups A, C, G, and F, and without Lancefield antigen. Furthermore, the collection includes isolates with all types of hemolysis. The diagnostic power of *moac* is demonstrated by the herein described PCR test that identified all of the phenotypically diverse *S. anginosus* and *S. constellatus* strains, discriminating them from other streptococci with 100% accuracy. To the best of our knowledge, this PCR method is the only one described that distinguishes *S. constellatus* subsp. *pharyngis* and *S. intermedius*. In addition to the reliability, the use of the moac as a marker may have a further valuable advantage; the *moac* gene is predicted to code for a transmembrane protein, a property that could be exploited for the development of an antibody based test for species determination.

Further valuable diagnostic information may lie in the *emm* gene. Examination of the Vellore collection allowed assessment of *emm* typing for use in species determination. Most of the 254 SDSE strains included in this study were positive for the *emm* gene by PCR. However, 2 strains of this species were negative for it, indicating that a negative *emm*-PCR is not an accurate exclusion criterion in typing of SDSE. In contrast to SDSE strains, all anginosus group strains, from both the Vellore and the Leipzig collection, were negative in *emm*-PCR. This examination of phenotypically diverse strains strongly suggests that a negative result from *emm*-PCR is a common, if not a general, property of the anginosus group. Oligonucleotide hybridization experiments did not show any indications for the presence of *emm*-homologs in such isolates (data not shown). Moreover, and contrary to observations with *S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi*, binding experiments with plasma proteins suggest that anginosus strains lack surface proteins that exert the typical functions of M proteins (data not shown).

Despite the absence of this key virulence factor, the anginosus group includes opportunistic pathogens that can cause bacteremia and a variety of severe purulent infections in the oral cavity, the urogenital tract, and internal organs. This circumstance raises the question, what are the factors that allow streptococci of the anginosus group to colonize at the site of infection and to survive under the hostile conditions of the suppurative focus of infection and the bloodstream? Little is known about the pathogenesis of anginosus group infections, but they seem to be governed by unique, unknown mechanisms. Unique mechanisms of pathogenesis and the decreased susceptibility of anginosus group strains to certain antimicrobial drugs (*15*) create a need for well-tailored treatments and thus for accurate diagnosis. In this context, indications that treatment with metronidazole facilitates infections with streptococci of the anginosus group deserve particular attention (*26*,*38*–*40*). Accumulating indications of the considerable clinical relevance of the anginosus group and the propensity of these bacteria to develop antimicrobial drug resistance suggest that they may be a group of emerging pathogens that should be monitored.

Acknowledgments

The excellent technical assistance of Annett Hennig-Rolle, Nina Janze, and Katja Mummenbrauer is gratefully acknowledged. We also thank the curators of the CDC database for streptococcal *emm* genes for their help in identifying new *emm* types.

This work was supported by the Presidential Fund of the Helmholtz Foundation in the framework of the Virtual Institute: Oral Streptococci, by a grant of the European Community's Sixth Framework Program under contract no. FP6-2004-INCO-DEV-3-032390, and by a DAAD-DST Bilateral Grant to K.N.B. and G.S.C.

Dr Reißmann is a biologist at the Helmholtz-Centre for Infection Research. She conducts research on streptococcal pathogens, developing new diagnostics approaches and investigating their mechanisms of pathogenesis.

References

1. Lancefield RC, Hare R. The serological differentiation of pathogenic and non-pathogenic streptococci from parturient woman. J Exp Med. 1935;61:335–49. DOI: 10.1084/jem.61.3.335
2. Hill HR, Caldwell GG, Wilson E, Hager D, Zimmerman RA. Epidemic of pharyngitis due to streptococci of Lancefield group G. Lancet. 1969;2:371–4. DOI: 10.1016/S0140-6736(69)92713-5
3. Efstratiou A. Outbreaks of human infection caused by pyogenic streptococci of Lancefield groups C and G. J Med Microbiol. 1989;29:207–19. DOI: 10.1099/00222615-29-3-207
4. Nohlgard C, Björklind A, Hammar H. Group G streptococcal infections on a dermatological ward. Acta Derm Venereol. 1992;72:128–30.
5. Baracco GJ, Bisno AL. Group C and group G streptococcal infections: epidemiologic and clinical aspects. In: Fischetti VA, editor. Gram-positive pathogens. 2nd ed. Washington: American Society for Microbiology Press; 2006. p. 223–9.
6. Broyles LN, Van Beneden C, Beall B, Facklam R, Shewmaker PL, Malpiedi P, et al. Population-based study of invasive disease due to beta-hemolytic streptococci of groups other than A and B. Clin Infect Dis. 2009;48:706–12. DOI: 10.1086/597035
7. Cleary PP, Peterson J, Chen C, Nelson C. Virulent human strains of group G streptococci express a C5a peptidase enzyme similar to that produced by group A streptococci. Infect Immun. 1991;59:2305–10.
8. Davies MR, McMillan DJ, Beiko RG, Barroso V, Geffers R, Sriprakash KS, et al. Virulence profiling of *Streptococcus dysgalactiae* subspecies *equisimilis* isolated from infected humans reveals 2 distinct genetic lineages that do not segregate with their phenotypes or propensity to cause diseases. Clin Infect Dis. 2007;44:1442–54. DOI: 10.1086/516780
9. Bisno AL, Craven DE, McCabe WR. M proteins of group G streptococci isolated from bacteremic human infections. Infect Immun. 1987;55:753–7.
10. Schnitzler N, Podbielski A, Baumgarten G, Mignon M, Kaufhold A. M or M-like protein gene polymorphisms in human group G streptococci. J Clin Microbiol. 1995;33:356–63.
11. Fischetti VA. Streptococcal M protein: molecular design and biological behavior. Clin Microbiol Rev. 1989;2:285–314.
12. Centers for Disease Control and Prevention. Protocol for *emm*-typing [cited 2009 Mar 12]. http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm
13. Beres SB, Sesso R, Pinto SW, Hoe NP, Porcella SF, Deleo FR, et al. Genome sequence of a Lancefield group C *Streptococcus zooepidemicus* strain causing epidemic nephritis: new information about an old disease. PLoS One. 2008;3:e3026. DOI: 10.1371/journal.pone.0003026
14. Galperine T, Cazorla C, Blanchard E, Boineau F, Ragnaud JM, Neau D. *Streptococcus canis* infections in humans: retrospective study of 54 patients. J Infect. 2007;55:23–6. DOI: 10.1016/j.jinf.2006.12.013
15. Ruoff KL. *Streptococcus anginosus* ("*Streptococcus milleri*"): the unrecognized pathogen. Clin Microbiol Rev. 1988;1:102–8.
16. Guthof O. Pathogenic strains of *Streptococcus viridans*; streptocci found in dental abscesses and infiltrates in the region of the oral cavity [in German]. Zentralbl Bakteriol. 1956;166:553–64.
17. Moore-Gillon JC, Eykyn SJ, Phillips I. Microbiology of pyogenic liver abscess. Br Med J (Clin Res Ed). 1981;283:819–21. DOI: 10.1136/bmj.283.6295.819
18. Parker MT, Ball LC. Streptococci and aerococci associated with systemic infection in man. J Med Microbiol. 1976;9:275–302. DOI: 10.1099/00222615-9-3-275
19. Bateman NT, Eykyn SJ, Phillips I. Pyogenic liver abscess caused by *Streptococcus milleri.* Lancet. 1975;1:657–9. DOI: 10.1016/S0140-6736(75)91760-2
20. Van der Auwera P. Clinical significance of *Streptococcus milleri.* Eur J Clin Microbiol. 1985;4:386–90. DOI: 10.1007/BF02148688
21. Murray HW, Gross KC, Masur H, Roberts RB. Serious infections caused by *Streptococcus milleri.* Am J Med. 1978;64:759–64. DOI: 10.1016/0002-9343(78)90514-4
22. Shlaes DM, Lerner PI, Wolinsky E, Gopalakrishna KV. Infections due to Lancefield group F and related Streptococci (*S. milleri, S. anginosus*). Medicine (Baltimore). 1981;60:197–207. DOI: 10.1097/00005792-198105000-00003
23. Jacobs JA, Pietersen HG, Stobberingh EE, Soeters PB. Bacteremia involving the "*Streptococcus milleri*" group: analysis of 19 cases. Clin Infect Dis. 1994;19:704–13.
24. Jacobs JA, Pietersen HG, Walenkamp GH, Stobberingh EE, Soeters PB. Intervertebral infection caused by *Streptococcus milleri*. A case report. Clin Orthop Relat Res. 1994; (302):183–8.
25. Whiley RA, Beighton D, Winstanley TG, Fraser HY, Hardie JM. *Streptococcus intermedius, Streptococcus constellatus*, and *Streptococcus anginosus* (the *Streptococcus milleri* group): association with different body sites and clinical infections. J Clin Microbiol. 1992;30:243–4.
26. Tresadern JC, Farrand RJ, Irving MH. *Streptococcus milleri* and surgical sepsis. Ann R Coll Surg Engl. 1983;65:78–9.
27. Laine P, Salo A, Kontio R, Ylijoki S, Lindqvist C, Suuronen R. Failed dental implants - clinical, radiological and bacteriological findings in 17 patients. J Craniomaxillofac Surg. 2005;33:212–7. DOI: 10.1016/j.jcms.2004.12.004
28. Facklam R. What happened to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev. 2002;15:613–30. DOI: 10.1128/CMR.15.4.613-630.2002
29. Hamrick HJ, Mangum ME. Beta-hemolytic *Streptococcus milleri* group misidentified as *Streptococcus pyogenes* on throat culture. Pediatr Infect Dis J. 1999;18:75–6. DOI: 10.1097/00006454-199901000-00022
30. Dierksen KP, Tagg JR. Haemolysin-deficient variants of *Streptococcus pyogenes* and *S. dysgalactiae* subsp. *equisimilis* may be overlooked as aetiological agents of pharyngitis. J Med Microbiol. 2000;49:811–6.
31. Johnson DR, Kaplan EL. False-positive rapid antigen detection test results: reduced specificity in the absence of group A streptococci in the upper respiratory tract. J Infect Dis. 2001;183:1135–7. DOI: 10.1086/319286
32. Friedrichs C, Rodloff AC, Chhatwal GS, Schellenberger W, Eschrich K. Rapid identification of viridans streptococci by mass spectrometric discrimination. J Clin Microbiol. 2007;45:2392–7. DOI: 10.1128/JCM.00556-07
33. Takahashi T, Kaneko M, Mori Y, Tsuji M, Kikuchi N, Hiramune T. Phylogenetic analyses of *Staphylococcus* based on the 16S rDNA sequence and assignment of clinical isolates from animals. J Vet Med Sci. 1997;59:775–83. DOI: 10.1292/jvms.59.775
34. Lupas A, Van Dyke M, Stock J. Predicting coiled coils from protein sequences. Science. 1991;252:1162–4. DOI: 10.1126/science.252.5009.1162
35. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001;305:567–80. DOI: 10.1006/jmbi.2000.4315

36. Brahmadathan KN, Koshi G. Importance of group G streptococci in human pyogenic infections. J Trop Med Hyg. 1989;92:35–8.
37. Whiley RA, Hall LM, Hardie JM, Beighton D. A study of small-colony, beta-haemolytic, Lancefield group C streptococci within the anginosus group: description of *Streptococcus constellatus* subsp. *pharyngis* subsp. *nov*., associated with the human throat and pharyngitis. Int J Syst Bacteriol. 1999;49:1443–9.
38. Onderdonk AB, Cisneros RL. Comparison of clindamycin and metronidazole for the treatment of experimental intraabdominal sepsis produced by *Bacteroides fragilis* and *Streptococcus intermedius.* Curr Ther Res. 1985;38:893–8.
39. Hatoff DE. Perineal Crohn's disease complicated by pyogenic liver abscess during metronidazole therapy. Gastroenterology. 1983;85:194–5.
40. Allison HF, Immelman EJ, Forder AA. Pyogenic liver abscess caused by *Streptococcus milleri*. Case reports. S Afr Med J. 1984;65:432–5.

Address for correspondence: D. Patric Nitsche-Schmitz, Helmholtz Centre for Infection Research, Inhoffenstraße 7, D-38124 Braunschweig, Germany; email: patric.nitsche@helmholtz-hzi.de

Alfred Russel Wallace and the Antivaccination Movement in Victorian England

Thomas P. Weber

Alfred Russel Wallace, eminent naturalist and codiscoverer of the principle of natural selection, was a major participant in the antivaccination campaigns in late 19th-century England. Wallace combined social reformism and quantitative arguments to undermine the claims of provaccinationists and had a major impact on the debate. A brief account of Wallace's background, his role in the campaign, and a summary of his quantitative arguments leads to the conclusion that it is unwarranted to portray Victorian antivaccination campaigners in general as irrational and antiscience. Public health policy can benefit from history, but the proper context of the evidence used should always be kept in mind.

In 2009, the scientific community commemorated the 200th birthday of Charles Darwin and the 150th anniversary of the publication of On the Origin of Species by Means of Natural Selection. These occasions also directed the view of a wider public to the unjustly neglected figure of Alfred Russel Wallace (1823–1913) (Figure), explorer and codiscoverer of the principle of natural selection. In the past few years, Wallace's work has in fact enjoyed increasing attention among the historians of science, as several new biographies and studies prove (*1*–*5*). But unlike Darwin, Wallace always was and probably will remain a serious challenge to the history of science: he stubbornly refuses to fit into the mold of the typical scientific hero. Wallace made without any doubt lasting contributions to biologic science, but the second half of his life was by and large devoted to what from today's perspective are utterly lost causes: He became a passionate advocate of spiritualism, supported land nationalization, and fervently objected to compulsory smallpox vaccination.

The motives behind Wallace's campaigns are sometimes difficult to fathom. He published copiously because this served for a long time as his major source of income, but these writings only show the public face of Wallace. Unlike Darwin, Wallace did not leave behind a large number of private letters and other personal documents; therefore, his more private thoughts, motives, and deliberations will probably remain unknown.

I provide a short introduction to Wallace's life and work and then describe his contributions to the British antivaccination campaigns. Wallace's interventions were influential; he was popular and well liked inside and outside scientific circles and, despite his controversial social reformism, commanded deep respect for his achievements and his personal qualities until the end of his long life.

I also briefly analyze the similarities and differences between the Victorian and contemporary vaccination debates. It has recently been argued that comparative historical analysis can play a major role in public health policy (*6*,*7*). In contemporary vaccination controversies, history is frequently used as a source of arguments (*8*,*9*), but the historical argument often is not based on up-to-date historical understanding. The polarizing controversies surrounding vaccination have never completely gone away, and the nearly unbroken tradition of debate apparently entices participants to reuse old arguments without making certain that their context is still valid. Vaccination involves national and international politics and the deeply personal sphere of child care. It is thus probably inevitable that culturally influenced ideas of bodily integrity and health from time to time are at odds with so-called vaccination technocracies (*10*).

Author affiliation: Ispra, Italy

DOI: 10.3201/eid1604.090434

Alfred Russel Wallace

Alfred Russel Wallace's humble origins contrast sharply with Charles Darwin's privileged background. Wallace was born on January 8, 1823, in the Welsh village of Llanbadoc into an impoverished middle-class family. In 1836, when his parents could no longer support him, he was taken out of school to earn a living. He joined his brother John in London to work as a builder. In London, he regularly attended meetings at the Hall of Science in Tottenham Court Road, where followers of the utopian socialist Robert Owen lectured. Thus, as an adolescent, he became acquainted with radical sciences such as phrenology (*11*). In 1841, when Wallace was working as a land surveyor in Wales, a slump in business enabled him to devote more time to his developing interests in natural history. A few years later, while working as a teacher in Leicester, Wallace met the 19-year-old amateur entomologist Henry Walter Bates, who introduced him to beetle collecting. Wallace returned to Wales, but he stayed in touch with Bates; in their letters they discussed natural history and recent books. In 1847, inspired by reading the best-selling and scandalous Vestiges of the History of Creation, an anonymously published book that offered a naturalistic, developmental history of the cosmos and life, Wallace and Bates decided to travel to the Amazon River basin to study the origin of species, paying for their journey by working as professional specimen collectors.

Wallace spent the next 14 years of his life, interrupted only by a stay in England from October 1852 until early April 1854, collecting specimens in the Amazon Basin and the Malay Archipelago. As with Darwin, the geographic variation of supposedly stable species nurtured in Wallace the idea of organic change. An 1855 paper, On the Law Which Has Regulated the Introduction of New Species, is Wallace's first formal statement of his understanding of the process of biological evolution. In this paper, he derives the law that "every species has come into existence coincident both in time and space with pre-existing closely allied species." In February 1858, while having a severe malaria attack, Wallace connected the ideas of Thomas Malthus (1766–1834) on the regulation of populations with his earlier reasoning and developed a concept that was similar to Darwin's mechanism of natural selection. Eager to share his discovery, Wallace wrote an essay on the subject as soon as he had recovered and sent it off to Darwin. This innocent act by Wallace set off the well-known and often recounted story of Darwin's hurried writing and publication of On the Origin of Species.

Wallace returned to England in 1862 after the initial storm of reaction to Darwin's theory had blown over. Together with Thomas Henry Huxley (1825–1895), he became one of the most vocal defenders of the theory of evolution. In the years up to 1880 he also wrote a large number of essays, letters, reviews and monographs that secured his position as one of the foremost naturalists in the United Kingdom; this status, however, did not translate into a permanent position or even some semblance of financial security. Only in 1881, after an intervention by Darwin and other eminent scientists, did he receive a Civil List Pension of 200£ per year. After 1880, having finished most of his major monographs, Wallace more and more directed his attention toward social issues and turned into a social radical—his conversion to spiritualism had already occurred in the 1860s. He remained faithful to his radical course until his death in 1913.

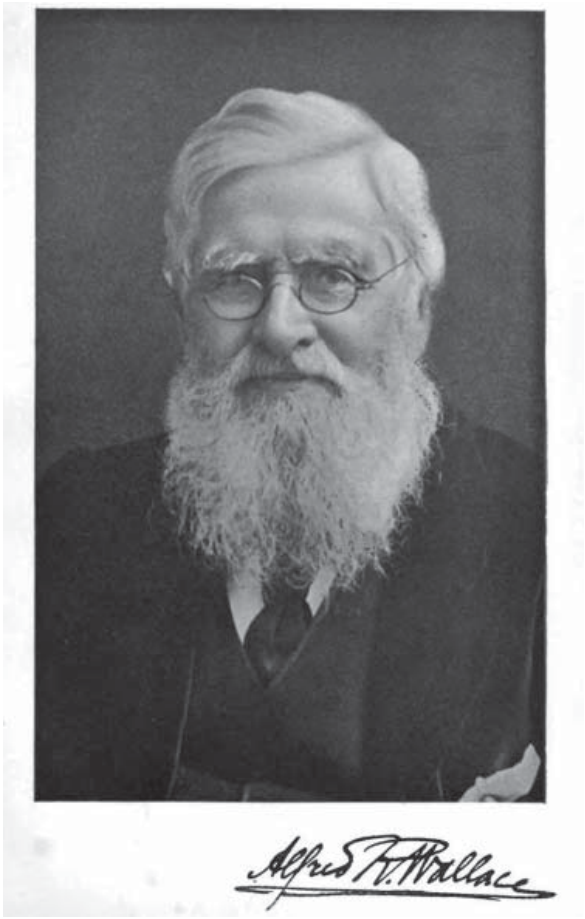


Figure. Alfred Russel Wallace (1823–1913). Perhaps best remembered today in history of science as the codiscoverer of the principle of natural selection, Wallace also played a prominent role in the antivaccination movement in late 19th century England.

The first Vaccination Act in England was passed in 1840; it outlawed variolation (i.e., the practice of infecting a person with actual smallpox) and provided vaccination that used vaccines developed from cow pox or vaccinia virus free of charge. The 1853 Act made vaccination mandatory and included measures to punish parents or guardians

who failed to comply. Changes in the law passed in 1867 permitted the authorities to enforce vaccination more efficiently. The law allowed the repeated prosecution of parents who failed to have their child vaccinated. The 1871 Act authorized the appointment of vaccination officers, whose task it was to identify cases of noncompliance. In 1889, in response to widespread public resistance, Parliament appointed a Royal Commission to draft recommendations to reform the system. The Commission published its conclusions in 1896. It suggested allowing conscientious objection, an exemption which passed into law in 1898. In the early 20th century, ≤200,000 exemptions were granted annually, representing ≈25% of all births (*12*).

The first vaccination act mainly incited resistance from heterodox medical practitioners who were forced out of business. Large-scale popular resistance began after the 1867 Act with its threat of coercive cumulative penalties. The social and political diversity of the British antivaccination movement is vividly described by Durbach (*12*). Many of the ≈200 organizations were quite eccentric, even by the standards of the time. However, Durbach's analysis and other analyses (*13*) show that it is not correct to portray antivaccinationists indiscriminately as antirational, antimodern, and antiscientific. Just considering the details of the vaccination practice of the mid-19th century does much to make many criticisms understandable. For instance, the widespread arm-to-arm vaccination, used until 1898, carried substantial risks, and the instruments used (*14*) could contribute to severe adverse reactions. Furthermore, many antivaccinationists appealed, like their opponents, to enlightenment values and expertly used quantitative arguments.

Wallace himself apparently did not hold strong opinions about vaccination until the mid-1880s. He had received a vaccination as a young man before he left for South America, and all 3 of his children were vaccinated as well. Wallace was recruited some time in 1884 to the antivaccination movement through the efforts of his fellow spiritualist William Tebb (1830–1917), a radical liberal who in 1880 had cofounded the London Society for the Abolition of Compulsory Vaccination. Wallace's commitment to the antivaccination cause was without doubt motivated by his social reformism, which in turn was underpinned by spiritualism and Swedenborgianism (*3*,*15*). These metaphysical foundations led him to a holistic view of health; he was convinced that smallpox was a contagious disease, but he also was certain that differences in susceptibility caused by nutritional or sanitary deficiencies played a major role in the epidemiology of the disease.

Despite his strong metaphysical commitments, Wallace, however, always remained a devoted empiricist and was among the first to use a statistics-based critique of a public health problem. Some of the groundwork for Wallace's quantitative critique was laid by the highly regarded, but controversial, physicians Charles Creighton (1847–1927) and Edgar Crookshank (1858–1928). They attacked simplistic interpretations of and conclusions from Edward Jenner's work (*16*) and demonstrated how difficult it is to determine vaccination success and vaccination status and to know what kind of contagion was actually used in an inoculation or vaccination. In works such as Vaccination Proved Useless and Dangerous (1889) or Vaccination a Delusion, Its Penal Enforcement a Crime (1898), Wallace mounted his attack on several claims: 1) that death from smallpox was lower for vaccinated than for unvaccinated populations; 2), that the attack rate was lower for vaccinated populations: and 3) that vaccination alleviates the clinical symptoms of smallpox.

Both provaccinationists and antivaccinationists relied heavily on time series of smallpox mortality rate data, which showed a general decline over the 19th century overlaid by several smaller epidemic peaks and the large pandemic peak of 1870–1873. Their conclusions from these data differed according to the way these data were subdivided into periods (*17*). For example, if it were assumed that vaccination rates increased in 1867, when cumulative penalties were introduced and fewer dared to challenge the vaccination law, and not in 1871, when the smallpox pandemic accelerated, then the rate of decline of smallpox mortality rates was lower when vaccination was more prevalent. Wallace concluded from his analysis that smallpox mortality rates increased with vaccination coverage, whereas his opponents concluded the exact opposite. Wallace argued that the problem of determining vaccination status was serious and undermined the claims of his opponents. He asserted that the physicians' belief in the efficacy of vaccination led to a bias in categorizing persons on the basis of interpretation of true or false vaccination scars. Additionally, epidemiologic data for vaccination status were seriously incomplete. Depending on the sample, the vaccination status of 30%–70% of the persons recorded as dying from smallpox was unknown. Furthermore, if a person contracted the disease shortly after a vaccination, it was often entirely unclear if the patient should be categorized as vaccinated or unvaccinated. Provaccinationists argued that the error introduced by this ambiguity was most likely to be random and thus would not affect the estimate of the efficiency of the vaccine. In contrast, Wallace believed that doctors would have been more willing to report a death from smallpox in an unvaccinated patient and that this led to a serious bias and an overestimation of vaccine efficiency.

Wallace's holistic conception of health influenced his argument as well. He was convinced that susceptibility to the disease of smallpox was not distributed equally across social classes. Weakened, poor persons living in squalor were in his opinion less likely to get vaccinated.

At the same time they would have higher smallpox mortality rates because their living conditions made them more susceptible to the disease. He supported his hypothesis that susceptibilities differ with the observation that the mortality rate of unvaccinated persons had increased to 30% after the introduction of vaccination, while the vaccinated had enjoyed a slight survival advantage. This demonstrated to Wallace that factors other than vaccination must have played a major role.

Conclusions

The numerical arguments used by Wallace and his opponents were based on an actuarial type of statistics, i.e., the analysis of life tables and mortalities. Inferential statistics that could be more helpful in identifying potential causes did not yet exist. The statistical approach to the vaccination debate used by Wallace and his opponents could simply not resolve the issue of vaccine efficiency; thus, each side was free to choose the interpretation that suited its needs best. However, despite its indecisive outcome, the debate was a major step in defining what kind of evidence was needed (*17*). It is also unjustified to portray the debate as a controversy of science versus antiscience because the boundaries between orthodox and heterodox science we are certain of today were far less apparent in the Victorian era (*18*). What the scope and methods of science were or should be were topics still to be settled. It is thus unwarranted to portray the 19th-century antivaccination campaigners generally as blindly religious, misguided, or irrational cranks. This judgment certainly does not apply to Alfred Russel Wallace.

Wallace was modern, but he represented an alternative version of modernity, a version that has been sidelined in historiography until recently but has lately been acknowledged as a central cultural feature of the late 19th century (*19*). Movements such as spiritualism were not resurrections of ancient traditions but used interpretations of the most recent natural science, such as experimental psychology, evolutionary biology, and astronomy (*20*), or electromagnetism (*21*). Some, like Wallace, also contested the social role that emerging professional sciences should play. Wallace strongly favored a natural science that also addressed moral, political, social, and metaphysical concerns, and with this inclination he ran against the tide that was more concerned with developing a barrier between politics and disinterested, objective science. In the case of vaccination, Wallace argued that liberty and science need to be taken into account, but that liberty is far more important than science. Wallace only appears to have been such a heretical figure if a large portion of the social, political, and intellectual reality of Victorian and Edwardian England is blotted out of the picture.

To argue that, then as now, the controversies are between religiously motivated, irrational eccentrics and rational, disinterested science is historically inaccurate and distracts from substantial differences in social, political, and economic context between then and now. The Victorian vaccination legislation was part of an unfair, thoroughly class-based, coercive, and disciplinary healthcare and justice system: poor, working-class persons were subjected to the full force of the law while better-off persons were provided with safer vaccines and could easily avoid punishment if they did not comply. The National Health Service, established in 1948, was planned to bring more social justice to health care. The new health system no longer was stigmatizing and coercive. The development has not stopped there: today, there is an increasingly strong emphasis on individual choice and involvement in decision making in the healthcare system in Great Britain. Patients have become customers. The contemporary vaccination controversy has to be seen against the opportunities and challenges offered within this new environment. It has become evident that population-based risk assessments of vaccine safety often fail to convince in this new context (*10*). Parents instead evince a clinical, individual-based attitude when assessing the risks of vaccination—their own children are often judged not to be average.

In Great Britain, such attitudes are reinforced by the recent developments, mentioned above, in the healthcare system that encourage choice and autonomy and also by individualized perspectives concerning parenting and child development. Such a clinical perspective of parents can, however, cut both ways. The individually witnessed causal relationship between therapy and recovery in the case of tetanus and diphtheria was instrumental in the widespread public acceptance of immunization (*17*). A similar mechanism is at play in the contemporary controversies: perceived causal relationships between vaccination and the appearance of complications undermine the claims that vaccines are generally safe.

This analysis also illustrates that contemporary vaccination controversies take place in specific historical contexts. Colgrove (*22*) depicts in detail how vaccination became an accepted public health intervention in the United States and what factors have fueled and influenced historical and contemporary controversies. For example, compared with most countries in Europe, the risk of costly litigation for pharmaceutical companies in the United States is much higher and the role of the state is seen as far more restricted. This specific background influences forms of provaccination and antivaccination campaigning, but it also needs to be taken into account that the increasing availability of Internet resources accessible from everywhere may contribute to making the arguments and the debate more uniform across the globe.

Modern vaccines save lives. But worries surrounding vaccination need to be taken seriously. And the lessons

taught by history are, as usual, complex. As pointed out forcefully by Leach and Fairhead (*10*), vaccine delivery systems must suit social, cultural, and political realities. Paternalistic and coercive attitudes were harmful in the 19th century and are even less appropriate in the 21st century.

Dr Weber is a biologist working in the fields of public health and consumer protection. He also publishes regularly in the history of science and has a particular research interest in the history of evolutionary biology.

References

1. Raby P. Alfred Russel Wallace. A life. Princeton (NJ): Princeton University Press; 2002.
2. Shermer M. In Darwin's shadow. The life and science of Alfred Russel Wallace. New York: Oxford University Press; 2002.
3. Fichman M. An elusive Victorian. The evolution of Alfred Russel Wallace. Chicago: The University of Chicago Press; 2004.
4. Slotten R. The heretic at Darwin's court. The life of Alfred Russel Wallace. New York: Columbia University Press; 2004.
5. Smith CH, Beccaloni G, eds. Natural selection and beyond: the intellectual legacy of Alfred Russel Wallace. New York: Oxford University Press; 2008.
6. Scally G, Womack J. The importance of the past in public health. J Epidemiol Community Health. 2004;58:751–5. DOI: 10.1136/jech.2003.014340
7. Berridge V. History matters? History's role in health policy making. Med Hist. 2008;52:311–26.
8. Wolfe RM, Sharp LK. Anti-vaccinationists past and present. BMJ. 2002;325:430–2. DOI: 10.1136/bmj.325.7361.430
9. Spier RE. Perception of risk of vaccine adverse events: a historical perspective. Vaccine. 2001;20:S78–84. DOI: 10.1016/S0264-410-X(01)00306-1
10. Leach M, Fairhead J. Vaccine anxieties. Global science, child health & society. London: Earthscan; 2007.
11. Jones G. Alfred Russel Wallace, Robert Owen and the theory of natural selection. Br J Hist Sci. 2002;35:73–96.
12. Durbach N. Bodily matters. The anti-vaccination movement in England, 1853–1907. Durham (NC) Duke University Press; 2005.
13. Keelan JE. The Canadian anti-vaccination leagues 1872–1892 [dissertation]. Toronto (Ontario, Canada): University of Toronto; 2004.
14. Baxby D. Smallpox vaccination techniques; from knives and forks to needles and pins. Vaccine. 2002;20:2140–9.
15. Scarpelli G. 'Nothing in nature that is not useful'. The anti-vaccination crusade and the idea of *harmonia naturae* in Alfred Russel Wallace. Nuncius. 1992;7:109–30.
16. Creighton C. Jenner and vaccination: a strange chapter in medical history. London: Swan Sonnenschein & Co.; 1889.
17. Fichman M, Keelan JE. Resister's logic: the anti-vaccination arguments of Alfred Russel Wallace and their role in the debates over compulsory vaccination in England, 1870–1907. Stud Hist Philos Biol Biomed Sci. 2007;38:585–607.
18. Barton R. "Men of science": language, identity and professionalization in the mid-Victorian scientific community. Hist Sci. 2003;41:73–119.
19. Owen A. The place of enchantment. British occultism and the culture of the modern. Chicago: The University of Chicago Press; 2004.
20. Weber TP. Carl du Prel (1839–1899): explorer of dreams, the soul, and the cosmos. Stud Hist Philos Sci. 2007;38:593–604. DOI: 10.1016/j.shpsa.2007.06.005
21. Noakes R. The 'world of the infinitely little': connecting physical and psychical realities circa 1900. Stud Hist Philos Sci. 2008;39:323–34. DOI: 10.1016/j.shpsa.2008.06.004
22. Colgrove J. State of immunity. The politics of vaccination in twentieth-century America. Berkeley (CA): The University of California Press; 2006.

Address for correspondence: Thomas P. Weber, via Marsala 17, 21014 Laveno Mombello, Italy; email: tp_weber@yahoo.de

Innovative Uses for Syndromic Surveillance

Erin K. O'Connell, Guoyan Zhang, Fermin Leguen, Anthoni Llau, and Edhelene Rico

To determine if expanded queries can be used to identify specific reportable diseases/conditions not detected by using automated syndrome categories, we developed new categories to use with the Electronic Surveillance System for the Early Notification of Community Based Epidemics. Results suggest innovative queries can enhance clinicians' compliance with reportable disease requirements.

Surveillance and control of communicable diseases are critical for the health status of a community. Traditional passive surveillance refers to health authorities' receipt of reports of diseases or conditions submitted by physicians, laboratories, and other healthcare providers as required by public health legislation. However, reportable diseases are often underreported to health departments (*1*,*2*). Syndromic surveillance has been defined as "an investigational approach where health department staff, assisted by automated data acquisition and generation of statistical alerts, monitor disease indicators in real-time or near real-time to detect outbreaks of disease earlier than would otherwise be possible with traditional surveillance" (*3*). Since 2005, the Miami–Dade County Health Department has used the Electronic Surveillance System for the Early Notification of Community Based Epidemics (ESSENCE) as part of its comprehensive syndromic surveillance system. The system categorizes chief complaints into 11 syndromes: botulism-like, exposure, fever, gastrointestinal illness, hemorrhagic illness, influenza-like illness, injury, neurologic, rash, respiratory, and shock-coma. Of the county's 23 acute-care hospitals, the 17 largest, which account for 90% of the county's emergency department visits, participate in ESSENCE. Staff epidemiologists rotate duties and dedicate 2 hours a day, including weekend, to syndromic surveillance activities. Monday through Friday daily reports are sent to community partners.

Syndromic surveillance was primarily designed to detect disease outbreaks and unusual public health events earlier than could be detected by traditional public health surveillance methods. However, if an outbreak or cluster of illness is too small, the method used currently for syndromic surveillance cannot trigger a statistical alert. We wanted to ascertain the value of syndromic surveillance in improving regular communicable disease surveillance and reporting. The possibility of using ESSENCE to detect specific diseases emerged when varicella (chickenpox) became a newly reportable disease in Florida in 2006; few cases were being reported despite the fact that guidelines had been mailed to all healthcare practitioners. We used a query in ESSENCE to search for "chicken pox or varicella" in the chief complaint field and contacted the hospital Infection Control Practitioners to verify if identified events could be confirmed as reportable conditions. After we did preassessment of the underreporting of chickenpox in 2007 (*4*), three additional query categories for daily investigation were created in ESSENCE.

Author affiliation: Miami–Dade County Health Department, Miami, Florida, USA

DOI: 10.3201/eid1604.090688

The Study

The following category queries were performed during March–December 2008: 1) Severe or time-sensitive diseases/conditions: anthrax, botulism, smallpox, and meningitis; 2) outbreaks not detected by regular alerts in ESSENCE: diarrhea, vomiting, and food poisoning with spatial-temporal clustering; and 3) other reportable diseases/conditions, consisting of varicella (chickenpox), carbon monoxide poisoning, ciguatera, cryptosporidiosis, cyclosporiasis, dengue fever, encephalitis, hepatitis, malaria, measles, mercury poisoning, mumps, pertussis, salmonellosis, shigellosis, and rabies.

When descriptions of the diseases/conditions were found in the chief complaint field, staff contacted the hospital's Infection Control Practitioner. If the disease was confirmed, further investigation was performed. Potential outbreaks were detected from clustering by age, gender, race/ethnicity, resident ZIP code, hospital, time of visit, and chief complaint.

A total of 740,320 emergency department visits (mean 2,419 visits/day) were monitored in ESSENCE during the study period; 1,813 (0.25%) of those had information leading to 1 of the queried reportable diseases in the chief complaint (mean 5.9 visits/day). After further investigation, we found 58.0% (1,052/1,813) of these additional queries were relevant after excluding unrelated terms, such as "malabsorption" instead of "malaria" or "chicken bone in the throat" instead of "chicken pox."

On August 31, 2008, the newly designed query for severe or time-sensitive diseases or conditions detected a group of 5 women who had arrived at the same hospital within a 2-hour period with a chief report of either meningococcemia or exposure to meningococcemia (Table 1). This cluster of potentially epidemiologically linked patients did not initiate an automated alert in a syndrome in ESSENCE, and the hospital did not report it by phone immediately. Therefore, without the query, the health department would

Table 1. Emergency department visits with meningococcemia in the chief complaint, Miami–Dade County, Florida, USA, August 31, 2008*

Time of visit	Patient age, y	Chief complaint
9:48 PM	3	Exposure to meningococcemia
9:47 PM	8	Exposure to meningococcemia
9:45 PM	11	Exposure to meningococcemia
11:50 PM	9	Exposure to meningococcemia
11:58 PM	10	Meningococcemia

*All 5 patients were girls who visited the same hospital and came from the same ZIP code area.

not have been aware of these patients in a timely manner. The findings from the query enabled health department staff to give postexposure prophylaxis to 36 persons identified as close contacts.

Gastrointestinal outbreaks are an example of outbreaks not detected by regular alerts in ESSENCE that were often detected through the use of the specialized query. One outbreak identified was among persons staying in the same homeless shelter (Table 2), another was among residents at an assisted living facility, and a third was among a group of persons who visited a restaurant. After the outbreaks were confirmed with the Infection Control Practitioner, recommendations for infection control and prevention were made to each facility.

The most common terms found under other reportable diseases or conditions were meningitis, hepatitis, chicken pox, and postexposure prophylaxis for rabies (Table 2). These 4 conditions accounted for 68.2% (717/1,052) of the queried chief complaints. When we contacted providers with regard to query findings, it was apparent that some providers were not familiar with reporting requirements for chicken pox and animal bites requiring postexposure prophylaxis for potential rabies exposure.

Conclusions

Results suggest ESSENCE can enhance healthcare practitioners' compliance with reportable disease requirements for individual diseases and potential outbreaks. Results demonstrated how expanded queries can detect potential outbreaks or diseases not found in automated syndrome categories. Without the specialized queries, we would have missed the opportunity to implement proper disease control measures required for these events.

Indiana State Health Department investigators found that certain keywords such as "exposure" and "meningitis" may uncover trends previously undetected and they continue to explore similar data-mining techniques (*5*). Syndromic surveillance systems use statistical algorithms to alert users when the number of reports for a syndrome exceeds the norm (*6*). Current spatial–temporal algorithms are used to detect large-scale outbreaks over a certain extended period (*7*,*8*). However, this method has not been successful for detecting many small clusters of patients with similar characteristics visiting the emergency department from the same home ZIP code, hospital, or within a short period, such as a few minutes. By contacting the Infection Control Practitioner when reportable disease names are found in the chief complaint field, the health department has developed a stronger relationship with hospitals.

One of the limitations of the study was that even when queries were performed with parsers, there were often misspellings, typographical errors, and abbreviations that can lead to a failure to capture all possible events (*9*). Because the level of investigation can vary from making a phone call to a participating hospital to dispatching a team to interview patients, the cost of time spent on each disease may need to be weighed before initiating action (*10*). Replication of this study depends on a health department's capabilities to contact the hospital for follow-up. Future research will examine the information gathered from this new project, and we expect that better disease reporting compliance will result from this innovative use of syndromic surveillance.

Table 2. Disease/conditions found in the chief complaint field from specialized query, Miami–Dade County, Florida, USA, March–December 2008

Disease/condition	No. (%) cases
Food poisoning	262 (24.9)
Postexposure prophylaxis for rabies	251 (23.9)
Meningitis	224 (21.3)
Hepatitis	162 (15.4)
Chicken pox	80 (7.6)
Carbon monoxide poisoning	31 (3.0)
Salmonellosis	13 (1.2)
Malaria	9 (0.9)
Pertussis	8 (0.8)
Mumps	5 (0.5)
Other*	5 (0.5)
Total	1,052 (100)

*Ciguatera, anthrax, dengue fever, measles.

Acknowledgments

We thank the staff of the Johns Hopkins University Applied Physics Laboratory for their technical expertise for South Florida ESSENCE. We also appreciate the assistance of the Infection Control Practitioners at the participating Miami–Dade County hospitals. Finally, we thank Rene Borroto-Ponce and Lizbeth Londoño and the staff of Epidemiology, Disease Control and Immunization Services at the Miami–Dade County Health Department for their support.

Setup and maintenance of ESSENCE is financially supported by the Centers for Disease Control and Prevention PHPB9 Bioterrorism Surveillance and Epidemiology grant.

Ms O'Connell works as an epidemiologist at the Miami–Dade County Health Department. Her research interests are maternal and child health, infectious disease, and syndromic surveillance.

References

1. Doyle TJ, Glynn M, Groseclose S. Completeness of notifiable infectious disease reporting in the United States: an analytical literature review. Am J Epidemiol. 2002;155:866–74. DOI: 10.1093/aje/155.9.866
2. Konowitz PM, Petrossian G, Rose D. The underreporting of disease and physician's knowledge of reporting requirements. Public Health Rep. 1984;99:31–5.
3. Centers for Disease Control and Prevention. Framework for evaluating public health surveillance systems for early detection of outbreaks: recommendations from the CDC working group. MMWR Recomm Rep. 2004;53:1–11.
4. O'Connell E, Zhang G, Llau A, Leguen F. Innovative uses for ESSENCE to improve standard communicable disease reporting practices in Miami–Dade County. Advances in Disease Surveillance. 2008;5:122.
5. Grannis S, Wade M, Gibson J, Overhage JM. The Indiana Public Health Emergency Surveillance System: ongoing progress, early findings, and future directions. AMIA Annu Symp Proc. 2006;304–8.
6. Hurt-Mullen K, Coberly J. Syndromic surveillance on the epidemiologist's desktop: making sense of much data. MMWR Morb Mortal Wkly Rep. 2005;54:141–6.
7. Lombardo JS, Burkom H, Pavlin J. ESSENCE II and the framework for evaluating syndromic surveillance systems. MMWR Morb Mortal Wkly Rep. 2004;53:159–65.
8. Heffernan R, Mostashari F, Das D, Karpati A, Kulldorf M, Weiss D. Syndromic surveillance in public health practice, New York City. Emerg Infect Dis. 2004;10:858–64.
9. Shapiro AR. Taming variability in free text: application to health surveillance. MMWR Morb Mort Wkly Rep. 2004;53:95–100.
10. Duchin JS. Epidemiological response to syndromic surveillance signals. J Urban Health. 2003;80:i115–6. DOI: 10.1093/jurban/jtg115

Address for correspondence: Erin K. O'Connell, Miami–Dade County Health Department Epidemiology, Disease Control and Immunization Services, 8600 NW 17th St, Suite 200, Miami, FL 33126, USA; email: erin_oconnell@doh.state.fl.us

Plasmodium knowlesi in Human, Indonesian Borneo

Melanie Figtree, Rogan Lee, Lisa Bain, Tom Kennedy, Sonia Mackertich, Merrill Urban, Qin Cheng, and Bernard J. Hudson

Plasmodium knowlesi is now established as the fifth *Plasmodium* species to cause malaria in humans. We describe a case of *P. knowlesi* infection acquired in Indonesian Borneo that was imported into Australia. Clinicians need to consider this diagnosis in a patient who has acquired malaria in forest areas of Southeast Asia.

Plasmodium knowlesi is now recognized as a cause of potentially fatal human malaria in forest areas of Southeast Asia. We describe a case of *P. knowlesi* malaria acquired in Indonesia and imported to Australia.

The Patient

A 39-year-old man from Australia came to a suburban hospital in Sydney, New South Wales, Australia, with a 2-week history of morning fevers and mild headaches. His symptoms started 13 days after he left Indonesian Borneo (Kalimantan). The patient had spent an average of 10 days per month for the past 18 months working adjacent to a forest area in South Kalimantan Province, Indonesian Borneo. The most recent visit was toward the end of the rainy season. He did not use any personal vector avoidance measures (mosquito nets, long clothing, insect repellent) or receive malaria chemoprophylaxis. The patient did not travel to any other malaria-endemic areas during this 18-month period.

He did not have a remarkable medical history. On examination, he was febrile (38.9°C) and had a heart rate of 88 beats/min, blood pressure of 128/78 mm Hg, normal respiration rate, and oxygen saturation 99% on room air. Physical examination were unremarkable. Laboratory investigations showed mild thrombocytopenia (106×10^9 cells/L, reference range 150–450 $\times 10^9$ cells/L), mild leukopenia (3.7×10^9 cells/L, reference range 4.3–10 $\times 10^9$ cells/L), and unremarkable results for levels of hemoglobin (142 g/L, reference range 130–180 g/L), bilirubin (12 μmol/L, reference value <20 μmol/L), and creatinine (95 μmol/L, reference range 40–120 μmol/L).

Malaria parasites were seen on Giemsa-stained thick and thin blood films (parasitemia level 185 parasites/μL). Parasite morphologic features resembled those of *P. malariae* with typical trophozoite band forms and heavily pigmented schizonts found inside smaller erythrocytes (Figure). Some parasites had morphologic features similar to those of *P. falciparum*. These similarities included ring forms and mature trophozoites with stippling of erythrocytes (Figure, panel B). A rapid diagnostic test result for histidine-rich protein 2 of *P. falciparum* was negative.

Given increased reports of *P. knowlesi* in Malaysian Borneo, we conducted molecular studies to identify the species. Results of multiplex PCRs for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* were negative (*1*). Results of a PCR using *P. knowlesi*–specific primers (*2*) were positive for undiluted and diluted (1:50) blood samples. Sequencing of a small subunit rRNA gene product showed 100% identity with *P. knowlesi* (National Center for Biotechnology Information accession no. GU049678).

The patient was treated with atovaquone/proguanil, 250 mg/100 mg, 4×/day for 3 days. His fever resolved and the thrombocyte count returned to the reference level within 48 hours. He did not show any complications.

Conclusions

Naturally acquired human infection with *P. knowlesi* was first described in Malaysian Borneo in 1965 after an unusual sequence of events (*3*). Extensive investigation at this time failed to demonstrate zoonotic transmission of simian malaria to humans. More recently, molecular techniques have identified human infections with *P. knowlesi*, establishing it as the fifth *Plasmodium* species that infects humans (*2*). *P. knowlesi* accounts for most (70%) human malaria infections requiring hospitalization in Sarawak, Malaysian Borneo (*4*), and is widespread throughout Malaysia (*5*). Reports have also described human infections in Thailand (*6*), along the border of the People's Republic of China and Myanmar (*7*), Singapore (*8*), and the Philippines (*9*). A recent epidemiologic study reported that 4/22 malaria cases diagnosed by microscopy as *P. falciparum*, *P. vivax*, or mixed *P. falciparum*/*P. vivax* infections were identified retrospectively by PCR to be mixed infections that included *P. knowlesi* (*10*). Presumably, *P. knowlesi* may account for a higher proportion of cases if those diagnosed morphologically as *P. malariae* were investigated.

Human malaria was considered to be caused by only 4 *Plasmodium* species in the premolecular biology era. Simian and human malaria parasites, including *P. knowlesi* and *P. malariae*, are often indistinguishable morphologically. Clues to identification of *P. knowlesi* by light microscopy

Author affiliations: Royal North Shore Hospital, Sydney, New South Wales, Australia (M. Figtree, T. Kennedy, S. Mackertich, B.J. Hudson); Westmead Hospital, Sydney (R. Lee); Australian Army Malaria Institute, Enoggera, Queensland, Australia (L. Bain, Q. Cheng); and Mona Vale Hospital, Sydney (M. Urban)

DOI: 10.3201/eid1604.091624

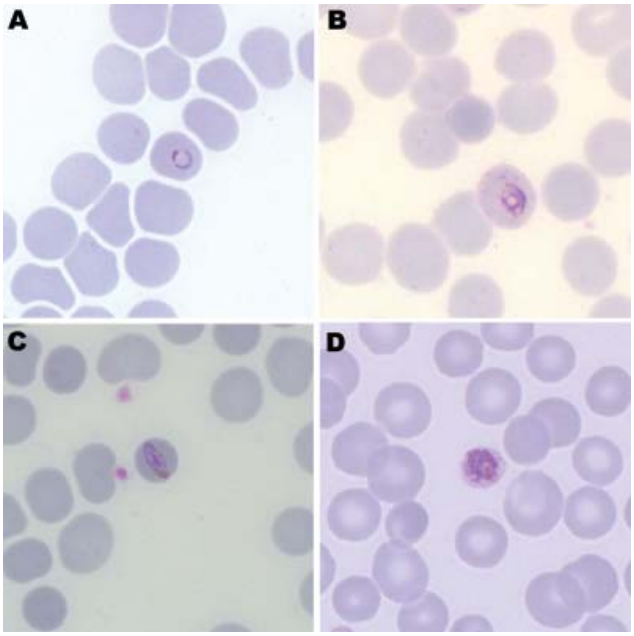


Figure. Giemsa-stained thin blood films of patient infected with *Plasmodium knowlesi*, showing a ring form (A), a trophozoite with Sinton and Mulligan stippling (B), a band form resembling *P. malariae* (C), and an early schizont (D). Original magnification ×100.

that are useful, if present, include early trophozoites with fine ring forms, double chromatin dots, and 2–3 parasites per erythrocyte (resembling *P. falciparum*), trophozoites with a bird's-eye appearance, mature trophozoites with a band appearance resembling *P. malariae* (Figure, panel C), and mature schizonts with a higher average merozoite count (16/erythrocyte) than in *P. malariae* (12/erythrocyte) (*2,11*).

Commercially available rapid diagnostic tests do not distinguish *P. knowlesi* from other forms of human malaria parasites. Lactate dehydrogenase produced by the 4 other *Plasmodium* spp. (pLDH) that cause human malaria is also present in *P. knowlesi*. Antibodies specific for pLDH of *P. falciparum* and *P. vivax* cross-react with pLDH of *P. knowlesi* (*12*) and therefore cannot be used to reliably distinguish *P. knowlesi* from mixed infections.

Distinction of *P. knowlesi* from *P. malariae* has useful management implications for patients and public health control measures. *P. knowlesi* potentially can cause severe disease and death, whereas *P. malariae* is generally benign. Daneshvar et al. recently published a prospective study of *P. knowlesi* infection in humans (*4*). They reported thrombocytopenia in 100% (107/107) of persons infected with *P. knowlesi* and lower mean ± SD thrombocyte counts (71 ± 35 × 10^9 cells/L) than in persons infected with *P. falciparum* (108 ± 59 × 10^9 cells/L) or *P. vivax* (118 ± 51 × 10^9 cells/L). Mean parasitemia level was 1,387 parasites/μL; 30.8% (33/107) of the case-patients had <500 parasites/μL. Severe infection was found in 7 (6.5%) of 107 patients, and the case-fatality rate was 1.8% (2/107) among hospitalized patients (*4*).

Deaths and severe disease caused by *P. knowlesi* result from pulmonary and hepatorenal failure (*5*). Severity of *P. knowlesi* infection is related to potentially high parasitemia levels produced by its rapid and unique 24-hour erythrocytic cycle and its ability to infect all stages of erythrocytes (*13*). Sequestration is not thought to occur during *P. knowlesi* infection, and neurologic complications seen during *P. falciparum* infection have not been described. Although our patient was treated with atovaquone/proguanil, patients with similar uncomplicated cases have responded well to treatment with chloroquine (*4*).

Public health control is challenging in areas where zoonotic human malaria is endemic (*14*). Standard public health measures for malaria prevention (insecticide-treated nets, indoor residual spraying, and intermittent preventive treatment in the reservoir population) are likely to be less effective than for typical forms of human malaria. Nevertheless, travelers to malaria-endemic areas should be encouraged to practice mosquito bite protection measures and chemoprophylaxis.

P. knowlesi malaria is transmitted from long-tailed (*Macaca fasicularis*) and pig-tailed (*M. nemestrina*) macaques to humans by *Anopheles latens* mosquitoes (in the Kapit Division of Malaysian Borneo) when humans visit forest or forest fringe areas. However, transmission does not seem to occur readily in villages (*2,15*). Increased recognition of *P. knowlesi* indicates that human infection is possible by with other simian malaria parasites (*P. cynomolgi* and *P. inui*).

We report a patient with *P. knowlesi* infection that was acquired in Indonesia and imported to Australia. Fortunately, this patient had a low parasitemia level and mild disease. A high degree of clinical suspicion is likely to increase the number of *P. knowlesi* cases diagnosed in patients with malaria acquired in forest areas of Southeast Asia.

Acknowledgment

We thank John Barnwell for providing *P. knowlesi* DNA used as PCR controls.

Dr Figtree is microbiology registrar at Royal North Shore Hospital, Sydney. Her research interests include the molecular diversity of *P. vivax*.

References

1. Padley D, Moody AH, Chiodini PL, Saldanha J. Use of a rapid, single-round, multiplex PCR to detect malarial parasites and identify the species present. Ann Trop Med Parasitol. 2003;97:131–7. DOI: 10.1179/000349803125002977

2. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet. 2004;363:1017–24. DOI: 10.1016/S0140-6736(04)15836-4
3. Chin W, Contacos PG, Coatney GR, Kimball HR. A naturally acquired quotidian-type malaria in man transferable to monkeys. Science. 1965;149:865. DOI: 10.1126/science.149.3686.865
4. Daneshvar C, Davis TM, Cox-Singh J, Rafa'ee MZ, Zakaria SK, Divis PC, et al. Clinical and laboratory features of human *Plasmodium knowlesi* infection. Clin Infect Dis. 2009;49:852–60. DOI: 10.1086/605439
5. Cox-Singh J, David TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, et al. *Plasmodium knowlesi* malaria in humans in widely distributed and potentially life threatening. Clin Infect Dis. 2008;46:165–71. DOI: 10.1086/524888
6. Jongwutiwes S, Putaporntip C, Takuya I, Tetsutaro S, Hiroji K. Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. Emerg Infect Dis. 2004;10:2211–3.
7. Zhu HM, Li J, Zheng H. Human natural infection of *Plasmodium knowlesi* [in Chinese]. Zhongguo Ji Sheng Chong Xue Ji Sheng Chong Bing Za Zhi. 2006;24:70–1.
8. Ng OT, Ooi EE, Lee CC, Lee PJ, Ng LC, Wong PS, et al. Naturally acquired human *Plasmodium knowlesi* infection, Singapore. Emerg Infect Dis. 2008;14:814–6. DOI: 10.3201/eid1405.070863
9. Luchavez J, Espino F, Curameng P, Espina R, Bell D, Chiodini P, et al. Human infections with *Plasmodium knowlesi*, the Philippines. Emerg Infect Dis. 2008;14:811–3. DOI: 10.3201/eid1405.071407
10. Berens-Rihas N. *Plasmodium knowlesi* found in several samples from Indonesia. ProMed. 2009 Jun 21 [cited 2010 Jan 12]. http://www.promedmail.org, archive no. 20090621.2278.
11. Lee KS, Cox-Singh J, Singh B. Morphological features and differential counts of *Plasmodium knowlesi* parasites in naturally acquired human infections. Malar J. 2009;8:73.
12. McCutchan TF, Piper RC, Makler MT. Use of malaria rapid diagnostic test to identify *Plasmodium knowlesi* infection. Emerg Infect Dis. 2008;14:1750–2.
13. Knowles RM, DasGupta BM. A study of monkey-malaria and its experimental transmission to man. Indian Medical Gazette. 1932;67:301–20.
14. Cox-Singh J, Singh B. Knowlesi malaria: newly emergent and of public health importance? Trends Parasitol. 2008;24:406–10. DOI: 10.1016/j.pt.2008.06.001
15. Vythilingam I, Tan CH, Asmad M, Chan ST, Lee KS, Singh B. Natural transmission of *Plasmodium knowlesi* to humans by *Anopheles latens* in Sarawak, Malaysia. Trans R Soc Trop Med Hyg. 2006;100:1087–8. DOI: 10.1016/j.trstmh.2006.02.006

Address for correspondence: Melanie Figtree, Department of Microbiology and Infectious Diseases, Royal North Shore Hospital, Pacific Hwy, St. Leonards, Sydney, New South Wales 2065, Australia; email: melfigtree@yahoo.com.au

etymologia

Clostridium difficile

[klos-trid′e-əm di-fi-sil′]

Clostridium, the genus name of these gram-positive, spore-forming, anaerobic bacteria, comes from Greek *klōstēr* (spindle) because, under the microscope, the colonies resemble spindles used in cloth weaving and long sticks with a bulge at the end. The species name *difficile* is a form of the Latin adjective *difficilis* because, when first identified (by Hall and O'Toole in 1935), the organism was difficult to isolate and grew slowly in pure culture. However, likely because of the familiarity of a French term with the same spelling and meaning, the French pronunciation has become widely used. These bacteria are part of the commensal intestinal flora in humans, and toxigenic strains of the organism can cause pseudomembranous colitis, a severe infection of the colon, after normal gut flora have been eradicated in patients who have received antimicrobial drugs.

Source: Kelly CP, Pothoulakis C, LaMont JT; *Clostridium difficile* colitis. N Engl J Med. 1994;330:257–62; Wells J. My phonetic blog. 2006. www.phon.ucl.ac.uk/home/wells/blog0606.htm; www.statemaster.com/encyclopedia/Clostridium-difficile; Dorland's illustrated medical dictionary, 31st ed. Philadelphia: Saunders Elsevier; 2007.

Fluoroquinolone Resistance and *Clostridium difficile*, Germany

Nils Henning Zaiß, Wolfgang Witte, and Ulrich Nübel

We characterized 670 *Clostridium difficile* isolates collected from patients in 84 hospitals in Germany in 2008. PCR ribotyping showed high prevalence of ribotype 001 and restricted dissemination of ribotype 027 strains. Fluoroquinolone resistance and associated gyrase mutations were frequent in various ribotypes, but no resistance to metronidazole or vancomycin was noted.

During the past decade, incidence rates of *Clostridium difficile* infections (CDI) have increased noticeably worldwide (*1*). In the United States and Canada, this increase has been associated with the emergence of a possibly more virulent strain designated North American pulsotype 1 (NAP1), or PCR ribotype 027. Strains with identical typing patterns have also been reported from several countries in Europe (*1*). In Germany, the first clusters of infections with *C. difficile* ribotype 027 were identified in the southwest region in 2007 (*2*,*3*). Although incidence rates increased in Germany after 2000 (*4*), an association with particular strains remains unclear, and no nationwide surveillance data on *C. difficile* genotype prevalence exist.

We report the distribution and associated antimicrobial drug susceptibility of prevalent *C. difficile* strains in Germany. Isolates were characterized by PCR ribotyping (*5*) by using the ribotype nomenclature of the Cardiff Anaerobe Reference Laboratory (Cardiff, Wales, UK). Antimicrobial drug susceptibility to therapeutic drugs (metronidazole and vancomycin) and to fluoroquinolones (moxifloxacin and levofloxacin) was determined by using the Etest method (breakpoints according to European Committee on Antimicrobial Susceptibility Testing guidelines).

The Study

A surveillance study, performed from January through December 2008, reported 5,640 CDI cases in a sample of 1.6 million patients in hospitals in Germany (www.nrz-hygiene.de). Six percent of cases were defined as severe CDI based on the following criteria: readmission to a healthcare facility due to recurrent CDI, admission to an intensive care unit, surgical intervention (colectomy), or death within 30 days after diagnosis. By projecting these case rates to all of Germany (17 million patients 2008; www.destatis.de), we estimated 58,000 CDI cases (including 3,500 severe cases) in 2008. The 670 isolates investigated in this study, which caused severe infections in 84 hospitals throughout Germany in 2008, represent ≈20% of those severe CDI cases. Among these isolates, 57 ribotypes were identified, and 312 isolates were characterized as PCR ribotype 001 (47%), followed by 53 (8%) isolates each of ribotypes 078 and 027. Figure 1 shows the distribution of the 5 most common ribotypes in proportion to the number of submitting hospitals.

Ribotype 001 was by far the most prevalent ribotype found, causing severe CDI in 70% of all collaborating hospitals (Figure 1). A high prevalence of ribotype 001 was also reported in a recent study investigating 2 hospitals in southern Germany (*6*). As depicted in Figure 2, strains of ribotype 001 are endemic to hospitals all over Germany. In contrast, the dissemination of ribotype 027 strains, which could be identified in 16 hospitals (19%), is restricted mostly to the southwest region of Germany; only 2 sporadic cases were observed in the eastern region (Figure 2). The second most prevalent ribotype was 078 (23%). An increasing incidence of infections with 078 was observed in several European countries, and a zoonotic source is widely debated (*7*). Isolates of ribotype 014 and 046 were identified in 15% and 10% of all participating hospitals respectively and are widespread in neighboring countries (*7*).

Antimicrobial drug susceptibility testing showed that all tested isolates were susceptible to metronidazole and

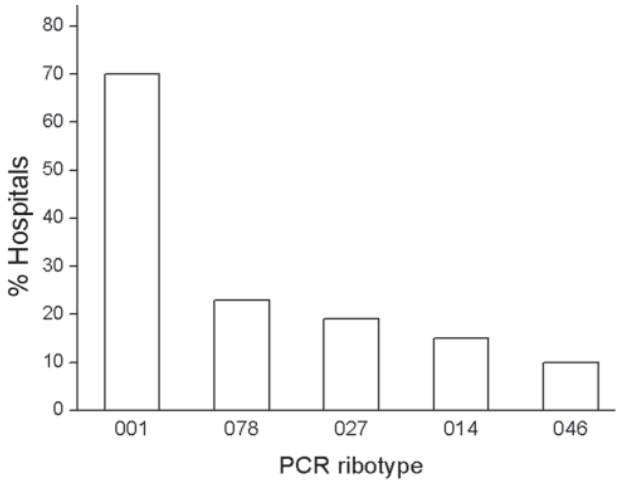


Figure 1. Prevalent PCR ribotypes of *Clostridium difficile* in hospitals in Germany, 2008. Eighty-four hospitals sent isolates from patients with severe *C. difficile* infections to the Robert Koch Institute. Ribotype 001, responsible for severe infections in 59 hospitals (70%), was the most prevalent ribotype, followed by ribotype 078 (19 hospitals, 23%), ribotype 027 (16 hospitals, 19%), ribotype 014 (13 hospitals, 15%), and ribotype 046 (8 hospitals, 10%).

Author affiliation: Robert Koch Institute, Wernigerode, Germany

DOI: 10.3201/eid1604.090859

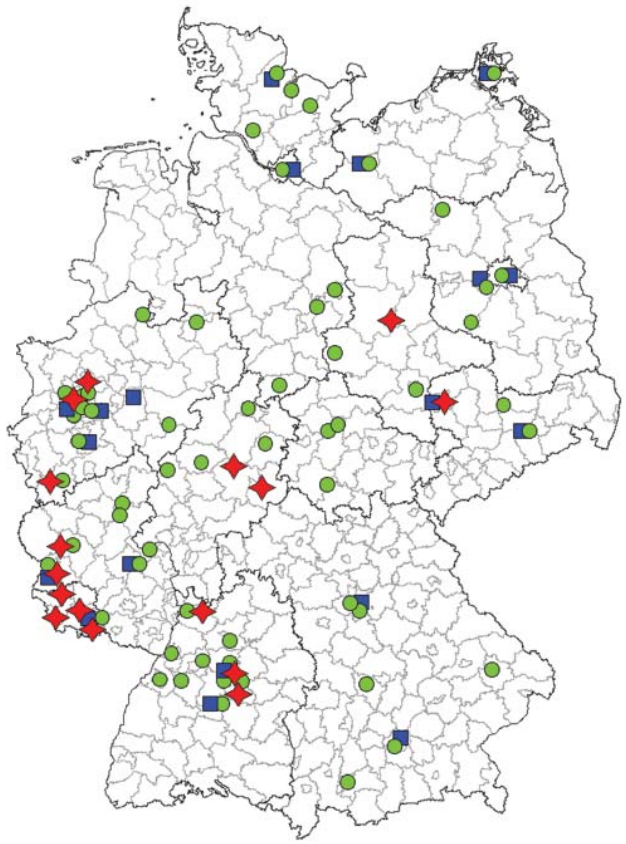


Figure 2. Approximate geographic dissemination of PCR ribotype 001, 078, and 027 of *Clostridium difficile* in hospitals in Germany in 2008. Green dots indicate hospitals with *C. difficile* ribotype 001 infections, blue squares hospitals with ribotype 078 infections, and red stars hospitals with ribotype 027 infections.

vancomycin. The mean MICs for metronidazole and vancomycin were calculated for each of the 7 most common ribotypes (Table 1). In contrast to a previous study from the United Kingdom, there were no significant differences in the mean MICs of metronidazole of common ribotypes 001, 078, and 027 compared with ribotypes 014, 046, 012, and 015 (p = 0.12, Student unpaired *t* test) (*8*).

Exposure to fluoroquinolones was described as an independent risk factor for CDI (*9*). Type 001 and 027 isolates from Germany showed widespread resistance to moxifloxacin and levofloxacin (Table 2). In several studies, acquisition of fluoroquinolone resistance in *C. difficile* has been associated with mutations in the active site of DNA gyrase (*10–12*). Sequence analysis of subunits *gyrA* and *gyrB* showed that most drug-resistant isolates (including ribotypes 001, 078, 027, 014, and 046) shared the same single transition mutation (ACT to ATT) in *gyrA*, resulting in the amino acid substitution Thr-82→Ile (Table 2). One isolate of type 001 possessed a Thr-82→Ile and Asp-71→Glu (GAC to GAA) mutation and 1 isolate of type 078, resistant to levofloxacin (MIC >32 mg/L) but susceptible to moxifloxacin (MIC = 2 mg/L), showed a Thr-82→Ala (ACT to GCT) change. In addition, high-level resistance (MIC >32 mg/L) to both fluoroquinolones was found in 2 isolates of type 014 and 078 associated with mutations in *gyrB*, although no mutations in gyrA were observed.

The *gyrA* mutations Thr-82→Ala and Asp-71→Glu have not been reported. However, several amino acid substitutions at both positions were noted before, indicating that Thr-82 and Asp-71 in *gyrA* play a major role in conferring fluoroquinolone resistance in *C. difficile (10–12)*. The Thr-82→Ile substitution especially has been associated with fluoroquinolone resistance in C. *difficile* by several groups (*10–12*). For instance, Spigaglia et al. described this substitution in 77 fluoroquinolone-resistant isolates affiliated with 19 ribotypes collected in 12 countries in Europe (*10*). Thr-82 in *C. difficile* corresponds to Ser-83 in the quinoline resistance–determining region of *Escherichia coli* (*11*), and mutations at homologous positions have been associated with fluoroquinolones resistance in several bacteria (*13*).

Fluoroquinolone resistance has been suggested to provide a selective advantage for the spread of epidemic *C. difficile* strains (*8*,*9*,*14*). However, resistance arose convergently in *C. difficile* ribotypes as a consequence of selective pressure resulting from widespread fluoroquinolone use. Because identical mutations were found even in many uncommon strains, gyrase mutations and associated fluoroquinolone resistance alone cannot explain the high prevalence of ribotypes 001 and 027.

Conclusions

We describe the dissemination and antimicrobial drug susceptibility of ribotypes causing severe CDI in Germany in 2008. We found that ribotype 001 was the most prevalent and widespread ribotype, found in 70% of submitting hospitals. Although dissemination of ribotype 027 was more restricted, recent studies in England and Canada demonstrated that ribotype 027 had the ability to replace type 001 within a few years. In England, ribotype 001 had accounted for ≈55% of all CDI cases in the late 1990s but was reduced to 8% in 2008; during the same period, the proportion of

Table 1. Mean MICs of *Clostridium difficile* PCR ribotypes for metronidazole and vancomycin

PCR ribotype (no.)	Metronidazole, mean MIC, mg/L	Vancomycin, mean MIC, mg/L
001 (303)	0.056	1.54
078 (46)	0.071	1.6
027 (51)	0.06	1.42
014 (21)	0.05	1.58
046 (14)	0.041	1.55
012 (14)	0.054	1.96
015 (6)	0.071	1.92

Table 2. Resistance to moxifloxacin and levofloxacin among the 5 PCR ribotypes of *Clostridium difficile* most common in Germany, 2008

PCR ribotype (no.)	No. (%) isolates, resistant to moxifloxacin (MIC ≥4 mg/L)	No. (%) isolates, resistant to levofloxacin (MIC ≥4 mg/L)	Amino acid substitution	
			GyrA	GyrB
001 (303)	301 (99)	301 (99)	Thr-82-Ile, Asp-71-Glu	
078 (46)	29 (63)	30 (65)	Thr-82-Ile, Thr-82-Ala	Asp-426-Asn
027 (51)	51 (100)	51 (100)	Thr-82-Ile	
014 (21)	2 (9)	2 (9)	Thr-82-Ile	Glu-466-Lys
046 (14)	14 (100)	14 (100)	Thr-82-Ile	

ribotype 027 had increased to 41% (*8*). Similarly, studies in Quebec showed a radical change from ribotype 001 strains accounting for 84% of isolates in 2000–2001 to 80% type 027 isolates in 2003–2004 (*14*). This change was associated with increasing rates of CDI illness and death. It therefore seems advisable to increase infection control measures to curb the spread of ribotype 027 in Germany.

Present data suggest that the dissemination of 027 strains in Germany is restricted to the southwest (Figure 2). Therefore, ribotype 027 cannot be the causative agent of increasing nationwide CDI incidence rates. Thus, increased infection control efforts should not be restricted to the exposure of ribotype 027 because severe CDI courses are caused by all toxigenic *C. difficile* strains.

To investigate if other traits (e.g., sporulation) or just random, stochastic events may determine the success of particular strains, a more detailed understanding of *C. difficile* population structure is necessary. Therefore, international investigations based on portable genotyping procedures (*15*) including isolates from both, CDI and asymptomatic carriage, would considerably improve current knowledge.

Acknowledgments

We are grateful to all laboratories and hospitals that contributed isolates to this study. We thank Heike Illiger and the staff at the sequencing unit of the Robert Koch Institute for excellent technical assistance.

This project was funded by the Antibiotic Resistance Surveillance program of the German Federal Ministry of Health.

Mr Zaiß is a doctoral student in the Division of Nosocomial Infections at the Robert Koch Institute in Wernigerode. He is currently working on his thesis about the molecular epidemiology of *C. difficile* in Germany.

References

1. Kuijper EJ, Coignard B, Tull P. Emergence of *Clostridium difficile*–associated disease in North America and Europe. Clin Microbiol Infect. 2006;12(Suppl 6):2–18. DOI: 10.1111/j.1469-0691.2006.01580.x
2. Zaiß NH, Weile J, Ackermann G, Kuijper E, Witte W, Nübel U. A case of *Clostridium difficile*–associated disease due to the highly virulent clone of *Clostridium difficile* PCR ribotype 027, March 2007 in Germany. Euro Surveill. 2007 Nov;12(11):E071115 1.
3. Kleinkauf N, Weiss B, Jansen A, Eckmanns T, Bornhofen B, Kuehnen E, et al. Confirmed cases and report of clusters of severe infections due to *Clostridium difficile* PCR ribotype 027 in Germany. Euro Surveill. 2007 Nov;12(11):E0711152
4. Vonberg RP, Schwab F, Gastmeier P. *Clostridium difficile* in discharged inpatients, Germany. Emerg Infect Dis. 2007;13:179–80.
5. Bidet P, Barbut F, Lalande V, Burghoffer B, Petit JC. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol Lett. 1999;175:261–6. DOI: 10.1111/j.1574-6968.1999.tb13629.x
6. Borgmann S, Kist M, Jakobiak T, Reil M, Scholz E, von Eichel-Streiber C, et al. Increased number of *Clostridium difficile* infections and prevalence of *Clostridium difficile* PCR ribotype 001 in southern Germany. Euro Surveill. 2008 Dec 4;13(49):pii: 19057
7. Hensgens MP, Goorhuis A, Notermans DW, van Benthem BH, Kuijper EJ. Decrease of hypervirulent *Clostridium difficile* PCR ribotype 027 in the Netherlands. Euro Surveill. 2009;14(45):19402.
8. Brazier JS, Raybould R, Patel B, Duckworth G, Pearson A, Charlett A, et al. Distribution and antimicrobial susceptibility patterns of *Clostridium difficile* PCR ribotypes in English hospitals, 2007–08. Euro Surveill. 2008 Oct 9;13(41):pii: 19000.
9. Pepin J, Saheb N, Coulombe MA, Alary ME, Corriveau MP, Authier S, et al. Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*–associated diarrhea: a cohort study during an epidemic in Quebec. Clin Infect Dis. 2005;41:1254–60. DOI: 10.1086/496986
10. Spigaglia P, Barbanti F, Mastrantonio P, Brazier JS, Barbut F, Delmee M, et al. Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. J Med Microbiol. 2008;57:784–9. DOI: 10.1099/jmm.0.47738-0
11. Ackermann G, Tang YJ, Kueper R, Heisig P, Rodloff AC, Silva J Jr, et al. Resistance to moxifloxacin in toxigenic *Clostridium difficile* isolates is associated with mutations in gyrA. Antimicrob Agents Chemother. 2001;45:2348–53. DOI: 10.1128/AAC.45.8.2348-2353.2001
12. Dridi L, Tankovic J, Burghoffer B, Barbut F, Petit JC. gyrA and gyrB mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob Agents Chemother. 2002;46:3418–21. DOI: 10.1128/AAC.46.11.3418-3421.2002
13. Hooper DC. Mechanisms of fluoroquinolone resistance. Drug Resist Updat. 1999;2:38–55. DOI: 10.1054/drup.1998.0068
14. Labbe AC, Poirier L, Maccannell D, Louie T, Savoie M, Beliveau C, et al. *Clostridium difficile* infections in a Canadian tertiary care hospital before and during a regional epidemic associated with the BI/NAP1/027 strain. Antimicrob Agents Chemother. 2008;52:3180–7. DOI: 10.1128/AAC.00146-08
15. Zaiß NH, Rupnik M, Kuijper EJ, Harmanus C, Michielsen D, Janssens K, et al. Typing *Clostridium difficile* strains based on tandem repeat sequences. BMC Microbiol. 2009;9:6. DOI: 10.1186/1471-2180-9-6

Address for correspondence: Nils Henning Zaiß, Robert Koch Institute, Burgstrasse 37, 38855 Wernigerode, Germany; email: zaissh@rki.de

Hypervirulent *Clostridium difficile* Strains in Hospitalized Patients, Canada[1]

Michael R. Mulvey, David A. Boyd, Denise Gravel, Jim Hutchinson, Sharon Kelly, Allison McGeer, Dorothy Moore, Andrew Simor, Kathryn N. Suh, Geoff Taylor, J. Scott Weese, Mark Miller, and the Canadian Nosocomial Infection Surveillance Program[2]

To determine the incidence rate of infections with North American pulsed-field types 7 and 8 (NAP7/NAP8) strains of *Clostrodium difficile*, ribotype 078, and toxinotype V strains, we examined data collected for the Canadian Nosocomial Infections Surveillance Program (CNISP) CDI surveillance project during 2004–2008. Incidence of human infections increased from 0.5% in 2004/2005 to 1.6% in 2008.

Clostridium difficile infections (CDIs) have increased in incidence and severity within the past decade in North America and Europe (*1*), in large part because of the emergence of the hypervirulent North American pulsed-field type 1 (NAP1/027/III) strains (*2–5*). Recently, interest has increased in the ribotype 078 strain. A 2007 North American study showed that ribotype 078 strains predominated in swine and cattle (83%–94% prevalence), but were rare in a group of hospitalized persons (4% prevalence) (*6*). However, in studies from Europe and the United States, 078/V strains were found at a prevalence ranging from 3% to 11% (*7–9*). In a subsequent study by the US group, analysis of the toxinotype V strains from humans and food animals showed that 83% of strains were either NAP7 or NAP8 (*10*). A Dutch group has recently shown that 078/V strains increased from 3% to 13% during February 2005–2008 and can be considered hypervirulent (*11*). Our study aimed to determine the incidence rate of infections attributed to hypervirulent NAP7/078/V and NAP8/078/V strains of *C. difficile* in hospitals in Canada.

The Study

The Canadian Nosocomial Infection Surveillance Program is a collaborative effort between the Canadian Hospital Epidemiology Committee, a subcommittee of the Association of Medical Microbiology and Infectious Disease Canada, the Centre for Infectious Disease Prevention and Control, and the National Microbiology Laboratory of the Public Health Agency of Canada. The Canadian Nosocomial Infection Surveillance Program conducted prospective surveillance including collection of stool specimens from patients showing the presence of CDI during November 2004–April 2005 and during March and April in 2007 and 2008.

An infection was considered healthcare-associated CDI if the patient's symptoms occurred at least 72 hours after hospital admission or if the symptoms resulted in readmission of a patient who had been hospitalized within the 2 months before the symptom onset date and who was not a resident in a long-term care facility or nursing home (*12*). An infection was considered community-onset CDI if the healthcare-associated definition was not met. Outcomes 30 days postinfection were recorded to capture severe cases, which were defined as infections in patients admitted to an intensive care unit, in patients who had undergone colectomy, or in patients who had died (*12*). Deaths were assessed by the Canadian Hospital Epidemiology Committee member and categorized into 3 groups: 1) death directly attributable to CDI, 2) death indirectly related to CDI by exacerbation of an existing disease condition, or 3) death not a result of CDI. The assessment was made from information obtained from medical charts, nurse logs, laboratory reports, and consultation with nursing and medical staff.

All stool specimens were cultured for *C. difficile,* and isolates were analyzed by PCR and pulsed-field gel electrophoresis (PFGE) at the National Microbiology Laboratory. PFGE, ribotyping, and toxinotyping were performed as described (*10,11*). MICs were determined by agar dilution or Etest. The primers used for PCR and sequencing are listed in Table 1. Macrorestriction patterns were analyzed with BioNumerics V4.5 (Applied Maths, Sint-Martens-Latem, Belgium).

Author affiliations: Public Health Agency of Canada, Winnipeg, Manitoba, Canada (M.R. Mulvey, D.A. Boyd); Public Health Agency of Canada, Ottawa, Ontario, Canada (D. Gravel); Health Sciences Centre, St. John's, Newfoundland and Labrador, Canada (J. Hutchinson and S. Kelly); Mount Sinai Hospital, Toronto, Ontario, Canada (A. McGeer); Montreal Children's Hospital, Montreal, Quebec, Canada (D. Moore); Sunnybrook Health Science Centre, Toronto (A. Simor); The Ottawa Hospital, Ottawa (K.N. Suh); University of Alberta Hospital, Edmonton, Alberta, Canada (G. Taylor); University of Guelph, Geulph, Ontario, Canada (J.S. Weese); and Jewish General Hospital, Montreal (M. Miller)

DOI: 10.3201/eid1604.091152

[1]Parts of this study were presented at the 48th Interscience Conference on Antimicrobial Agents and Chemotherapy/46th Infectious Disease Society of America meeting in Washington DC, USA, October 25–28, 2008.

[2]Members of the Canadian Nosocomial Infection Surveillance Program who contributed data are listed at the end of this article.

Table 1. Primers used in study of hospitalized patients with *Clostridium difficile* infection, Canada, 2004–2008

Primer	Sequence (5′ → 3′)	Specificity
*tcd*3	TGCAATTATAAAAACATCTTTAAAC	*tcdC* PaLoc negative regulator
*tcd*4	TATATCTAATAAAAGGGAGATTG	
cdtB-F1	TGGACAGGAAGAATAATTCCTTC	*cdtB* binary toxin subunit B
cdtB-R1	TGCAACTAACGGATCTCTTGC	
E5	CTCAAAACTTTTTAACGAGTG	*ermB* erythromycin/clindamycin resistance
E6	CCTCCCGTTAAATAATAGATA	
GyrAF	TTGAAATAGCGGAAGAAATGA	*gyrA* DNA gyrase subunit A
GyrAR	TTGCAGCTGTAGGGAAATC	
GyrBF	GAAGGTCAAACTAAAACAAA	*gyrB* DNA gyrase subunit B
GyrBR	GGGCTCCATCTACATCG	

Fifteen NAP7 and 4 NAP8 patterns were identified from isolates obtained from 2,794 patients (overall prevalence 0.68%). Table 2 lists the patients and epidemiologic information, and the Figure shows the corresponding genomic fingerprint patterns. During the study period, the incidence rate increased as follows: 8/1,785 (0.5%) in 2004–2005; 5/638 (0.8%) in 2007; and 6/371 (1.6%) in 2008. Of the 19 patients identified, 14 were men with an average age of 70.8 years (not including 1 pediatric case), and 4 were women with an average age of 52.2 years; the overall average age was 61.5 years (Table 2). CDI was considered as community onset in 7 (37%) of 19 cases, and severe CDI was manifested in 3 (15.8%) case-patients (1 was healthcare-associated CDI and 2 were community-onset CDI). At 30 days postinfection for CDI, 26.3% of all patients had died, 1 death a direct result of CDI (5.3%), and 1 indirectly related; 10.6% of total deaths were attributable to CDI.

Sequence analysis of the *tcdC* gene showed that all strains carried a C184T transition that introduces a stop codon leading to a presumptive truncated protein of 61 residues, and a 39-bp deletion located downstream of the alternative stop codon. This *tcdC* variant has been previously described for toxinotype V strains (*13*). Sixteen of the isolates were ribotype 078 and 3 isolates had unknown ribotypes. All 2004/2005 and 2007 isolates were toxinotype V. The 2008 isolates were not toxinotyped. All 19 strains were susceptible to metronidazole and vancomycin. Seven isolates were susceptible to clindamycin (MIC <8 μg/mL) and 12 were resistant (6 had MICs = 8, 2 had MICs = 16, and 4 had MICs ≥256). Only the 4 latter strains carried *ermB* and all were NAP8. Fourteen isolates

Table 2. Epidemiologic information from hospitalized patients with *Clostridium difficile* infection, Canada, 2004–2008*

Year and patient ID	Province	Age, y/sex	Source	Severe CDI†	Outcome‡
2004–2005					
O1-0059	Ontario	62/M	Healthcare-associated	No	Discharged
O2-0053	Ontario	35/M	Community-onset	No	Died-not attrib
O3-0042	Ontario	64/F	Community-onset	No	Discharged
Q1-0028	Quebec	66/M	Healthcare-associated	Yes	Died-attrib
H1-0040	Nova Scotia	70/M	Healthcare-associated	No	Discharged
S1-0054	Saskatchewan	72/M	Community-onset	No	Discharged
S1-0063	Saskatchewan	82/M	Community-onset	Yes	Discharged
O7-0121	Ontario	74/M	Healthcare-associated	No	Survived-hosp
2007					
O1-7-0011	Ontario	87/M	Community-onset	No	Survived-hosp
O4-7-0011	Ontario	82/M	Community-onset	Yes	Died-contrib
Q1-7-0017	Quebec	40/F	Healthcare-associated	No	Discharged
O8B-7-0002	Ontario	65/M	Healthcare-associated	No	Died-not attrib
Q5-7-0013	Quebec	71/M	Healthcare-associated	No	Discharged
2008					
B1-8-0052	British Columbia	44/F	Healthcare-associated	No	Discharged
B1-8-0059	British Columbia	73/M	Healthcare-associated	No	Discharged
A3-8-0022	Alberta	38/F	Community-onset	No	Discharged
O2B-8-0015	Ontario	75/F	Community-onset	No	Survived-hosp
Q1-8-0008	Quebec	81/M	Healthcare-associated	No	Died-not attrib
O5-8-0001	Ontario	2/M	Healthcare-associated	No	Discharged

*ID, identification; CDI, *Clostridium difficile* infection; Died-not attrib, death not attributable to CDI; Died-attrib, death directly attributable to CDI; Survived-hosp, patient survived but was still in a hospital at endpoint; Died-contrib, CDI indirectly contributed to death.
†Required admission to intensive care unit due to CDI, received a colectomy, or died.
‡At 30 days after diagnosis of CDI.

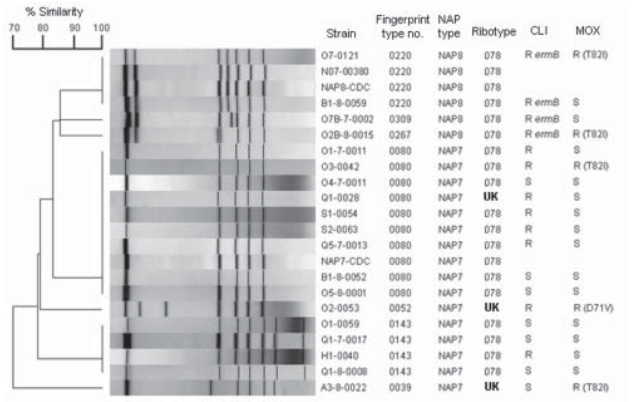


Figure. Dendrogram analysis of macrorestriction patterns (*Sma*I) of the NAP7 and NAP8 *Clostridium difficile* strains isolated from the patients listed in Table 2. *C. difficile* N07-00380 is a ribotype 078 control strain. *C. difficile* NAP7-CDC and NAP8-CDC control strains are toxinotype V. Isolates exhibiting high-level clindamycin resistance (≥256 μg/mL) and harboring *ermB* are indicated. The amino acid change found in the gyrA protein is shown for the moxifloxacin-resistant strain antimicrobial drug–resistance mechanisms.

that were susceptible to moxifloxacin (MIC <8 μg/mL) had identical *gyrA* and *gyrB* quinolone-resistance–determining regions (QRDR) sequences to the genes in *C. difficile* 630 (GenBank accession no. AM180355). Five moxifloxacin-resistant isolates (MIC > 8 μg/mL) had no mutations in the *gyrB* QRDR but each had 1 mutation in the *gyrA* QRDR. One with MIC = 8 had an Asp71Val mutation; 3 with MIC = 16 and 1 with MIC >32 had a Thr82Ile mutation. These mutations have been previously described in moxifloxacin-resistant *C. difficile* (*14*).

Conclusions

C. difficile NAP7 and NAP8/078/V strains are relatively rare in hospitalized patients with CDI in Canada, in contrast to their prevalence in Europe and the United States (*7–11*). However, incidence rates have tripled from 0.5% in 2004 to 1.6% in 2008 (p = 0.22). There was a high association with a community onset, although dataset was too small to statistically confirm that increased cases were more likely to be community onset; 2 (40%) of 5 deaths were attributable to CDI. Although the number of strains studied here was small, data are consistent with other studies that indicate a community association for NAP7 and NAP8/078/V strains (*9–11*). The prevalence of these strains in Canada may be higher than suggested here if they are a common cause of community-associated CDI, as studies have indicated (*10,11*). The role of animals in acquisition of NAP7 and NAP8/078/V strains was not evaluated because animal and food contact data were not available.

Molecular typing of *C. difficile* is typically performed by using ribotyping in Europe and PFGE/macrorestriction analysis in North America; both groups may use toxinotyping, which strictly looks at PaLoc variation. We showed a high correlation between NAP7, NAP8, ribotype 078, and toxinotype V strains by the 3 typing methods, which enabled results of separate studies to be compared. Furthermore, *tcdC* analysis provides an additional diagnostic tool for these strains because the gene has a 39-bp deletion and a C184T-transition in all isolates we studied.

Continued surveillance is warranted in humans, animals, and retail meat to determine whether NAP7 and 8/078/V strains will continue to emerge in patients hospitalized in Canada and to determine whether the sources of these infections are related to animals or food. Surveillance is especially important given that these strains appear to be hypervirulent as has been reported for NAP1/027/III strains (*11*).

Members of the Canadian Nosocomial Infection Surveillance Program who participated in the surveillance for *C. difficile* infection: Elizabeth Bryce, Vancouver General Hospital, Vancouver, British Columbia; John Conly, Foothills Medical Centre, Calgary, Alberta; John Embil, Health Sciences Centre, Winnipeg, Manitoba; Joanne Embree, Health Sciences Centre, Winnipeg; Sarah Forgie, Stollery Children's Hospital, Edmonton, Alberta; Charles Frenette, McGill University Health Centre, Montreal, Quebec; Camille Lemieux, University Health Network, Toronto, Ontario; Elizabeth Henderson, Peter Lougheed Centre, Calgary; Michael John, London Health Sciences Centre, London, Ontario; Lynn Johnston, QEII Elizabeth Health Sciences Centre, Halifax, Nova Scotia; Pamela Kibsey, Victoria General Hospital, Victoria, British Columbia; Joanne Langley, IWK Health Centre, Halifax; Mark Loeb, Hamilton Health Sciences Corporation, Hamilton, Ontario; Anne Matlow, Hospital for Sick Children, Toronto; Sophie Michaud, CHUS-Hôpital Fleurimont, Sherbrooke, Quebec; Marianne Ofner, Centre for Communicable Diseases and Infection Control, Public Health Agency of Canada, Ottawa, Ontario; Virginia Roth, The Ottawa Hospital, Ottawa; Eva Thomas, Children's and Women's Health Center, Vancouver; William Thompson, South East Regional Health Authority, Moncton, New Brunswick; Nathalie Turgeon, Hôtel-Dieu de Québec du CHUQ, Quebec, Quebec; Mary Vearncombe, Sunnybrook Health Sciences Centre, Toronto; Karl Weiss, Maisonneuve-Rosemont Hospital, Montreal; Alice Wong, Royal University Hospital, Saskatoon, Saskatchewan; Dick Zoutman, Kingston General Hospital, Kingston, Ontario.

Acknowledgments

We gratefully thank Krista Wilkinson for critical reading of the manuscript. Expert technical assistance was provided by Romeo Hizon, Tim Du, and Stuart McCorrister. *C. difficile* isolates

representing NAP7 and NAP8 were kindly provided by B. Limbago (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Dr Mulvey is chief of the Antimicrobial Resistance and Nosocomial Infections Section of the National Microbiology Laboratory of the Public Health Agency of Canada. His research interests include the molecular epidemiology of antimicrobial-resistant bacterial pathogens.

References

1. Kelly CP, LaMont JT. *Clostridium difficile*—more difficult than ever. N Engl J Med. 2008;359:1932–40. DOI: 10.1056/NEJMra0707500
2. McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N Engl J Med. 2005;353:2433–41. DOI: 10.1056/NEJMoa051590
3. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, et al. A predominantly clonal multi-institutional outbreak of *Clostridium difficile*–associated diarrhea with high morbidity and mortality. N Engl J Med. 2005;353:2442–9. DOI: 10.1056/NEJMoa051639
4. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 2005;366:1079–84. DOI: 10.1016/S0140-6736(05)67420-X
5. MacCannell DR, Louie TJ, Gregson DB, Laverdiere M, Labbe A-C, Laing F, et al. Molecular analysis of *Clostridium difficile* ribotype 027 isolates from eastern and western Canada. J Clin Microbiol. 2006;44:2147–52. DOI: 10.1128/JCM.02563-05
6. Keel K, Brazier S, Post KW, Weese S, Songer JG. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J Clin Microbiol. 2007;45:1963–4. DOI: 10.1128/JCM.00224-07
7. Goorhuis A, Debast SB, van Leengoed LAMG, Harmanus C, Notermans DW, Bergwerff AA, et al. *Clostridium difficile* PCR ribotype 078: an emerging strain in humans and in pigs? J Clin Microbiol. 2008;46:1157–8. DOI: 10.1128/JCM.01536-07
8. Rupnik M, Widmer A, Zimmermann O, Eckert C, Barbut F. *Clostridium difficile* toxinotype V, ribotype 078, in animals and humans. J Clin Microbiol. 2008;46:1963–4. DOI: 10.1128/JCM.00598-08
9. Limbago B. Long CM, Thompson AD, Killgore GE, Hannett G, Havill N, et al. Isolation and characterization of *Clostridium difficile* responsible for community-associated disease. In: Abstracts of the Second International *Clostridium difficile* Symposium, Maribor, Slovenia, June 6–9, 2007 [cited 2009 Jun 10]. http://clostridia.net/ICDS.htm
10. Jhung MA, Thompson AD, Killgore GE, Zukowski WE, Songer G, Warny M, et al. Toxinotype V *Clostrdium difficile* in humans and food animals. Emerg Infect Dis. 2008;14:1039–45. DOI: 10.3201/eid1407.071641
11. Goorhuis A, Bakker D, Corver J, Debast SB, Harmanus C, Notermans DW, et al. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin Infect Dis. 2008;47:1162–70. DOI: 10.1086/592257
12. Gravel D, Miller M, Simor A, Taylor G, Gardam M, McGeer A, et al. Health-care associated *Clostridium difficile* infection in adults admitted to acute care hospitals in Canada: a Canadian Nosocomial Infection Surveillance Program study. Clin Infect Dis. 2009;48:568–76. DOI: 10.1086/596703
13. Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J Clin Microbiol. 2002;40:3470–5. DOI: 10.1128/JCM.40.9.3470-3475.2002
14. Dridi L, Tankovic A, Burghoffer B, Barbut F, Petit J-C. *gyrA* and *gyrB* mutations are implicated in cross-resistance to ciprofloxacin and moxifloxacin in *Clostridium difficile*. Antimicrob Agents Chemother. 2002;46:3418–21. DOI: 10.1128/AAC.46.11.3418-3421.2002

Address for correspondence: Michael R. Mulvey, Public Health Agency of Canada, 1015 Arlington St, Winnipeg, Manitoba, Canada, R3E 3R2; email: michael_mulvey@phac-aspc.gc.ca

Porcine-Origin Gentamicin-Resistant *Enterococcus faecalis* in Humans, Denmark

Jesper Larsen, Henrik C. Schønheyder, Camilla H. Lester, Stefan S. Olsen, Lone J. Porsbo, Lourdes Garcia-Migura, Lars B. Jensen, Magne Bisgaard, and Anette M. Hammerum

During 2001–2002, high-level gentamicin-resistant (HLGR) *Enterococcus faecalis* isolates were detected in 2 patients in Denmark who had infective endocarditis and in pigs and pork. Our results demonstrate that these isolates belong to the same clonal group, which suggests that pigs are a source of HLGR *E. faecalis* infection in humans.

Infective endocarditis is a life-threatening infection that involves the endocardial surface or vascular structures in proximity to the heart. Its intrinsic resistance to a number of antimicrobial drugs makes enterococcal infective endocarditis cumbersome to treat. For decades, the mainstay has been the combination of a cell wall–active agent (ampicillin, penicillin, or vancomycin) and gentamicin (*1*). However, high-level resistance to gentamicin hinders the bactericidal activity of combination therapy and increases the likelihood of clinical and microbiologic failure and even death (*2*).

High-level gentamicin-resistant (HLGR) *Enterococcus faecalis* has been associated with the hospital setting and prior healthcare exposure, which suggests the existence of a healthcare reservoir (*3*). Nevertheless, enterococci are gut commensals in humans and warm-blooded animals; therefore, reservoirs of HLGR *E. faecalis* conceivably might exist in the community not directly linked to the healthcare setting.

Author affiliations: University of Copenhagen, Frederiksberg, Denmark (J. Larsen, M. Bisgaard); Aarhus University Hospital, Aalborg, Denmark (H.C. Schønheyder); Statens Serum Institut, Copenhagen, Denmark (C.H. Lester, S.S. Olsen, A.M. Hammerum); Technical University of Denmark, Søborg, Denmark (L.J. Porsbo); and Technical University of Denmark, Copenhagen (L. Garcia-Migura, L.B. Jensen).

DOI: 10.3201/eid1604.090500

During 2000–2002, the proportion of HLGR *E. faecalis* isolates increased from 2% to 6% in the pig population in Denmark (*4–6*), which coincided with the emergence of HLGR *E. faecalis* isolates among patients with infective endocarditis in North Denmark Region. We undertook this study to determine whether pigs are a source of *E. faecalis* infections in humans.

The Study

Two HLGR *E. faecalis* isolates were collected by blood culture (BacT/Alert; bioMérieux, Marcy l'Etoile, France) from separate patients with infective endocarditis in North Denmark Region during 2001–2002 (Table). The regional clinical microbiology laboratory at Aalborg Hospital performed all original antimicrobial susceptibility testing by using Etest (bioMérieux) according to the manufacturer's recommendations. We retrieved patient information recorded in a regional research database and used it to classify cases as definite, possible, or rejected according to the modified Duke criteria (*8*). A nosocomial origin of infection was determined in accordance with the US Centers for Disease Control and Prevention (*9*).

Patient A, an 81-year-old man with a previous stroke and a urinary catheter, was admitted twice within 1 month with HLGR *E. faecalis* bacteremia, a systolic murmur, transitory cerebral ischemia, and petechial elements. Aortic valve endocarditis remained a clinical diagnosis because no echocardiography was performed (possible infective endocarditis). The patient died from a new stroke 1.5 months later.

Patient B, a 70-year-old woman with non–insulin-dependent diabetes mellitus, had recurrent bacteremia with HLGR *E. faecalis* and a diagnosis of aortic valve endocarditis confirmed by transesophageal echocardiography (definite infective endocarditis). Aortic valve replacement was performed, and she died 1.5 years later from an unrelated cause. Both cases of infective endocarditis were deemed to be community acquired.

During 2001–2002, a total of 20 HLGR isolates were collected from 19 pigs and from 1 sample of pork as part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) (Table) (*5,6*). DANMAP also collected fecal samples from humans in the community during 2002–2006. The protocol was approved by the regional Scientific Ethics Committee before the investigation ([KF] 01-006/02). Two HLGR isolates were recovered from community-dwelling persons (Table): a 24-year-old woman (community healthcare worker) and a 2-year-old boy in whom inflammatory bowel disease had been diagnosed and who had received antiinflammatory therapy (mesalazine) within the preceding 30 days. Both regularly ate meat, including pork, and had no contact with food-producing animals during the 7 days before sampling.

Table. Origins of gentamicin-resistant *Enterococcus faecalis* isolates and MICs for gentamicin, Denmark, 2001–2002*

Identification no.	Other name	Origin	Sampling date	MIC for gentamicin, μg/mL	MLST type
124832		IE patient A	2001 Nov 18	>2,048	16
48250		IE patient B	2002 May 2	>2,048	16
7330318-4	D6	Pig	2001 Mar 29	>2,048	**16**
7330612-2	D29	Pig	2001 Jul 25	2,048	**16**
7330948-5	D34	Pig	2001 Nov 23	2,048	**16**
7330115-2		Pig	2001 Jan 29	>2,048	16
7330445-1		Pig	2001 May 21	2,048	16
7430040-2		Pig	2002 Feb 18	1,024	16
7430049-2		Pig	2002 Feb 22	1,024	16
7430275-3		Pig	2002 May 16	>2,048	16
7430291-2		Pig	2002 May 21	2,048	16
7430297-2		Pig	2002 May 22	>2,048	16
7430315-3		Pig	2002 May 23	2,048	35
7430317-5		Pig	2002 May 23	2,048	16
7430328-3		Pig	2002 May 27	>2,048	16
7430405-1		Pig	2002 Jun 24	>2,048	16
7430411-3		Pig	2002 Jun 25	1,024	16
7430416-2		Pig	2002 Jun 24	1,024	16
7430416-3		Pig	2002 Jun 24	>2,048	16
7430803-2		Pig	2002 Nov 18	>2,048	16
7430821-4		Pig	2002 Nov 21	>2,048	16
19116		Pork	2002 Oct 6	>2,048	16
1448		CD human	2003 Oct 1	>2,048	16
3382		CD human	2005 Dec 9	>2,048	16

*MLST, multilocus sequence typing; IE, infective endocarditis; CD, community dwelling. MLST types in **boldface** have been published (*7*).

MICs were determined for gentamicin (128–2,048 μg/mL) by using the Sensititre system (Trek Diagnostic Systems, East Grinstead, UK) according to current guidelines for inoculation and incubation recommended by the Clinical and Laboratory Standards Institute (*10*). MICs were consequently ≥1,024 μg/mL (Table).

Multilocus sequence typing (MLST) was performed for the 24 HLGR isolates as described (*11*). The 2 isolates from patients A and B, 18 of 19 isolates from pigs, the isolate from pork, and the 2 isolates from community-dwelling persons belonged to MLST type ST16 (Table). The remaining isolate from pigs belonged to MLST type ST35 (Table).

The major MLST type ST16 was further characterized by pulsed-field gel electrophoresis (PFGE) by using *Sma*I (*12*). The PFGE patterns were analyzed by using BioNumerics version 5.1 (Applied Maths, Kortrijk, Belgium), and isolates were grouped into PFGE clonal types by using Dice coefficients and a value of >82% relatedness (*13*). The PFGE patterns had a minimum of 86% relatedness and were clustered into 1 major clonal group.

The HLGR ST16 isolates were further screened by PCR for the *aac(6')Ie-aph(2'')Ia* gene, the *aph(2'')Ib* and *aph(2'')Id* genes and the *aph(2'')Ic* gene to assess the genetic background of gentamicin resistance. All 24 HLGR ST16 isolates carried the *aac(6')Ie-aph(2'')Ia* gene and none of the other genes encoding gentamicin resistance.

Conclusions

Our study provides evidence of the existence of a widespread community reservoir of HLGR ST16 in pigs in Denmark during 2001–2002, which coincided with emergence of HLGR ST16 isolates among IE patients in North Denmark Region. One isolate was present in pork, which supports foodborne transmission, although direct transmission from animals to humans is also possible.

Our study has potential limitations. First, the method used by DANMAP (susceptibility testing of 1 colony per sample, rather than resistance prevalence per sample) may underestimate the extent of the HLGR reservoir in food-producing animals, meat products, and community-dwelling persons. Second, HLGR isolates from patients with infective endocarditis emanated from 2001 and 2002 and therefore do not represent recent trends.

Our findings support the results of a recent study in the United States that identified HLGR *E. faecalis* isolates with similar PFGE patterns (≤3-band difference) from pork and fecal swabs of outpatients (*14*). These pig-related HLGR isolates, as well as our collection of HLGR ST16 isolates, carry the *aac(6')Ie-aph(2'')Ia* gene encoding gentamicin resistance.

Pig-related *E. faecalis* isolates belonging to ST16 carry pathogenicity island genes (*7*). These genes are more frequently detected among *E. faecalis* isolates recovered from blood or from fecal swabs of inpatients than among isolates from fecal swabs of healthy persons, which sug-

gests that they are associated with invasiveness and virulence in humans (*15*).

With an annual production of >22 million slaughter pigs (*4–6*), Denmark has a large potential reservoir of HLGR ST16. Although HLGR ST16 was not detected in other food-producing animals and meat products, this type may not be exclusive to pigs. We found HLGR ST16 isolates in 2 community-dwelling persons during 2003–2005. Preference for eating pork, close contact with the healthcare setting, underlying disease, or a combination thereof may have predisposed these persons to become colonized by this potential pathogen.

HLGR ST16 appears to be transmitted from pigs to humans, although other routes of transmission also may exist. Further studies are needed to better understand the human and veterinary epidemiology of this zoonosis. Areas of study should include recent trends of HLGR among invasive *E. faecalis*; size of the reservoir in pigs; its association with antimicrobial drug use in pigs; and whether other animals, immunocompromised persons, or healthy persons constitute a community reservoir of HLGR ST16.

Acknowledgments

We thank Karin S. Pedersen for genotyping and PCR assays and Lena Mortensen for so meticulously handling the clinical isolates.

This work was supported by grant 271-06-0241 from the Danish Medical Research Council, the Danish Ministry of Family and Consumer Affairs, and the Danish Ministry of the Interior and Health as part of the Danish Integrated Antimicrobial Resistance and Research Program and the European Union Sixth Framework Program, Approaches to Control Multiresistant Enterococci: Studies on Molecular Ecology, Horizontal Gene Transfer, Fitness and Prevention, under contract LSHE-CT-2007-037410.

Dr Larsen is a postdoctoral trainee in infectious diseases at the University of Copenhagen, Frederiksberg C. His research interests are the evolution of opportunistic pathogens and their epidemiology.

References

1. Baddour LM, Wilson WR, Bayer AS, Fowler VG Jr, Bolger AF, Levison ME, et al. Infective endocarditis: diagnosis, antimicrobial therapy, and management of complications: a statement for healthcare professionals from the Committee on Rheumatic Fever, Endocarditis, and Kawasaki Disease, Council on Cardiovascular Disease in the Young, and the Councils on Clinical Cardiology, Stroke, and Cardiovascular Surgery and Anesthesia, American Heart Association: endorsed by the Infectious Diseases Society of America. Circulation. 2005;111:e394–434. DOI: 10.1161/CIRCULATIONAHA.105.165564
2. Aslangul E, Ruimy R, Chau F, Garry L, Andremont A, Fantin B. Relationship between the level of acquired resistance to gentamicin and synergism with amoxicillin in *Enterococcus faecalis*. Antimicrob Agents Chemother. 2005;49:4144–8. DOI: 10.1128/AAC.49.10.4144-4148.2005
3. Claesson C, Hallgren A, Nilsson M, Svensson E, Hanberger H, Nilsson LE. Susceptibility of staphylococci and enterococci to antimicrobial agents at different ward levels in four north European countries. Scand J Infect Dis. 2007;39:1002–12. DOI: 10.1080/00365540701472064
4. Danish Integrated Antimicrobial Resistance Monitoring and Research Program. DANMAP 2000—consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark [cited 2009 Sep 1]. http://www.danmap.org/pdfFiles/Danmap_2000.pdf
5. Danish Integrated Antimicrobial Resistance Monitoring and Research Program. DANMAP 2001—use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark [cited 2009 Sep 1]. http://www.danmap.org/pdfFiles/Danmap_2001.pdf
6. Danish Integrated Antimicrobial Resistance Monitoring and Research Program. DANMAP 2002—use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark [cited 2009 Sep 1]. http://www.danmap.org/pdfFiles/Danmap_2002.pdf
7. Shankar N, Baghdayan AS, Willems R, Hammerum AM, Jensen LB. Presence of pathogenicity island genes in *Enterococcus faecalis* isolates from pigs in Denmark. J Clin Microbiol. 2006;44:4200–3. DOI: 10.1128/JCM.01218-06
8. Li JS, Sexton DJ, Mick N, Nettles R, Fowler VG Jr, Ryan T, et al. Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis. 2000;30:633–8. DOI: 10.1086/313753
9. Garner JS, Jarvis WR, Emori TG, Horan TC, Hughes JM. CDC definitions for nosocomial infections, 1988. Am J Infect Control. 1988;16:128–40. DOI: 10.1016/0196-6553(88)90053-3
10. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: Fifteenth Informational Supplement M100-S15. Wayne (PA): The Institute; 2005.
11. Ruiz-Garbajosa P, Bonten MJ, Robinson DA, Top J, Nallapareddy SR, Torres C, et al. Multilocus sequence typing scheme for *Enterococcus faecalis* reveals hospital-adapted genetic complexes in a background of high rates of recombination. J Clin Microbiol. 2006;44:2220–8. DOI: 10.1128/JCM.02596-05
12. Turabelidze D, Kotetishvili M, Kreger A, Morris JG Jr, Sulakvelidze A. Improved pulsed-field gel electrophoresis for typing vancomycin-resistant enterococci. J Clin Microbiol. 2000;38:4242–5.
13. Morrison D, Woodford N, Barrett SP, Sisson P, Cookson BD. DNA banding pattern polymorphism in vancomycin-resistant *Enterococcus faecium* and criteria for defining strains. J Clin Microbiol. 1999;37:1084–91.
14. Donabedian SM, Thal LA, Hershberger E, Perri MB, Chow JW, Bartlett P, et al. Molecular characterization of gentamicin-resistant enterococci in the United States: evidence of spread from animals to humans through food. J Clin Microbiol. 2003;41:1109–13. DOI: 10.1128/JCM.41.3.1109-1113.2003
15. Ruiz-Garbajosa P, Canton R, Pintado V, Coque TM, Willems R, Baquero F, et al. Genetic and phenotypic differences among *Enterococcus faecalis* clones from intestinal colonisation and invasive disease. Clin Microbiol Infect. 2006;12:1193–8. DOI: 10.1111/j.1469-0691.2006.01533.x

Address for correspondence: Jesper Larsen, Department of Disease Biology, University of Copenhagen, Stigbøjlen 4, DK-1870 Frederiksberg C, Denmark; email: jesl@life.ku.dk

Merkel Cell Polyomavirus in Cutaneous Swabs

Vincent Foulongne, Nicolas Kluger, Olivier Dereure, Grégoire Mercier, Jean-Pierre Molès, Bernard Guillot, and Michel Segondy

To assess the usefulness of using cutaneous swabs to detect Merkel cell polyomavirus (MCPyV) DNA, we analyzed swabs from persons with Merkel cell carcinoma (MCC), others with skin diseases, and healthy volunteers. MCPyV was detected in at least 1 sample from virtually all participants. Viral loads were higher in samples from patients with MCC.

Merkel cell polyomavirus (MCPyV) is a recently identified human virus initially discovered in tumor tissues of patients with Merkel cell carcinoma (MCC), a rare but aggressive skin cancer (*1*). Several studies have confirmed that MCPyV DNA is present in 70%–80% of MCC tumors (*1–4*) and that tumoral cells in most patients show a monoclonal integration of the viral genome and expression of the large T antigen (*1,5–7*). Furthermore, the integrated viral genome may harbor mutations in the T-antigen coding sequence, resulting in truncation of the corresponding helicase protein (*8*). These data support a causal role of MCPyV in tumorogenesis. However, the tumorogenic potential of MCPyV remains unclear because the MCPyV genome cannot be detected in 20%–30% of MCC tumors, whereas MCPyV DNA has been consistently identified in persons with other disorders or in the skin of healthy persons (*9–12*).

Most studies of MCPyV DNA detection in skin samples have been performed on biopsy samples. We assessed the relevance of using cutaneous swabbing instead of full-skin tissue samples for viral MCPyV DNA detection. Overall, our results show that MCPyV DNA was detected in cutaneous swabs from almost all study participants, which indicated that MCPyV is likely a ubiquitous virus.

The Study

Cutaneous swabs were obtained from 46 persons: 5 patients with MCC (median age 76 years, range 68–78 years); 16 patients with various skin diseases (median age 76.5 years, range 41–90 years); and 25 clinically healthy volunteers (median age 35 years, range 22–58 years) who were recruited from among Montpellier University Hospital staff (Table). All study participants gave written informed consent, and the study was approved by the local ethics committee. Cutaneous swabs were collected from the face and, for most study participants, from the trunk (chest, back, or abdomen) and upper and lower limbs. Lesions in patients with MCC or other cutaneous disorders were also swabbed. Additionally, buccal mucosa swabs were obtained from 42 patients.

Swabs were suspended in 400 μL of phosphate-buffered saline, and DNA was extracted from 300 μL of the suspension with an automatic EasyMag apparatus (bioMérieux, Marcy l'Etoile, France). The elution volume was 50 μL, and 10 μL of eluate was used for subsequent PCRs. An unused swab, processed in the same way as the cutaneous swab, was included in each run as the negative control. MCPyV DNA was detected by PCR by using large T antigen (LT3) and viral capsid protein (VP1) primers (*1*); MCPyV DNA levels were measured by real-time PCR as described (*11*). Total DNA level in swabs was measured by using the LightCycler Control DNA Kit (Roche Diagnostics, Meylan, France), and results were expressed as MCPyV DNA copies/ng DNA.

We analyzed categorical data by using the χ^2 test. Continuous variables were compared by using the Kruskal-Wallis test for multiple groups and the Fligner-Policello rank tests for pairwise comparisons. Bonferroni correction was applied when appropriate. A p value <0.05 was considered significant.

Overall, MCPyV DNA was detected in at least 1 swab from all but 2 study participants (1 patient with Kaposi sarcoma and another with a leg ulcer). MCPyV DNA was detected in 141 (79.6%) of 177 cutaneous swabs and in 2 (4.8%) of 42 buccal mucosa swabs ($p<0.001$) (Table). The viral genome was detected in 27 (93.1%) of 29 cutaneous swabs from patients with MCC, in 58 (74.3 %) of 78 from patients with other cutaneous diseases, and in 56 (80.0%) of 70 from clinically healthy volunteers ($p = 0.10$). MCPyV DNA was more frequently detected on swabs taken from the face (43/47, 91.5%) than from the trunk (40/47, 85.1%) or limbs (48/69, 69.6%), but the difference was significant only between face and limb swabs ($p = 0.005$). The MCPyV genome was absent from swabs taken from 3 leg ulcers.

Levels of total human DNA were significantly higher in buccal mucosa swabs than in cutaneous swabs (2,170 pg/μL vs. 62 pg/μL; $p<0.001$). MCPyV DNA levels were higher in cutaneous swabs obtained from patients with MCC (median 861 copies/ng DNA) than in those from patients with other skin diseases (median 45 copies/ng DNA; $p<0.001$) or from the clinically healthy volunteers (median 43 copies/ng DNA; $p<0.001$) (Figure). MCPyV DNA levels were 5 and 11 copies/ng of DNA in each of the 2 positive buccal swabs.

Affiliations: University of Montpellier I, Montpellier, France

DOI: 10.3201/eid1604.091278

Table. Merkel cell polyomavirus DNA detection rates in swabs

Swab type	Patients with MCC* (no. positive/no. tested)	Patients with other skin diseases† (no. positive/no. tested)	Clinically healthy volunteers (no. positive/no. tested)
Buccal mucosa	1/5	0/13	1/24
Cutaneous			
Overall	27/29	58/78	56/70
Face	6/6	14/16	23/25
Trunk	11/11	16/20	13/16
Upper limb	1//2	10/15	9/14
Lower limb	7/8	10/15	11/15
Lesion	2/2	8/12	Not applicable

*MCC, Merkel cell carcinoma.
†Patients with Kaposi sarcoma (n = 6), cutaneous drug side-effect (n = 3), leg ulcer (n = 3), bullous pemphigoid (n = 2), psoriasis (n = 1), and mycosis fungoides (n = 1).

Conclusions

Our results demonstrate that MCPyV DNA can be efficiently detected by cutaneous swabbing. This easy method could be a useful tool for future epidemiologic or molecular studies targeting MCPyV. Indeed, this noninvasive procedure may be easily performed without the potential risk for side effects related to biopsy collection and is more acceptable than a biopsy for patients who do not have cutaneous disease. The high prevalence of MCPyV DNA at the skin surface, contrasted with its low prevalence in buccal mucosa and its absence in skin ulcers (where the epidermis is absent), strongly suggests that MCPyV is localized in the epidermis. As an alternative hypothesis, MCPyV could be released onto the skin surface through sebaceous or sudoral secretions. The relative high levels of viral DNA contrasted with the low amount of total DNA in cutaneous swabs might indeed support this hypothesis.

A lower prevalence of MCPyV DNA in skin biopsy samples among a similar subset of patients has been reported (*11*). However, this finding may be explained by the more limited amounts of superficial skin layers in skin biopsy samples compared with those in cutaneous swabs. Because disinfection of the skin before a biopsy may eliminate potential viral DNA from the epidermis, cutaneous swabbing may produce a more thorough sample for testing.

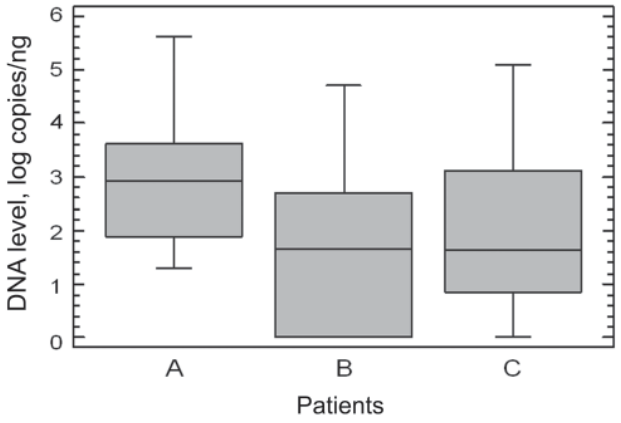


Figure. Comparison of Merkel cell polyomavirus DNA levels in cutaneous swabs obtained from 46 study participants. A) Patients with Merkel cell carcinoma; B) patients with other skin diseases; C) clinically healthy volunteers. Data are presented as box-and-whisker plots. Boxes represent the interquartile range, lines within the boxes represent the median value, and whiskers represent 1.5 × the interquartile range.

Our results are in accordance with those from a study by Loyo et al. that showed widespread distribution of MCPyV (*12*) and with those from a study by Wieland et al., who detected MCPyV DNA on the forehead skin of 62% of healthy persons (*13*). These findings strongly suggest that MCPyV is likely present in the skin of almost all adults. Furthermore, another recent study on MCPyV seroprevalence concluded that MCPyV circulates widely in the human population because specific MCPyV antibodies can be detected in as much as 80% of persons tested (*14*). Our results indeed support these serologic data and would be indicative of a persistent, although asymptomatic, infection with MCPyV in the epidermis of most persons.

Our results concur with those of other studies that found higher levels of MCPyV DNA in samples from MCC patients than from other subsets of patients (*11,12*). Similarly, higher MCPyV-specific antibody titers in patients with MCC have been reported (*14*). These consistent data suggest that MCPyV is likely involved in MCC pathomechanisms. However, high MCPyV DNA levels and high antibody titers (*11,12,14*) may be found in persons without MCC. The clinical relevance of increased viral load or antibody titers, therefore, remains unclear.

Our detection of MCPyV DNA in 2 buccal mucosa swabs is in line with recent reports of the widespread viral DNA in the human body (*12,13,15*). Even though these data might point toward a mucosal, respiratory, or fecal–oral route of transmission, the skin carriage we observed suggests that a cutaneous route should also be considered. Cutaneous swabbing could be an effective, less invasive method of detecting MCPyV DNA, providing a useful tool for future epidemiologic and molecular studies.

This work was funded in part by a grant from the Clinical Research Program of the Montpellier University Hospital (AOI 2008, Protocole UF8425).

Dr Foulongne is a virologist at the Montpellier University Hospital and associate professor at Montpellier University I. His research interests include clinical virology, epidemiology, and molecular diagnostics of recently identified human viruses.

References

1. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008;319:1096–100. DOI: 10.1126/science.1152586
2. Becker JC, Houben R, Ugurel S, Trifler U, Pföhler C, Schrama D. MC polyomavirus is frequently present in Merkel cell carcinoma of European patients. J Invest Dermatol. 2009;129:248–50. DOI: 10.1038/jid.2008.198
3. Garneski KM, Warcola AH, Feng Q, Kiviat NB, Leonard JH, Nghiem P. Merkel cell polyomavirus is more frequently present in North American than Australian Merkel cell carcinoma tumors. J Invest Dermatol. 2009;129:246–8. DOI: 10.1038/jid.2008.229
4. Kassem A, Schöpflin A, Diaz C, Weyers W, Stickeler E, Werner M, et al. Frequent detection of Merkel cell polyomavirus in human Merkel cell carcinomas and identification of a unique deletion in the VP1 gene. Cancer Res. 2008;68:5009–13. DOI: 10.1158/0008-5472.CAN-08-0949
5. Shuda M, Arora R, Kwun HJ, Sarid R, Fernandez-Figueras MT, Tolstov Y, et al. Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors. Int J Cancer. 2009;125:1243–9. DOI: 10.1002/ijc.24510
6. Sastre-Garau X, Peter M, Avril MF, Laude H, Couturier J, Rozenberg F, et al. Merkel cell carcinoma of the skin: pathological and molecular evidence for a causative role of MCV in oncogenesis. J Pathol. 2009;218:48–56. DOI: 10.1002/path.2532
7. Busam KJ, Jungbluth AA, Rekthman N, Coit D, Pulitzer M, Bini J, et al. Merkel cell polyomavirus expression in Merkel cell carcinoma and its absence in combined tumors and pulmonary neuronedocrine carcinomas. Am J Surg Pathol. 2009;33:1378–85.
8. Shuda M, Feng H, Kwun HJ, Rosen ST, Gjoerup O, Moore PS, et al. T antigen mutations are a human tumor-specific signature for Merkel cell polyomavirus. Proc Natl Acad USA. 2008;105:16272–7. DOI: 10.1073/pnas.0806526105
9. Kassem A, Technau K, Kurz AK, Pantulu D, Löning M, Kayser G, et al. Merkel cell polyomavirus sequences are frequently detected in nonmelanoma skin cancer of immunosupressed patients. Int J Cancer. 2009;125:356–61. DOI: 10.1002/ijc.24323
10. Dworkin AM, Tseng ST, Allain DC, Iwenofu O, Peters SB, Toland AE. Merkel cell polyomavirus in cutaneous squamous cell carcinoma of immunocompetent individuals. J Invest Dermatol. 2009;129:2868–74. DOI: 10.1038/jid.2009.183
11. Foulongne V, Dereure O, Kluger N, Molès JP, Guillot B, Segondy M. Merkel cell polyomavirus DNA detection in lesional and nonlesional skin from patients with Merkel cell carcinoma or other skin diseases. Br J Dermatol. 2009 Jul 6; [Epub ahead of print].
12. Loyo M, Guerrero-Preston, Brait M, Hoque M, Chuang A, Kim MS, et al. Quantitative detection of Merkel cell virus in human tissus and possible mode of transmission. Int J Cancer. 2009 Jul 8; [Epub ahead of print].
13. Wieland U, Mauch C, Kreuter A, Krieg T, Pfister H. Merkel cell polyomavirus DNA in persons without Merkel cell carcinoma. Emerg Infect Dis. 2009;15:1496–8. DOI: 10.3201/eid1509.081575
14. Tolstov YL, Pastrana DV, Feng H, Becker JC, Jenkins FJ, Moschos S, et al. Human Merkel cell polyomavirus infection II. MCV is a common human infection that can be detected by conformational capsid epitope immunoassays. Int J Cancer. 2009;125:1250–6. DOI: 10.1002/ijc.24509
15. Bialasiewicz S, Lambert SB, Whiley DM, Nissen MD, Sloots TP. Merkel cell polyomavirus DNA in respiratory specimens from children and adults. Emerg Infect Dis. 2009;15:492–4. DOI: 10.3201/eid1503.081067

Address for correspondence: Vincent Foulongne, Laboratory of Virology, Hôpital St-Eloi, 34295 Montpellier CEDEX 5, France; email: v-foulongne@chu-montpellier.fr

Novel *Corynebacterium diphtheriae* in Domestic Cats

Aron J. Hall, Pamela K. Cassiday, Kathryn A. Bernard, Frances Bolt, Arnold G. Steigerwalt, Danae Bixler, Lucia C. Pawloski, Anne M. Whitney, Masaaki Iwaki, Adam Baldwin, Christopher G. Dowson, Takako Komiya, Motohide Takahashi, Hans P. Hinrikson, and Maria L. Tondella

Novel nontoxigenic *Corynebacterium diphtheriae* was isolated from a domestic cat with severe otitis. Contact investigation and carrier study of human and animal contacts yielded 3 additional, identical isolates from cats, although no evidence of zoonotic transmission was identified. Molecular methods distinguished the feline isolates from known *C. diphtheriae*.

The clinical relevance of *Corynebacterium diphtheriae* recovered from a cat with otitis is poorly understood. Historically, humans have been thought to be its sole reservoir, and the few human cases reported annually in the United States are generally associated with international travel (*1*). Therefore, when *C. diphtheriae* was isolated from the ears of a cat, an investigation was initiated to evaluate potential sources of the cat's infection and potential public health risks and to preliminarily characterize the *C. diphtheriae* isolate.

The cat, an 8-month-old female domestic shorthair, was examined at a West Virginia veterinary hospital on 5 occasions during January–June 2007. Pertinent findings included severe bilateral otitis, vestibular signs, mild ataxia, anorexia, and failure to gain weight; the cat had a history of ear, eye, and lung infections. Results of diagnostic tests showed no evidence of systemic disease and were negative for feline immunodeficiency and leukemia viruses and feline infectious peritonitis. Culture of an otic swab collected from the cat in May 2007 yielded 4 organisms: *C. diphtheriae*, *Streptococcus equi zooepidemicus*, *Staphylococcus* spp., and *Achromobacter xylosoxidans*. The cat was treated with oral clindamycin, otic enrofloxacin, and an ear-flushing solution.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (A.J. Hall, P.K. Cassiday, A.G. Steigerwalt, L.C. Pawloski, A.M. Whitney, H.P. Henrikson, M.L. Tondella); West Virginia Department of Health and Human Resources, Charleston, West Virginia, USA (A.J. Hall, D. Bixler); Public Health Agency of Canada, Winnipeg, Manitoba, Canada (K.A. Bernard); University of Warwick, Coventry, UK (F. Bolt, A. Baldwin, C.G. Dowson); and National Institute of Infectious Diseases, Tokyo, Japan (M. Iwaki, T. Komiya, M. Takahashi)

DOI: 10.3201/eid1604.091107

The Study

In June 2007, investigators visited the veterinary clinic and the household of the index cat and conducted a contact investigation and carrier study. Interviews of 2 household members and 8 veterinary staff members indicated no recent respiratory illness, skin infection, or risk factors for diphtheria (e.g., travel to countries to which diphtheria is endemic or contact with known case-patients). Half of these 10 contacts had received diphtheria vaccination within the previous 5 years. Cultures of oropharyngeal swab samples obtained from each person were negative, including cystine tellurite blood agar, which is selective for *C. diphtheriae*. Household members also were interviewed about medical history of a convenience sample of household animals (4 cats, including the index cat; 2 dogs; and 1 horse). Each animal was briefly examined, and oropharyngeal, otic, or ocular swab samples were collected. Otitis was observed in all 4 cats and 1 dog. The horse reportedly had had an eye infection ≈5 years earlier. No other abnormal findings were noted. Animal specimens yielded 3 additional isolates of *C. diphtheriae*: 1 from each ear of the index cat and 1 from the left ear of a 2-year-old domestic medium-hair cat. Both cats had been born on the premises and had remained with the same household since birth.

Feline *C. diphtheriae* and reference isolates used are described in the Table. Tinsdale agar plate growth (Remel, Lenexa, KS, USA) gave rise to black colonies with a brown halo, typical of cysteinase-producing *C. diphtheriae*, *C. ulcerans*, or *C. pseudotuberculosis*. After 24 hours on blood agar, 1–2-mm grey-white or opaque, rounded, convex colonies with no hemolysis were observed. Microscopically, the bacteria were gram-positive, club-shaped rods, 1 μm in diameter, arranged singly or at angles. Biochemical profiles to determine species and biotype were done by using an API Coryne strip (bioMérieux, Durham, NC, USA, and St-Laurent, Quebec, Canada). Query of API Coryne code 0010304 obtained for all isolates by APIWEB (https://apiweb.biomerieux.com) indicated a decreased level of confidence of *C. diphtheriae* biotype *mitis* or *belfanti* (89.5%) because of a maltose-negative result. Isolates were further characterized morphologically and biochemically by using tube substrates (*2*) and were identified by using a standard taxonomic scheme (*3*). Feline isolates were biochemically identical with each other and phenotypically consistent with *C. diphtheriae* biotype *belfanti*, except for the lack

Table. Feline *Corynebacterium diphtheriae* isolates and reference strains used for comparison, West Virginia, 2008*

Strain	Culture collection	Source	Diphtheria toxin	GenBank accession no.		
				16S rRNA	*rpoB*	*tox* gene
CD443	ATCC BAA-1774	Cat 1, right ear	Nontoxigenic	FJ409572	FJ415317	FJ376656
CD448	ND	Cat 1, right ear	Nontoxigenic	FJ409573	ND	FJ422272
CD449	ND	Cat 1, left ear	Nontoxigenic	FJ409574	ND	FJ422273
CD450	ND	Cat 2, left ear	Nontoxigenic	FJ409575	FJ415318	FJ422274
C. diphtheriae biotype *mitis*	NCTC 10356†	Human nose	Nontoxigenic	GQ118340	GQ409648	ND
C. diphtheriae biotype *gravis*	NCTC 10648	Unknown	Toxigenic	ND	ND	ND
C. diphtheriae biotype *gravis*	NCTC 11397^T ATCC 27010^T	Unknown	Nontoxigenic	GQ118341	GQ409649	ND
C. diphtheriae biotype *gravis*	NCTC 13129 ATCC 700971	Human throat	Unknown	GQ118344	GQ409650	ND
C. pseudotuberculosis	NCTC 3450^T	Sheep gland	Unknown	GQ118342	GQ409651	ND
C. ulcerans	NCTC 12077	Human throat	Unknown	GQ118343	ND	ND
C. ulcerans	NCTC 7910	Human throat	Unknown	GQ118345	ND	ND

*CD, Centers for Disease Control and Prevention identifier number; ATCC, American Type Culture Collection; ND, not deposited in this study; NCTC, National Collection of Type Cultures, London, UK. Additional strains used as controls for specific assays: toxigenic *C. diphtheriae* biotype *belfanti* isolates used for real-time PCR of *tox* gene were 718, G4182, C59, C60, C75, C76, C77; toxigenic *C. diphtheriae* ATCC 27012 used as positive control for Elek; *C. diphtheriae* NCTC 10481 and *C. ulcerans* CD199 used as positive and negative controls for Vero cell assay.
†NCTC 10356 is described in the NCTC catalogue as *C. diphtheriae* biotype *mitis*; however, analyses in this study found this strain to be nitrate negative and therefore consistent with *C. diphtheriae* biotype *belfanti*. Thus, it was used in this study as a *belfanti* reference strain.

of maltose fermentation, which was considered an unusual finding (*3*).

Antimicrobial drug susceptibility testing was performed according to the Clinical and Laboratory Standards Institute's recommended methods and interpretative criteria (*4*). All 4 feline isolates were sensitive to ampicillin, cefepime, cefotaxime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, clindamycin, daptomycin, erythromycin, ertapenam, gatifloxacin, gentamicin, levofloxacin, linezolid, meropenem, moxifloxacin, penicillin, quinupristin/dalfopristin, rifampin, telithromycin, tetracycline, tigecycline, trimethoprim/sulfamethoxazole, and vancomycin. Cellular fatty acid composition analysis was performed as described (*5*) by using the Sherlock system (MIDI, Inc., Newark, DE, USA), except that version 4.5 of the operating software was used. The cellular fatty acid composition profiles were consistent for *C. diphtheriae, C. ulcerans*, or *C. pseudotuberculosis*, including a substantial proportion (28%–30% of total) of C16:1ω7c (*5*). All feline isolates produced 7–15 meq/L of propionic acid among fermentation products, a feature associated with C. *diphtheriae* (*2*).

Results from use of the modified Elek test (*6*) indicated that all feline isolates were negative for production of diphtheria toxin; however, an atypical precipitation was observed after 36 h of incubation. Lack of toxin expression was corroborated by negative Vero cell assay results (*7*) and confirmed by using Western blot. Real-time PCR selective for the *C. diphtheriae* and *C. ulcerans* toxin gene (*tox*) (*8*) was positive for all feline isolates. However, real-time PCR for A and B subunits of *tox* (*9*) amplified subunit A but not subunit B. Sequence analysis of the *tox* gene was performed as previously outlined (*10*) and compared with a reference *tox* gene, GenBank accession no. K01722. The 4 feline *tox* sequences were identical to each other but con-

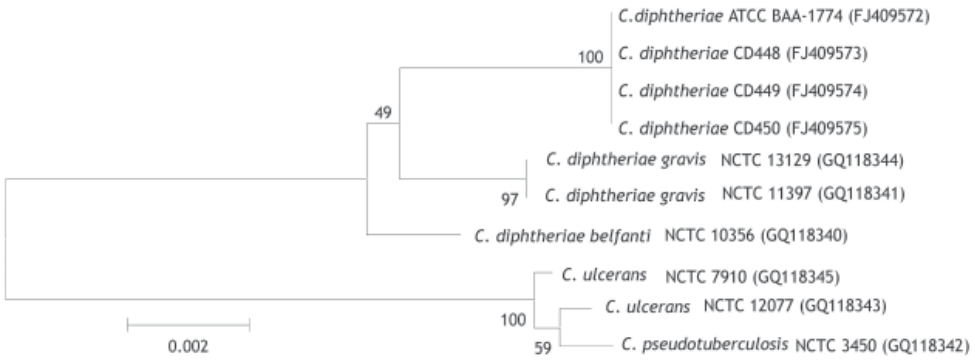


Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence analysis of *Corynebacterium diphtheriae* isolates, including 4 feline isolates from West Virginia, 2008 (ATCC BAA-1774, CD 448, CD 449, CD 450). The tree was constructed from a 1,437-bp alignment of 16S rRNA gene sequences by using the neighbor-joining method and Kimura 2-parameter substitution model. Bootstrap values (expressed as percentages of 1,000 replicates) >40% are illustrated at branch points. Feline isolates had 100% identity with each other and ≥99.1% identity with *C. diphtheriae* biotypes *gravis* and *belfanti*. GenBank accession nos. given in parentheses. ATCC, American Type Culture Collection; CD, Centers for Disease Control and Prevention identifier number; NCTC, National Collection of Type Cultures. Scale bar indicates number of substitutions per site.

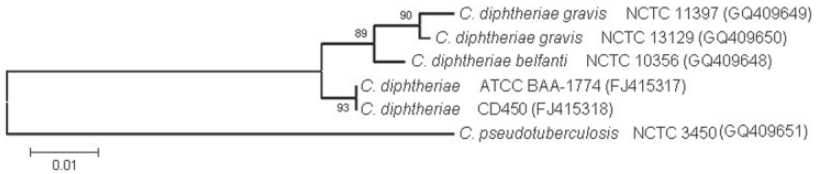


Figure 2. Jukes-Cantor–derived phylogenetic tree based on sequence analysis of a selected region of the *rpoB* gene of *Corynebacterium* isolates, including 2 feline isolates from West Virginia, 2008 (ATCC BAA-1774, CD 450). Feline isolates had 100% identity with each other and 97.7% identity with *C. diphtheriae* biotypes *gravis* and *belfanti*. GenBank accession nos. given in parentheses. ATCC, American Type Culture Collection; CD, Centers for Disease Control and Prevention identifier number; NCTC, National Collection of Type Cultures. Scale bar indicates number of substitutions per site.

tained multiple nucleotide substitutions and deletions compared with the reference gene. By NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the feline *tox* had higher sequence identity (97%–98%) to the *tox* sequences of *C. ulcerans*, compared with those from C. diphtheriae (94%–95%). A deletion at nt 55, coupled with a cytosine-to-thymine substitution at nt 74, prematurely terminated the peptide at aa 25.

Species characterization was corroborated by using 16S rRNA (*11*) and partial *rpoB* (*12*) gene sequencing. By 16S rRNA gene sequence analysis, the feline strains had 100% identity with each other and ≥99.1% identity with various reference sequences for *C. diphtheriae* biotype *gravis* and *belfanti* sequences, including NCTC 11397^T. Partial *rpoB* sequence analyses indicated 100% identity among the feline isolates and 97.7% identity with *C. diphtheriae* NCTC 11397^T. Neighbor-joining phylogenetic trees based on both 16S rRNA (Figure 1) and partial *rpoB* gene sequencing (Figure 2) positioned the feline isolate sequences within the *C. diphtheriae* clade but clearly distinguished them from the other *C. diphtheriae* isolates. Comprehensive molecular analyses to characterize differences between biotype *belfanti* strains, including these feline isolates, with other *C. diphtheriae* biotypes, are the subject of a separate publication (C.G. Dowson, pers. comm.).

Conclusions

We identified a potentially novel biotype of *C. diphtheriae* recovered from domestic cats in West Virginia but found no evidence of zoonotic transmission. Although rare, isolation of *C. diphtheriae* from animals has been reported, including *C. diphtheriae* biotype *belfanti* from a skin lesion of a cow (*13*) and toxigenic *C. diphtheriae* biotype *gravis* from a wound of a horse (*14*). *C. ulcerans* is a known animal pathogen, and zoonotic transmission of toxigenic *C. ulcerans* from companion animals has been reported, often associated with predisposing concurrent illnesses (*15*).

The feline strains isolated during this investigation differed phenotypically from previously described biotypes but were otherwise regarded as typical of *C. diphtheriae*. However, isolates were nontoxigenic and harbored a modified *tox* gene with sequence differences from *Corynebacterium* spp. capable of expressing diphtheria toxin. On the basis of published criteria (*11*), the feline strain might represent a novel subspecies of *C. diphtheriae* because it shares <98% sequence homology to the type strain within the *rpoB* gene. Potential for zoonotic transmission of this novel, cat-associated *C. diphtheriae* and associated public health implications are unknown. Additional studies are needed to further characterize these isolates and determine their appropriate taxonomy. Large-scale screening of domestic cat populations is recommended to determine the prevalence of *C. diphtheriae* and its pathogenic potential and to identify additional isolates for more formal description and classification.

Acknowledgments

We gratefully recognize Amy Isaac, Gary Kinder, and Katrina Kretsinger for collaborative assistance in the epidemiologic investigation and Tamara Burdz, Christi Clark, Tiffany Jackson, Brenda Keavey, Betty Ng, Chris Paddock, and Deborah Wiebe for laboratory assistance.

Work performed at University of Warwick was funded in part by the Biotechnology and Biological Sciences Research Council, Micropathology Ltd, and the Medical Research Fund.

Dr Hall is a public health veterinarian who completed this study while serving as an Epidemic Intelligence Service Officer of the Centers for Disease Control and Prevention (CDC), assigned to the state of West Virginia. Currently he is an epidemiologist on the CDC Viral Gastroenteritis Team. His research interests focus on public health issues involving interactions between humans, domestic animals, wildlife, and the environment.

References

1. Vitek CR, Wharton M. Diphtheria toxoid. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines. 5th ed. Philadelphia: Elsiever; 2008. p. 139–56.
2. Bernard KA, Munro C, Wiebe D, Ongsansoy E. Characteristics of rare or recently described *Corynebacterium* species recovered from human clinical material in Canada. J Clin Microbiol. 2002;40:4375–81. DOI: 10.1128/JCM.40.11.4375-4381.2002

3. Funke G, Bernard KA. Coryneform gram-positive rods. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of clinical microbiology. 9th ed. Washington: ASM Press; 2007. p. 485–514.
4. Clinical Laboratory Standards Institute. M45-A. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. Wayne (PA): The Institute; 2006.
5. Bernard KA, Bellefeuille M, Ewan EP. Cellular fatty acid composition as an adjunct to the identification of asporogenous, aerobic gram-positive rods. J Clin Microbiol. 1991;29:83–9.
6. Engler KH, Glushkevich T, Mazurova IK, George RC, Efstratiou A. A modified Elek test for detection of toxigenic corynebacteria in the diagnostic laboratory. J Clin Microbiol. 1997;35:495–8.
7. Miyamura K, Nishio S, Ito A, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. I. Studies on factors affecting the toxin and antitoxin titration. J Biol Stand. 1974;2:189–201. DOI: 10.1016/0092-1157(74)90015-8
8. Schuhegger R, Lindermayer M, Kugler R, Heesemann J, Busch U, Sing A. Detection of toxigenic *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* strains by a novel real-time PCR. J Clin Microbiol. 2008;46:2822–3. DOI: 10.1128/JCM.01010-08
9. Mothershed EA, Cassiday PK, Pierson K, Mayer LW, Popovic T. Development of a real-time fluorescence PCR assay for rapid detection of the diphtheria toxin gene. J Clin Microbiol. 2002;40:4713–9. DOI: 10.1128/JCM.40.12.4713-4719.2002
10. Cassiday PK, Pawloski LC, Tiwari T, Sanden GN, Wilkins PP. Analysis of toxigenic *Corynebacterium ulcerans* strains revealing potential for false-negative real-time PCR results. J Clin Microbiol. 2008;46:331–3. DOI: 10.1128/JCM.01251-07
11. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol. 1991;173:697–703.
12. Khamis A, Raoult D, La Scola B. *rpoB* gene sequencing for identification of *Corynebacterium* species. J Clin Microbiol. 2004;42:3925–31. DOI: 10.1128/JCM.42.9.3925-3931.2004
13. Corboz L, Thoma R, Braun U, Zbinden R. Isolation of *Corynebacterium diphtheriae* subsp. *belfanti* from a cow with chronic active dermatitis [in German]. Schweiz Arch Tierheilkd. 1996;138:596–9.
14. Henricson B, Segarra M, Garvin J, Burns J, Jenkins S, Kim C, et al. Toxigenic *Corynebacterium diphtheriae* associated with an equine wound infection. J Vet Diagn Invest. 2000;12:253–7.
15. Bonmarin I, Guiso N, Le Fleche-Mateos A, Patey O, Patrick AD, Levy-Bruhl D. Diphtheria: a zoonotic disease in France? Vaccine. 2009;27:4196–200. DOI: 10.1016/j.vaccine.2009.04.048

Address for correspondence: Aron J. Hall, Division of Viral Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop A47, Atlanta, GA 30333, USA; email: ajhall@cdc.gov

Sympatric Occurrence of 3 Arenaviruses, Tanzania

Joëlle Goüy de Bellocq, Benny Borremans, Abdul Katakweba, Rhodes Makundi, Stuart J.E. Baird, Beate Becker-Ziaja, Stephan Günther, and Herwig Leirs

To determine the specificity of Morogoro virus for its reservoir host, we studied its host range and genetic diversity in Tanzania. We found that 2 rodent species other than *Mastomys natalensis* mice carry arenaviruses. Analysis of 340 nt of the viral RNA polymerase gene showed sympatric occurrence of 3 distinct arenaviruses.

Arenaviruses are RNA viruses, primarily rodent borne, that include the etiologic agents of lymphocytic choriomeningitis and hemorrhagic fevers in humans. On the basis of their antigenic properties, arenaviruses have been divided in 2 groups: New World and Old World (*1*). In Africa, 2 arenaviruses are known to be highly pathogenic to humans: Lassa virus in West Africa and the recently described Lujo virus from southern Africa (*2*). Rodents from the subfamily Murinae are the principal hosts of the Old World arenaviruses. The multimammate mouse, *Mastomys natalensis*, is the reservoir host of Lassa virus in western Africa (*3*) and Mopeia virus, for which human pathogenicity has not been reported, in eastern Africa (*4*,*5*).

Previously, a serosurvey of small mammals from Tanzania identified a hot spot of arenavirus circulation in Morogoro (*6*). Molecular screening detected a new arenavirus in *M. natalensis* mice: Morogoro virus, closely related to Mopeia virus (*6*). This virus seems a promising model for studying virus–host dynamics and testing rodent control measures for arenaviruses for which *M. natalensis* mice are host. However, before being used as a model, the degree of specificity of Morogoro virus for its reservoir host must be assessed because secondary reservoir species may play a role in the transmission and maintenance of the virus in natural habitats. Our objective, therefore, was to determine the limit of specificity of the Morogoro virus.

Author affiliations: University of Antwerp, Antwerp, Belgium (J. Goüy de Bellocq, B. Borremans, H. Leirs); Sokoine University of Agriculture, Morogoro, Tanzania (A. Katakweba, R. Makundi); Research Center in Biodiversity and Genetic Resources, Vairão, Portugal (J. Goüy de Bellocq, S.J.E. Baird); Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany (B. Becker-Ziaja, S. Günther); and University of Aarhus, Kongens Lyngby, Denmark (H. Leirs)

DOI: 10.3201/eid1604.091721

The Study

From October 13 through December 3, 2008, a total of 555 small mammals were trapped in Morogoro, Tanzania (6.84°S, 37.65°E). This period corresponds to the end of the dry season, when the density *of M. natalensis* mice is usually high (*7*). Sherman traps were set in habitats where these mice were expected to occur in high density (*7*). Dried blood samples were preserved on calibrated, prepunched filter papers. Blood samples (1 punch ≈15μL/rodent) were eluted in 300 μL of phosphate-buffered saline and tested for antibodies to arenaviruses by indirect immunofluorescence antibody (IFA) testing using Morogoro virus as antigen. In addition, total RNA was extracted from another punch of blood by using 300 μL of AVL buffer (QIAGEN, Venlo, the Netherlands). The lysate, plus 300 μL of ethanol, was centrifuged in a silica column (Zymo Research, Orange, CA, USA). The column was washed with 400 μL of AW1 and AW2 buffers (QIAGEN). RNA was eluted with 15 μL of water. A 1-step reverse transcription–PCR (RT-PCR) selective for the Morogoro virus RNA polymerase (large [L]) gene was performed as described (*6*); it was based on a pan–Old World arenavirus RT-PCR approach (*8*), but primers were adjusted to detect the Morogoro virus (*6*). Products were shown by agarose gel electrophoresis. A subset of the amplicons was purified and unidirectionally sequenced by using MoroL3359-fwd primer (*6*). Amplicons derived from *Lemniscomys rosalia* and *Mus minutoides* mouse blood samples were bidirectionally sequenced. Nucleotide and amino acid sequences were aligned by using BioEdit software (*9*). Old World arenaviruses and 2 representatives of New World arenaviruses were used to estimate nucleotide and amino acid pairwise divergence (p-distance) with MEGA 4 (*10*). A phylogram was reconstructed by using the neighbor-joining algorithm in MEGA 4.

We trapped 511 *M. natalensis* mice and 44 individuals from 7 other small mammal species (Table 1). IFA results were positive for 58 blood samples from *M. natalensis*, 1 from *L. rosalia*, and 1 from *M. minutoides* mice (Table 1). *M. rosalia* mice were trapped in woodlands, whereas *M. minutoides* mice were trapped in vegetable gardens and fallow fields. These results are consistent with recently reported results from a study in the same locality 20 years ago, in which mice from the genera *Lemniscomys* and *Mus* were seropositive for arenaviruses according to IFA with Lassa virus as antigen (*6*). In our 2008 study, the antibody prevalence for *M. natalensis* mice was 12.1%, which is low compared with 50% antibody prevalence reported for 2004 and 2007 (*6*), suggesting high fluctuation of interannual or seasonal prevalence of Morogoro virus in its host.

Table 1. Arenavirus antibodies and arenaviruses in blood samples of small mammals around Morogoro, Tanzania, October 13–December 3, 2008

Species	No. trapped	Antibodies*		Arenaviruses†		Total positive, no. (%)
		No. examined	No. (%) positive	No. examined	No. (%) positive	
Acomys spinosissimus	1	1	0	1	0	0
Crocidura sp.	20	11	0	12	0	0
Dasymys incomtus	1	1	0	1	0	0
Lemniscomys rosalia‡	3	3	1 (33.3)	3	1 (33.3)	2 (66)
Mastomys natalensis	511	480	58 (12.1)	489	41 (8.4)	93 (19)
Mus minutoides‡	7	5	1 (20)	5	1 (20)	2 (40)
Rattus rattus	1	1	0	1	0	0
Gerbilliscus robustus	11	11	0	11	0	0
Total	555	513	60 (11.7)	523	43 (8.2)	97 (18.5)

*Detected by indirect immunofluorescent antibody testing.
†Large segment. Detected by reverse transcription–PCR.
‡Species identification was confirmed by sequencing mitochondrial cytochrome b gene.

Using RT-PCR selective for the arenavirus L gene, we obtained positive results for 43 mice: 41 *M. natalensis*, 1 *L. rosalia*, and 1 *M. minutoides*. In total, 6 samples were positive according to IFA and RT-PCR. We sequenced 33 RT-PCR amplicons. The sequences derived from *M. natalensis* mice (286 bp used for the analysis) showed 97.1%–100% amino acid homology with the Morogoro prototype L sequence (GenBank accession no. EU914104). In contrast, the 2 sequences derived from the blood samples of *L. rosalia* and *M. minutoides* mice showed only 69.3% and 65.2% aa homology with the Morogoro prototype L sequence. These sequences (320 bp) were compared with sequences of the Old World arenaviruses (Table 2). The virus amino acid sequence from *M. minutoides* mice clustered at 93.7% homology with that of the Kodoko virus, in the lymphocytic choriomeningitis clade (Figure; Table 2): thus, the arenavirus of *M. minutoides* mice seems to be a strain of the Kodoko virus originally isolated from 2 *M. minutoides* mice in Guinea (*11*). Our finding supports *M. minutoides* mice as the true reservoir of Kodoko virus in Africa.

The amino acid sequence of the virus isolated from *L. rosalia* mice clusters with the Ippy virus sequence (Figure). Ippy virus was isolated in the Central African Republic from *Arvicanthis niloticus* rodents (*12*). For the portion of L gene sequenced (320 bp), the level of amino acid divergence between the 2 is 17.3%, higher than the level of divergence between other Old World arenavirus species (e.g., 14.5% aa divergence between Mobala and Lassa viruses; Table 2). Thus, the arenavirus found in *L. rosalia* mice appears to be a new species of Old World arenavirus. The genus *Lemniscomys* is more closely related to the genus *Arvicanthis* than to the genera *Mus* and *Mastomys*.

Table 2. Nucleotide and amino acid p-distances of 2 arenaviruses in blood of *Mus minutoides* and *Lemniscomys rosalia* mice in Morogoro, Tanzania, October 13–December 3, 2008, compared with Old World and 2 New World arenaviruses*

Virus sequence†	Old World arenaviruses											New World arenaviruses	
	Dandedong	Ippy	Kodoko	Mobala	Mopeia	Morogoro	Lassa	LCMV	Lujo	Lemn	Minu	Pirital	Pichinde
Dandedong		0.282	0.073	0.218	0.255	0.282	0.209	0.027	0.355	**0.245**	**0.091**	0.418	0.409
Ippy	0.345		0.264	0.227	0.209	0.209	0.191	0.300	0.345	**0.173**	**0.273**	0.491	0.482
Kodoko	0.244	0.354		0.200	0.227	0.255	0.227	0.091	0.336	**0.236**	**0.073**	0.409	0.409
Mobala	0.318	0.315	0.315		0.136	0.127	0.145	0.227	0.327	**0.209**	**0.236**	0.482	0.464
Mopeia	0.310	0.295	0.351	0.256		0.055	0.145	0.273	0.345	**0.182**	**0.255**	0.482	0.464
Morogoro	0.327	0.324	0.360	0.262	0.241		0.136	0.282	0.364	**0.209**	**0.282**	0.491	0.473
Lassa	0.286	0.283	0.366	0.286	0.271	0.262		0.209	0.364	**0.200**	**0.236**	0.473	0.436
LCMV	0.182	0.354	0.223	0.339	0.324	0.345	0.330		0.373	**0.264**	**0.109**	0.436	0.409
Lujo	0.351	0.393	0.360	0.372	0.387	0.405	0.399	0.357		**0.373**	**0.336**	0.500	0.500
Lemn	**0.333**	**0.315**	**0.336**	**0.292**	**0.313**	**0.307**	**0.301**	**0.327**	**0.351**		**0.227**	0.482	0.464
Minu	**0.211**	**0.360**	**0.241**	**0.301**	**0.348**	**0.348**	**0.339**	**0.244**	**0.354**	**0.324**		0.436	0.409
Pirital	0.440	0.467	0.429	0.420	0.452	0.443	0.455	0.432	0.476	0.473	0.417		0.173
Pichinde	0.426	0.446	0.432	0.458	0.476	0.467	0.473	0.452	0.446	0.443	0.435	0.268	

*Nucleotides below diagonal; amino acids above diagonal. LCMV, lymphocytic choriomeningitis virus; Lemn, virus sequenced from *Lemniscomys rosalia* mice (indicated by **boldface**); Minu, virus sequenced from *Mus minutoides* mice (indicated by **boldface).**
†Strains and GenBank accession numbers of the sequences used: Dandenong (0710-2678, EU136039), Ippy (Dak An B 188 d, DQ328878), Kodoko (KD42, EF179865), Mobala (Acar 3080, DQ328876), Mopeia (Mozambique, DQ328875), Morogoro (3017/2004, EU914104), Lassa (Josiah, AY628202), LCMV (Armstrong,,AY847351), Lujo (NC_012777), Pirital (VAV 488, AY216505), Pichinde (AN3739, NC_006439). Sequences of arenaviruses in *L. rosalia* and *M. minutoides* mice have been deposited in GenBank under accession nos. GU182412 and GU182413, respectively.

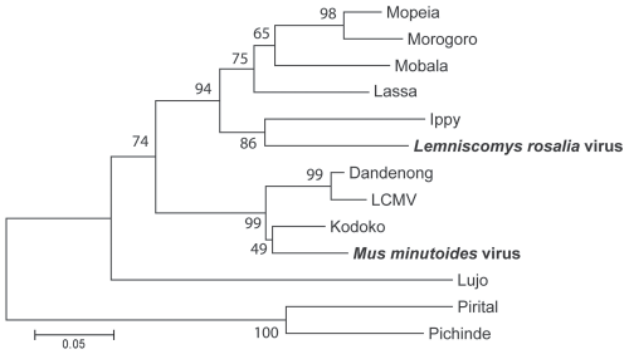


Figure. Neighbor-joining tree of Old World arenaviruses, showing position of 2 arenaviruses found in blood samples of *Lemniscomys rosalia* and *Mus minutoides* mice (**boldface**), based on the analysis of partial sequences of the RNA polymerase gene. Phylogeny was estimated by neighbor-joining of amino acid pairwise distance in MEGA 4 (*10*). Numbers represent percentage bootstrap support (1,000 replicates). Two New World arenaviruses, Pirital and Pichinde, were used as outgroups. See Table 2 for virus strains and GenBank accession numbers. Scale bar indicates amino acid substitutions per site. LCMV, lymphocytic choriomeningitis virus.

Conclusions

In high-density habitats of *M. natalensis* mice, where Morogoro arenavirus transmission occurs, sympatric murine species do not seem to be secondary reservoirs for the virus. In contrast, 2 mouse species, *L. rosalia* and *M. minutoides*, seem to be reservoirs of 2 other Old World arenaviruses, 1 of which may be a new species. Our study emphasizes the complementary nature of serologic and genetic-based approaches for arenavirus detection. Because of the cross-reactivity of Morogoro antigens with immune serum from individuals infected with other arenaviruses, a serology-only approach might have led to the conclusion that an extended set of hosts exists for the Morogoro virus. Because of its high cost, a genetics-only approach might never have indicated the hot spot of arenavirus around Morogoro that was shown by IFA (*6*). However, critically, genetics then enable cross-reactivity to be decomposed.

Our study demonstrates the presence of 3 Old World arenaviruses in a single location. To date, only 5 Old World arenavirus species and 17 New World arenaviruses have been recognized by the International Committee for Taxonomy of Viruses (*13*). Although the likely presence of additional arenaviruses in Africa has long been suggested (*14*,*15*), the discovery of new Old World arenaviruses is rare. Our study illustrates that arenaviruses in Africa may be highly diverse and demonstrates the efficiency of the recently developed pan–Old World arenavirus RT-PCR for identifying new Old World arenaviruses (*8*). To isolate and describe the new arenavirus of *L. rosalia* mice and the strain of Kodoko virus, additional sampling and genotyping are being conducted. In particular, determining the sequence of the S segment will further clarify evolutionary relationships within the Old World group.

Acknowledgments

We thank the academic authorities of the Sokoine University of Agriculture, Morogoro, who provided us with the necessary permits and working facilities. We are also grateful to our colleagues Khalid Kibwana and Shabani Lutea for their help with field work.

Financial support was provided by the University of Antwerp (grant no. 2964 to J.G.B.) and the Fund for Scientific Research-Flanders (grant no. 1.5.180.09 to J.G.B.).

Dr Goüy de Bellocq is a postdoctoral researcher at the Fund for Scientific Research-Flanders. Her research interests are the evolutionary ecology and epidemiology of arenaviruses and macroparasites in rodents.

References

1. Charrel RN, De Lamballerie X, Emonet S. Phylogeny of the genus *Arenavirus*. Curr Opin Microbiol. 2008;11:362–8. DOI: 10.1016/j.mib.2008.06.001
2. Briese T, Paweska JT, McMullan LK, Hutchison SK, Street C, Palacios G, et al. Genetic detection and characterization of Lujo virus, a new hemorrhagic fever–associated arenavirus from southern Africa. PLoS Pathog. 2009;5:e1000455.5556.
3. Lecompte E, Fichet-Calvet E, Daffis S, Koulémou K, Sylla O, Kourouma F, et al. *Mastomys natalensis* and Lassa fever, West Africa. Emerg Infect Dis. 2006;12:1971–4.
4. Johnson KM, Taylor P, Elliott LH, Tomori O. Recovery of a Lassa-related arenavirus in Zimbabwe. Am J Trop Med Hyg. 1981;30:1291–3.
5. Wulff H, McIntosh BM, Hamner DB, Johnson KM. Isolation of an arenavirus closely related to Lassa virus from *Mastomys natalensis* in south-east Africa. Bull World Health Organ. 1977;55:441–4.
6. Günther S, Hoofd G, Charrel R, Röser C, Becker-Ziaja B, Lloyd G, et al. Mopeia virus–related arenavirus in natal multimammate mice, Morogoro, Tanzania. Emerg Infect Dis. 2009;15:2008–12. DOI: 10.3201/eid1512.090864
7. Leirs H. Population ecology of *Mastomys natalensis* (Smith, 1834). Implications for rodent control in Africa. Agricultural ed. no. 35. Brussels: Belgian Administration for Development Cooperation; 1994.
8. Vieth S, Drosten C, Lenz O, Vincent M, Omilabu S, Hass M, et al. RT-PCR assay for detection of Lassa virus and related Old World arenaviruses targeting the L gene. Trans R Soc Trop Med Hyg. 2007;101:1253–64. DOI: 10.1016/j.trstmh.2005.03.018
9. Hall TA. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8.
10. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol. 2007;24:1596–9. DOI: 10.1093/molbev/msm092
11. Lecompte E, ter Meulen J, Emonet S, Daffis S, Charrel RN. Genetic identification of Kodoko virus, a novel arenavirus of the African pigmy mouse (*Mus Nannomys minutoides*) in West Africa. Virology. 2007;364:178–83. DOI: 10.1016/j.virol.2007.02.008
12. Swanepoel R, Leman PA, Shepherd AJ, Shepherd SP, Kiley MP, McCormick JB. Identification of Ippy as a Lassa fever–related virus. Lancet. 1985;1:639. DOI: 10.1016/S0140-6736(85)92175-0

13. Salvato MS, Clegg JCS, Buchmeier MJ, Charrel RN, Gonzales JP, Lukashevich IS, et al. V: Family Arenaviridae. In: Van Regenmortel MHV, Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy. Eighth report of the International Committee on Taxonomy of Viruses. San Diego (CA): Elsevier Academic Press;2005;725–38.
14. Gonzalez JP. Les arenavirus d'Afrique: un nouveau paradigme d'evolution. Bulletin de l'Institut Pasteur. 1986;84:67–85.
15. Kiley MP, Swanepoel R, Mitchell SW, Lange JV, Gonzalez JP, McCormick JB. Serological and biological evidence that Lassa-complex arenaviruses are widely distributed in Africa. Med Microbiol Immunol (Berl). 1986;175:161–3. DOI: 10.1007/BF02122440

Address for correspondence: Joëlle Goüy de Bellocq, Evolutionary Ecology Group, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium; email: joelle.gouydebellocq@ua.ac.be

Evolution of Porcine Kobuvirus Infection, Hungary

Gábor Reuter, Sándor Kecskeméti, and Péter Pankovics

Porcine kobuvirus was first identified in early 2007 in Hungary. Originally thought to be confined to the intestine, almost 2 years later the virus was found in the blood of clinically healthy pigs on the same farm. Porcine kobuvirus may be widely distributed on pig farms worldwide.

Picornaviruses (family *Picornaviridae*) are small, nonenveloped viruses with single-stranded, positive-sense genomic RNA; they are divided into 12 genera: *Aphthovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Parechovirus, Sapelovirus, Senecavirus, Teschovirus, Tremovirus*, and *Kobuvirus* (*1*). The genus *Kobuvirus* consists of 2 officially recognized species, *Aichi virus* and *Bovine kobuvirus,* and 1 candidate species, porcine kobuvirus (*2–4*). Aichi virus (strain A846/88) was first isolated in 1989 in Japan, from a fecal sample from a person with acute gastroenteritis (*2*). Aichi viruses have also been detected in human fecal samples in other countries in Asia (*5*); Europe (*6,7*), including Hungary; South America (*6*); and North Africa (*8*). Bovine kobuvirus (strain U-1) has been detected in culture medium derived from cattle serum suspected to be contaminated with cattle feces (*3*) and in fecal samples from cattle in 2003 in Japan (*3*) and in 2008 in Thailand (*9*) and Hungary (*10*).

Porcine kobuvirus was first identified and the complete genome (S-1-HUN; EU787450) characterized from fecal samples from domestic pigs in Hungary (*4,11*). Porcine kobuvirus has also been recently (2009) reported from the People's Republic of China (*12*). Kobuvirus genomes are ≈8.2–8.4 kb. Genome organization includes leader (L) protein following structural (VP0, VP3, and VP1) and nonstructural (2A-2C and 3A-3D) regions (*1,3,4,11*). Genetic identity on the coding region between Aichi, U-1, and S-1-HUN viruses varies from 35% (L protein) to 74% (3D region) (*3,11*).

Kobuvirus infection has been thought to be confined to the intestine. To our knowledge, detection of kobuvirus in the infected host species serum (viremia) has not been reported. We report the endemic circulation and in vivo evolution of porcine kobuvirus on a pig farm where the virus was originally discovered and virus escape from the gastrointestinal tract, resulting in viremia in domestic pigs.

The Study

In February 2007, a total of 39 (65%) of 60 fecal samples from clinically healthy domestic pigs on a farm in eastern Hungary were positive (by reverse transcription–PCR) for porcine kobuvirus (*11*) (Table 1). In November 2008, 21 months later, we obtained 60 fecal and 60 serum sample pairs, again from apparently clinically healthy domestic pigs on the same farm. We divided these pigs, all <6 months of age with no diarrhea, into 4 age groups (Table 1). Blood samples were taken from the jugular vein by using closed vacuum sets. Fecal and serum samples were processed and tested separately for porcine kobuvirus by using the same methods (RNA isolation, reverse transcription–PCR) and the same primers for screening (UNIV-kobu-F/UNIV-kobu-R for 3D) and for the complete genome analysis described previously (*11*). PCR products were sequenced directly in both directions with the BigDye Reaction Kit (Applied Biosystems, Warrington, UK) by using the PCR primers and an automated sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Stafford, TX, USA). The genome was assigned GenBank accession no. GQ249161. Kobuvirus-positive fecal samples were inoculated into Vero cells.

Porcine kobuvirus RNA was detected in 32 (53.3%) of the 60 fecal samples and 16 (26.6%) of the 60 serum samples collected in November 2008 (Table 1). Porcine kobuvirus was found in at least 1 fecal sample from each age group; from serum samples, viral RNA was detected in 1 sample from the 10-day group, 7 samples from the 3–4-week group, and 8 samples from the 6-month group. Porcine kobuvirus was identified in 9 sample pairs (feces and serum) collected at the same time. Only the serum or fecal samples were kobuvirus positive, for 7 and 23 animals, respectively. The 173-nt sequences of kobuvirus 3D region were genetically identical in all serum and fecal samples collected in 2007 and 2008.

The complete genetic sequence of strain kobuvirus/swine/K-30-HUN/2008/Hungary, detected in a fecal specimen from a 3–4-week-old pig in 2008, was determined and compared with the prototype strain kobuvirus/swine/S-1-HUN/2007/Hungary (Table 2). In the nonstructural 2A-3D regions, 74 (1.7%; 1.14%–2.43% between 3D and 3C) nucleotide substitutions could be detected between the prototype strains collected in 2007 (S-1-HUN) and 2008 (K-30-HUN), which led to 13 (0.9%) nonsynonymous amino acid changes (2, 2, 5, 2, and 2 aa in the 2A, 2B, 2C, 3C, and 3D regions). In structural regions, 36 (1.42%) nt substitutions were detected, leading to 6 (0.71%) nonsynonymous amino acid changes (3–3 aa in VP0 and VP3 regions). The highest

Author affiliations: ÁNTSZ Regional Institute of State Public Health Service, Pécs, Hungary (G. Reuter, P. Pankovics); and Central Agricultural Office, Debrecen, Hungary (S. Kecskeméti)

DOI: 10.3201/eid1604.090937

Table 1. Incidence of porcine kobuvirus RNA in samples from domestic pigs, Hungary*

	Specimen, by date		
	2007 Feb	2008 Nov	
Pig age	Feces	Feces	Serum
<10 d	15 (100)	8 (53.3)	1 (6.7)
<3–4 wk	14 (93.3)	13 (86.7)	7 (46.7)
<3 mo	3 (20)	5 (33.3)	0
<6 mo	7 (46.7)	6 (40)	8 (53.3)
Total	39/60 (65)	32/60 (53.3)	16/60 (26.6)

*Values are no. (%) or no. positive/no. tested (%). All animals were on the same farm. For each type of sample and age group, 15 specimens were tested. In November 2008, specimens (feces and serum) were collected in pairs from the same animals.

and the lowest rates of nucleotide mutations were seen in coding regions, L and 3D, respectively (Table 2). For amino acid levels, the highest changes in percentage within the 21-month period were found in 2C (1.49%), 2A (1.47%), and VP3 (1.34%) (Table 2). No amino acid changes were detected in regions VP1, 3A, and 3B. No cytopathic effect was seen in Vero cells after serial passages.

Conclusions

We report the endemic circulation and natural interhost evolution of porcine kobuvirus among domestic pigs on a pig farm within a 21-month period. This follow-up study indicates that domestic pigs are generally infected with porcine kobuvirus. It is likely that porcine kobuvirus is not restricted geographically and is widely distributed on pig farms worldwide. According to a veterinarian's clinical report, no clinical signs were associated with the endemic kobuvirus infection. In this closed-animal community, the susceptible young host generations were continuously infected; the length of the cycle interval when the virus is outside the host (nonreplicative viral form) is probably short. However, the number of nucleotide changes is consistent with rates observed for other picornaviruses (*13–15*), suggesting good adaptation of the virus–host relationship. This adaptation is also supported by the level of the nucleotide changes in different gene regions. More nucleotide mutations were found in nonstructural than in structural regions, which leads to a nearly equal probability for nonsynonymous amino acid changes in these 2 regions.

No available data confirm transmission mode, transmission efficiency, and pathogenesis of kobuviruses. Aichi virus has been reported to be shed in feces and maybe transmitted by food (especially seafood) in humans (*2,5,7*), suggesting fecal–oral transmission. However, in the pigs, viral RNA or infectious kobuvirus particles were also present in serum of virus-infected pigs. This finding suggests that porcine kobuvirus (and possibly kobuviruses in general) escaped the gastrointestinal tract into the circulatory system in immunocompetent virus-infected hosts, resulting in viremia. Further study is needed to investigate whether the viremia was acquired passively or actively. In addition to direct fecal–oral transmission, the possibility of kobuvirus transmission through breast-feeding (milk) and of bloodborne, foodborne, and zoonotic infections remains.

Similar explanations for the pathogenesis of many picornaviruses exist. Escape from the gastrointestinal tract into the bloodstream was probably the situation for the bovine kobuvirus detected in culture medium supplemented with cattle serum (*3*), which was suspected of possibly being contaminated with feces containing bovine kobuviruses. Porcine kobuvirus viremia open these data to another

Table 2. Natural interhost in vivo mutation changes in sequences of 2 porcine kobuvirus strains, Hungary*

	Nucleotides			Amino acids		
Region	Length	Difference	% Difference	Length	Difference	% Difference
5′ UTR	576	1	0.17	–	–	–
L	585	15	2.56	195	1	0.51
VP0	1,098	19	1.73	366	3	0.82
VP3	669	8	1.19	223	3	1.34
VP1	762	9	1.18	254	0	0
2A	408	6	1.47	136	2	1.47
2B	585	7	1.19	195	2	1.02
2C	1,005	23	2.28	335	5	1.49
3A	270	6	2.22	90	0	0
3B	102	2	1.96	34	0	0
3C	576	14	2.43	192	2	1.04
3D	1,407	16	1.14	469	2	0.42
3′ UTR	167	1	0.6	–	–	–
Nonstructural†	4,353	74	1.7	1,451	13	0.89
Structural‡	2,529	36	1.42	843	6	0.71
Complete genome	8,210	127	1.54	2,489	20	0.80

*Strain length shown is for kobuvirus/swine/K-30-HUN/2008/Hungary (accession no. GQ249161) and is compared with prototype strain kobuvirus/swine/S-1-HUN/2007/Hungary (length not shown; accession no. EU787450), from samples collected from pigs on the same farm, November 2008 and February 2007 respectively. UTR, untranslated region; L, leader region.
†2A-3D.
‡VP0–VP1.

interpretation for bovine kobuvirus infections and for Aichi virus pathogenesis in humans. Aichi virus was identified in low incidence in fecal samples from humans with gastroenteritis, but seroprevalence was high (*6,7*). Knowledge about viremia and natural virus evolution is crucial for understanding the pathogenesis, transmission, immunology, and clinical manifestations of kobuvirus infection in general and especially in humans.

This work was supported by grants from the Hungarian Scientific Research Fund (OTKA, F048433) and the project "Enteric Virus Emergence, New Tools" (EVENT, SP22-CT-2004-502571) funded by the European Union.

Dr Reuter is a medical virologist at the Regional Laboratory of Virology, ÁNTSZ Regional Institute of State Public Health Service, Pécs, Hungary. His research interests include molecular epidemiology of viral diseases, especially of viral gastroenteritis and enterically transmitted and food-borne viruses.

References

1. International Committee on Taxonomy of Viruses. ICTV official taxonomy: updates since the 8th report [cited 2010 Feb 10]. http://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/default.aspx
2. Yamashita T, Kobayashi S, Sakae K, Nakata S, Chiba S, Ishihara Y, et al. Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. J Infect Dis. 1991;164:954–7.
3. Yamashita T, Ito M, Kabashima Y, Tsuzuki H, Fujiura A, Sakae K. Isolation and characterization of a new species of kobuvirus associated with cattle. J Gen Virol. 2003;84:3069–77. DOI: 10.1099/vir.0.19266-0
4. Reuter G, Boldizsár Á, Kiss I, Pankovics P. Candidate new species of *Kobuvirus* in porcine hosts. Emerg Infect Dis. 2008;14:1968–70. DOI: 10.3201/eid1412.080797
5. Pham NT, Khamrin P, Nguyen TA, Kanti DS, Phan TG, Okitsu S, et al. Isolation and molecular characterization of Aichi viruses from fecal specimens collected in Japan, Bangladesh, Thailand, and Vietnam. J Clin Microbiol. 2007;45:2287–8. DOI: 10.1128/JCM.00525-07
6. Oh DY, Silva PA, Hauroeder B, Deidrich S, Cardoso DD, Schreier E. Molecular characterization of the first Aichi viruses isolated in Europe and in South America. Arch Virol. 2006;151:1199–206. DOI: 10.1007/s00705-005-0706-7
7. Ambert-Balay K, Lorrot M, Bon F, Giraudon H, Kaplon J, Wolfer M, et al. Prevalence and genetic diversity of Aichi virus strains in stool samples from community and hospitalized patients. J Clin Microbiol. 2008;46:1252–8. DOI: 10.1128/JCM.02140-07
8. Sdiri-Loulizi K, Gharbi-Khélifi H, de Rougemont A, Chouchane S, Sakly N, Ambert-Balay K, et al. Acute infantile gastroenteritis associated with human enteric viruses in Tunisia. J Clin Microbiol. 2008;46:1349–55. DOI: 10.1128/JCM.02438-07
9. Khamrin P, Maneekarn N, Peerakome S, Okitsu S, Mizuguchi M, Ushijama H. Bovine kobuviruses from cattle with diarrhea. Emerg Infect Dis. 2008;14:985–6. DOI: 10.3201/eid1406.070784
10. Reuter G, Egyed L. Bovine kobuvirus in Europe. Emerg Infect Dis. 2009;15:822–3. DOI: 10.3201/eid1505.081427
11. Reuter G, Boldizsár Á, Pankovics P. Complete nucleotide and amino acid sequences and genetic organization of porcine kobuvirus, a member of a new species in genus *Kobuvirus*, family *Picornaviridae.* Arch Virol. 2009;154:101–8. DOI: 10.1007/s00705-008-0288-2
12. Yu J-M, Jin M, Zhang Q, Li H-Y, Li D-D, Xu Z-Q, et al. Candidate porcine *Kobuvirus,* China. Emerg Infect Dis. 2009;15:823–5. DOI: 10.3201/eid1505.081518
13. Domingo E, Holland JJ. RNA virus mutations and fitness for survival. In: Ornston LN, Balows A, Greenberg EP, editors. Annual review of microbiology. Palo Alto (CA): Annual Reviews;1997;51:151–78.
14. Cottam EM, Haydon DT, Paton DJ, Gloster J, Wilesmith JW, Ferris NP, et al. Molecular epidemiology of the foot-and-mouth disease virus outbreak in the United Kingdom in 2001. J Virol. 2006;80:11274–82. DOI: 10.1128/JVI.01236-06
15. Jorba J, Campagnoli R, De L, Kew O. Calibration of multiple poliovirus molecular clocks covering an extended evolutionary range. J Virol. 2008;82:4429–40. DOI: 10.1128/JVI.02354-07

Address for correspondence: Gábor Reuter, Regional Laboratory of Virology, National Reference Laboratory of Gastroenteric Viruses, ÁNTSZ Regional Institute of State Public Health Service, Szabadság út 7. H-7623 Pécs, Hungary; email: reuter.gabor@ddr.antsz.hu

Influenza Virus Transmission from Horses to Dogs, Australia

Peter D. Kirkland, Deborah S. Finlaison, Ellie Crispe, and Aeron C. Hurt

During the 2007 equine influenza outbreak in Australia, respiratory disease in dogs in close contact with infected horses was noted; influenza (H3N8) virus infection was confirmed. Nucleotide sequence of the virus from dogs was identical to that from horses. No evidence of dog-to-dog transmission or virus persistence in dogs was found.

Respiratory disease in dogs caused by type A influenza virus was first noted in racing greyhounds in Florida in January 2004 (*1*). This subtype H3N8 virus has a presumptive but unidentified equine origin. The geographic extent of infection in racing greyhounds and in pet dogs suggest that this virus has become enzootic to the United States (*1*,*2*).

In the United Kingdom, pneumonia in dogs and influenza (H3N8) virus have been retrospectively linked, and subtype H3N8 infections have been identified serologically in dogs likely to have been in close contact with horses during the 2003 outbreak of equine influenza (*3*,*4*). A 78-bp segment of the hemagglutinin (HA) gene identified in dogs with pneumonia had complete homology with local equine strains (*3*). Unlike the situation in the United States, no evidence of continuing circulation of an influenza virus of equine origin in the canine population has been found in the United Kingdom.

In Australia, in late 2007, an outbreak of equine influenza virus (EIV) infection occurred in horses. During this outbreak, respiratory disease was noted in dogs of various ages and breeds that were kept near infected horses. Investigations were undertaken to exclude influenza virus infection.

The Study

The first reported case was in a dog near a large stable; the dog became inappetant and lethargic and had had a slight nasal discharge and a persistent cough for several days. Over the next 2–3 weeks, dogs in or near stables with infected horses, including dogs whose owners were handling infected horses or dogs (n = 6) that were only housed with infected dogs, were examined. Samples were also collected from dogs kept with horses at 5 other locations 20–60 km from the first case. Of the 40 dogs, examined, 10 had clinical signs consistent with influenza (anorexia, lethargy, and, for some, a harsh cough that persisted for several weeks). All affected dogs recovered.

Nasal swabs and serum were collected from each of the 40 dogs; 23 were seropositive according to influenza type A blocking ELISA (*5*) and hemagglutinin inhibition (HI) assay (*5*) using A/equine/Sydney/2007 virus as antigen (Table). HI titers were 16–256 (geometric mean 122). Results were discordant for 5 dogs: for 2, HI titer was high but ELISA results were negative; for 3, ELISA results were positive but HI titer was negative. These discrepancies may have been resolved had later sampling been possible. Convalescent-phase serum samples were collected 14–16 days later from 26 of the dogs; seroconversion was noted for 4 of the 5 dogs with discordant ELISA and HI results. Testing of 19 dogs 2 years later showed no change in HI titer, although ELISA results were negative for each. Each seropositive dog had been in close proximity to EIV-infected horses but not always in direct contact. No evidence of lateral transmission was found for dogs that did not have contact with horses.

Nasal swabs from 1 clinically healthy dog had a positive result in an influenza A real-time reverse transcription–PCR assay (*5*) on 2 consecutive days. The dog remained clinically healthy and was seropositive (titer 64) on day 16 after the first positive swab was collected. Attempts to isolate virus from these swabs were unsuccessful.

Nucleic acid sequencing was conducted for the HA, neuraminidase (NA), and matrix (M) genes amplified by PCR from the RNA purified from 2 samples from this dog (A/canine/Sydney/6525/2007 and A/canine/Sydney/6692/2007) and from a nasal swab from an infected horse (A/equine/Sydney/6085/2007) in the same stable (GenBank accession nos. GU045761–GU045769). Sequences were aligned with representative sequences from GenBank by using Clustal W (www.clustal.org/) before phylogenetic trees with bootstrapping were generated (n = 1,000; random seed n = 111) with MegAlign (Lasergene; DNAStar, Madison, WI, USA). Complete nucleotide homology was found for each of the HA, NA, and M gene sequences from the 2 dogs and the sequence from the infected horse in the same stable (A/equine/Sydney/6085/2007).

When influenza subtype H3N8 sequences from horses and dogs were compared with other subtype H3N8 sequences in GenBank, the HA, NA, and M sequences

Author affiliations: Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales, Australia (P.D. Kirkland, D.S. Finlaison); Warwick Farm Equine Centre, Warwick Farm, New South Wales, Australia (E. Crispe); and WHO Collaborating Centre for Reference and Research on Influenza, North Melbourne, Victoria, Australia (A.C. Hurt)

DOI: 10.3201/eid1604.091489

were most similar to strains A/equine/Kanazawa/1/2007 and A/equine/Ibaraki/1/2007, which were isolated during the 2007 equine influenza outbreak in Japan (Figure). The HA, NA, and M gene sequences from the dogs in Australia were positioned on separate clades of the phylogenetic trees, as opposed to those from subtype H3N8 viruses from dogs in the United States, which all grouped closely together (Figure).

Conclusions

Researchers in Japan have described transmission of EIV from 3 experimentally infected horses to 3 dogs indi-

Table. Clinical signs and serologic findings for 40 dogs exposed to equine influenza virus, Australia, October 2007*

				Day of sample collection			Titer					
							ELISA			HI assay		
Dog	Breed	Age/sex	Clinical signs	1st	2nd	PCR	1st	2nd	3rd†	1st	2nd	3rd†
1	Cattle dog x	6 mo/F	Cough	5	NS	Neg	70	NA	NA	64	NA	NA
2	Whippet	6 mo/F	Cough, inappetance, lethargy, nasal discharge	5	25	Neg	79	69	Neg	128	128	128
3	Rottweiler	UK	Cough, lethargy	10	25	Neg	Neg	65	Neg	Neg	Neg	Neg
4	Dalmation	3 y/MN	Cough, inappetance, lethargy	11	NS	Neg	74	NA	NA	64	NA	NA
5	Kelpi x	9.5 y/FN	Cough, lethargy	12	26	Neg	66	74	NA	64	128	NA
6	Border collie	5 y/MN	Inappetance, lethargy	13	27	Neg	60	75	Neg	64	32	64
7	Cattle dog	4.5 y/M	Cough, lethargy	13	27	Neg	64	57	Neg	256	128	128
8	German shepherd	9 y/M	Inappetance, lethargy	14	30	Neg	Neg	Neg	NA	Neg	Neg	NA
9	Jack Russell	9 y/MN	Cough	14	30	Neg	Neg	65	NA	Neg	Neg	NA
10	Lowchen	2 y/MN	Cough, inappetance, lethargy, nasal discharge	26	NS	Neg	Neg	NA	Neg	Neg	NA	Neg
11	Cattle dog x	18 mo/F	None	10	26	Neg	77	75	NA	64	64	NA
12	Fox terrier x poodle	15 y/FN	None; lived with dog 4	11	NS	Neg	Neg	Neg	NA	Neg	Neg	NA
13	Kelpie x	4 y/MN	None	12	26	Neg	64	71	Neg	128	64	128
14	German shepherd	1.5 y/MN	None	12	26	Neg	71	67	Neg	128	64	64
15	Kelpie x labrador	10 y/FN	None	12	26	Neg	71	80	Neg	256	32	Neg
16	Cattle x kelpie	3 y/MN	None	12	27	Neg	64	61	Neg	128	32	64
17	Unknown	UK	None	12	27	Neg	66	66	Neg	256	64	32
18	Border collie	1 y/M	None	12	27	Neg	78	59	Neg	128	64	128
19	Jack Russell x	2.5 y/MN	None	12	26	Neg	49	71	NA	32	16	NA
20	Greyhound	18 mo/F	None	12	26	Neg	Neg	Neg	NA	256	128	NA
21	Greyhound	2 y. M	None	12	26	Neg	Neg	55	NA	Neg	Neg	NA
22	Greyhound	5 y/M	None	12	25	Neg	Neg	Neg	NA	Neg	Neg	NA
23	Greyhound	18 mo/F	None	12	25	Neg	Neg	Neg	NA	Neg	Neg	NA
24	Greyhound	18 m/F	None	12	25	Neg	Neg	Neg	NA	Neg	Neg	NA
25	Cattle x kelpie	4 y/FN	None	12	26	Neg	51	73	Neg	128	64	64
26	Poodle	5 mo/M	None, lived with dog 7	13	27	Neg	Neg	Neg	Neg	8	Neg	Neg
27	Jack Russell	5 y/F	None, lived with dog 5	13	27	Neg	Neg	Neg	NA	Neg	Neg	NA
28	Cattle x hunterway	4.5 y/M	None	14	30	Pos	Neg	50	NA	Neg	64	NA
29	Border collie	4 y/FN	None	15	NS	Neg	Neg	NA	NA	Neg	NA	NA
30	Cattle	13 y/MN	None	15	NS	Neg	Neg	NA	NA	>32	NA	NA
31	Jack Russell	UK	None	15	NS	Neg	76	NA	NA	64	NA	NA
32	Rottweiler	UK	None	15	NS	Neg	76	NA	Inc	128	NA	128
33	Fox terrier	4 y/MN	None	15	NS	Neg	78	NA	NA	32	NA	NA
34	Labrador	13 y/FN	None	15	NS	Neg	51	NA	NA	64	NA	NA
35	x	UK	None	18	NS	Neg	Neg	NA	Neg	Neg	NA	Neg
36	x	UK	None	18	NS	Neg	73	NA	Neg	64	NA	64
37	x	UK	None	18	NS	Neg	Neg	NA	Neg	Neg	NA	Neg
38	x	UK	None	18	NS	Neg	Neg	NA	NA	Neg	NA	NA
39	x	UK	None	18	NS	Neg	67	NA	Neg	256	NA	128
40	Poodle x	9 y/UK	None, lived with dog 10	26	NS	Neg	Neg	NA	Neg	Neg	NA	Neg

*HI, hemagglutination inhibition; x, cross-breed; NS, not sampled; neg, negative; NA, not applicable; pos, positive; UK, unknown; MN, male neutered; FN, female neutered.
†Samples collected ≈2 y after second sample.

vidually housed with each horse (*6*). Their findings were mostly consistent with ours, but there were some differences. Both studies showed direct linkage between active influenza virus infection in dogs and horses. Because some naturally infected dogs were only in the vicinity of stables and not in direct contact with horses, we believe that EIV may be readily transmitted from horses to dogs in close proximity. The mechanism of spread remains unclear, although in the United Kingdom aerosol transmission was believed to be a major means of spread to dogs (*4*). Studies conducted during the equine influenza outbreak in Australia indicate that the levels of virus excretion from horses not previously exposed to the virus can be extremely high (A.J. Read et al., unpub. data). Although humans readily spread virus from horse to horse, either directly during handling or by fomite transmission, human transmission of EIV to dogs that were not in the immediate vicinity of infected horses was not found. Similarly, dog-to-dog transmission was not found when infected dogs were transported and kept with other dogs in urban locations where there was no opportunity for contact with horses.

Although clinical signs were not observed for any of the dogs in Japan, ≥35% of the naturally infected dogs in Australia exhibited clinical signs, some quite severe and protracted. Nevertheless, virus was rarely detected in nasal secretions of the dogs in Australia, and there was no evidence of horizontal transmission to other dogs. The lack of clinical signs in experimentally infected dogs may be because of the small numbers of dogs or because of inoculum attenuation after passage in embryonated chicken eggs. That the experimentally infected dogs in Japan also had lower HI titers than did naturally infected dogs may be relevant.

Finally, when 19 of the dogs in Australia were tested 2 years after infection and without opportunity for reexposure, with only 1 exception, the HI antibody titers had not changed. This finding supports the interpretation that antibodies detected in dogs in the United Kingdom (*3*,*4*) had been acquired during the equine influenza outbreak several years earlier.

The nucleotide gene sequences encoding the 2 surface proteins (HA and NA) and the M protein from the infected dog in Australia matched those from the horse with which it had contact and did not have any of the nucleotide changes that have been identified in viruses from dogs in the United States (*2*). Such changes may be critical to, or a consequence of, the adaptation of EIVs to dogs and may play a role in enhancing the infectivity of these viruses for dogs because there is no evidence of continuing circulation of virus in dogs in Australia.

Acknowledgments

We are indebted to the staff of the Virology Laboratory for technical support and to the NSW Department of Primary Industries for funding. The Melbourne WHO Collaborating Centre for Reference and Research in Influenza is supported by the Australian Government Department of Health and Ageing.

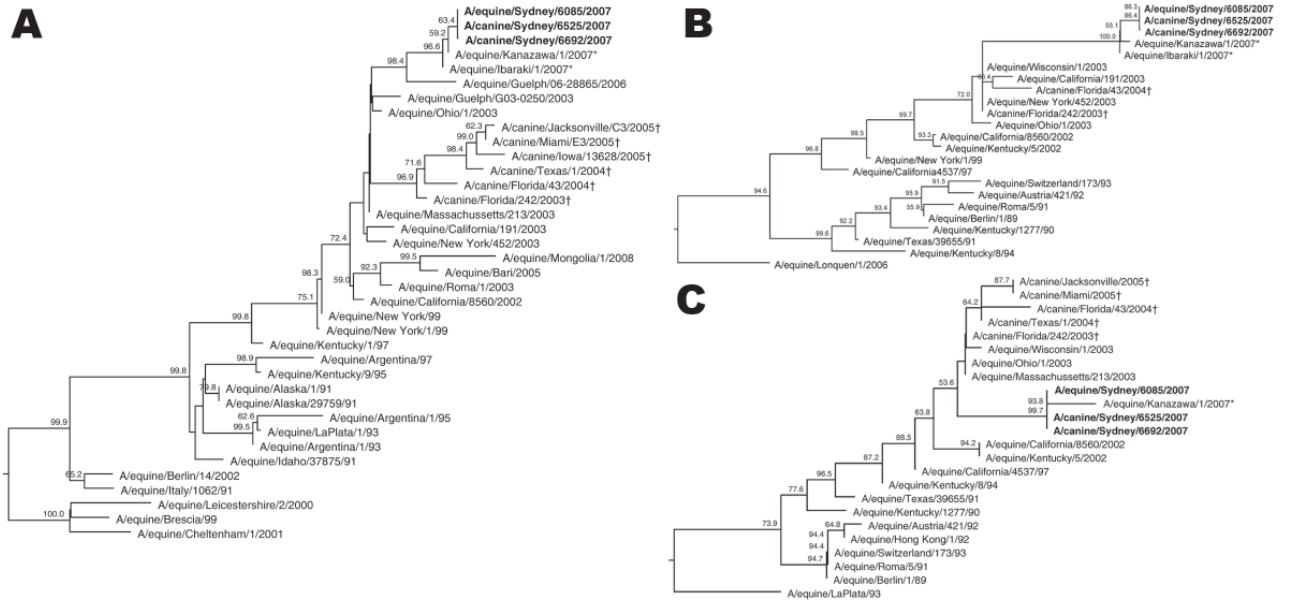


Figure. Phylogenetic trees of influenza subtype H3N8 viruses. Phylogenetic analyses conducted on A) hemagglutinin genes, B) neuraminidase genes, and C) matrix genes. Sequences from dogs are from the same animal on successive days. **Boldface** indicates viruses identified in dogs and horses in Australia, 2007, asterisks (*) indicate viruses from horses in Japan, and daggers (†) indicate viruses from dogs in the United States. Bootstrap values >50 are indicated at branch nodes. Bootstrap trials = 1,000; seed = 111.

Dr Kirkland is a veterinary virologist and head of the virology laboratory at the Elizabeth Macarthur Agricultural Institute. His research interests include the epidemiology and pathogenesis of viral diseases of animals, development of rapid diagnostic assays, and new and emerging diseases.

References

1. Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, et al. Transmission of equine influenza virus to dogs. Science. 2005;310:482–5. DOI: 10.1126/science.1117950
2. Payungporn S, Crawford PC, Kouo TS, Chen L, Pompey J, Castleman WL, et al. Influenza A virus (H3N8) in dogs with respiratory disease, Florida. Emerg Infect Dis. 2008;14:902–8. DOI: 10.3201/eid1406.071270
3. Daly JM, Blunden AS, MacRae S, Miller J, Bowman SJ, Kolodziejek J, et al. Transmission of equine influenza to English foxhounds. Emerg Infect Dis. 2008;14:461–4. DOI: 10.3201/eid1403.070643
4. Newton R, Cooke A, Elton D, Bryant N, Rash A, Bowman S, et al. Canine influenza: cross-species transmission from horses. Vet Rec. 2007;161:142–3.
5. Selleck PW, Kirkland PD. 2009. Avian influenza. In: Australian and New Zealand standard diagnostic procedures for animal diseases, Sub-Committee on Animal Health Laboratory Standards for Animal Health Committee, Australia [cited 2010 Feb 14]. http://www.scahls.org.au
6. Yamanaka T, Nemoto M, Tsujimura K, Kondo T, Matsumura T. Interspecies transmission of equine influenza virus (H3N8) to dogs by close contact with experimentally infected horses. Vet Microbiol. 2009; 139:351–5.

Address for correspondence: Peter D. Kirkland, Virology Laboratory, Elizabeth Macarthur Agricultural Institute, Private Bag 4008, Narellen, New South Wales 2567, Australia; email: peter.kirkland@industry.nsw.gov.au

Limited Susceptibility of Chickens, Turkeys, and Mice to Pandemic (H1N1) 2009 Virus

Donata Kalthoff, Christian Grund, Timm C. Harder, Elke Lange, Thomas W. Vahlenkamp, Thomas C. Mettenleiter, and Martin Beer

To determine susceptibility of chickens, turkeys, and mice to pandemic (H1N1) 2009 virus, we conducted contact exposure and inoculation experiments. We demonstrated that chickens were refractory to infection. However, oculo-oronasally inoculated turkeys and intranasally inoculated mice seroconverted without clinical signs of infection.

The current outbreak of pandemic (H1N1) 2009 continues to expand in humans, with occasional spillover into domestic pigs. Pandemic (H1N1) 2009 virus causes only mild disease compared with pandemic influenza viruses from the 20th century. However, this characteristic might change if pandemic (H1N1) 2009 viruses acquire virulence markers by reassorting with influenza viruses that cause severe disease in humans, such as highly pathogenic avian influenza viruses (HPAIVs) of the H5N1 subtype. Such reassortment might occur in humans but appears more likely in so-called mixing vessels. Pigs, which had been described as potential mixing vessels (*1*), are highly susceptible to infection with pandemic (H1N1) 2009 virus (*2,3*). In addition, pigs have been infected subclinically by HPAIV (H5N1) in countries to which HPAIV (H5N1) is endemic (*4*). However, whether poultry can be infected with pandemic (H1N1) 2009 virus is not well understood. Therefore, we determined the susceptibility of chickens, turkeys, and mice to pandemic (H1N1) 2009 virus.

The Study

All animal experiments were reviewed and approved by the responsible state ethics committee (LALLF M-V/TSD/7221.3–2.1.-014/09). Five chickens (12 weeks of age, specific pathogen–free [spf]) were inoculated oculo-oronasally by dripping a 10^6 50% tissue culture infectious dose ($TCID_{50}$) of virus A/Regensburg/D6/09/H1N1 on the cornea, nares, and oropharynx. Ten chickens (3 months of age, spf) were inoculated intravenously with 10^4 $TCID_{50}$. Five chickens (15 weeks of age, spf) were housed (permanent contact behind bars and daily direct contact for 30 min) with pigs infected with pandemic (H1N1) 2009 virus (*3*).

A second transmission experiment was performed with the same contact exposure experimental setup, which included 8 infected pigs and 5 turkeys (4 weeks of age). In addition, 28 chickens (1 week of age) and 28 turkeys (1 week of age) were inoculated with 200 μL of virus suspension (10^6 $TCID_{50}$) directly into the left air sac. Six fattening turkeys (16 weeks of age) from a local flock were inoculated oculo-oronasally with 10^6 $TCID_{50}$ of A/Regensburg/D6/09 (H1N1) virus. Six fattening turkeys were inoculated oculo-oronasally with 10^8 $TCID_{50}$ of the more recently isolated pandemic (H1N1) 2009 A/Bayern/74/2009 virus. Cloacal and oropharyngeal swab samples from poultry infected oculo-oronasally, through the air sac, or by contact with pigs were collected daily. Samples were tested by using a real-time reverse transcription–PCR with subtype H1N1–specific primers (http://offlu.net) (Table).

Additional studies were conducted to determine the 50% lethal dose of strain A/Regensburg/D6/2009 pandemic (H1N1) 2009 virus for mice. Sixteen BALB/c mice (6 weeks of age) were inoculated intranasally with 10^2–10^5 $TCID_{50}$/animal (30 μL), and 4 mice were inoculated intraperitoneally with 10^5 $TCID_{50}$/animal (30 μL).

None of the inoculated animals became ill after infection by any tested route; the intravenous pathogenicity index of the virus for chickens was 0. All swab samples from poultry after contact with infected pigs or from poultry inoculated through the air sac were negative for virus (Table). Virus excretion by pigs was detected (*3*); real-time reverse transcription–PCR cycle threshold values ≥26 (contact experiment with chickens) or ≥17 (contact experiment with turkeys). Virus RNA was detected (cycle threshold values 27–39) in swab samples obtained 1–6 days postinoculation from the oropharynx of turkeys inoculated with 10^8 $TCID_{50}$ of the A/Bayern/74/2009 (H1N1) strain. Small amounts of virus RNA also were detected in the lung and left air sac from a few chicks and poults early after infection through the air sac (Table), which most likely represent residual inoculum. Although contact exposure pigs were infected (*3*), seroconversion was not detected in any of the tested contact exposure poultry species. Poultry inoculated intravenously or through the air sac and chickens inoculated oculo-oronasally were negative for antibodies against influenza A virus nucleoprotein.

Fattening turkeys seroconverted after oculo-oronasal inoculation with 10^6 $TCID_{50}$ or 10^8 $TCID_{50}$ (Table). In addition, 7 of 8 mice inoculated with 10^4 $TCID_{50}$ or 10^5 $TCID_{50}$ by the oculo-oronasal route seroconverted; 4 mice inocu-

Author affiliation: Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

DOI: 10.3201/eid1604.091491

lated with 10^5 $TCID_{50}$ by the intraperitoneal route showed no detectable antibody response to the virus (Table).

Conclusions

Our results demonstrate lack of susceptibility of chickens and minor susceptibility of turkeys for infection by pandemic (H1N1) 2009 virus. Transmission of swine influenza viruses to poultry, particularly to turkeys, has been demonstrated (*5*), and experimental infection of chickens with virus subtype H3N2 resulted in a low replication rate, primarily in the alimentary tract (*6*). In contrast, our data indicate that pandemic (H1N1) 2009 virus cannot productively infect chickens at the ages of 1 week (air sac inoculation), 12 weeks (contact exposure, intravenous inoculation), or 15 weeks (oculo-oronasal inoculation), or turkeys at the ages of 1 week (air-sac inoculation) and 4 weeks (contact exposure) because neither virus excretion nor seroconversion was observed during the 10-day observation period. Fattening turkeys from a local flock seroconverted after oculo-oronasal inoculation, but virus RNA was detected in respiratory samples only in turkeys inoculated with a high dose of the A/Bayern/74/2009 (H1N1) virus (Table).

On the basis of our experiments, risk for transmission of pandemic (H1N1) 2009 virus strain to chicken and turkeys and subsequent possible reassortment with other avian influenza viruses should be low. However, we observed a slightly higher susceptibility of older turkeys to high doses of pandemic (H1N1) 2009 virus, which is consistent with recent reports of infected layer turkey flocks in Chile and Canada (*7,8*). Analysis of specific virus strains involved in these outbreaks would be useful for confirming these observations and analyzing different strains. Host factors (age, physiologic state, stress levels, and concurrent infections) influencing susceptibility of turkeys should be investigated. Our data, which demonstrate absence of illness in poultry after inoculation with pandemic (H1N1) 2009 virus, are consistent with those of recent experimental infections of poultry, including turkeys (*9–11*). Seroconversion was detected only in turkeys by using a hemagglutination-inhibition test in 1 study (*9*), which might indicate higher sensitivity of competitive ELISAs used in our study.

As in other studies (*12,13*), characterization of pandemic (H1N1) 2009 virus strains in BALB/c mice showed differences in lethal dose and clinical signs dependent on the virus strain. None of the infected BALB/c mice in our study showed clinical signs. These findings may have resulted from the fact that mice were infected intranasally without anesthesia and with a dose $<10^6$ $TCID_{50}$/animal or because of a different phenotype of the virus strain used. However, replication competence of pandemic (H1N1) 2009 virus in mice without prior adaptation was indicated by seroconversion, at least for the higher infectious doses (Table). Intraperitoneal inoculation did not cause development of influenza virus–specific antibodies. This finding cannot be explained by receptor specificity of pandemic (H1N1) 2009 virus because Childs et al. (*14*) showed that representative pandemic (H1N1) 2009 viruses bound not only to most α2–6-linked sialyl moieties, irrespective of the backbone chain length and type, but also to a considerable range of α2–3-linked sialyl residues. Although virus rep-

Table. Susceptibility of chicken, turkeys, and mice to pandemic (H1N1) 2009 virus*

Animal and dose (virus strain)	No. animals	Route of inoculation	Swab samples	Tissue samples, no. positive/no. tested†	Seroconversion in NP-ELISA (no. positive/no. tested)‡
Chicken	5	Oculo-oronasal	–	NA	–
Chick	28	Intra–air sac	–	Day 2: 2/3; Day 4: 2/3; Day 8: 1/3	–
Chicken	10	Intravenous	NA	NA	–
Chicken	5	Contact with infected pigs	–	NA	–
Turkey, 10^6 $TCID_{50}$ (A/Regensburg/D6/2009)	6	Oculo-oronasal	–	NA	+ (3/6)
Turkey, 10^8 $TCID_{50}$ (A/Bayern/74/2009)	6	Oculo-oronasal	+ (respiratory), – (cloacal)	NA	+ (4/6)
Poult	28	Intra–air sac	–	Day 2: 3/3; Day 4: 1/3; Day 6: 1/3	–
Turkey	5	Contact with infected pigs	–	NA	–
Mouse, 10^2–10^3 $TCID_{50}$	8	Oculo-oronasal	NA	NA	–
Mouse, 10^4 $TCID_{50}$	4	Oculo-oronasal	NA	NA	+ (3/4)
Mouse, 10^5 $TCID_{50}$	4	Oculo-oronasal	NA	NA	+ (4/4)
Mouse, 10^5 $TCID_{50}$	4	Intraperitoneal	NA	NA	–

*NP, nucleoprotein; –, negative; NA, not applicable; +, positive; $TCID_{50}$, 50% tissue culture infectious dose.
†Three juvenile chickens and turkeys inoculated through the air sac were killed on days 0, 2, 4, 6, 8, and 14 posinoculation, and left and right lung lobes were removed for analysis by real-time reverse transcription–PCR. Tissues from the left (inoculation site) lung lobe of 5 chicks and 4 poults killed at the indicated days postinfection were positive for virus RNA with high cycle threshold values (>35), and 1 additional poult was positive for virus RNA with a cycle threshold value of 27 on day 2 postinfection.
‡Seroconversion was verified by testing for antibodies against virus NP by commercially available ELISAs (ELISA Avian Influenza Type A; Pourquier, Montpellier, France, and FlockChek AI MultiS-Screen; IDEXX, Ludwigsburg, Germany).

lication may be reduced after intraperitoneal inoculation, development of neutralizing antibodies after inoculation by this route has been reported (*15*).

Our results demonstrate lack of susceptibility and absence of serologic responses of chickens and turkeys to contact infection with an early human-origin isolate of pandemic (H1N1) 2009 virus. However, direct inoculation of fattening turkeys resulted in seroconversion and detection of virus RNA in oropharyngeal swab samples. Generation of new variants and reassortants caused by double infections with other influenza A viruses and pandemic (H1N1) 2009 virus in poultry other than turkeys appears unlikely. However, further adaptation of pandemic (H1N1) virus strains in turkeys cannot be excluded.

Acknowledgments

We thank Mareen Grawe for excellent technical assistance; Stephan Becker, Jennifer Uhlendorf, Mikhail Matrosovich, and Markus Eickmann for isolating and providing virus strain A/Regensburg/D6/2009; and Brunhilde Schweiger and Barbara Biere for providing virus strain A/Bayern/74/2009.

This study was supported by European Union FP7 project European Management Platform for Emerging and Re-emerging Infectious Disease Entities (no. 223498).

Dr Kalthoff is a veterinarian at the Friedrich-Loeffler-Institut in Greifswald-Insel Riems, Germany. Her research interests are virus pathogenesis and vaccine development.

References

1. Scholtissek C, Burger H, Kistner O, Shortridge KF. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology. 1985;147:287–94. DOI: 10.1016/0042-6822(85)90131-X
2. Brookes SM, Irvine RM, Nunez A, Clifford D, Essen S, Brown IH, et al. Influenza A (H1N1) infection in pigs. Vet Rec. 2009;164:760–1.
3. Lange E, Kalthoff D, Blohm U, Teifke JPT, Breithaupt A, Maresch C, et al. Pathogenesis and transmission of the novel swine origin influenza virus A/H1N1 after experimental infection of pigs. J Gen Virol. 2009;90:2119–23.
4. Takano R, Nidom CA, Kiso M, Muramoto Y, Yamada S, Shinya K, et al. A comparison of the pathogenicity of avian and swine H5N1 influenza viruses in Indonesia. Arch Virol. 2009;154:677–81. DOI: 10.1007/s00705-009-0353-5
5. Choi YK, Lee JH, Erickson G, Goyal SM, Joo HS, Webster RG, et al. H3N2 influenza virus transmission from swine to turkeys, United States. Emerg Infect Dis. 2004;10:2156–60.
6. Thomas C, Manin TB, Andriyasov AV, Swayne DE. Limited susceptibility and lack of systemic infection by an H3N2 swine influenza virus in intranasally inoculated chickens. Avian Dis. 2008;52:498–501. DOI: 10.1637/8210-011408-RESNOTE.1
7. PRO/AH/EDR. Influenza pandemic (H1N1) 2009, animal health (03): Chile, avian, RFI [cited 2010 Jan 14]. http://www.promedmail.org, archive no. 20090821.2961.
8. PRO/AH/EDR. Influenza pandemic (H1N1) 2009, animal health (14): Canada (ON), avian [cited 2010 Jan 14]. http://www.promedmail.org, archive no. 20091020.3602.
9. Terregino C, De Nardi R, Nisi R, Cilloni F, Salviato A, Fasolato M, et al. Resistance of turkeys to experimental infection with an early 2009 Italian human influenza A(H1N1)v virus isolate. Euro Surveill. 2009;14:19360.
10. Swayne DE, Pantin-Jackwood M, Kapczynski D, Spackman E, Suarez DL. Susceptibility of poultry to pandemic (H1N1) 2009 virus. Emerg Infect Dis. 2009;15:2061–3. DOI: 10.3201/eid1512.091060
11. Russell C, Hanna A, Barrass L, Matrosovich M, Núñez A, Brown IH, et al. Experimental infection of turkeys with pandemic (H1N1) 2009 influenza virus (A/H1N1/09v). J Virol. 2009;83:13046–7. DOI: 10.1128/JVI.01645-09
12. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature. 2009;460:1021–5.
13. Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, Zeng H, et al. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science. 2009;325:484–7.
14. Childs RA, Palma AS, Wharton S, Matrosovich T, Liu Y, Chai Wet al. Receptor-binding specificity of pandemic influenza A (H1N1) 2009 virus determined by carbohydrate microarray. Nat Biotechnol. 2009;27:797–9. DOI: 10.1038/nbt0909-797
15. Bertram EM, Lau P, Watts TH. Temporal segregation of 4–1BB versus CD28-mediated costimulation: 4–1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection. J Immunol. 2002;168:3777–85.

Address for correspondence: Martin Beer, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493 Greifswald-Insel Riems, Germany; email: martin.beer@fli.bund.de

Pandemic (H1N1) 2009 Infection in Swine Herds, Manitoba, Canada

Tim Pasma and Tomy Joseph

In Manitoba, Canada, several swine herds were infected by pandemic (H1N1) 2009 virus in the summer of 2009. Results of several investigations concluded that outbreaks of infection with this virus are similar in duration to outbreaks of infections with swine influenza viruses A (H1N1) and A (H3N2).

On April 21, 2009, the US Centers for Disease Control and Prevention announced the finding of a novel strain of influenza virus A (H1N1), now known as pandemic (H1N1) 2009 virus, in 2 children in southern California (*1*). By June 11, this virus had spread so quickly and extensively among humans that the World Health Organization declared a phase 6 pandemic (*2*). The disease in humans is a self-limiting, uncomplicated respiratory illness with fever; however, severe disease and deaths have occurred (*3*). Clinical signs in humans are generally mild and include fever, slight cough, sneezing, and nasal discharge. Vomiting and diarrhea also have been reported in up to 38% of cases (*3*).

Pandemic (H1N1) 2009 virus also has affected swine. On May 2, 2009, the virus was isolated from a swine herd in Alberta, Canada (*4*). The disease in swine has been reported as mild in field and experimental conditions. Clinical signs in pigs include fever, slight cough, sneezing, and nasal discharge. Diarrhea was also reported in experimentally infected pigs; however, this symptom may have been secondary to the influenza infection (*5*). In experimental infection of pigs, clinical signs peaked on days 4–5 postinfection (*5*).

In Manitoba, Canada, pandemic (H1N1) 2009 virus was first detected in a swine herd on June 30, 2009. During the following months, more outbreaks in Manitoba were reported in farrowing, nursery, and finishing herds. Our study aimed to determine the length of time that virus was shed in swine herds after a field outbreak of pandemic (H1N1) 2009.

Author affiliation: Manitoba Agriculture, Food and Rural Initiatives, Winnipeg, Manitoba, Canada

DOI: 10.3201/eid1604.091636

The Study

We studied 5 herds in which pandemic (H1N1) 2009 was diagnosed. We collected information about the production type and number of animals housed in the barn, influenza vaccination status of the herd, date of influenza-like illness in any barn employees before the outbreak, date of the outbreak as determined by onset of clinical signs, and sampling date and number of positive swabs. In each herd, 32 nasal swabs were taken from randomly selected pigs as soon as possible after diagnosis. The procedure was repeated every 7 days until all the samples tested showed negative results.

Nasal swabbing was performed by using a polyester swab with a plastic handle that was placed in a viral transport medium (Starswab Multitrans Collection & Transport System; Starplex Scientific Inc, Etobicoke, Ontario, Canada). The swabs were refrigerated and submitted to the Veterinary Services Diagnostic Laboratory at Manitoba Agriculture, Food and Rural Initiatives (Winnipeg, Manitoba, Canada). Samples were tested by using a generic real-time PCR specific for the genomic RNA segment 7 (matrix gene) of the influenza A virus provided by the National Centre for Foreign Animal Disease (Winnipeg, Manitoba, Canada) (*3*) and an H1 differential PCR (*6*) provided by the National Microbiology Laboratory (Winnipeg, Manitoba, Canada).

We tested 5 herds (herds A–E) in which pandemic (H1N1) 2009 virus was diagnosed (Table). Herds A, B, and D were finishing herds, herd C was a nursery herd, and herd E was a farrowing herd. Herd sizes ranged from 850 to 4,100 pigs. For herds C and D, human illness 16–92 days before the outbreak was reported. The owners of herds A and B reported that they received pigs from a previously infected herd. For persons in contact with herd E, no ill persons were reported, and no pigs from previously affected herds were received before the outbreak. Pigs in herd E were vaccinated for swine influenza A (H1N1) and (H3N2) viruses with an autogenous vaccine.

Clinical signs in pigs were reported to be mild, with no deaths. However, herd D, co-infected with porcine reproductive and respiratory syndrome virus, *Mycoplasma hyopneumoniae*, and porcine circovirus, reported a 1% outbreak-associated death rate. No vomiting or diarrhea was reported in any pigs infected with the virus.

Nasal swabbing of the pigs demonstrated that pandemic (H1N1) 2009 virus was no longer detected in swine 10–20 days after clinical signs appeared. When tested again the week before slaughter (day 67), herd B showed no evidence of virus shedding.

Conclusions

We demonstrated that field infections of pandemic (H1N1) 2009 in swine are similar in duration to infec-

Table. Findings from study of 5 tested swine herds in which pandemic (H1N1) 2009 was diagnosed, Manitoba, Canada, 2009

Herd	Type/size	Vaccine	Human illness	Sample 1	Sample 2	Sample 3
A	Finishing, 2,080	None	No (animal spread)	Day 20, 0/32 positive		
B	Finishing, 3,872	None	No (animal spread)	Day 5, 3/32 positive	Day 19, 0/32 positive	Day 67, 0/31 positive
C	Nursery, 4,100	None	Day –16, manager and family sick	Day 19, 0/32 positive		
D	Finishing, 850	None	Day –92, manager and family sick	Day 10, 0/32 positive		
E	Farrowing, 3,100	Subtypes H1N1 and H3N2 (autogenous)	None reported	Day 10, 7/32 positive	Day 17, 0/32 positive	

tions with other swine influenza viruses. In the herds studied, the virus caused mild illness identical to the clinical signs typical of swine influenza (*7*). Sampling by nasal swab indicated that pandemic (H1N1) 2009 virus sheds for up to 20 days after clinical signs appear. Our findings support the laboratory work of Lange et al., who established that pigs experimentally infected with this strain intermittently shed the virus 6–11 days postinfection and ceased excretion by day 11 (*5*). Shedding of the circulating strains of swine influenza in nasal secretions stops by 5–7 days postinfection (*7*–*9*). Our study also indicates that autogenous vaccine prepared with circulating H1N1 subtype may not protect pigs from pandemic (H1N1) 2009 infection.

Our study has several limitations. Other swine viruses, such as porcine reproductive and respiratory syndrome virus, may interfere with the detection of swine influenza viruses from nasal swabs (*8*), and we did not test for other viruses. In addition, the virus can be difficult to diagnose in nursery pigs because of maternal antibodies and low levels of exposure (*8*), which may have affected the samples from the nursery herd. The small sample size and the unknown sensitivity of the PCR in this specific application also limit the findings of our study.

The swine herds we studied quickly cleared the virus after infection. This study supports the recommendations developed by the Canadian Food Inspection Agency (*10*) and the World Organisation for Animal Health (OIE) (*11*). These guidelines state that pigs infected with pandemic (H1N1) 2009 virus should be managed similarly to herds infected with any swine influenza virus. On the basis of our study findings, restrictions of trade or slaughter of pigs as a public health intervention are irrational actions.

Only 10 countries have reported pandemic (H1N1) 2009 infection in commercial swine to the OIE (*12*). Whether pandemic (H1N1) 2009 will become established in swine populations worldwide remains to be seen. All countries should implement vigilant surveillance for, and monitor for changes in the structure and behavior of, the virus.

Acknowledgments

We thank Linda Duffy, Cherry McCormick, Cheryl Sachvie, Cherie Scammell-Chandler, and Tracy Scammell-LaFleur for their assistance in processing samples.

Dr Pasma is a veterinarian with Animal Health and Welfare, Chief Veterinary Office/Food Safety Knowledge Centre, Manitoba Agriculture, Food and Rural Initiatives, Winnipeg, Manitoba, Canada. His research interest is in the epidemiology of swine influenza.

Dr Joseph is a virologist with Veterinary Diagnostic Services Laboratory, Livestock Knowledge Centre, Manitoba Agriculture, Food and Rural Initiatives, Winnipeg. His research interests include diagnostic virology and the molecular basis of pathogenesis of avian and swine influenza viruses.

References

1. Centers for Disease Control and Prevention. Swine influenza A (H1N1) infection in two children—southern California, March–April 2009. MMWR Morb Mortal Wkly Rep. 2009;58:400–2.
2. World Health Organization. Global alert and response. Current WHO phase of pandemic alert [cited 29 Jan 2010]. http://www.who.int/csr/disease/avian_influenza/phase/en
3. Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med. 2009;360:2605–15. DOI: 10.1056/NEJMoa0903810
4. Howden KJ, Brockhoff EJ, Caya FD, McLeod LJ, Lavoie M, Ing JD, et al. An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. Can Vet J. 2009;50:1153–61.
5. Lange E, Kalthoff D, Blohm U, Teifke JP, Breithaupt A, Maresch C, et al. Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs. J Gen Virol. 2009;90:2119–23. DOI: 10.1099/vir.0.014480-0
6. Leblanc JJ, Li Y, Bastien N, Forward KR, Davidson RJ, Hatchette TF. Switching gears for an influenza pandemic: validation of a duplex RT-PCR for simultaneous detection and confirmation of pandemic (H1N1) 2009. J Clin Microbiol. 2009;47:3805–13.
7. Olsen CW, Brown IH, Easterday BC, Van Reeth K. Swine influenza. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ, editors. Diseases of swine. 9th ed. Ames (IA): Blackwell Publishing; 2006. p. 469–82.
8. Gillespie TG. Diagnosing endemic swine influenza virus in nursery pigs using cross-sectional serologic profiling. Swine Health and Production. 1999;7:81–3.

9. Janke BH. Diagnosis of swine influenza. Swine Health and Production. 2000;8:79–84.
10. Canadian Food Inspection Agency. News release. Management of pandemic H1N1 in swine herds [cited 2009 Oct 5]. http://www.inspection.gc.ca/english/corpaffr/newcom/2009/20090724e.shtml
11. World Organisation for Animal Health. Press releases. Pandemic (H1N1) 2009: the OIE reiterates its recommendations to animal health authorities worldwide [cited 2009 Oct 5]. http://www.oie.int/eng/press/en_090713.htm
12. World Organisation for Animal Health. WAHID interface. Weekly disease information [cited 2009 Dec 3]. http://www.oie.int/wahis/public.php?page=weekly_report_index&admin=0

Address for correspondence: Tim Pasma, Chief Veterinary Office/Food Safety Knowledge Centre, Manitoba Agriculture, Food and Rural Initiatives, 545 University Crescent, Winnipeg, Manitoba R3T 5S6, Canada; email: tim.pasma@gov.mb.ca

Pandemic (H1N1) 2009 in Breeding Turkeys, Valparaiso, Chile

Christian Mathieu, Valentina Moreno, Patricio Retamal, Alvaro Gonzalez, Alejandro Rivera, Jorge Fuller, Cecilia Jara, Claudio Lecocq, Miriam Rojas, Alfonso García, Marcela Vasquez, Michel Agredo, Cristian Gutiérrez, Hector Escobar, Rodrigo Fasce, Judith Mora, Julio García, Jorge Fernández, Claudio Ternicier, and Patricia Avalos

Pandemic (H1N1) 2009 virus was detected in breeding turkeys on 2 farms in Valparaiso, Chile. Infection was associated with measurable declines in egg production and shell quality. Although the source of infection is not yet known, the outbreak was controlled, and the virus was eliminated from the birds.

Influenza A pandemic (H1N1) 2009 virus is a novel highly transmissible agent that contains a unique combination of gene segments from different swine lineages (*1*); it has circulated in humans since April 2009 (*2*). First detected in North America, the virus was disseminated worldwide in just a few weeks, prompting the World Health Organization to raise its global health alert to the pandemic stage (*2,3*).

By December 2009, a total of 14 countries had reported that the pandemic strain was infecting swine, generating concern about the role of other susceptible species in the viral epidemiology. Fortunately, only a mild respiratory disease developed in the ill swine, and outbreaks were controlled with biosafety measures, avoiding dissemination to humans and animals (*4*). In Chile, the first case of human infection with the pandemic strain was confirmed on May 17, 2009; infection increased within 6 months to a total of 12,276 cases, with 147 deaths (*5*).

On July 23, 2009, in the Valparaiso Region of Chile, 1 flock (A1) from a commercial turkey breeding farm (farm A) started to show a measurable decrease in egg production and shell quality (Figure 1). During the following 2 weeks, similar signs were observed in 3 other flocks (A2, A3, A4) at farm A and 2 flocks (B1 and B4) on another turkey breeding farm (farm B) 50 km away, both belonging to the same company. However, neither respiratory signs nor increased death rates were observed. Because an influenza virus was suspected, on August 13 the situation was reported to the Chilean Agricultural and Livestock Service (SAG) for diagnosis.

The Study

On August 14, the first blood samples were taken from turkeys of all affected flocks and submitted for serodiagnosis (Table). The agar gel immunodiffusion assay (AGID) detected antibodies to influenza A virus in 140 (62%) of 227 turkeys sampled. Farm A had a higher proportion of positives (80%) than farm B (32%) (odds ratio [OR] 8.4; 95% confidence interval [CI] 4.6–15.5).

Because of this finding, SAG adopted several control measures. Involved premises were quarantined and biosecurity standards were intensified, an epidemiologic investigation of the outbreak was initiated, and postmortem examinations of some carcasses were conducted. Each farm was considered an epidemiologic unit because each contained several flocks located closer than 1,000 m inside the same farm and were managed under independent biosecurity measures. On August 16, sampling for viral RNA detection by real-time reverse transcription–PCR (rRT-PCR) (*6*) was conducted, including not only affected flocks but surrounding premises; cloacal and tracheal swabs and embryonated eggs were collected from hatcheries. In addition, neighboring turkey farms were sampled for serologic analysis.

By August 18, results of this screening showed infection only in breeder turkeys from the first affected flocks of farms A and B. The rRT-PCR identified RNA corresponding to the influenza A matrix gene (Table) but not RNA of H5 or H7 genes. In some cloacal and tracheal swabs,

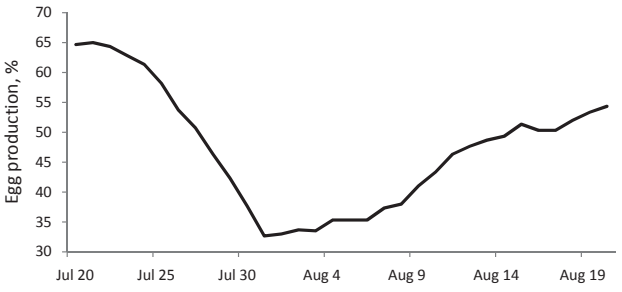


Figure 1. Average egg production of 3 pandemic (H1N1) 2009–infected turkey flocks (A1, A2, and A3) during July 20–August 20, 2009, Valparaiso, Chile. Production was calculated as a daily egg-laying rate (%).

Author affiliations: Servicio Agrícola y Ganadero, Santiago, Chile (C. Mathieu, V. Moreno, A. Gonzalez, A. Rivera, J. Fuller, C. Jara, C. Lecocq, M. Rojas, A. García, M. Vasquez, M. Agredo, C. Gutiérrez, H. Escobar, C. Ternicier, P. Avalos); Universidad de Chile, Santiago (P. Retamal); and Instituto de Salud Pública, Santiago (R. Fasce, J. Mora, J. García, J. Fernández)

DOI: 10.3201/eid1604.091402

Table. Laboratory results of testing conducted on turkey farms affected by the pandemic (H1N1) 2009 influenza outbreak, Valparaiso Region, Chile, 2009*

		Turkeys		Results				
				AGID		rRT-PCR		
Farm	Flock no.	No.	Age, wk	No. animals sampled	No. positive	No. animals sampled	No. positive	Ct
A	1	7,032	53	28	18	26	3	31.4
	2	4,410	77	28	25	43	1	34.8
	3	7,556	49	28	17	19	0	
	4	6,922	37	56	52	42	0	
B	1	7,004	45	28	2	30	3	34.9
	4	5,950	57	59	26	47	6	27.1

*AGID, agar gel immunodiffusion assay; rRT-PCR, real-time reverse transcription–PCR to detect matrix protein; Ct, cycle threshold values of positive results (averages).

viral RNA was detected, with cycle threshold values of 22.3–36.0. In the homogenates of embryo lungs and tracheas, viral RNA was not detected, suggesting that no vertical transmission occurred in this outbreak. Necropsies of 3 birds showed salpingitis, peritonitis, and interruption of follicular development. No other lesions were observed, and birds that had symptoms at the beginning of the outbreak were recovering and returning to normal laying rates after 3 weeks (Figure 1). Pools of feces belonging to 12 wild birds, including ducks (*Anas georgica*) and coots (*Fulica armillata*), collected near farm A were negative for influenza virus by rRT-PCR.

In addition, the SAG laboratory had been working on subtyping the agent by developing hemagglutinin and neuraminidase inhibition tests (*7*) using 5 AGID-positive serum samples. Subtype H1N1 was found after all 15 hemagglutinin and 9 neuraminidase subtypes were tested.

On August 19, SAG authorities coordinated with the Chilean Public Health Institute (ISP) for the virus isolation by using specific pathogen-free chicken embryos and MDCK cells (*8*) and for sequencing the viral genome (*9*) because the diagnosis for the pandemic agent was centered in the ISP facilities. In addition, an RNA sample was sent to the National Veterinary Services Laboratories (US Department of Agriculture), the World Organization for Animal Health reference laboratory for avian influenza.

On August 27, the viral sequences had been informed by ISP and National Veterinary Services Laboratories (GenBank accession nos. GQ866230, GQ866231, GQ866229, GQ866225, GQ866227, GQ866226, GQ866232, and GQ866228), having an almost complete identity with the novel pandemic strain. These results suggested transmission of pandemic (H1N1) 2009 virus from humans to birds.

In the follow-up to this outbreak during September and October, SAG implemented an rRT-PCR assay developed by Southeast Poultry Research Laboratory (Athens, GA, USA) for the detection of the pandemic (H1N1) 2009 N1 gene (*10*). When egg production began progressively recovering in flocks, cloacal and tracheal swabs were analyzed for viral detection by using rRT-PCR assays. The last evidence of infection was obtained on August 31 (Figure 2), suggesting that the virus was eliminated from turkeys after 2–4 weeks.

Conclusions

The source of infection for animals is still under investigation, but no clinical signs consistent with influenza-like illness were reported in staff working on the farms. Viral dissemination might have occurred either from the same infectious source, i.e., a farm worker or through fomites transported between premises. In any case, the biosafety system implemented in the company was working mainly to prevent infections from wild animals but not from humans. Transmission among animals was expected to have occurred through direct contact with secretions and feces from infected to susceptible birds.

The low cycle threshold values found by rRT-PCR in some samples suggest that the virus can replicate in turkeys, although previous attempts to experimentally infect turkeys with this strain had been unsuccessful (*10*). Some unknown predisposing factor expressed in the farm environment has transformed these birds into reservoirs of the pandemic strain. This event, also detected in Canada and the United States (*4*), could be explained by the reported presence of both avian and mammalian influenza A recep-

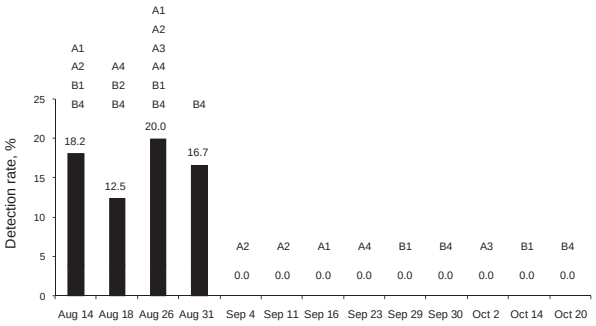


Figure 2. Pandemic (H1N1) 2009 virus detection rates (%) in affected turkey flocks from farms A and B during August 14–October 20, 2009, Valparaiso, Chile. Tracheal and cloacal swabs were analyzed by real-time reverse transcription–PCR to detect matrix and N1 genes. In each sampling date, detection rates appear in numbers, and sampled flocks are indicated by letters and numbers.

tors in tissues from turkeys. Indeed, these turkeys are susceptible to a wide variety of influenza A viruses, including those from wild birds and swine, which are detected in these birds frequently (*11*). The role of turkeys in the epidemiology of pandemic (H1N1) 2009 strain remains to be elucidated.

In this outbreak, the infection in turkeys was mild, with reproductive symptoms and natural recovery. Some authors have reported a similar situation when these birds were experimentally infected with the triple reassortant H3N2 subtype isolated from pigs (*11,12*). Because some segments of subtype H3N2 are in the lineage of pandemic (H1N1) 2009 virus (*2*), the genetic bases for the interspecies transmission of this strain might be found on these segments. The turkeys involved in the outbreak were held under strict biosafety measures and sent to slaughter after laboratory verification of viral disappearance. The control strategy promptly and completely eliminated the risk to humans and animals.

Dr Mathieu works in the virology laboratory of the Chilean Agricultural and Livestock Service and is a Food and Agricultural Organization of the United Nations (FAO)–Instituto Interamericano de Cooperación para la Agricultura (IICA) consultant on the diagnosis of avian influenza.

References

1. World Health Organization. Pandemic (H1N1) 2009 [cited 2009 Sept 2]. http://www.who.int/csr/disease/swineflu/en/index.html
2. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A (H1N1) influenza viruses circulating in humans. Science. 2009;325:197–201. DOI: 10.1126/science.1176225
3. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, et al. Pandemic potential of a strain of influenza A (H1N1): early findings. Science. 2009;324:1557–61. DOI: 10.1126/science.1176062
4. World Organisation for Animal Health. Weekly disease information. Follow-up reports. 2009 [cited 2009 Dec 3]. http://www.oie.int/wahis/public.php?page=weekly_report_index&admin=0
5. Ministerio de Salud. Report of human influenza A–H1N1situation, 2009 [in Spanish] [cited 2009 Dec 6]. http://www.minsal.cl
6. Spackman E, Senne DA, Bulaga LL, Myers TJ, Perdue ML, Garber LP, et al. Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis. 2003;47:1079–82. DOI: 10.1637/0005-2086-47.s3.1079
7. World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals, 2009. Chapter 2.3.4: avian influenza [cited 2009 Dec 6]. http://www.oie.int/fr/normes/mmanual/A_summry.htm
8. World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals, 2009. Chapter 2.8.8: swine influenza [cited 2009 Dec 6]. http://www.oie.int/fr/normes/mmanual/A_summry.htm
9. World Health Organization. Sequencing primers and protocol. Pandemic (H1N1) 2009 guidance documents [cited 2009 Sept 6]. http://www.who.int/csr/resources/publications/swineflu/sequencing_primers/en/index.html
10. Swayne DE, Pantin-Jackwood M, Kapczynski DR, Spackman E, Suarez DL. Susceptibility of poultry to pandemic (H1N1) 2009 virus [letter]. Emerg Infect Dis. 2009;15:2061–3. DOI: 10.3201/eid1512.091060
11. Yassine HM, Al-Natour MQ, Lee CW, Saif YM. Interspecies and intraspecies transmission of triple reassortant H3N2 influenza A viruses. Virol J. 2007;4:129. DOI: 10.1186/1743-422X-4-129
12. Pillai SP, Pantin-Jackwood M, Jadhao SJ, Suarez DL, Wang L, Yassine HM, et al. Pathobiology of triple reassortant H3N2 influenza viruses in breeder turkeys and its potential implication for vaccine studies in turkeys. Vaccine. 2009;27:819–24. DOI: 10.1016/j.vaccine.2008.11.076

Address for correspondence: Patricia Avalos, Departamento Laboratorios y Estaciones Cuarentenarias Agrícola y Pecuaria, Servicio Agrícola y Ganadero, Ruta 68, Km 12, Lo Aguirre, Pudahuel, Chile; email: patricia.avalos@sag.gob.cl

16S rRNA Methyltransferase RmtC in *Salmonella enterica* Serovar Virchow

Katie L. Hopkins, Jose A. Escudero, Laura Hidalgo, and Bruno Gonzalez-Zorn

We screened *Salmonella* and *Escherichia coli* isolates, collected 2004–2008 in the United Kingdom, for 16S rRNA methyltransferases. *rmtC* was identified in *S. enterica* serovar Virchow isolates from clinical samples and food. All isolates were clonally related and bore the *rmtC* gene on the bacterial chromosome. Surveillance for and research on these resistance determinants are essential.

Aminoglycosides are used in treating a wide range of infections caused by both gram-negative and gram-positive bacteria and have been classified by the World Health Organization as critically important antimicrobial drugs in human medicine (*1*). They inhibit bacterial protein synthesis by binding irreversibly to the bacterial 16S ribosomal subunit, which thereby leads to cell death. Resistance to these antimicrobial agents usually results from production of aminoglycoside-modifying enzymes (such as acetyltransferases, phosphorylases, and adenyltransferases), reduced intracellular antimicrobial drug accumulation, or mutation of ribosomal proteins or rRNA. An additional mechanism, methylation of the aminoacyl site of 16S rRNA, confers high-level resistance to clinically important aminoglycosides such as amikacin, tobramycin, and gentamicin. Six types of 16S rRNA methyltransferase genes conferring resistance to these antimicrobial agents, *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, and *npmA*, have been identified (*2*,*3*). *armA* and *rmtB* are spread in enterobacteria worldwide, and the presence of other methyltransferase genes have not previously been reported in Europe (*3*). With the exceptions of *armA* and *rmtB* in porcine *Escherichia coli* from Spain and the People's Republic of China, respectively (*4*,*5*), all methyltransferase genes described have been identified in human clinical samples, for which a possible role for food in transmission of these determinants remains largely unknown. Despite large surveys performed to identify 16S rRNA methyltransferases, the *rmtC* gene has been detected in only 2 *Proteus mirabilis* clinical isolates from Japan and Australia in 2006 and 2008, respectively (*3*,*6*,*7*). In this study, 81,632 *Salmonella* and 10,700 *Escherichia coli* isolates obtained from the Health Protection Agency (HPA) Centre for Infections culture collection (isolated from January 2004 through December 2008) were screened for the presence of 16S rRNA methyltransferases.

Author affiliations: Health Protection Agency Centre for Infections, London, UK (K.L. Hopkins); and Universidad Complutense de Madrid, Madrid, Spain (J.A. Escudero, L. Hidalgo, B. Gonzalez-Zorn)

DOI: 10.3201/eid1604.090736

The Study

Salmonella enterica (56 isolates) and *Escherichia coli* (24 isolates) were selected from the HPA collection based on their resistance to amikacin (breakpoint concentration routinely used in HPA *Salmonella* Reference Unit = 4 μg/mL). Because 16S rRNA methyltransferases confer high-level resistance to amikacin, 13 *S. enterica* isolates were selected on the basis of ability to grow on Isosensitest agar containing 500 μg/mL amikacin, whereas none of the *E. coli* isolates grew under these conditions. All isolates belonged to serotype Virchow. Further antimicrobial susceptibility testing by microdilution by using dehydrated Sensititer plates following the CLSI guidelines confirmed high-level resistance to 4,6-disubstituted 2-deoxystreptamines (Table 1). PCR screening of the 13 isolates for *armA*, *rmtA*, *rmtB*, *rmtC*, and *rmtD* (*8*) identified *rmtC*. Nucleotide sequencing of the amplicons confirmed an *rmtC* gene with 100% identity with those originally identified in *Proteus mirabilis* strain ARS68 isolated from an inpatient in Japan (*6*) and *P. mirabilis* strain JIE273 from Australia (*7*). To our knowledge, this is the third report of *rmtC*-bearing bacteria. Class one integrons were amplified (*9*), and sequenced. Isolates resistant to neomycin bore the *aac(6')-Ib* gene cassette, whereas the *dfrA1* gene was responsible for resistance to trimethoprim.

Twelve of the 13 *S. enterica* strains were originally isolated over a 4-year period from patients with clinical infection; 1 strain was obtained from frozen produce. Seven of 12 strains were obtained from patients with histories of foreign travel; 4 of the 7 patients had reported recent travel to India (Table 2). *P. mirabilis* strain JIE273 was also isolated from a patient who had recently returned from India (*7*). Investigations to ascertain the presence of *rmtC* genes in India are under way. To identify a possible link between the isolates, chromosomal DNA was embedded in agarose plugs prepared according to the pulsed-field gel electrophoresis (PFGE) protocol of PulseNet Europe (*10*). PFGE patterns showed only 1–2-band differences (Figure 1) and correlated with phage typing data (Table 1). All clinical isolates were recovered from feces, except a blood isolate recovered from a patient with invasive salmonellosis (Table 2). The temporal and geographic distribution of the isolates suggested independent acquisition of infections in most cases and possibly epidemiologically linked cases, e.g., strains 9 and 10 (Table 2; Figure 2).

Table 1. Phage types for *Salmonella enterica* serovar Virchow isolates bearing *rmtC* and MICs of selected antimicrobial agents*

Isolate	Phage type	GEN	KAN	AMK	TOB	ARB	NEO	TMP	CPX	AMP
HO 5164 0340	ND	>512	>512	>512	>512	>512	64	≤0.5	0.5	1
HO 5366 0426	30	>512	>512	>512	>512	>512	2	>32	0.25	≤0.05
HO 6018 0151	30	>512	>512	>512	>512	>512	2	>32	0.25	1
HO 6316 0322	30	>512	>512	>512	>512	>512	32	≤0.5	0.25	1
HO 6398 0463	30	>512	>512	>512	>512	>512	32	≤0.5	0.25	1
HO 7078 0136	30	>512	>512	>512	>512	>512	2	>32	0.25	≤0.05
HO 7310 0210	31	>512	>512	>512	>512	>512	4	>32	0.25	1
HO 7468 0335	25	>512	>512	>512	>512	>512	2	>32	0.5	1
HO 7474 0467	25	>512	>512	>512	>512	>512	4	>32	0.25	≤0.05
HO 7496 0137	25	>512	>512	>512	>512	>512	2	>32	0.25	1
HO 7512 0259	25	>512	>512	>512	>512	>512	4	>32	0.25	1
HO 8354 0857	25	>512	>512	>512	>512	>512	4	>32	0.25	≤0.05
HO 8512 0713	25	>512	>512	>512	>512	>512	4	>32	0.25	1

*MICs are given in μg/mL. GEN, gentamicin; KAN, kanamycin; AMK, amikacin; TOB, tobramycin; ARB, arbekacin; NEO, neomycin; TMP, trimethoprim; CPX, ciprofloxacin; AMP, ampicillin; ND, not determined.

PCR with primers ISEcp1R-F and *rmtC*-down (*7*) showed that the *rmtC* gene and immediate upstream sequences (GenBank accession nos. FJ984623–FJ984634 for human isolates and GQ131574 for the food isolate) were identical to those previously identified in *P. mirabilis* (*6*,*7*), in which IS*Ecp1* has been shown to play a role in the expression and transposition of the *rmtC* gene (*11*). However, the complete IS*Ecp1* element could not be amplified by using primers IS*Ecp1* 5′ and IS*Ecp1* reverse, which suggests either partial deletion of this element or involvement of a different IS*Ecp1*-like element in spread of *rmtC* in *Salmonella* (*6,12*). Attempts to isolate *rmtC* by conjugal transfer to rifampin-resistant *E. coli* 20R764 were unsuccessful, as was electroporation into *E. coli* LMG194 and ElectroMAX DH10B cells (both Invitrogen, Paisley, UK) by using plasmid preparations. An ≈100-kb *rmtC*-bearing plasmid was previously transferred from *P. mirabilis* ARS68 by electroporation but could not be mobilized by conjugation (*6*), and attempts to transfer the *rmtC* plasmid from *P. mirabilis* JIE273 by electroporation and conjugation failed (*7*). This finding contrasts with some qualities of the other methyltransferases, such as *armA* and *rmtB*, which are mostly located on conjugative plasmids (*8,13*).

The location of the *rmtC* gene was determined with PFGE by using I-*Ceu*I nuclease treatment. Agarose plugs were digested with 9.5 U I-*Ceu*I nuclease (New England Biolabs, Beverly, MA, USA). Separated DNA fragments were transferred onto a nylon membrane (GE Healthcare, Madrid, Spain) and hybridized with 16S rDNA and *rmtC* probes labeled with DIG-11-dUTP. Hybridization, labeling, and detection were performed according to the manufacturer's recommendations (Roche Applied Science, Mannheim, Germany). A DNA band hybridized with both probes, showing that the *rmtC* gene was located on the chromosome. Results of hybridization of plasmid extractions (Plasmid Midi kit; QIAGEN, Inc., Chatworth, CA, USA) with the *rmtC* probe were negative (data not shown).

Conclusions

We describe the occurrence of 16S rRNA methyltransferase *rmtC* in *Salmonella* isolates and the *rmtC* gene in

Table 2. Epidemiologic information from *rmtC*-positive *Salmonella enterica* serovar Virchow isolates, United Kingdom, 2004–2008*

Isolate	Date received	Map no.	Location	Sample type	Symptoms	Travel history
HO 5164 0340	2005 Apr 20	1	Reading	Feces	ND	ND
HO 5366 0426	2005 Sept 8	2	London	Feces	Diarrhea	Unknown destination
HO 6018 0151	2006 Jan 6	3	Wexham	Feces	ND	ND
HO 6316 0322	2006 Aug 3	4	Nottinghamshire	Feces	Diarrhea	No recent travel
HO 6398 0463	2006 Sept 29	5	London	Blood	Fever and diarrhea	India
HO 7078 0136	2007 Feb 16	6	Orpington	Feces	Diarrhea	India
HO 7310 0210	2007 July 30	7	Wrexham	Feces	Diarrhea	Unknown destination
HO 7468 0335	2007 Nov 16	8	Bedford	Feces	Enteritis	India
HO 7474 0467	2007 Nov 21	9	West Sussex	Feces	Diarrhea	ND
HO 7496 0137	2007 Dec 6	10	West Sussex	Feces	Diarrhea	ND
HO 7512 0259	2007 Dec 18	11	Surrey	Feces	Diarrhea	India
HO 8354 0857	2008 Aug 27	12	Kent	ND	Diarrhea	Unknown destination
HO 8512 0713	2008 Dec 16	13	Spalding	Food	NA	NA

*ND, not determined; NA, not applicable.

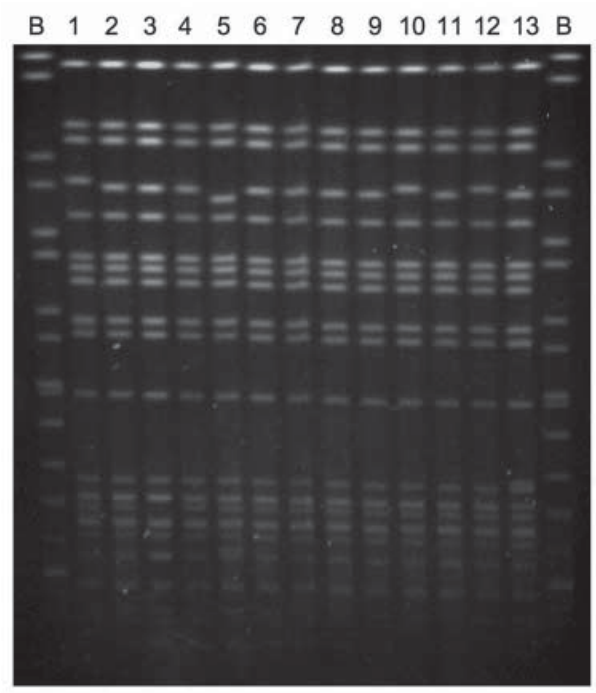


Figure 1. Pulsed-field gel electrophoresis patterns of *rmtC*-positive *Salmonella enterica* serovar Virchow isolates. Lanes: B, *S.* Braenderup H9812 size standard; 1, H0 5164 0340; 2, H0 5366 0426; 3, H0 6018 0151; 4, H0 6136 0322; 5, H0 6398 0463; 6, H0 7078 0136; 7, H0 7310 0210; 8, H0 7468 0335; 9, H0 7474 0467; 10, H0 7496 0137; 11, H0 7512 0259; 12, H0 8354 0857; and 13, H0 8512 0713.

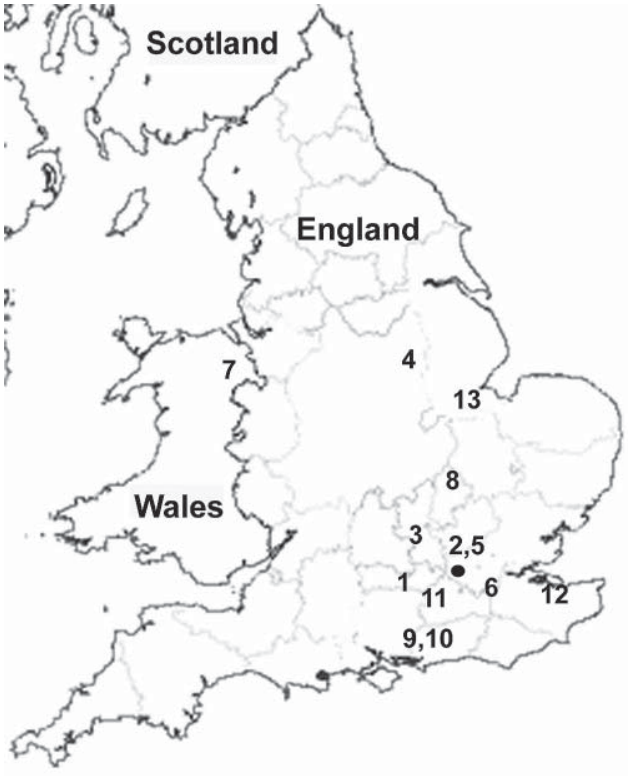


Figure 2. Map of United Kingdom showing geographic location of the 13 *Salmonella enterica* serovar Virchow isolates bearing *rmtC*. Each number represents 1 isolate in chronologic order of isolation as shown in Table 2.

Europe. We also report that a producer of 16S rRNA methyltransferase was isolated from food.

The overall isolation frequency of 16S rRNA methyltransferase–producing *S. enterica* is low (13/81,632 strains) in the United Kingdom, and these genes were absent in *E. coli*. However, spread of multidrug-resistant isolates that express 16S rRNA methyltransferases, amplified by the association of these genes with the IS*Ecp1* element, raises clinical concern that further spread is likely. Ongoing surveillance of 16S rRNA methyltransferases in isolates found in food products and in humans and animals is crucial to delay the spread of resistance to these classes of antimicrobial agents.

Addendum

While this manuscript was under revision, an *S. enterica* ser. Virchow isolate bearing the *rmtC* gene isolated from a child with a history of travel to India was reported in the United States (*14*).

Acknowledgment

We thank Michel Doumith of the HPA Antimicrobial Resistance Monitoring and Reference Laboratory for technical advice and assistance with the electroporation experiments.

This study was supported by work package 29 of the MedVet-Net Network of Excellence (FOOD-CT-2004-506122). Strain requests should be addressed to the Health Protection Agency Centre for Infections (katie.hopkins@hpa.org.uk).

Dr Hopkins is a researcher in the Gastrointestinal, Emerging and Zoonotic Infections Department at the Health Protection Agency Centre for Infections (London, UK). Her research interests focus on mechanisms of antimicrobial resistance in enteric pathogens and development and application of DNA-based typing methods for epidemiologic investigations.

References

1. World Health Organization. Critically important antimicrobials for human medicine: categorization for the development of risk management strategies to contain antimicrobial resistance due to nonhuman antimicrobial use: report of the second WHO expert meeting, 29–31 May 2007, Copenhagen, Denmark. Geneva: The Organisation; 2007 [cited 2009 Jan 15]. http://www.who.int/foodborne_disease/resistance/antimicrobials_human.pdf
2. Yamane K, Wachino J, Doi Y, Kurokawa H, Arakawa Y. Global spread of multiple aminoglycoside resistance genes. Emerg Infect Dis. 2005;11:951–3.

3. Fritsche TR, Castanheira M, Miller G, Jones R, Armstrong E. Detection of methyltransferases conferring high-level resistance to aminoglycosides in enterobacteriaceae from Europe, North America, and Latin America. Antimicrob Agents Chemother. 2008;52:1843–5. DOI: 10.1128/AAC.01477-07
4. Gonzalez-Zorn B, Teshager T, Casas M, Porrero MC, Moreno MA, Courvalin P, et al. *armA* and aminoglycoside resistance in *Escherichia coli.* Emerg Infect Dis. 2005;11:954–6.
5. Chen L, Chen Z, Liu J, Zeng Z, Ma J, Jiang H. Emergence of RmtB methylase–producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. J Antimicrob Chemother. 2007;59:880–5. DOI: 10.1093/jac/dkm065
6. Wachino J, Yamane K, Shibayama K, Kurokawa H, Shibata N, Suzuki S, et al. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrob Agents Chemother. 2006;50:178–84. DOI: 10.1128/AAC.50.1.178-184.2006
7. Zong Z, Partridge S, Iredell J. RmtC 16S rRNA methyltransferase in Australia. Antimicrob Agents Chemother. 2008;52:794–5. DOI: 10.1128/AAC.01399-07
8. Doi Y, Arakawa Y. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clin Infect Dis. 2007;45:88–94. DOI: 10.1086/518605
9. Lévesque C, Piché L, Larose C, Roy P. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob Agents Chemother. 1995;39:185–91.
10. Peters TM, Maguire C, Threlfall E, Fisher I, Gill N, Gatto A. The Salm-gene project—a European collaboration for DNA fingerprinting for food-related salmonellosis. Euro Surveill. 2003;8:46–50.
11. Wachino J, Yamane K, Kimura K, Shibata N, Suzuki S, Ike Y, et al. Mode of transposition and expression of 16S rRNA methyltransferase gene *rmtC* accompanied by ISEcp1. Antimicrob Agents Chemother. 2006;50:3212–5. DOI: 10.1128/AAC.00550-06
12. Doi Y, Adams-Haduch J, Paterson D. Genetic environment of 16S rRNA methylase gene *rmtD.* Antimicrob Agents Chemother. 2008;52:2270–2. DOI: 10.1128/AAC.00037-08
13. Gonzalez-Zorn B, Catalan A, Escudero JA, Dominguez L, Teshager T, Porrero C, et al. Genetic basis for dissemination of *armA.* J Antimicrob Chemother. 2005;56:583–5. DOI: 10.1093/jac/dki246
14. Folster JP, Rickert R, Barzilay EJ, Whichard JM. Identification of the aminoglycoside resistance determinants *armA* and *rmtC* among non-Typhi *Salmonella* isolates from humans in the United States. Antimicrob Agents Chemother. 2009;53:4563–4. DOI: 10.1128/AAC.00656-09

Address for correspondence: Bruno Gonzalez-Zorn, Departamento de Sanidad Animal, Facultad de Veterinaria and VISAVET, Universidad Complutense de Madrid, 28040, Spain; email: bgzorn@vet.ucm.es

Reemergence of Dengue in Mauritius

Mohammad I. Issack, Vidula N. Pursem, Timothy M.S. Barkham, Lee-Ching Ng, Masafumi Inoue, and Shyam S. Manraj

Dengue reemerged in Mauritius in 2009 after an absence of >30 years, and >200 cases were confirmed serologically. Molecular studies showed that the outbreak was caused by dengue virus type 2. Phylogenetic analysis of the envelope gene identified 2 clades of the virus. No case of hemorrhagic fever was recorded.

Mauritius is a tropical island nation of 1,865 km^2 in the southwestern Indian Ocean, ≈2,000 km off the coast of eastern Africa. It has a population of 1.25 million, with ≈68% of Indian origin and 27% of predominantly African or mixed ancestry. It is classified by the World Bank as an upper-middle-income country. The economy is diversified; textiles, tourism, sugar cane, banking, and business process outsourcing are the main sectors. Most Mauritians have a sedentary lifestyle.

In 2008, a total of 930,000 visitors traveled to Mauritius for tourism and business; 98% arrived by air (*1*). They were mostly from western Europe (60.5%), Réunion island (10.3%), South Africa (9.1%), and India (4.7%).

Mauritius was, for many years, essentially free from indigenous dengue and chikungunya disease until 2005 and 2006 when outbreaks of chikungunya occurred, with *Aedes albopictus* mosquitoes as the vector (*2*). In the region, a well-documented epidemic of dengue fever caused by dengue virus type 2 (DENV-2) occurred on Réunion island in 1977–1978, and 2 outbreaks were caused by the same dengue serotype in the Seychelles in 1976–1977 and 1978–1979 (*3*,*4*). Although no record was made of laboratory-confirmed cases in the 1970s in Mauritius, a subsequent seroepidemiologic study suggests that cases of dengue also occurred in the country around that time, and it is reasonable to postulate that they were also caused by DENV-2 (*5*). Since then, apart from the occasional imported case, no evidence of dengue transmission has been reported for >30 years in Mauritius. However, dengue fever reemerged in the country in 2009, and we report on the laboratory investigation of the outbreak.

Author affiliations: Central Health Laboratory, Candos Mauritius (M.I. Issack, V.N. Pursem, S.S. Manraj); Tan Tock Seng Hospital, Singapore (T.M.S. Barkham); Environmental Health Institute, Singapore (L.-C. Ng); and Institute of Molecular and Cell Biology, Singapore (M. Inoue)

DOI: 10.3201/eid1604.091582

The Outbreak

At the end of April 2009, a physician from a private clinic in Port-Louis, the capital city, reported having seen several patients with fever, malaise, diarrhea, increased levels of liver enzymes, and marked thrombocytopenia. Rash and arthralgia were not mentioned, and no test was requested for dengue or chikungunya viruses. On June 1, another physician requested dengue serologic testing for a patient who had fever for the past 10 days, rigors, generalized aches and pains, and a petechial rash. The patient had lived in Malaysia several years previously but had no history of recent travel. His liver enzyme levels were increased, and his thrombocyte count was 15,000 cells/μL. At the Central Health Laboratory (CHL) in Mauritius, the patient's serum was positive for immunoglobulin (Ig) G and IgM against dengue with the Hexagon Dengue rapid immunochromatography test (Human GmbH, Wiesbaden, Germany) and negative for Platelia dengue nonstructural protein 1 (NS1) (Bio-Rad Laboratories, Marnes-la-Coquette, France).

The next day, 6 patients who had previously been admitted with fever at the above-mentioned private clinic were traced by the Department of Public Health. They all lived in the same suburb of the capital city. Serum samples from all 6 patients showed IgG and IgM against dengue. The Ministry of Health and Quality of Life immediately initiated an action plan which included mosquito control measures by fogging and larviciding, environmental cleaning, and a public awareness campaign on how to eliminate mosquito breeding sites. The first 10 positive serum specimens, all of which had dengue antibodies were sent for confirmation to the National Health Laboratory Services in South Africa, where all were subsequently found to be positive for dengue IgG and IgM by hemagglutination-inhibition test and ELISA, respectively.

On June 5, dengue NS1 antigen was detected at CHL from serum specimens from 3 patients. Four days later, serum samples from 11 patients that were positive for dengue antigen were sent to Tan Tock Seng Hospital in Singapore for reverse transcription–PCR (RT-PCR) testing. Approximately 24 hours later, the laboratory reported that DENV RNA was detected in 7 of the samples by real-time RT-PCR, with previously described primers using SYBR Green and gel electrophoresis (*6*). The serum samples were later found to be positive for DENV-2 by multiplex RT-PCR with serotype-specific primers, and detection with serotype-specific probes by using a Luminex xMAP-based assay (Luminex, Austin, TX, USA) (unpub. technique).

Subsequently, nucleotide sequencing and phylogenetic analysis of the envelope gene from the PCR products of the 7 positive serum specimens showed that, although all the viruses belonged to the Cosmopolitan genotype, 2 separate clades were present. Four samples clustered with isolates from India, and the remaining 3 were most closely related

to an isolate from Sri Lanka (Figure). Overall, during June, dengue NS1 antigen was detected in the serum specimens of 194 patients. In 40 other cases, the serum specimens tested positive for dengue IgM by immunochromatography or capture ELISA (Panbio, Brisbane, Queensland, Australia) but negative for NS1 antigen. All patients were clustered in suburbs of Port-Louis or had traveled there. Only 5 and 3 new cases of dengue were diagnosed in July and August, respectively, and no case was reported in September. The case-patients ranged in age from1 to 91 years; median age was 36 years, and 52.5% were male.

Conclusions

Dengue has reemerged in Mauritius after >30 years, but the outbreak was short-lived because of the institution of control measures and the arrival of cooler and drier weather. In the affected areas, monthly mean maximum temperature dropped from 28.3°C in June to 26.4°C in August, and total monthly rainfall amount fell from 126.4 mm in May to 44.8 mm in August. The outbreak was also restricted to some suburbs of the capital city, possibly because of of relatively warm temperatures and high population density. The reemergence was probably caused by introduction of DENV-2 by unrecognized infective travelers. The high bootstrap value of 94% in the phylogenetic analysis suggests at least 2 separate importations of DENV-2 occurred. In 2008, an imported case of dengue was diagnosed in a child returning from India, but control measures were rapidly instituted and no local transmission occurred.

No case of dengue hemorrhagic fever was recorded in this outbreak, probably because the population has not been exposed previously to another serotype. The vector of the outbreak was likely to have been *Ae. albopictus* mosquitoes, which are widely distributed in Mauritius (*Ae. aegypti* was eradicated from the country in the early 1950s as a result of a DDT indoor-spraying campaign in 1949–1951 to control malaria) (*7*). However, the rapid increase in the number of observed cases in June is more consistent with an *Ae. aegypti*–borne dengue outbreak, and a new comprehensive entomologic study is needed to exclude the possibility that *Ae. aegypti* has recently been reintroduced into Mauritius.

Whether DENV-2 will persist in Mauritius throughout the winter and lead to more cases next summer, despite maintenance of intensive mosquito control programs, is uncertain However, all practical measures must be taken to prevent introduction and transmission of another DENV serotype in Mauritius to minimize the risk for dengue hemorrhagic fever. In particular, surveillance of travelers from dengue-endemic regions should be instituted. The thermal scanner, recently installed at Mauritius' only airport, could be used to screen passengers for dengue fever because a study from Taiwan suggested that fever screening at airports was a cost-effective means of identifying many imported dengue cases (*8*). Moreover, the present policy of monitoring all persons arriving from malaria-endemic areas for fever and parasitemia could be extended to include testing for dengue in febrile travelers arriving from dengue-endemic areas. The recently opened CHL molecular biology unit needs to be ready by next summer to detect and serotype dengue viruses to enable prompt diagnosis and epidemiologic evaluation of any new case.

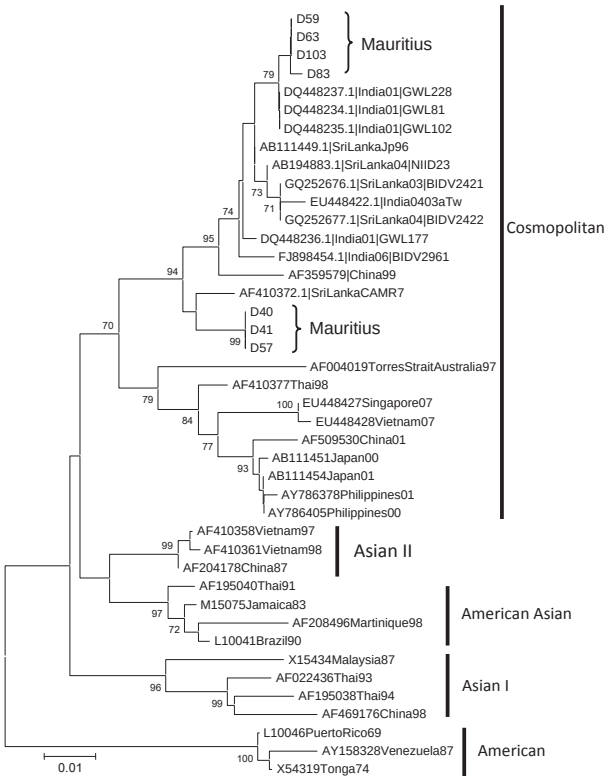


Figure. Phylogenetic relationships of dengue virus isolates from Mauritius inferred by envelope (E) gene sequence by using the maximum-likelihood method as implemented in PAUP* version 4.0b10 (http://paup.csit.fsu.edu/about.html). Primers used for amplification of product for sequencing were 5′-AATCCAGATG TCATCAGGAAAC-3′ and 5′-CCTATAGATGTGAACACTCCTCC-3′. The E gene sequences were consolidated from overlapping, bidirectional sequences. Scale bar indicates nucleotide substitutions per site.

Acknowledgments

We thank Devendranauth Gooljar for collecting the data on serologically confirmed cases and Deoraj Caussy for reviewing the manuscript. We are grateful to the Mauritius Meteorological Services for providing rainfall and temperature data. We also thank Sharon Lo for performing the nucleotide sequencing.

Dr Issack is a consultant pathologist and head of the bacteriology section at the Central Health Laboratory of the Ministry of

Health in Mauritius. His main research interests include the epidemiology of infectious diseases and antimicrobial drug resistance.

References

1. Central Statistics Office. Digest of international travel and tourism statistics—2008 [cited 2009 Dec 8]. Government of Mauritius; 2009. http://www.gov.mu/portal/goc/cso/report/natacc/tourism08/toc.htm
2. Beesoon S, Funkhouser E, Kotea N, Spielman A, Robich RM. Chikungunya fever, Mauritius, 2006. Emerg Infect Dis. 2008;14:337–8. DOI: 10.3201/eid1402.071024
3. Coulanges P, Clercy Y, Jousset FX, Rodhain F, Hannoun C. Dengue at Reunion: isolation of a strain at the Pasteur Institute of Madagascar [in French]. Bull Soc Pathol Exot Filiales. 1979;72:205–9.
4. Calisher CH, Nuti M, Lazuick JS, Ferrari JD, Kappus KD. Dengue in the Seychelles. Bull World Health Organ. 1981;59:619–22.
5. Schwarz TF, Dobler G, Gilch S, Jäger G. Hepatitis C and arboviral antibodies in the island populations of Mauritius and Rodrigues. J Med Virol. 1994;44:379–83. DOI: 10.1002/jmv.1890440411
6. Barkham TM, Chung YK, Tang KF, Ooi EE. The performance of RT-PCR compared with a rapid serological assay for acute dengue fever in a diagnostic laboratory. Trans R Soc Trop Med Hyg. 2006;100:142–8. DOI: 10.1016/j.trstmh.2005.05.015
7. Gopaul AR. The common man-biting mosquitoes of Mauritius. The Mauritius Institute Bulletin. 2003;XI:9–19.
8. Shu PY, Chien LJ, Chang SF, Su CL, Kuo YC, Liao TL, et al. Fever screening at airports and imported dengue. Emerg Infect Dis. 2005;11:460–2.

Address for correspondence: Mohammad I. Issack, Central Health Laboratory, Victoria Hospital, Candos, Mauritius; email: missack@intnet.mu

One Flu for One Health

To the Editor: The emergence and spread of influenza A pandemic (H1N1) 2009 virus from the animal reservoir to humans raise questions about the future approach to influenza virus infections. The scientific community has evidence demonstrating that influenza virus genes migrate across continents and animal species and assemble themselves in combinations that are a threat to animal and human health, resulting in panzootics like that caused by influenza A virus (H5N1) or pandemics like that caused by pandemic (H1N1) 2009 virus. The latter virus emerged from the animal reservoir, containing a unique combination of genes donated by viruses originating from 3 species and 2 hemispheres. In a globalized environment, mapping gene movement across species and national borders and identifying mutations and gene constellations with pandemic potential or virulence determinants are essential to enact prevention and control strategies at a global level. This conclusion is in agreement with, and possibly the best example of, the One Health (http://un-influenza.org/node/2341) vision: a multidisciplinary collaborative approach to improving the health of humans, animals, and the environment. One Health is endorsed by the United Nations Food and Agriculture Organization, the World Organisation for Animal Health, and the World Health Organization.

Vast improvements in capacity building have been achieved as a result of the influenza A (H5N1) global crisis. Thousands of viral isolates with zoonotic potential have been obtained through surveillance efforts, although the genetic information has not been exploited fully. In addition, investigating how influenza viruses circulate in certain species, including dogs, pigs, and horses, has been neglected. This neglect is evidenced by the fact that, at the time of this writing, GenBank contained 4,001 full genome sequences of influenza viruses isolated from humans, 2,590 of viruses isolated from birds, and only 325 from swine, 85 from horses, 2 from mink, 4 from dogs, 2 from cats, 2 from tigers, and 3 from seals.

We invite donors and international agencies to invest in a novel approach to influenza virus infections, to abandon prefixed compartments linked to geographic origin or species of isolation, and to analyze the influenza gene pool as one entity. We propose capitalizing on existing achievements and investments to develop an international network and a permanent observatory, which will improve our understanding of the dynamics of the influenza virus gene pool in animals and humans. A greater understanding will generate important information to support both public and animal health. Ideally, a small consortium, including representatives of major international organizations, could take leadership and liaise with major institutions involved in influenza surveillance and research to develop a feasibility study and roadmap to achieve this goal. The One Flu initiative could result in international synergies, the bridging of gaps between medical and veterinary scientists, permanent monitoring of virus evolution and epidemiology, and the best exploitation of investments in capacity building. Above all, this collaboration could be a challenge and opportunity to implement the One Health vision, and possibly act as a model for other emerging zoonotic diseases.

Ilaria Capua and Giovanni Cattoli

Author affiliations: World Organisation for Animal Health, United Nations Food and Agriculture Organization, and National Reference Laboratory for Newcastle Disease and Avian Influenza, Legnaro, Italy; and World Organisation for Animal Health Collaborating Centre for Diseases at the Human Animal Interface, Legnaro

DOI: 10.3201/eid1604.091593

Address for correspondence: Ilaria Capua, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro, Padova, Italy; email: icapua@izsvenezie.it

Pandemic (H1N1) 2009 Risk for Nurses after Trivalent Vaccination

To the Editor: We report results of the effect of inactivated seasonal influenza vaccination on risk of pandemic (H1N1) 2009 in a cohort of nurses in Canada who participated in a recent randomized controlled trial that compared the effectiveness of surgical masks with that of N95 respirators in preventing influenza (*1*). From September 23, 2008, through December 8, 2008, a total of 446 nurses from 8 hospitals in the province of Ontario were enrolled. They were then randomly assigned an intervention; 225 were assigned to wear surgical masks, and 221 were assigned to wear the N95 respirator. The mean age of participants was 36.2 years; 94% were women. A total of 128 (30.3%) received the trivalent influenza vaccine. Vaccination status was similar between the groups: 68 (30.2%) persons in the surgical mask group and 62 (28.1%) persons in the N95 respirator group had received the 2008–2009 trivalent inactivated influenza vaccine. The nurses were monitored from January 12, 2009, through April 23, 2009.

Blood specimens for serologic analysis were obtained before enrollment and at the end of the follow-up period. End-of-study serum samples were collected from April 23 through May 15, 2009. Serologic infection

was defined by a ≥4-fold increase in influenza-specific hemagglutinin inhibition assay titer between baseline and convalescent-phase serum samples by using turkey erythrocytes and A/TN/1560/2009(H1N1), a representative pandemic influenza virus.

Of the 422 nurses included in the analysis, 42 (10.0%) showed seroconversion to pandemic (H1N1) 2009. Of 128 nurses who received the trivalent influenza vaccine, 9 (7.0%) showed seroconversion vs. 33 (11.2%) of those that did not (relative risk 0.63, 95% confidence interval 0.31–1.27, p = 0.19).

Although the point estimate was protective, the confidence interval is wide and does not exclude an increase in risk. Our sample size limits inferences that can be drawn. Heterotypic antibodies may have contributed to the relatively high rate of seroconversion. A rise in antibody titer is considered by some as an outcome associated with bias, unlike virus identification. Nevertheless, these data suggest a possible positive effect of seasonal influenza vaccine reducing risk of infection with pandemic (H1N1) 2009.

This study was funded by the Public Health Agency of Canada.

Mark Loeb, David J.D. Earn, Marek Smieja, and Richard Webby

Author affiliations: McMaster University, Hamilton, Ontario, Canada (M. Loeb, D.J.D. Earn, M. Smieja); and St. Jude Children's Research Hospital, Memphis, Tennessee, USA (R. Webby)

DOI: 10.3201/eid1604.091588

Reference

1. Loeb M, Dafoe N, Mahony J, John M, Sarabia A, Glavin V, et al. Surgical mask vs N95 respirator for preventing influenza among health care workers: a randomized trial. JAMA. 2009;302:1865–71. DOI:10.1001/jama.2009.1466

Address for correspondence: Mark Loeb, McMaster University, 1200 Main St W, Hamilton, Ontario L8N 3Z5, Canada; email: loebm@mcmaster.ca

Patients with Pandemic (H1N1) 2009 in Intensive Care Units, Israel

To the Editor: We report results of an active surveillance system established by the Tel Aviv District Health Office in Israel. This surveillance system monitors the daily status of patients with laboratory-confirmed pandemic (H1N1) 2009 virus infection in each of the district's intensive care units (ICUs), including pediatric ICUs.

Follow-up is maintained by daily phone conversations with medical staff until disease outcome is concluded by discharge, transfer to a long-term rehabilitation facility, or death. Medical records, as well as daily laboratory reports, are collected to confirm or to rule out pandemic (H1N1) 2009 infection.

During July 10–October 10, 2009, our prospective cohort included 17 patients with pandemic (H1N1) 2009 laboratory-confirmed infection who were residents of the district; 12 (70.6%) were male patients. The median age was 44 years (interquartile range 13–72 years). By October 10, 2009, six patients had been discharged, 7 had died, 2 had been transferred to long-term rehabilitation facilities, and 2 remained hospitalized.

Twelve (70.6%) patients had an underlying medical condition, mainly chronic lung disease (6 patients) or chronic cardiovascular disease (5 patients). Two patients were morbidly obese (body mass index ≥35), and 1 patient was pregnant. Additionally, 3 patients (17.6%) were infected while hospitalized.

Thirteen patients (76.5%) had acute respiratory distress syndrome caused by diffuse viral pneumonitis. Other notable manifestations were acute renal failure (6 patients), sepsis/septic shock (5 patients), and neurologic complications such as Guillain-Barré syndrome, encephalitis, and seizures (3 patients).

Documented nosocomial sepsis, often of multiple gram-negative bacteria (9 patients), was the most frequent complication during the course of the disease. Other frequent characteristics were the use of high positive end-expiratory pressure during mechanical ventilation (4 patients) and the need for tracheostomy (5 patients).

Average time from disease onset to hospital admission was 3 days. Time from hospital admission to ICU admission for those patients who died was longer than for those who survived, with a median of 2 days compared with 0.5 day, respectively, albeit not significant (p = 0.26). Average hospitalization was 23.4 days; average length of stay in the ICU was 16.7 days (71.4% of the average hospitalization time).

As mentioned previously, 7 patients (41.2%) died; 5 (71.4%) were male, similar to their cohort's proportion. One significant difference (p = 0.02) was found between the age of survivors (mean 26.0 years, 95% confidence interval 7.6–44.3) and the age of nonsurvivors (mean 59.3 years, 95% confidence interval 39.6–79.0). The most prominent case–fatality rate was for elderly patients, >65 years of age (3 of 4 patients) followed by patients between 20 and 64 years of age (4 of 9 patients); these subgroups constituted 23.5% and 52.9% of the cohort, respectively.

Estimated incidence rate was 13.8 patients and 5.7 deaths in ICUs per million residents in the Tel Aviv district. Again, the elderly subgroup was

dominant, with the highest estimated rate of illness (23.1 per million residents) and death rate (17.3 per million residents). The denominator of these rates was calculated from the population data published by the Israeli Central Bureau of Statistics for 2007 and 2008. Upon that basis, the population data for the end of the third quarter of 2009 was estimated.

During the described surveillance period, 5.7% of ICU beds in the district were, on average, continuously occupied by patients infected with pandemic (H1N1) 2009. The occupancy peak was 6.5 of 53.8 standardized ICU beds (12.1%) per million residents during the week ending August 28, 2009 (Figure).

In conclusion, our analysis of patients having the most severe pandemic (H1N1) 2009 infection indicates a need for prolonged periods of hospitalizations, especially in ICUs, for young adults and elderly patients. Death or prolonged adverse complications were frequent outcomes. We found that the impact of patients with pandemic (H1N1) 2009 on the ICUs in our district during the summer wave was surprisingly similar in length and intensity to the impact that was recently reported in Australia and New Zealand during the winter wave (*1*). The maximum number of ICU beds occupied per million residents, reported for all regions of Australia and New Zealand combined, was 7.4 during the week ending July 27, 2009 (vs. 6.5 as described above). We also found that the mean age of those who died was older than that in previous reports (*2*–*6*).This finding may present a need for policymakers to reconsider current vaccination priorities (*7*) while facing the winter wave of influenza in the Northern Hemisphere.

Acknowledgments

We thank the medical team and administrative staff of the intensive care units in our district. We are grateful for the daily ongoing cooperative effort because it allows us to continuously monitor and evaluate the evolving pandemic status.

Eran Kopel, Ziva Amitai, Itamar Grotto, Ehud Kaliner, and Irina Volovik

Author affiliations: Ministry of Health, Tel Aviv, Israel

DOI: 10.3201/eid1604.091696

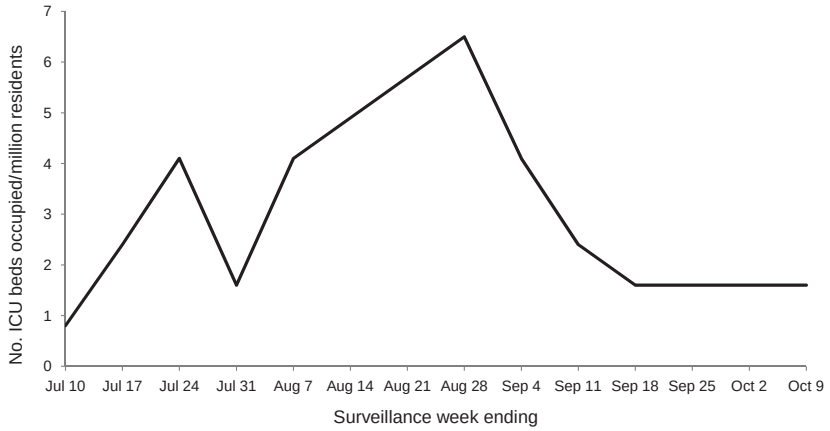


Figure. Number of intensive care unit (ICU) beds occupied by patients with pandemic (H1N1) 2009 infection in district ICUs during the described surveillance period, Tel Aviv, Israel. During this period, 5.7% of ICU beds, on average, were continuously occupied by patients with pandemic (H1N1) 2009 infection. The occupancy peak was 6.5 of 53.8 standardized ICU beds per million residents (12.1%) during the week ending August 28, 2009. Data are per million residents.

References

1. ANZIC Influenza Investigators, Webb SA, Pettilä V, Seppelt I, Bellomo R, Bailey M, Cooper DJ, et al. Critical care services and 2009 H1N1 influenza in Australia and New Zealand. N Engl J Med. 2009;361:1925–34. DOI: 10.1056/NEJMoa0908481
2. Kumar A, Zarychanski R, Pinto R, Cook DJ, Marshall J, Lacroix J, et al. Critically ill patients with 2009 influenza A (H1N1) infection in Canada. JAMA. 2009;302:1872–9. DOI: 10.1001/jama.2009.1496
3. Domínguez-Cherit G, Lapinsky SE, Macias AE, Pinto R, Espinosa-Perez L, de la Torre A, et al. Critically ill patients with 2009 influenza A (H1N1) in Mexico. JAMA. 2009;302:1880–7. DOI: 10.1001/jama.2009.1536
4. Jain S, Kamimoto L, Bramley AM, Schmitz AM, Benoit SR, Louie J, et al. Hospitalized patients with 2009 H1N1 influenza in the United States, April–June 2009. N Engl J Med. 2009;361:1935–44.
5. Rello J, Rodríguez A, Ibañez P, Socias L, Cebrian J, Marques A, et al. Intensive care adult patients with severe respiratory failure caused by influenza A (H1N1)v in Spain. Crit Care. 2009;13:R148. DOI: 10.1186/cc8044
6. Centers for Disease Control and Prevention. Intensive-care patients with severe novel influenza A (H1N1) virus infection–Michigan, June 2009. MMWR Morb Mortal Wkly Rep. 2009;58:749–52.
7. Centers for Disease Control and Prevention. Use of influenza A (H1N1) 2009 monovalent vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. MMWR Recomm Rep. 2009;58:1–8.

Address for correspondence: Eran Kopel, Ministry of Health, Tel Aviv District Health Office, 12 Ha'arba'ah, Tel Aviv 61203, Israel; email: eran.kopf@telaviv.health.gov.il

Risk for Transmission of Pandemic (H1N1) 2009 Virus by Blood Transfusion

To the Editor: Influenza A pandemic (H1N1) 2009 virus emerged in early 2009 in Mexico and has since spread worldwide. In Japan, the first outbreak of the novel influenza was reported in May 2009 (*1*) and became pandemic in November. Although no cases of transfusion-transmitted influenza have been published, evidence exists of brief viremia before onset of symptoms (*2*,*3*). The possibility of transmission of this virus through transfusion of donated blood is of concern. The Japanese Red Cross Blood Centers have intercepted blood products with accompanying postdonation information indicating possible pandemic (H1N1) 2009 infection and attempted to identify the viral genome in those products by using nucleic acid amplification technology (NAT).

During June–November 2009, blood samples were collected from plasma and erythrocyte products that had been processed from donations; postdonation information indicated diagnosis of pandemic (H1N1) 2009 infection soon after donation. Viral RNA was extracted from plasma samples and erythrocyte fractions by using a QIAamp Virus Biorobot MDx kit (QIAGEN, Valencia, CA, USA) and a High Pure Viral Nucleic Acid Large Volume kit (Roche Diagnostics, Indianapolis, IN, USA), respectively. RNA samples were subjected to real-time reverse transcription–PCR (RT-PCR) of hemagglutinin (HA) and matrix (M) genes of influenza A by using PRISM 7900 (Applied Biosystems, Foster City, CA, USA). The RT-PCR of HA was specific for pandemic (H1N1) 2009 virus, whereas the RT-PCR of M was designed to detect both pandemic (H1N1) 2009 and seasonal influenza A viruses. The sequences of probes and primers were synthesized according to the protocols developed by the Japanese National Institute of Infectious Diseases (*4*). Either 200 μL of a plasma sample or 100 μL of packed erythrocytes was used for each test, and the test was performed 2× for each gene in each sample. Before the investigation using donated blood samples, the sensitivity of the NAT system was checked by spiking experiments. Viral particles of pandemic (H1N1) 2009 virus (A/California/04/2009 [H1N1]), donated by the National Institute of Infectious Diseases, were spiked into plasma and erythrocyte samples from healthy volunteers. Viral RNA was detected in the plasma samples spiked with viral particles corresponding to 300 genome equivalents/mL and in the packed erythrocyte samples spiked with viral particles corresponding to 3,000 genome equivalents/mL.

NAT was conducted by using 96 plasma and 67 erythrocyte samples obtained from 96 blood donors who had symptoms of influenza within 7 days postdonation. For 20 donors, pandemic (H1N1) 2009 was diagnosed within 1 day postdonation and, for another 20, within 2 days postdonation (Figure). Pandemic (H1N1) 2009 virus was not found in any of the samples tested, but it was consistently detected in the external positive control. These results suggest that the viremia with pandemic (H1N1) 2009 virus, if any, is very low and can be missed by current NAT or that the viremic period is too brief to identify viremia. Although the risk for transmission of pandemic influenza by transfusion seems to be low, further investigation is needed to elucidate this risk.

Chieko Matsumoto, Rieko Sobata, Shigeharu Uchida, Takao Hidaka, Syunya Momose, Satoru Hino, Masahiro Satake, and Kenji Tadokoro

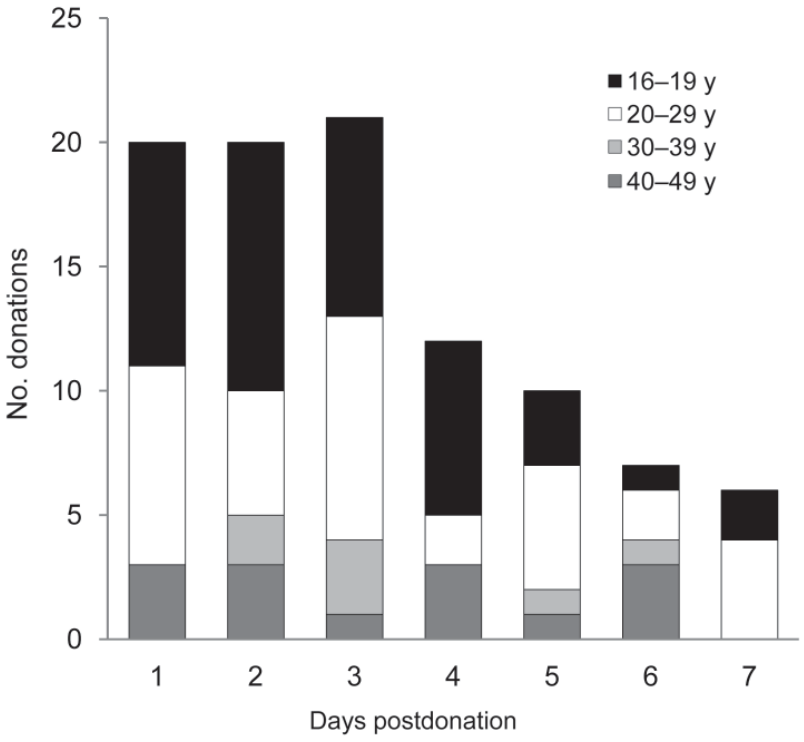


Figure. Number of blood donations from persons for whom pandemic (H1N1) 2009 infection was diagnosed postdonation and time between donation and diagnosis, by donor age, Japan.

Author affiliation: Japanese Red Cross Society Blood Service Headquarters, Tokyo, Japan

DOI: 10.3201/eid1604.091795

References

1. Shimada T, Gu Y, Kamiya H, Komiya N, Odaira F, Sunagawa T, et al. Epidemiology of influenza A (H1N1)v virus infection in Japan, May–June 2009. Euro Surveill. 2009;14:pii:19244.
2. Likos AM, Kelvin DJ, Cameron CM, Rowe T, Kuehnert MJ, Norris PJ. Influenza viremia and the potential for blood-borne transmission. Transfusion. 2007;47:1080–8. DOI: 10.1111/j.1537-2995.2007.01264.x
3. Khakpour M, Saidai A, Naficy K. Proved viraemia in Asian influenza (Hong Kong variant) during incubation period. BMJ. 1969;4:208–9. DOI: 10.1136/bmj.4.5677.208
4. Kageyama T. H1N1 novel influenza. In: Pathogen detection manual (version 1) [in Japanese]. Tokyo: National Institute of Infectious Diseases; 2009.

Address for correspondence: Chieko Matsumoto, Infectious Disease Research Department, Japanese Red Cross, Central Blood Institute, JRC 2-1-67 Tatsumi, Koto-ku, Tokyo 135-8521, Japan; email: c-matsumoto@jrc.or.jp

Rapid Emergence of Oseltamivir Resistance

To the Editor: The influenza A pandemic (H1N1) 2009 virus has spread globally since it first appeared in Mexico in April 2009. This third influenza pandemic since the Spanish influenza pandemic of 1918 (*1*) has caused at least 400,000 infections within 6 months; estimated mortality rate is 1.2% (*2*). Emergence of oseltamivir resistance in the pandemic (H1N1) 2009 virus is a rising challenge to global control of the pandemic. So far, 39 oseltamivir-resistant pandemic (H1N1) 2009 viruses have been reported worldwide (*3*). Among the 32 resistant strains reported in October 2009, a total of 13 (41%) were associated with postexposure chemoprophylaxis and 16 (50%) were from samples of patients receiving oseltamivir (*3*). We report rapid emergence of resistance (H275Y mutation) in a patient, 4 days after early treatment with standard doses of oseltamivir for pandemic (H1N1) 2009 pneumonia.

On September 1, 2009, a 20-year-old man with mental retardation consulted the emergency department of Kaohsiung Veterans General Hospital after 1 day of fever, sore throat, and nonproductive cough. A rapid diagnostic antigen test (Quick Vue Influenza test; Quidel, San Diego, CA, USA) showed the man to be positive for influenza A. He was hospitalized for bilateral pneumonitis and treated with oseltamivir (75 mg 2×/day for 5 days), ampicillin/sulbactam, and erythromycin. However, a progressive increase in bilateral perihilar interstitial infiltration developed on the third day, accompanied by increasing dyspnea. Influenza A pandemic (H1N1) 2009 virus was isolated from the patient's nasopharyngeal secretions on days 1 and 4 by using MDCK cells. After DNA sequence analysis of the neuraminidase gene, the mutation of H275Y was not found in the first isolate, but sequence analysis of the second isolate detected mixed populations (C/T) in the 823-nt position of the neuraminidase gene. Only a single pattern (T) was found from the cultured viruses, indicating a mixed quasispecies of oseltamivir-resistant and -susceptible viruses emerging after 4 days of oseltamivir treatment. The oseltamivir-resistant viruses become dominant in the cell culture–propagated viruses. Chan et al. reported a similar case in which the original clinical specimens contained a mixed population of variants, and oseltamivir-resistant viruses become dominant after the passage in MDCK cells (*4*).

On his 9th day in the hospital, the patient was intubated because of acute respiratory distress syndrome (Figure) and given levofloxacin. Urine samples were negative for *Pneumococcus* and *Legionella* spp. antigens. The patient improved and was extubated on hospital day 16.

Paired serologic test results were negative for *Mycoplasma pneumoniae* and *Legionella* spp. antibody; however, immunoglobulin G for *Chlamydia pneumoniae* increased 4-fold. By 37 days after illness onset, clinical signs and symptoms resolved and bilateral lineoreticular infiltration was reduced.

On August 8, 2009, Taiwan had the most devastating typhoon (Typhoon Morakot) in 50 years. The patient reported here had stayed in a typhoon evacuation camp for 1 week before his influenza signs and symptoms developed. Although 4 sporadic cases of pandemic (H1N1) 2009 infections were reported from the same camp, none of the isolated viruses harbored the H275Y mutation in the neuraminidase gene. No evidence of virus transmission was found among healthcare personnel, family members, and camp members who had been in close contact with the patient.

Oseltamivir has been recommended by the US Centers for Disease Control and Prevention for the treatment of

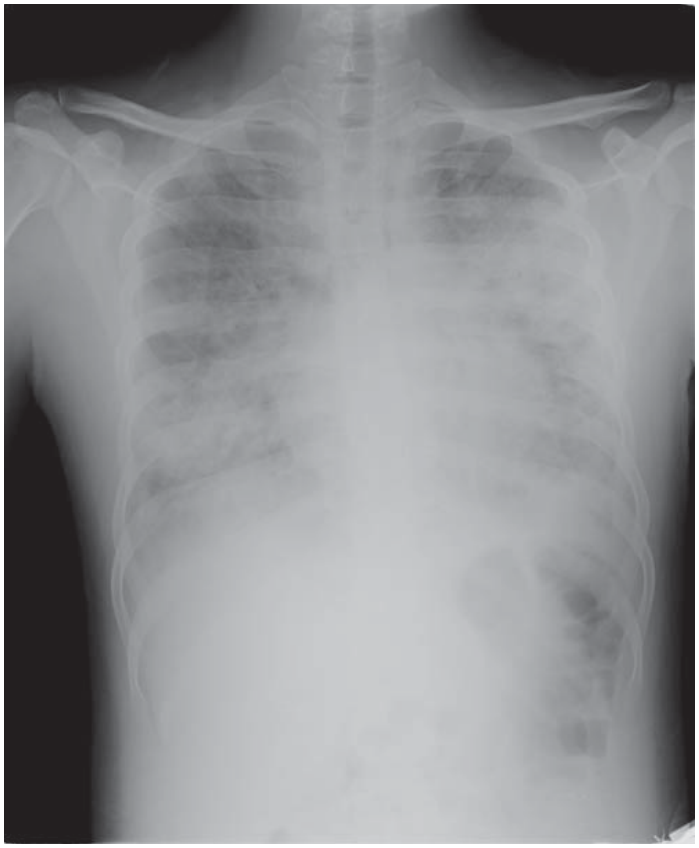


Figure. Radiograph (anteroposterior view) of patient with acute respiratory distress syndrome and oseltamivir-resistant pandemic (H1N1) 2009 virus.

infection caused by pandemic (H1N1) 2009 virus (*5*). The first 2 cases of oseltamivir resistance of pandemic H1N1 (2009) virus were reported in August 2009 (*6*). For these cases, oseltamivir-resistant virus was isolated on days 11 and 23 after the initial isolation of oseltamivir-susceptible viruses, for each patient, respectively. In contrast, in the case reported here, resistance to oseltamivir developed rapidly, after only 4 days of treatment.

In severe cases of pandemic (H1N1) 2009 infections, mortality rates are highest for patients who are pregnant, <2 years of age, or obese, or who have chronic lung disease (*7*). The patient reported here was previously healthy except for mental retardation; his body mass index was 23.9 kg/m^2. Progression of pneumonia to acute respiratory distress syndrome occurred despite early initiation of the standard dose of oseltamivir, within 48 hours after illness onset and initial susceptibility of the virus. Clinical deterioration might have resulted from the rapid emergence of an oseltamivir-resistant pandemic (H1N1) 2009 virus with a H275Y mutation, which is known to confer a high level of oseltamivir resistance while retaining zanamivir susceptibility (*8*), or it might have resulted from co-infection with *C. pneumoniae*. A 4-month study found concurrent bacterial infections in 29% of fatal cases of pandemic (H1N1) 2009 virus (*9*).

Oseltamivir resistance can emerge rapidly during treatment of pandemic (H1N1) virus infection. Healthcare providers should be aware that resistance may emerge in otherwise apparently healthy persons as early as day 4 of treatment with standard doses of oseltamivir.

Cheng Len Sy, Susan Shin-Jung Lee, Ming-Tsan Liu, Hung-Ching Tsai, and Yao-Shen Chen

Author affiliations: Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan (C.L. Sy, S.S.-J. Lee, H.-C. Tsai, Y.-S. Chen); National Yang-Ming University, Taipei, Taiwan (S.S.-J. Lee, H.-C. Tsai, Y.-S. Chen); and Centers for Disease Control, Taipei (M.-T. Liu)

DOI: 10.3201/eid1604.091706

References

1. Nicholls H. Pandemic influenza: the inside story. PLOS Biology. 2006;4:156–60. Epub 2006 Feb 14. DOI: 10.1371/journal.pbio.0040156
2. World Health Organization. Pandemic (H1N1) 2009: update 70 [cited 2009 Oct 22]. http://www.who.int/csr/don/2009_10_16/en/index.html
3. World Health Organization. Oseltamivir-resistant pandemic (H1N1) 2009 influenza virus, October 2009. Wkly Epidemiol Rec. 2009;84:453–9.
4. Chen H, Cheung CL, Tai H, Zhao P, Chan JFW, Cheng VCC, et al. Oseltamivir-resistant influenza A pandemic (H1N1) 2009 virus, Hong Kong, China. Emerg Infect Dis. 2009;15:1970–2.
5. Centers for Disease Control and Prevention. Updated interim recommendations for the use of antiviral medications in the treatment and prevention of influenza for the 2009–2010 season [cited 2009 Oct 22]. http://www.cdc.gov/h1n1flu/recommendations.htm
6. Centers for Disease Control and Prevention (CDC). Oseltamivir-resistant novel influenza A (H1N1) virus infection in two immunosuppressed patients—Seattle, Washington, 2009. MMWR Morb Mortal Wkly Rep. 2009;58:893–6.
7. World Health Organization. Clinical features of severe cases of pandemic influenza [cited 2009 Oct 22]. http://www.who.int/csr/disease/swineflu/notes/h1n1_clinical_features_20091016/en/
8. Escuret V, Frobert E, Bouscambert-Duchamp M, Sabatier M, Grog I, Valette M, et al. Detection of human influenza A (H1N1) and B strains with reduced sensitivity to neuraminidase inhibitors. J Clin Virol. 2008;41:25–8. DOI: 10.1016/j.jcv.2007.10.019

9. Centers for Disease Control and Prevention. Bacterial coinfections in lung tissue specimens from fatal cases of 2009 pandemic influenza A (H1N1)—United States, May–August 2009. MMWR Morb Mortal Wkly Rep. 2009;58:1071–4.

Address for correspondence: Yao-Shen Chen, Kaohsiung Veterans General Hospital, Infectious Diseases, No. 386 Ta-chung 1st Rd, Zuo-ying District, Kaohsiung 813, Taiwan; email: yschen@vghks.gov.tw

Dual Seasonal Patterns for Influenza, China

To the Editor: Since 2000, the People's Republic of China has had a nationwide surveillance network for influenza, which as of 2005 has been reported on the Chinese Center for Disease Control and Prevention website (www.cnic.org.cn/ch/). This surveillance has shown a remarkable dual pattern of seasonal influenza on mainland China. Whereas a regular winter pattern is noted for northern China (similar to that in most parts of the Northern Hemisphere), the pattern in southern China differs. In southern China, influenza is prevalent throughout the year; it has a clear peak in the summer and a less pronounced peak in the winter. Because this dual seasonal pattern of influenza has not been reported outside China and is relevant to pandemic (H1N1) 2009, we describe surveillance data for rates of consultation for influenza-like illness (ILI) and influenza subtypes in patients with ILI. We emphasize the spread of influenza from southern to northern China.

Before it was extended in June 2009, the National Influenza Surveillance Network had been composed of 63 influenza laboratories and 197 sentinel hospitals across 31 provinces of mainland China. In 13 of 16 northern provinces, surveillance began from the week including October 1 and ended in the week including March 31 of the following year. In the 3 northern provincial areas of Liaoning, Tianjin, and Gansu and in all southern provinces, surveillance was conducted throughout the year. Data consisted of information about ILI cases and virus subtypes. The sentinel hospitals defined ILI cases according to World Health Organization criteria: sudden onset of fever >38°C, cough or sore throat, and absence of other diagnoses (*1*). The number of ILI cases and the total number of outpatients at the sites (ILI consultation rate) were recorded each day and reported to the National Influenza Surveillance Information System each week.

Sentinel hospitals were required to collect 5–15 nasopharyngeal swabs each week from ILI patients who had not taken antiviral drugs and who had fever (≥38°C) for no longer than 3 days. The swabs were sent to the corresponding influenza laboratories for virus isolation and identification; results were reported to the National Influenza Surveillance Information System within 24 hours.

From the National Influenza Surveillance Network, a database of surveillance information from April 2006 to March 2009 was established. For influenza surveillance purposes, mainland China was divided into northern and southern parts, basically following the Qinling Mountain range in the west and the Huai River in the east. The prominent influenza peaks in the winter in the north and summer in the

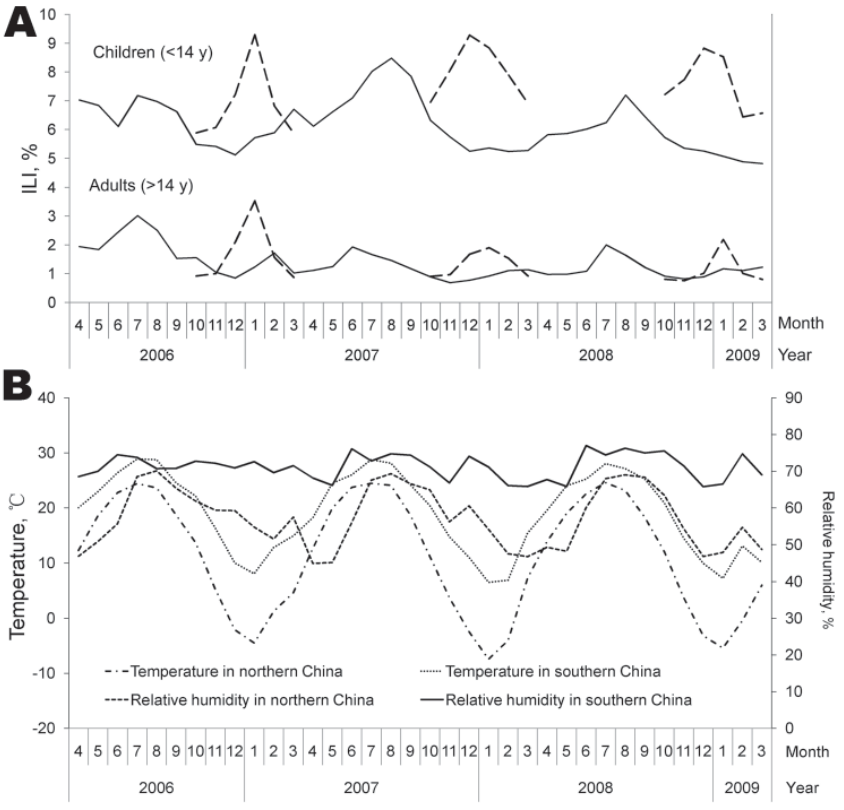


Figure. Epidemic patterns, by month, from the surveillance data of the influenza-like illness (ILI) consulting rate and influenza subtypes in ILI patients in mainland China, per month, April 2006–March 2009. A) ILI percentages for northern (dashed lines) and southern (solid lines) mainland China, by age group. B) Average temperature and relative humidity. A color version of this figure is available online (www.cdc.gov/EID/content/16/4/725-F.htm).

south were clear for adults and for children (Figure, panel A); the level of ILI was 3–5× for children. The influenza subtypes causing the 3 peaks in the north were preceded by a peak of the same subtypes in the south. During winter 2006–07, the influenza subtype was seasonal H1N1 and to a lesser extent H3N2. In winter 2007–08, the virus was B/Yamagata; and in 2008–09, it was again seasonal influenza A (H1N1), which was almost absent in the south during April–December 2007. Antigenic characteristics of the influenza virus from the north were similar to those from the south in the same epidemic episode (*2*). Furthermore, influenza A (H3N2) was in southern China throughout the year, whereas in northern China, this subtype only showed a clear peak in the first 2 winters of the study period. Subtype B/Victoria and B (unsubtyped) were both in northern and southern China in irregular and low numbers. Data from the 3 northern provincial areas with year-round surveillance confirmed that influenza cases during April–September were negligible (data not shown).

The influenza subtypes of seasonal influenza A (H1N1) and B/Yamagata that have caused the past 3 summer peaks in southern China were followed by an epidemic of the same subtypes in northern China during the subsequent winter. This finding may indicate that these peaks are regular epidemic phenomena for seasonal influenza in China. Another possible explanation is that other subtypes were cocirculating with the predominant subtype at the time of epidemics.

The dual pattern of seasonal peaks for influenza is well-known for the Northern and Southern Hemispheres, but apparently it is also possible on 1 side of the equator. China is a large country with climatic differences between north and south. Although most of southern China is above the Tropic of Cancer, it is warmer and more humid than northern China (Figure, panel B), which may explain the different seasonal patterns within mainland China (*3*). Knowledge of the dual patterns of influenza in China is relevant for determining effective control measures, and knowledge of the underlying mechanisms of such patterns is relevant to understanding the epidemiology of influenza in general.

This study was supported by the National Science Fund for Distinguished Young Scholars (grant no. 30725032), the Special Program for Prevention and Control of Infectious Diseases (grant no. 2008ZX10004-012), and the Natural Science Foundation of China (grants nos. 30810103903 and 30972521).

Yue-Long Shu,[1] Li-Qun Fang,[1] Sake J. de Vlas, Yan Gao, Jan Hendrik Richardus, and Wu-Chun Cao[1]

Author affiliations: Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China (Y.L. Shu, Y. Gao); Beijing Institute of Microbiology and Epidemiology, Beijing (L.-Q. Fang); University Medical Center Rotterdam, Rotterdam, the Netherlands (S.J. de Vlas, J.H. Richardus); and State Key Laboratory of Pathogen and Biosecurity, Beijing (W.-C. Cao)

DOI: 10.3201/eid1604.091578

References

1. World Health Organization. WHO recommended surveillance standards, Second edition [cited 2009 Aug 19]. http://www.who.int/csr/resources/publications/surveillance/WHO_CDS_CSR_ISR_99_2_EN/en/
2. Zhang Y, et al. Antigenic and genetic study of influenza virus circulated in China in 2006 [in Chinese]. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi. 2007;21:304–6.
3. Chan PK, Mok HY, Lee TC, Chu IM, Lam WY, Sung JJ. Seasonal influenza activity in Hong Kong and its association with meteorological variations. J Med Virol. 2009; 81:1797–806.

[1]These authors contributed equally to this article.

Address for correspondence: Wu-Chun Cao, Beijing Institute of Microbiology and Epidemiology, State Key Laboratory of Pathogen and Biosecurity, 100071, Beijing, People's Republic of China; email: caowc@nic.bmi.ac.cn

Avian Influenza Prevalence in Pigs, Egypt

To the Editor: Since 1996, avian influenza virus (H5N1) has spread to ≥65 countries (*1*). The disease represents a serious threat for the poultry industry and public health. Egypt has the highest human infection and fatality rates outside Asia (*2*). Some isolates of influenza virus (H5N1) in Egypt are resistant to oseltamivir (*3*), and in others, virulent mutations have developed, leading to case-fatality rates of 100% (*4*).

Pigs have the largest epidemiologic role in the evolution of new influenza viruses (*5*). Recombination between the newly emerged influenza virus subtypes H1N1 and H5N1 in pigs would have catastrophic results. We therefore investigated the seroprevalence of influenza virus (H5N1) in pigs in Egypt.

In May 2008, we collected 1 serum sample and 1 nasal swab from each of 240 pigs (11 herds) in Cairo slums. May was selected because it directly follows the season of bird migration and the seasonal storms usually accompanied by airborne diseases. Cairo slums were selected because 1) pigs there feed on organic remains, including dead birds, and thus have a higher chance of becoming infected; 2) Cairo is at the base of the Nile Delta, where most subtype H5N1 foci occurred; and 3) Cairo is near Fayum, the main stopover site for migrating birds.

To detect anti–avian influenza antibodies in the serum, we used hemagglutination inhibition (HI) assays with 2 inactivated antigens: subtype H5N2 from the Veterinary Laboratories Agency, UK; and a local subtype H5N1 prepared according to the protocol used in the central national laboratories. To detect viral RNA in the nasal swabs, we used real-time PCR, as was recommended for detection of influenza (H5N1) infection during outbreaks in Southeast Asia (*6*).

Although all nasal samples reacted negatively to influenza A/H5 by real-time PCR, only 4 serum samples showed positive results by HI when using subtype H5N2 antigen; titers were 32 for 3 samples and 64 for 1. Seven additional positive serum samples were detected when antigen prepared from local subtype H5N1 virus was used; titers ranged from 16 (6 samples) to 512 (1 sample). Also during this 2-week sampling period, titers of 32 for 3 samples and 128 for 1 were obtained. Seroprevalence rate of avian influenza for the 240 pigs was 1.67% and 4.6% when the nonlocal or local viral antigens, respectively, were used. Of the 11 positive pigs, 8 were from 1 herd and 3 were from 3 other herds.

Failure to detect viral RNA in the upper respiratory tract indicates the absence of acute infections in the investigated pigs. Inability of the virus to persist in the pigs was reported (*7*). Contrary to the HI results, results of routine examination of the 240 pigs found no abnormalities. Absence of clinical signs in infected pigs was reported (*8*) and was attributed to their low susceptibility to influenza (H5N1) (*7*). The results indicate that infection rate for pigs in Egypt is clearly higher than that for pigs in China and Vietnam (*8*,*9*). This increase may be attributed to different spatial and temporal factors leading to increased infection risk among sampled pigs**,** higher antigenicity of native isolates, or most probably to the disease situation in Egypt. The detection of 8 positive reactors from 1 herd indicates a subtype H5N1 focus there as was reported in Indonesia (*8*). The difference in the number of reactors when using different antigens indicates the difference in antigenicity. These data are supported by field observations regarding low protection level (≈35% in some reports) of imported vaccines (A. El-Sayed, unpub. data). The relatively low seroprevalence of avian influenza in pigs may be misleading because of the poor immunogenicity of some avian influenza lines and lack of sensitivity for detecting low titers of induced antibodies (*10*). It may be also explained by the use of a virus antigen other than that existing in the population, as was done in the present study.

Human risk for influenza (H5N1) infection in Egypt seems to be associated mainly with infected birds. It has not yet been associated with infected pigs.

Acknowledgments

We thank Elham Elebiary and Lamia Omar for their excellent help.

This study was financed by Cairo University.

Amr El-Sayed, Walid Awad, Adel Fayed, Hans-Peter Hamann, and Michael Zschöck

Author affiliations: Cairo University, Giza, Egypt (A. El-Sayed, W. Awad, A. Fayed); and Hessian State Laboratory, Giessen, Germany (H.P. Hamann, M. Zschöck)

DOI: 10.3201/eid1604.091316

References

1. Center for Infectious Disease Research and Policy. Avian influenza (bird flu): implications for human disease [cited 2010 Jan 29]. http://www.cidrap.umn.edu/cidrap/content/influenza/avianflu/biofacts/avflu_human.html
2. World Health Organization. Confirmed human cases of avian influenza A/(H5N1); 2009 [cited 2009 Apr 4]. http://www.who.int/csr/disease/avian_influenza/country/en/
3. Kage B. Bird flu virus mutates into Tamiflu-resistant strain in Egypt; WHO does not expect pandemic [cited 2007 Jan 24]. http://www.flunews.org/1058_bird_flu_Tamiflu.html
4. Niman L. Third H5N1 fatality in Gharbiya cluster in Nile Delta. Recombinomics commentary, December 27, 2006 [cited 2010 Feb 11]. http://www.recombinomics.com/News/12270601/H5N1_Gharbiya_Fatal_3.html
5. Ma W, Kahn RE, Richt JA. The pig as a mixing vessel for influenza viruses: human and veterinary implications. J Mol Genet Med. 2008;3:158–66.
6. Tran TH, Nguyen L, Nguyen D, Luong S, Pham M, Nguyen C, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med. 2004;350:1179–88. DOI: 10.1056/NEJMoa040419
7. Lipatov AS, Kwon YK, Sarmento LV, Lager KM, Spackman E, Suarez DL, et al. Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses. PLoS Pathog. 2008;4:e1000102. DOI: 10.1371/journal.ppat.1000102
8. Cyranoski D. Bird flu spreads among Java's pigs. Nature. 2005;435:390–1. DOI: 10.1038/435390a
9. Choi YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, Buranathal C, et al. Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol. 2005;79:10821–5. DOI: 10.1128/JVI.79.16.10821-10825.2005
10. de Jong MD, Hien TT. Avian influenza A (H5N1). J Clin Virol. 2006;35:2–13. DOI: 10.1016/j.jcv.2005.09.002

Address for correspondence: Amr El-Sayed, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt; email: aaelsayed2000@yahoo.de

Triple Reassortant Swine Influenza A (H3N2) Virus in Waterfowl

To the Editor: In 1998, a new lineage of triple reassortant influenza A (H3N2) virus (TR-H3N2) with genes from humans (hemmaglutinin [HA], neuraminidase [NA], and polymerase basic 1 [PB1]), swine (matrix [M], nonstructural [NS], and nucleoprotein [NP]), and birds (polymerase acidic [PA] and PB2) emerged in the U.S. swine population. Subsequently, similar viruses were isolated from turkeys (*1*,*2*), minks, and humans in the United States and Canada (*3*,*4*). In 2007, our national influenza surveillance resulted in isolation of 4 swine-like TR-H3N2 viruses from migratory waterfowl (3 from mallards [*Anas platyrrhynchos*] and 1 from a northern pintail [*Anas acuta*] of 266 birds sampled) in north-central South Dakota. We report on the characterization of these TR-H3N2 viruses and hypothesize about their potential for interspecies transmission.

Two of these isolates, A/mallard/South Dakota/Sg-00125/2007 (H3N2) and A/northern pintail/South Dakota/Sg-00126/2007 (H3N2), were recovered from the birds sampled in north-central South Dakota, 45°44′30″N, 98°16′30″W; 2 isolates, A/mallard/South Dakota/Sg-00127/2007(H3N2) and A/mallard/South Dakota/Sg-00128/2007(H3N2), were sampled at 45°46′30″N, 98°15′30″W. Viral RNA was extracted, reverse transcribed, and amplified; all segments were sequenced in entirety and submitted to GenBank under the identified virus names. Phylogenetic analysis showed significant nucleotide identities (99%–100%), differing only in 4 nucleotide positions: 1 each from PB1, PA, NP, and NS genes. Among 4 substitutions, 3 were nonsynonymous (PA, NP, and NS), and 1 (PB1) was synonymous. A1725G substitution in PB1 was identified in 2 isolates. C419T change was identified in 3 isolates (Sg-00125, Sg-00126, and Sg-00128), resulting in substitution of threonine by phenylalanine. Three isolates (Sg-00125, Sg-00126, and Sg-00127) carried an A at residue 149 of the NP gene (leading to S50N change) and 1 isolate (Sg-00128) had a G at that position (encoding serine). G809A change was present in the NP gene of 3 isolates (Sg-00125, Sg-00127, and Sg-00128). Genomes of the 4 isolates had high nucleotide and amino acid identities (>98%) with North American swine TR-H3N2 virus (A/Swine/Iowa/533/99 [H3N2]). Phylogenetic analysis indicated that TR-H3N2 waterfowl and North American TR-H3N2 swine isolates belonged to a single cluster. The H3N2 subtypes from avian and swine isolates of our sequencing projects belonged to different clusters (Figure). Deduced amino acid sequences of all segments showed that these virus isolates shared common themes in virulence determinants to those previously reported for swine-like TR-H3N2 viruses (*5*).

Inasmuch as we identified a swine lineage virus in waterfowl, we first investigated laboratory contamination by using trace back and history of swine virus isolations during the time the surveillance samples were processed. No H3N2 subtype were isolated from swine sources in the Minnesota Veterinary Diagnostic Laboratory during this period. Furthermore, phylogenetic analysis of all HA segment sequences from isolates obtained in that 4-month period confirmed no contamination. We then investigated whether an ecologic niche existed for potential exposure of waterfowl to pigs. We identified a swine herd near the wildlife refuge area where the waterfowl sampling occurred. Pigs were housed outdoors, and the owner of this swine herd reported that geese and ducks inhabit the water ponds/stock dams/slough area to which the pigs had access. Contact with the local veterinarian and the South Dakota Veterinary Diagnostic Laboratory indicated no recent reports of influenza A (H3N2) episodes in the swine herd. In addition, this herd was not vaccinated for swine influenza.

The mode of transmission of swine-origin virus to waterfowl is not clear. In previously published cases, where swine influenza viruses have been identified in turkeys, the flocks were in close proximity to swine herds (*2*). Similarly, we identified a swine herd in north-central South Dakota where all 4 waterfowl were sampled. Respiratory secretions from the pigs possibly could have spread to birds through aerosols or droplets. It is also likely that swine and waterfowl shared common water sources, which contained feces from influenza-infected waterfowl or respiratory secretions from influenza-infected swine. This mode of influenza virus transmission from birds to pigs has been documented (*6*–*9*). Indeed, a waterborne source for transmission is most likely because influenza A virus can persist in water for several months depending on environmental factors such as pH, temperature, and salinity (*10*). Finally, because the swine herd in this area was housed outdoors in open pens, direct interaction with waterfowl was possible.

In late 2008, serum samples were collected from this swine herd. Hemagglutination inhibition test (*1*) showed that 10 of 19 samples reacted with all 4 waterfowl isolates; titers ranged from 10 to ≥640. Although low titers may have occurred because pigs were exposed to heterologous cross-reactive viruses, the high titers in most animals with positive serum samples suggest exposure to an influenza (H3N2) virus similar to that recovered from the waterfowl. Our data emphasize the need to investigate the possible role of waterfowl in the maintenance and transmission of influenza A viruses to humans and to lower mammalian species.

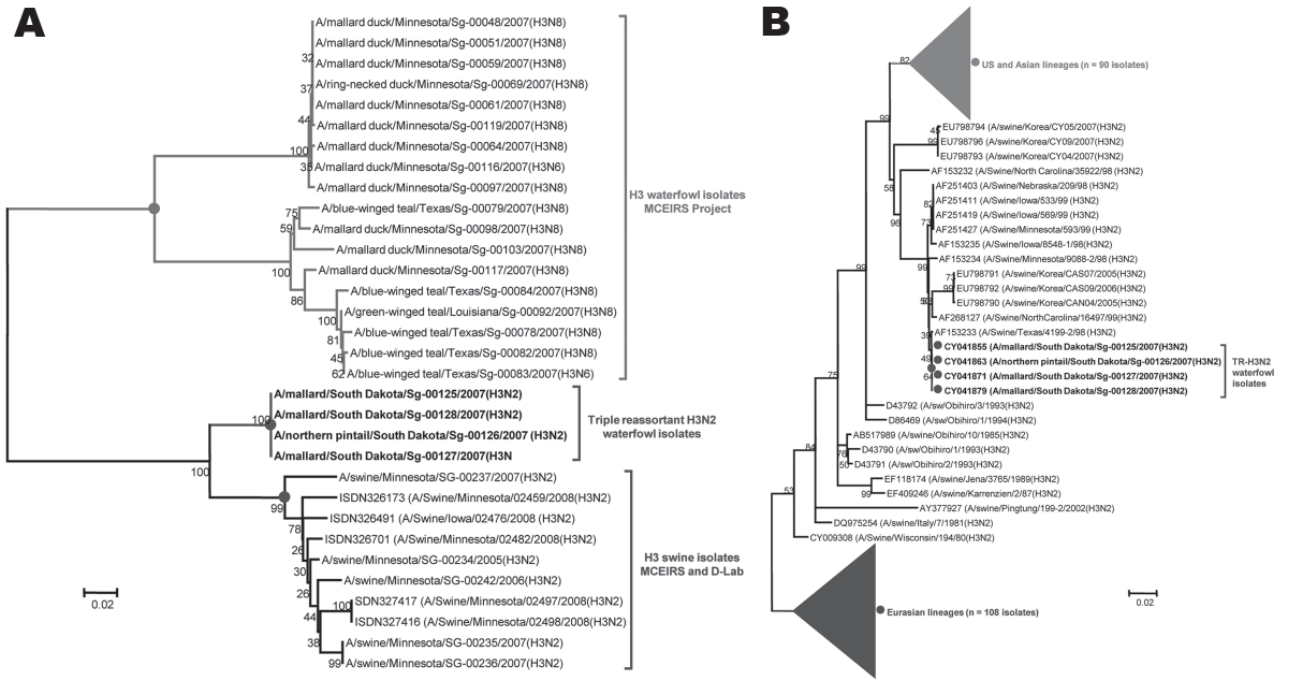


Figure. Phylogenetic analysis of hemagglutinin (HA) sequences from waterfowl strains isolated in this study (**boldface**), based on the HA gene sequences. The evolutionary associations were inferred in MEGA4.0 (www.megasoftware.net) by using the neighbor-joining algorithm with the Kimura 2-parameter gamma model and 1,000 bootstrap replications (shown on branch bifurcations). A) Evolutionary distances of waterfowl isolates from swine and avian HA (H3) sequences from the Minnesota Center of Excellence for Influenza Research and Surveillance (MCEIRS) sequencing project or Minnesota Veterinary Diagnostic Laboratory (D-Lab) database. B) Phylogeny of 230 strains, including Eurasian and North American lineages of influenza A (H3N2) viruses. Data suggest swine influenza virus (H3N2) ancestry in the waterfowl strains. GenBank accession numbers are shown. Scale bars indicate nucleotide substitutions per site.

This work was funded in whole or in part with funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract no. HHSN266200700007C.

Muthannan A. Ramakrishnan, Ping Wang, Martha Abin, My Yang, Sagar M. Goyal, Marie R. Gramer, Patrick Redig, Monte W. Fuhrman, and Srinand Sreevatsan

Author affiliations: University of Minnesota, St. Paul, Minnesota, USA (M.A. Ramakrishnan, P. Wang, M. Abin, M. Yang, S.M. Goyal, M.R. Gramer, P. Redig, S. Sreevatsan); and Sioux Nation Agriculture Center of Sioux Falls, Sioux Falls, South Dakota, USA (M.W. Fuhrman)

DOI: 10.3201/eid1604.091583

References

1. Gramer MR, Lee JH, Choi YK, Goyal SM, Joo HS. Serologic and genetic characterization of North American H3N2 swine influenza A viruses. Can J Vet Res. 2007 3:2016. Medline
2. Tang Y, Lee CW, Zhang Y, Senne DA, Dearth R, Byrum B, et al. Isolation and characterization of H3N2 influenza A virus from turkeys. Avian Dis. 2005;49:207–13. DOI: 10.1637/7288-101304R
3. Olsen CW. The emergence of novel swine influenza viruses in North America. Virus Res. 2002;85:199–210. DOI: 10.1016/S0168-1702(02)00027-8
4. Olsen CW, Karasin AI, Carman S, Li Y, Bastien N, Ojkic D, et al. Triple reassortant H3N2 influenza A viruses, Canada, 2005. Emerg Infect Dis. 2006;12:1132–5.
5. Yassine HM, Al-Natour MQ, Lee CW, Saif YM. Interspecies and intraspecies transmission of triple reassortant H3N2 influenza A viruses. Virol J. 2007;4:129. DOI: 10.1186/1743-422X-4-129
6. Karasin AI, Brown IH, Carman S, Olsen CW. Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J Virol. 2000;74:9322–7. DOI: 10.1128/JVI.74.19.9322-9327.2000
7. Karasin AI, West K, Carman S, Olsen CW. Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. J Clin Microbiol. 2004;42:4349–54. DOI: 10.1128/JCM.42.9.4349-4354.2004
8. Ma W, Vincent AL, Gramer MR, Brockwell CB, Lager KM, Janke BH, et al. Identification of H2N3 influenza A viruses from swine in the United States. Proc Natl Acad Sci U S A. 2007;104:20949–54. DOI: 10.1073/pnas.0710286104
9. Olsen CW, Karasin A, Erickson G. Characterization of a swine-like reassortant H1N2 influenza virus isolated from a wild duck in the United States. Virus Res. 2003;93:115–21. DOI: 10.1016/S0168-1702(03)00073-X

10. Brown JD, Goekjian G, Poulson R, Valeika S, Stallknecht DE. Avian influenza virus in water: infectivity is dependent on pH, salinity and temperature. Vet Microbiol. 2009;136:20–6. DOI: 10.1016/j.vetmic.2008.10.027

Address for correspondence: Srinand Sreevatsan, Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, 1365 Gortner Ave, 225 VTH, St Paul, MN 55108, USA; email: sreev001@umn.edu

Ventilator-associated Pneumonia and MRSA ST398, Italy

To the Editor: Methicillin-resistant *Staphylococcus aureus* (MRSA) sequence type (ST)398 has become increasingly common in livestock, particularly pigs, in some countries in Europe, such as Spain and Germany (*1*). In Italy, prevalences as high as 14% and 21.6% in pig-breeding facilities and meat-processing sites, respectively, have been recently reported (*1*).

Possible association of MRSA in animals with infection in humans has been investigated. One study showed a strong relationship between contact with pigs or calves and carriage by persons having direct contact with animals and families of persons who handle animals (*2*). Moreover, an MRSA prevalence ≥11.9% has been described by de Boer et al. (*3*) in meat, with 85% of isolates belonging to the ST398 lineage.

MRSA ST398 has been described as a lineage with limited virulence and ability to spread between humans, but severe clinical manifestations, such as wound infections and endocarditis, have been recently attributed to this clone (*1*,*4*). Cases of nosocomial ventilator-associated pneumonia have also been reported in Germany (*1*). Moreover, an outbreak of infection with MRSA ST398 occurred in a surgical ward of a hospital in the Netherlands in 2007 (*5*).

MRSA ST398 is an infrequent cause of human infections in Italy. No isolates belonged to this lineage in 2 studies of MRSA in Italy during 2006–2007 (*6*) or in hospitals during 1990–2007 (*7*). Only 1 invasive infection has been recently reported in a pig farm worker (*8*). We report a case of ventilator-associated pneumonia caused by MRSA ST398 in a patient in Palermo, Italy. The patient and his household members did not report any exposure to companion or livestock animals.

The case-patient was a 78-year-old man admitted to a cardiac intensive care unit (ICU) of ARNAS Ospedale Civico Di Cristina e Benfratelli in Palermo on January 31, 2009, because of a recent history of unstable angina pectoris and acute anemia caused by duodenal ulcers. After cardiocirculatory arrest, he was transferred to a general ICU on February 3. The patient had type 2 diabetes and ischemic-hypertensive cardiomyopathy. MRSA nasal colonization at admission was not investigated because the patient lacked risk factors for screening at admission, e.g., antimicrobial drug therapy, hospitalization for >48 hours or time in a long-term care facility within the past 6 months, need for long-term nursing care, presence of indwelling devices, or chronic skin lesions.

The clinical course of the patient's illness was characterized by serious hemodynamic instability and difficulty in weaning from mechanical ventilation. Two bronchial aspirate specimens were cultured on February 4 and 9, when he was being treated with a third-generation cephalosporin (ceftriaxone). These cultures showed *Staphylococcus epidermidis* and *S. saprophyticus*. On the 14th day in the ICU, clinical signs of ventilator-associated pneumonia developed in the patient. He had increased sputum production, fever (38.8°C), leukocytosis, and infiltrates were seen on a chest radiograph.

Empiric antimicrobial drug therapy with glycopeptides and a β-lactam/β-lactamase inhibitor combination was started. Culture of bronchial secretions yielded MRSA that was susceptible to glycopeptides, rifampin, linezolid, macrolides, and sulfamethoxazole and resistant to fluoroquinolones and tetracyclines. Three days later, linezolid was given, but the patient died after an acute myocardial infarction.

The isolate was identified genetically by *mec*A PCR. It was not typeable by pulsed-field gel electrophoresis after digestion with *Sma*I, negative for Panton-Valentine leukocidin, and carried staphylococcal cassette chromosome *mec* (SCC*mec*) type IVa (*9*). Multilocus sequence typing, performed according to a recommended procedure (http://saureus.mlst.net/misc/info.asp), identified the isolate as ST398.

A 1-year epidemiologic survey on MRSA isolates from 4 general hospitals in Palermo, which had begun on February 2009, did not identify any MRSA isolate carrying SCC*mec* type IV or V in patients admitted to the ICU until September 2009. However, colonization or infection by MRSA ST398 in the ICU patients before the study period could not be ruled out. Although an MRSA screening policy for the ICU staff members was not being carried out, a nosocomial chain of transmission appeared to be unlikely.

Our results indicate that a new zoonotic clone of MRSA is emerging as a potential cause of serious human infections. Screening at hospital admission would likely help efforts to determine whether exposure to pet animals and livestock had occurred. However, the absence of specific exposure to zoonotic clonal lineages, as in our case-patient, is a matter of concern in terms of screening and contact tracing policy for MRSA in-

fections. Prevalence of MRSA and distribution of MRSA sequence types in livestock in Italy are not known. However, surveys of foods of animal (pig) origin have showed an MRSA prevalence of 3.7% (*1*,*10*). In view of the low prevalence of MRSA ST398 in patients with no exposure to animals, food products currently seem to play a negligible role. However, this clone is likely spreading because of the large animal reservoir of ST398 and the global market for meat and livestock. The changing epidemiology of MRSA indicates that collaborative surveillance plans integrating human and animal information should be increased.

Caterina Mammina, Cinzia Calà, Maria R.A. Plano, Celestino Bonura, Antonietta Vella, Rachele Monastero, and Daniela M. Palma

Author affiliations: University of Palermo, Palermo, Italy (C. Mammina, C. Calà, M.R.A. Plano, C. Bonura, A. Vella); and ARNAS Ospedale Civico Di Cristina e Benfratelli, Palermo (R. Monastero, D.M. Palma)

DOI: 10.3201/eid1604.091584

References

1. European Food Safety Authority. Analysis of the baseline survey on the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in holdings with breeding pigs, in the EU, 2008. European Food Safety Authority Journal. 2009;7:1376.
2. van Loo I, Huijsdens X, Tiemersma E, de Neeling A, van de Sande-Bruinsma N, Beaujean D, et al. Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. Emerg Infect Dis. 2007;13:1834–9.
3. de Boer E, Zwartkruis-Nahuis JT, Wit B, Huijsdens XW, de Neeling AJ, Bosch T, et al. Prevalence of methicillin-resistant *Staphylococcus aureus* in meat. Int J Food Microbiol. 2009;134:52–6. DOI: 10.1016/j.ijfoodmicro.2008.12.007
4. Ekkelenkamp MB, Sekkat M, Carpaij N, Troelstra A, Bonten MJ. Endocarditis due to methicillin-resistant *Staphylococcus aureus* originating from pigs [in Dutch]. Ned Tijdschr Geneeskd. 2006;150:2442–7.
5. Wulf MW, Markestein A, van der Linden FT, Voss A, Klaassen C, Verduin CM. First outbreak of methicillin-resistant *Staphylococcus aureus* ST398 in a Dutch hospital, June 2007. Euro Surveill. 2008;13:8051.
6. Campanile F, Bongiorno D, Borbone S, Stefani S. Hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) in Italy. Ann Clin Microbiol Antimicrob. 2009;8:22. DOI: 10.1186/1476-0711-8-22
7. Marchese A, Gualco L, Maioli E, Debbia E. Molecular analysis and susceptibility patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) strains circulating in the community in the Ligurian area, a northern region of Italy: emergence of USA300 and EMRSA-15 clones. Int J Antimicrob Agents. 2009;34:424–8. DOI: 10.1016/j.ijantimicag.2009.06.016
8. Pan A, Battisti A, Zoncada A, Bernieri F, Boldini M, Franco A, et al. Community-acquired methicillin-resistant *Staphylococcus aureus* ST398 infection, Italy. Emerg Infect Dis. 2009;15:845–7. DOI: 10.3201/eid1505.081417
9. Zhang K, McClure JA, Elsayed S, Louie T, Conly JM. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus.* J Clin Microbiol. 2005;43:5026–33. DOI: 10.1128/JCM.43.10.5026-5033.2005
10. Normanno G, Corrente M, La Salandra G, Dambrosio A, Quaglia NC, Parisi A, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) in foods of animal origin product in Italy. Int J Food Microbiol. 2007;117:219–22. DOI: 10.1016/j.ijfoodmicro.2007.04.006

Address for correspondence: Caterina Mammina, Department of Sciences for Health Promotion G. D'Alessandro, Centre for Enteric Pathogens of Southern Italy, University of Palermo, Via del Vespro 133, I-90127 Palermo, Italy; email: diptigmi@unipa.it

Panton-Valentine Leukocidin–Positive MRSA, Shanghai, China

To the Editor: The development of methicillin resistance in community strains of *Staphylococcus aureus* is a notable step in the evolution of this pathogen. Unlike their equivalents in the hospital environment, community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains tend to cause infections in children and young adults who have few known healthcare risks (*1*). CA-MRSA strains usually possess the Panton-Valentine leukocidin (PVL) genes and staphylococcal cassette chromosome (SCC) *mec* type IV or V (*1*,*2*).

We studied 72 *S. aureus* isolates (49 MRSA and 23 methicillin-susceptible [MSSA]) by pulsed-field gel electrophoresis and by SCC*mec*, staphylococcal protein A (spa), and multilocus sequence typing (*1*,*3*). These isolates were recovered from clinical specimens (52 respiratory specimens, 9 wound, 4 urine, 2 blood, and 5 other body fluids) from 72 patients treated in 5 district hospitals in Shanghai, People's Republic of China, during October 2005 through January 2007. The isolates were randomly chosen. In the hospitals, ≈1,000 *S. aureus* isolates were recovered annually during the time period of our study. The 5 hospitals are estimated to serve a population of 3.4 million, equivalent to one fourth of the total population in Shanghai. Hospital D is a children's hospital. The other 4 hospitals (A, B, C, and E) have all the major clinical specialties, emergency departments, and outpatient clinics.

The isolates were identified as *S. aureus* by Gram stain, latex agglutination (Slide StaphPlus; bioMérieux, Marcy l'Etoile, France), and tube coagulase, mannitol, ornithine, and deoxyribonuclease reactions (*1*,*4*). Methicillin resistance in the isolates was

detected by cefoxitin disc screening and confirmed by *mecA* PCR (*1*,*4*). For patients with PVL-positive MRSA, the computerized discharge records in the hospitals were retrospectively reviewed to ascertain demographic and clinical information. A MRSA case was considered to be community associated if it was isolated from an outpatient or within 2 days of a patient's hospitalization. Exclusion criteria included a history of hospitalization for illness (except birth), surgery, or dialysis in the previous year or the presence of indwelling catheters or other medical devices (*1*). Conversely, healthcare-associated MRSA was defined by isolation >2 days after hospitalization or presence of any of the aforementioned healthcare risks.

PVL genes were detected in 9 (18.4%) of the 49 MRSA isolates (Table) and 4 (17.3%) of the 23 MSSA isolates. The 9 MRSA case-patients included 8 infants with pneumonia and 1 adult with prostatitis. Pulsed-field gel electrophoresis clustered 8 of the 9 PVL-positive MRSA isolates into 2 groups: 6 isolates as SH100 and 2 isolates as SH200. Strains of SH100 were *spa* type/MLST-SCC*mec* type t318/ST30-IV or t318/ST1114-V, and SH200 strains had t1376/ST88-V. Similar to the PVL-positive MRSA isolates, a limited number of *spa* types were found among the 40 PVL-negative MRSA isolates. These were t037/ST239-III (n = 19), t002/ST5-II (n = 14), t030/ST239-III (n = 5), t459/ST239-III (n = 1), and t1764/ST88-IV (n = 1).

In contrast, *spa* and sequence types (STs) among the 23 MSSA isolates were highly diverse. There were 20 *spa* types and 14 STs, giving a total of 20 distinct patterns. Three patterns (t091/ST7, t3388/ST630, t3389/ST15) had 2 isolates, and 17 patterns (t002/ST5, t1077/ST121, t127/ST1, t1376/ST88, t189/ST188, t2024/ST30, t2092/ST121, t2207/ST1206, t2471/ST25, t258/ST25, t3383/ST20, t3386/ST630, t377/ST630, t437/ST1205, t548/ST5, t701/ST6, t796/ST7) had 1 isolate only. The 4 PVL-positive MSSA isolates were t1376/ST88, t2471/ST25, t258/ST25, and t3383/ST20.

Mupirocin resistance rates among the PVL-positive and PVL-negative MRSA isolates were 33.3% (3/9) and 7.5% (3/40), respectively (p = 0.07). All MSSA isolates were susceptible to mupirocin.

It is notable that of the 9 PVL-positive MRSA isolates, 8 of them came from hospital D and all were from children 1–4 months of age. Others have noted that the epidemiology of MRSA differs for children and adults (*1*,*2*,*7*,*8*). Molecular typing showed that the PVL-positive CA-MRSA isolates were attributed to 2 clones with genotypes t318/ST30-IV (or t318/ST1114-V) and t1376/ST88-V. Detection of t318/ST30 strains in 4 patients with healthcare-associated infections suggested hospital transmission of this CA-MRSA clone, corroborating reports elsewhere (*9*). Worldwide, ST30 is a common CA-MRSA genetic lineage (*1*,*2*). Besides t318, strains related to the ST30 clone have been reported to be spa types t019, t021, and t1273 (*2*). ST88 PVL-positive MRSA is relatively less common but has been found in Wenzhou (People's Republic of China), Bangladesh, Belgium, and Nigeria (*2*,*7*,*8*,*10*).

Because the number of isolates tested in this study is relatively small, no firm conclusion could be drawn on the prevalence of PVL-positive CA-MRSA among *S. aureus* isolates. Nonetheless, our findings agree with previous reports that the genotypes of MSSA isolates are more diverse than are those for PVL-positive and -nega-

Table. Epidemiologic and microbiologic characteristics for Panton-Valentine leukocidin–positive MRSA infections in 9 case-patients, Shanghai, People's Republic of China, 2006*

Strain	Age/sex	Admission month	Onset	Diagnosis†	Resistance pattern‡	Resistance determinants§	ST§	SCC*mec*	*spa* type	PFGE
D8	1 mo/F	Feb	HA	Pneumonia	Chl, Ery, Gen, Mup	*aacA-aphD*, *ermC*, *ileS-2*	ST30	IV	t318	SH100
D9	1 mo/F	Feb	CA	Pneumonia	Gen, Mup	*aacA-aphD*, *ileS-2*	–	IV	t318	SH100
D13	1 mo/M	Mar	HA	Pneumonia	Gen	*aacA-aphD*	–	IV	t318	SH100
D14	3 mo/M	Mar	CA	Pneumonia	Gen	*aacA-aphD*	–	IV	t318	SH100
D16	1 mo/M	Mar	CA	Pneumonia	Gen, Mup	*aacA-aphD*, *ileS-2*	–	IV	t318	SH100
D12	3 mo/F	Mar	HA	Pneumonia	Ery	*ermC*	ST1114	V	t318	SH100
A18	29 y/M	Mar	HA	Prostatitis	None	–	ST30	IV	t3384	Singleton
D7	1 mo/F	Jan	CA	Pneumonia	Ery, Gen	*aacA-aphD*, *ermC*	ST88	V	t1376	SH200
D18	4 mo/M	Apr	CA	Pneumonia	Ery, Gen	*aacA-aphD*, *ermC*	–	V	t1376	SH200

*Strains are designated by hospital code and strain number. MRSA, methicillin-resistant *Staphylococcus aureus*; ST, sequence type; SCC, staphylococcal cassette chromosome; *spa*, staphylococcal protein A; PFGE, pulsed-field gel electrophoresis; HA, healthcare-associated; Chl, chloramphenicol; Ery, erythromycin; Gen, gentamicin, Mup, mupirocin; *acA-aphD*, aminoglycoside resistance gene encoding the bifunctional enzyme, 6'-aminoglycoside N-acetyltransferase/2″-aminoglycoside phosphotransferase; *ermC*, gene encoding macrolide-lincosamide-streptogramin B resistance; *ileS-2*, gene encoding high-level mupirocin resistance mediated by isoleucyl tRNA-synthetase; CA, community-associated.
†According to the diagnosis given in the computerized record. In the 8 children, MRSA was isolated from sputum specimens. One child also had MRSA isolated from pleural fluid. Because limited information was provided in the computerized records, some of the isolations may represent respiratory colonization. The outcomes of the 8 children were unknown.
‡According to disk diffusion test *(1,5)*.
§Determined by PCR as described (*4*,*6*). ST30 (allelic profile 2–2-2–2-6–3-2) and ST114 (139–2-2–2-6–139–2) belonged to clonal complex 30.

tive MRSA isolates and that genotypes for some CA-MRSA strains are shared by a few of the MSSA strains (*1*).

The experimental work was conducted in the Department of Microbiology, University of Hong Kong, and supported by grants from the University Development Fund Project–Research Centre of Emerging Infectious Diseases of the University of Hong Kong.

Li-Zhong Han, Pak-Leung Ho, Yu-Xing Ni, Hong Zhang, Yan-Qun Jiang, Hai-Qing Chu, Yang Sun, and Yi-Bo Zhang

Author affiliations: Ruijin Hospital, Shanghai, People's Republic of China (L.-Z. Han, Y.-X. Ni, Y.-B. Zhang); University of Hong Kong, Hong Kong Special Administrative Region, PRC (P.-L. Ho); Shanghai Children's Hospital, Shanghai (H. Zhang); Sixth People's Hospital of Shanghai, Shanghai (Y.-Q. Jiang); Shanghai Pulmonary Hospital, Shanghai (H.-Q. Chu); and Minhang Central Hospital, Shanghai (Y. Sun)

DOI: 10.3201/eid1604.081324

References

1. Ho PL, Chuang SK, Choi YF, Lee RA, Lit AC, Ng TK, et al. Community-associated methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*: skin and soft tissue infections in Hong Kong. Diagn Microbiol Infect Dis. 2008;61:245–50. DOI: 10.1016/j.diagmicrobio.2007.12.015
2. Tristan A, Bes M, Meugnier H, Lina G, Bozdogan B, Courvalin P, et al. Global distribution of Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus*, 2006. Emerg Infect Dis. 2007;13:594–600. DOI: 10.3201/eid1304.061316
3. Ho PL, Lai EL, Chow KH, Chow LS, Yuen KY, Yung RW. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in residential care homes for the elderly in Hong Kong. Diagn Microbiol Infect Dis. 2008;61:135–42. DOI: 10.1016/j.diagmicrobio.2007.12.017
4. Ho PL, Wang TK, Ching P, Mak GC, Lai E, Yam WC, et al. Epidemiology and genetic diversity of methicillin-resistant *Staphylococcus aureus* strains in residential care homes for elderly persons in Hong Kong. Infect Control Hosp Epidemiol. 2007;28:671–8. DOI: 10.1086/517951
5. Finlay JE, Miller LA, Poupard JA. Interpretive criteria for testing susceptibility of staphylococci to mupirocin. Antimicrob Agents Chemother. 1997;41:1137–9.
6. Perez-Roth E, Claverie-Martin F, Villar J, Mendez-Alvarez S. Multiplex PCR for simultaneous identification of *Staphylococcus aureus* and detection of methicillin and mupirocin resistance. J Clin Microbiol. 2001;39:4037–41. DOI: 10.1128/JCM.39.11.4037-4041.2001
7. Denis O, Deplano A, De Beenhouwer H, Hallin M, Huysmans G, Garrino MG, et al. Polyclonal emergence and importation of community-acquired methicillin-resistant *Staphylococcus aureus* strains harbouring Panton-Valentine leucocidin genes in Belgium. J Antimicrob Chemother. 2005;56:1103–6. DOI: 10.1093/jac/dki379
8. Yu F, Chen Z, Liu C, Zhang X, Lin X, Chi S, et al. Prevalence of *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes among isolates from hospitalised patients in China. Clin Microbiol Infect. 2008;14:381–4. DOI: 10.1111/j.1469-0691.2007.01927.x
9. Zaoutis TE, Toltzis P, Chu J, Abrams T, Dul M, Kim J, et al. Clinical and molecular epidemiology of community-acquired methicillin-resistant *Staphylococcus aureus* infections among children with risk factors for health care–associated infection: 2001–2003. Pediatr Infect Dis J. 2006;25:343–8. DOI: 10.1097/01.inf.0000207403.67197.cc
10. Ghebremedhin B, Olugbosi MO, Raji AM, Layer F, Bakare RA, Konig B, et al. Emergence of a community-associated methicillin-resistant *Staphylococcus aureus* strain with a unique resistance profile in Southwest Nigeria. J Clin Microbiol. 2009;47:2975–80. DOI: 10.1128/JCM.00648-09

Address for correspondence: Pak-Leung Ho, Division of Infectious Diseases, Department of Microbiology, Queen Mary Hospital, The University of Hong Kong, Pokfulam Rd, Pokfulam, Hong Kong Special Administrative Region, People's Republic of China; email: plho@hkucc.hku.hk

Clostridium difficile in Ground Meat, France

To the Editor: *Clostridium difficile* is a toxigenic enteropathogen responsible for 15%–20% of antimicrobial drug–associated diarrhea and for almost all cases of pseudomembranous colitis. Two protein toxins (TcdA and TcdB) play a major role in the pathogenesis of infections. *C. difficile* is also recognized as a cause of disease in several animal species, which could be potential reservoirs (*1*). In the past few years, the presence of *C. difficile* in raw diets for dogs and cats and in retail meat sold for human consumption has been reported in the United States and Canada at rates from 6% to 42% (*2*–*5*). To determine *C. difficile* contamination of meat in France, we evaluated 105 packages of ground beef (vacuum packed or not), 59 pork sausages, and 12 packages of feline raw diet meat purchased from 20 urban and suburban Paris retail stores and supermarkets during September 2007–July 2008.

C. difficile spores or vegetative forms in samples were found as described by Rodriguez-Palacios et al. (*4*). Briefly, 5 g of each sample was cultured in 100 mL of prereduced brain–heart infusion (BHI) broth supplemented with cefoxitin (10 μg/mL), cycloserine (250 μg/mL), and taurocholate (0.1%). After the samples were incubated under anaerobic conditions at 37°C for 72 h, subculturing with and without alcohol shock for spore selection was performed. The BHI broth culture was treated with 2 mL of absolute ethanol (1:1 vol/vol) for 30 min and centrifuged at 3,800 × *g* for 10 min, and the pellet was resuspended in 200 μL of prereduced BHI broth. Serial dilutions of the BHI broth and the pellet were injected onto Columbia cysteine agar supplemented with cefoxitin-cycloserine, taurocholate, and 5% horse blood and incubated anaerobically for 48 h at 37°C.

C. difficile colonies were identified classically, and susceptibilities to moxifloxacin, teicoplanin, vancomycin, metronidazole, linezolid, levofloxacin, telithromycin, erythromycin, and lincomycin were determined by the agar disk-diffusion methods described by the French Society for Microbiology (www.sfm.asso.fr). PCR amplifications of a species-specific internal fragment of the triose phosphate isomerase (*tpi)* gene, an internal fragment of the toxin B (*tcdB*) gene, and the 3′ region of the toxin A (*tcdA*) gene were performed as described by Lemee et al. (*6*). Strains were characterized by toxinotyping according to Rupnik et al. (*7*) and PCR-ribotyping as described by Bidet et al. (*8*).

The detection threshold of the enrichment method was established by spiking known uninfected samples (ground beef, pork sausage, and feline raw diets) with vegetative cells and spores of *C. difficile* (VPI 10463 strain). For ground beef samples, the detection thresholds for vegetative forms and spores were 2 CFU/5 g and 4.5 CFU/5 g of meat, respectively. For pork sausages, the detection thresholds were 14 CFU/5 g and 38 CFU/5 g of sample after 72 h, for vegetative forms and spores, respectively. For feline raw diets, the detection threshold of spores was 2 CFU/5 g of sample. In addition, toxin B was detected in the culture supernatants by the cytotoxicity assay onto MRC-5 cells. Toxin detection showed 100% agreement with the culture method.

C. difficile was not detected in pork sausages or in commercial feline raw diets. *C. difficile* was isolated from 2 (1.9%) of 105 ground beef, but only from those packages that were vacuum packed. The anaerobic atmosphere of vacuum packaging could facilitate the survival of *C. difficile* and the germination of spores. These 2 isolates were fully susceptible to moxifloxacin, teicoplanin, vancomycin, metronidazole, and linezolid but resistant to levofloxacin, telithromycin, erythromycin, and lincomycin. They harbored genes encoding for Tpi protein and for TcdA and TcdB. The 2 strains belonged to the toxinotype 0 and PCR-ribotype 012. Toxinotype 0 was already identified in meat samples in Canada (*4*). PCR-ribotype 012 belongs to the 10 ribotypes most frequently isolated from humans (*9*).

The prevalence of *C. difficile* in ground meat in France is low compared with the prevalence reported by other countries. In Canada, Rodriguez-Palacios et al. (*4*) studied 60 beef samples and found the prevalence of *C. difficile* to be 20%. These same authors, by using a broader sampling scheme (214 meat samples), isolated *C. difficile* from 6% of the samples (*5*). Also in Canada, Weese et al. (*10*) reported that 12% of ground beef and ground pork samples were contaminated. In the United States, *C. difficile* was isolated from 42% of meat samples (beef, pork, and turkey products) (*3*).

For a better understanding of the sources of *C. difficil*e in France, it would be interesting to determine its prevalence in different animal fecal samples and the toxinotypes associated with animals. The low prevalence in retail meat in France could result from hazard analysis critical control point principles and microbiologic quality controls implemented throughout the food production chain, which help reduce the spread of *C. difficile* and minimize the risk for infection for the consumer.

Sylvie Bouttier, Marie-Claude Barc, Benjamin Felix, Sylvie Lambert, Anne Collignon, and Frédéric Barbut

Author affiliations: Université Paris-Sud 11, Châtenay-Malabry, France (S. Bouttier, M.-C. Barc, S. Lambert, A. Collignon); and Hôpital Saint-Antoine, Paris, France (B. Félix, F. Barbut)

DOI: 10.3201/eid1604.091138

References

1. Jhung MA, Thompson AD, Killgore GE, Zubowski WE, Songer G, Warny M, et al. Toxinotype V *Clostridium difficile* in humans and food animals. Emerg Infect Dis. 2008;14:1039–45. DOI: 10.3201/eid1407.071641
2. Weese JS, Rousseau J, Arroyo L. Bacteriological evaluation of commercial canine and feline raw diets. Can Vet J. 2005;46:513–6.
3. Songer JG, Trinh HT, Killgore GE, Thompson AD, McDonald C, Limbago BM. *Clostridium difficile* in retail meat products, USA, 2007. Emerg Infect Dis. 2009;15:819–21. DOI: 10.3201/eid1505.081071
4. Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS. *Clostridium difficile* in retail ground meat, Canada. Emerg Infect Dis. 2007;13:485–7. DOI: 10.3201/eid1303.060988
5. Rodriguez-Palacios A, Reid-Smith RJ, Staempfli HR, Martin H, Daignault D, Janecko N, et al. Possible seasonality of *Clostridium difficile* in retail meat, Canada. Emerg Infect Dis. 2009;15:802–5. DOI: 10.3201/eid1505.081084
6. Lemee L, Dhalluin A, Testelin S, Mattrat MA, Maillard K, Lemeland JF, et al. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (toxin A), and *tcdB* (toxinB) genes for toxinogenic culture of *Clostridium difficile.* J Clin Microbiol. 2004;42:5710–4. DOI: 10.1128/JCM.42.12.5710-5714.2004
7. Rupnik M, Avesani V, Janc M, von Eichel-Streiber C, Delmée M. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J Clin Microbiol. 1998;36:2240–7.
8. Bidet P, Lalande V, Salauze B, Burghoffer B, Avesani V, Delmée M, et al. Comparison of PCR-ribotyping, arbitrarily primed PCR and pulsed-field gel electrophoresis for typing *Clostridium difficile.* J Clin Microbiol. 2000;38:2484–7.
9. Barbut F, Mastrantonio P, Delmée M, Brazier J, Kuijper E, Poxton I. European Study Group on *Clostridium difficile* (ESGCD). Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. Clin Microbiol Infect. 2007;13:1048–57. DOI: 10.1111/j.1469-0691.2007.01824.x
10. Weese JS, Avery BP, Rousseau J, Reid-smith RJ. Detection and enumeration of *Clostridium difficile* in retail beef and pork. Appl Environ Microbiol. 2009;75:5009–11. DOI: 10.1128/AEM.00480-09

Address for correspondence: Sylvie Bouttier, Faculté de Pharmacie, Université Paris-Sud 11, 5 rue Jean-Baptiste Clément, 92290 Châtenay-Malabry, France; email: sylvie.bouttier@u-psud.fr

WUPyV in Children with Acute Respiratory Tract Infections, China

To the Editor: WU polyomavirus (WUPyV) is a human polyomavirus first detected in respiratory samples in 2007 (*1*). It has since been detected worldwide (*2*–*7*), including the People's Republic of China (*8*), but whether it is a causative respiratory pathogen remains speculative. To investigate the potential role of WUPyV in respiratory diseases and its prevalence in Tianjin, China, we examined samples obtained from children with upper respiratory tract infections (URTIs) and lower respiratory tract infections (LRTIs) by using PCR for the VP2 and LTAg genes, as described previously (*1*). As a control, we also tested samples from patients who did not have respiratory diseases.

Case-patients were 174 inpatients, hospitalized for LRTIs March–April 2008 and September 2008–February 2009, and 68 outpatients treated for URTIs November–December 2009 at Tianjin Children's Hospital. Controls were 43 outpatients with illnesses other than respiratory diseases treated at Tianjin Xianshuigu Hospital. Most patients with LRTIs had pneumonia or bronchitis. Case-patient age ranged from 3 hours to 12 years; for 1 patient, age was unknown (Table). Nasopharyngeal aspirate samples were collected from hospitalized children, and throat swabs were obtained from outpatients with URTIs and from control children.

Of the 174 nasopharyngeal aspirate specimens tested, 28 (16%) had WUPyV VP2 gene–positive fragments; 24 had LTAg gene–positive fragments. Four VP2-positive but LTAg-negative fragments were sequenced; nucleotide sequences were identical to WUPyV strains in GenBank. Mean age of WUPyV-infected patients was 11.7 months (range 12 days–39 months); 10 patients (36%) were ≤6 months of age, 10 (36%) were 6 months–1 year, 7 (25%) were 1–2 years, and 1 (4%) was 2–5 years of age. The age distribution was similar to that of the original cohort. We found WUPyV-positive samples in most months, except for March 2008. Highest proportion of WUPyV-positive samples occurred in December 2008 (27%), followed by April 2008 (25%), November 2008 (22%), and February 2009 (19%).

We detected WUPyV VP2 fragment–positive specimens by multiplex PCR using the Seeplex RV Detection kit-1 (Seegene, Seoul, South Korea) for other respiratory viruses, including adenovirus, parainfluenza viruses 1, 2, 3 (PIV1, 2, 3), influenza viruses A and B, rhinovirus (rhinovirus V), human metapneumovirus, respiratory syncytial virus A and B (RSV A, B), and coronaviruses OC43/HKU1 and 229E/NL63, according to the manufacturers' instructions. First strands of cDNA were produced by using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). Human bocavirus (HBoV) was tested as described previously (*9*).

Twenty (71%) case-patients were co-infected with other respiratory viruses, most commonly RSV B (9/28, 32%), followed by HBoV (6/28, 21%), rhinovirus V and PIV3 (4 each of 28, 14%), human metapneumovirus (3/28, 11%), adenovirus and influenza A (2 each of 28, 7%), and 229E (1/28, 4%). Of 20 patients with co-infections, 14 (50%) were infected with 2 viruses; 2 (7%) with 3 viruses (WU, RSV B, PIV3; and WU, influenza virus, A, HBoV); 3 (11%), with 4 viruses (WU, RSV B, PIV3, influenza A; WU, RSV B, PIV3, HBoV; and WU, RSV B, rhinovirus V, HBoV); and 1 (4%), with 5 viruses (WU, RSV B, PIV3, BoV, rhinovirus V).

Three (4%) of 68 throat swabs from outpatients with URTIs were WUPyV positive, substantially lower than from inpatients with LRTIs. Among 3 WUPyV-positive case-patients, 2 were 2 years of age and 1 was 3. No WUPyV was detected in 43 control samples.

The prevalence of WUPyV in hospitalized children with acute respiratory tract infections in Tianjin was 16.1%, higher than 7.1% found in a study in the United States (*3*); 4.9% in Germany (*4*); 4.5% in Australia (*5*); 6.29% in Thailand (*6*); and 2.2%

Table. Age distribution of children infected with WU polyomavirus, Tianjin, People's Republic of China, 2008–2009*

Age	Inpatients with LRTIs	Outpatients with URTIs	Children without respiratory diseases
Mean ± SD, y	10.2 ± 16.1	5.96 ± 3.69	7.56 ± 4.25
Median age, y	0.4	5.0	7.0
Age group			
<6 mo	95 (54.6)	1 (1.5)	0
6 mo–<1 y	52 (29.9)	1 (1.5)	0
1–<2 y	15 (8.6)	3 (4.4)	2 (4.6)
2–5 y	9 (5.2)	25 (36.8)	11 (25.6)
>5 y	2 (1.1)	38 (55.9)	30 (69.8)
Unknown	1 (0.6)	0	0
Total	174 (100.0)	68 (100.0)	43 (100.0)

* LRTIs, lower respiratory tract infections; URTIs, upper respiratory tract infections. Values are no. (%) unless otherwise indicated.

in Lanzhou, China (*8*). Variation may be due to different geographic and age distributions of the virus. Another study reported that frequencies of WUPyV in URTIs (6.7%) and LRTIs cases (7.1%) were comparable (*10*). However, we found the incidence of WUPyV in patients with LRTIs (16.1%) was higher than in patients with URTIs (4.4%). Among WUPyV-infected patients with LRTIs, 71.4% were <1 year of age, which was comparable to populations investigated in other studies (*2*,*3*,*6*). Although ≈60% of outpatients with URTIs were >5 years of age, none was WUPyV positive. This finding suggests WUPyV may play a major role in young children, especially infants, with LRTIs.

Most WUPyV infection has been detected during later winter and early spring (*2*,*4*,*5*) although other research showed no seasonal distribution (*6*). We found 2 peaks, in April and December 2008 (L. Xiaoyan et al., unpub. data). We also detected 1 WUPyV-infected case in September 2008, which suggests WUPyV could also occur in summer months.

Frequency of WUPyV co-infection with other pathogens varied from 42.1% to 79.7% (*4*–*6*). Although we showed a co-infection rate of 71.4%, there were 8 (28.6%) of 28 patients with respiratory illness in whose specimens we detected only 1 virus, WUPyV. No WUPyV was detected in samples from 43 control patients, whereas in patients with LRTIs and URTIs, infection rates were 16.1% and 4.4%, respectively. These findings suggest WUPyV may be a potential pathogenic agent in children with acute respiratory tract infections. More comprehensive case–control investigations are needed to determine the association of WUPyV infections with respiratory diseases.

This work was supported by Tianjin Municipal Science and Technology Commission (grant no. 07SYSYSF05100).

Xiaoyan Li, Jinying Chen, Mei Kong, Xu Su, Ming Zou, Hua Zhang, and Yumin Han

Author affiliations: Tianjin Centers for Disease Control and Prevention, Tianjin, People's Republic of China (X. Li, M. Kong, X. Su, M. Zou); Tianjin Medical University, Tianjin (X. Li, J. Chen); Tianjin Children's Hospital, Tianjin (H. Zhang); and Tianjin Xianshuigu Hospital, Tianjin (Y. Han)

References

1. Gaynor AM, Nissen MD, Whiley DM, Mackay IM, Lambert SB, Wu G, et al. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog. 2007;3:e64. DOI: 10.1371/journal.ppat.0030064
2. Le BM, Demertzis LM, Wu G, Tibbets RJ, Buller R, Arens MQ, et al. Clinical and epidemiologic characterization of WU polyomavirus infection, St. Louis, Missouri. Emerg Infect Dis. 2007;13:1936–8.
3. Wattier RL, Vázquez M, Weibel C, Shapiro ED, Ferguson D, Landry ML, et al. Role of human polyomaviruses in respiratory tract disease in young children. Emerg Infect Dis. 2008;14:1766–8. DOI: 10.3201/eid1411.080394
4. Neske F, Blessing K, Ullrich F, Pröttel A, Wolfgang Kreth H, Weissbrich B. WU polyomavirus infection in children, Germany. Emerg Infect Dis. 2008;14:680–1. DOI: 10.3201/eid1404.071325
5. Bialasiewicz S, Whiley DM, Lambert SB, Jacob K, Bletchly C, Wang D, et al. Presence of the newly discovered human polyomaviruses KI and WU in Australian patients with acute respiratory tract infection. J Clin Virol. 2008;41:63–8. DOI: 10.1016/j.jcv.2007.11.001
6. Payungporn S, Chieochansin T, Thongmee C, Samransamruajkit R, Theamboolers A, Poovorawan Y. Prevalence and molecular characterization of WU/KI polyomaviruses isolated from pediatric patients with respiratory disease in Thailand. Virus Res. 2008;135:230–6. DOI: 10.1016/j.virusres.2008.03.018
7. Dalianis T, Ramqvist T, Andreasson K, Kean JM, Garcea RLKI. WU and Merkel cell polyomaviruses: a new era for human polyomavirus research. Semin Cancer Biol. 2009;19:270–5. DOI: 10.1016/j.semcancer.2009.04.001
8. Yuan XH, Jin Y, Xie ZP, Gao HC, Xu ZQ, Zheng LS, et al. Prevalence of human KI and WU polyomaviruses in children with acute respiratory tract infection in China. J Clin Microbiol. 2008;46:3522–5. DOI: 10.1128/JCM.01301-08
9. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005;102:12891–6. DOI: 10.1073/pnas.0504666102
10. Norja P, Ubillos I, Templeton K, Simmonds P. No evidence for an association between infections with WU and KI polyomaviruses and respiratory disease. J Clin Virol. 2007;40:307–11. DOI: 10.1016/j.jcv.2007.09.008

Address for correspondence: Xiaoyan Li, Tianjin Centers for Disease Control and Prevention, Institute of Microbiology, No. 76 Hualong St, Hedong District, Tianjin 300011, People's Republic of China; email: xiaoyanli1291@163.com

Intestinal Capillariasis, Western Mindanao, the Philippines

To the Editor: Capillariasis is caused by the foodborne nematode *Capillaria philippinensis*. Infection causes severe diarrhea and protein loss resulting in dehydration, cachexia, and eventually death. Infected patients may also have borborygmi, abdominal pain, weight loss, anorexia, vomiting, and bipedal edema (*1*).

C. philippinensis was first reported in 1963 in Bacarra, Ilocos Norte Province in the northern Philippines (*2*). Since then, additional endemic foci of *C. philippinensis* have been identified. The most recent focus is in Monkayo, Compostela Valley, in the southern Philippines (*3*). In the past several years, suspected unconfirmed cases have been reported from Zamboanga del Norte Province in western Mindanao. In 1999, an epidemic of gastroenteritis in Piñan Municipality was reported; it resulted in 42 deaths. The schistosomiasis team of the De-

partment of Health Regional Office conducted stool examinations and suspected the presence of *Capillaria* ova in symptomatic patients (*4*). In November 2007, several deaths caused by chronic diarrhea were reported in Siayan Municipality. These deaths were attributed to capillariasis, but their cause was never confirmed (*5*).

In February 2008, we obtained 205 stool specimens from residents of Katipunan who had a history of diarrhea of >2 weeks duration and abdominal disturbance. These samples were processed by using the formalin–ether concentration technique (*6*) and examined by expert microscopists. One hundred fifty-one (73.3%) persons were infected with ≥1 organism; 67 (32.5%) had 1 parasitic infection and 84 (40.8%) had multiple parasitic infections. Ninety-one (44.2%) persons had ≥1 soil-transmitted helminth infection, and 93 (45.2%) had ≥1 protozoan infection. Ten (4.9%) persons were confirmed to have *Capillaria* infections. The distribution of organisms observed is shown in the Table.

Among the 10 persons who had capillariasis, 8 were from Barangay (smallest adminstrative region) Matam, 1 from Barangay Dabiak, and 1 from Barangay Carupay, a nearby barangay. Six cases were in male patients and 4 were in female patients. Ages of infected persons ranged from 5 to 54 years (mean 29.2 years, SD 17.1 years). Three of the reported case-patients (a 5-year-old boy, an 8-year old boy, and a 48-year-old woman) were from the same household.

A total of 24 persons in Katipunan were interviewed regarding history of capillariasis and their eating habits. Fourteen residents reported having eaten kinilaw (raw freshwater fish soaked in vinegar and garnished with salt, ginger, and lime). Seven of the persons interviewed had a diagnosis of capillariasis, and 6 had ≥1 relative with a diagnosis of capillariasis. All of the previously diagnosed case-patients were treated with albendazole (400 mg tablets). Most patients were instructed to take 1 tablet 1×/day for 20 days; others were instructed to take 1 tablet 2×/day for 5 or 10 days.

The drug of choice for treating patients with capillariasis is mebendazole, 200 mg 2×/day for 20–30 days. An alternative treatment is albendazole, 400 mg 1×/day for 10 days (*7*,*8*). Variations in the treatment regimen used for patients with capillariasis at the study site suggest a need to train health professionals on the diagnosis, treatment, and follow-up of cases, and on disease prevention and control. Guidelines on proper laboratory techniques for diagnosis of capillariasis; treatment protocols and supportive measures; and protocols for detection, follow-up, and treatment for relapse cases must be developed.

Rates of infection with protozoans and soil-transmitted helminths at the study site are high, which indicate fecal contamination of food and water. A review of records from the Katipunan rural health unit indicated that 76% of households in this municipality have access to toilets. Only 11% of households have water connections (level III). Fifty-seven percent of households have access to communal faucets (level II), and 31% have access only to rivers or springs (*9*). Therefore, increased access to toilets and safe water is needed. Local ordinances concerning ownership and use of toilets must be strictly enforced, and evaluation and rehabilitation of existing toilet and water systems must be conducted.

In spite of efforts concerning information, education, and communication on capillariasis, many residents continue to eat raw or poorly cooked freshwater fish. Concurrent infection among household members, including those in younger age groups, was observed in this study. These findings result from the fact that consumption of kinilaw has become widely accepted and is consumed as a viand (choice food) by families. Thus, information, education, and communication campaigns must be intensified. A promising approach is through collaboration with other agencies. For example, the Department of Education in the Philippines may become involved in dissemination of information on capillariasis to students and in early detection and treatment of infected school children.

Table. Parasites detected in Katipunan, Zamboanga del Norte, the Philippines, February 2008*

Parasite	No. (%)
Trichuris trichiura	64 (31.1)
Entamoeba coli	49 (23.8)
Ascaris lumbricoides	46 (22.3)
Endolimax nana	14 (19.9)
Hookworm	34 (16.5)
Blastocystis hominis	21 (10.2)
Giardia lamblia	19 (0.2)
Entamoeba histolytica	14 (6.8)
Capillaria philippinensis	10 (4.9)

*A total of 205 parasites were detected by using the formalin–ether concentration technique.

Acknowledgments

We thank the National Center for Disease Prevention and Control, the National Epidemiological Center, and the Center for Health Development for Western Mindanao of the Department of Health, the local government units of the province of Zamboanga del Norte, and the municipality of Katipunan for technical assistance and logistics support; Johnson and Johnson Philippines, Inc., for providing partial support for conducting field work; and Joanne Ramirez and Edward Castelo for assistance with the field work.

Vicente Y. Belizario Jr, Francis Isidore G. Totañes, Winifreda U. de Leon, Julius R. Migriño Jr, and Lino Y. Macasaet

Author affiliations: University of the Philippines–National Institutes of Health, Manila, the Philippines (V.Y. Belizario Jr, F.I.G. Totañes, J.R. Migriño Jr); University of the Philippines College of Public Health, Manila (W.U. de Leon); and National Center for Disease Prevention and Control, Manila (L.Y. Macasaet)

DOI: 10.3201/eid1604.080483

References

1. Cross J, Belizario VY Jr. Capillariasis. In: Murrel KD, Fried B, editors. Food-borne parasitic zoonoses, fish and plant-borne parasites. Vol. 11. In: Black S, Seed RJ, editors. World class parasites. New York: Springer Science and Business Media, LLC; 2007. p. 209–32.
2. Chitwood MB, Velasquez C, Salazar NG. *Capillaria philippinensis* (Nematoda: Trichinellida) from intestine of man in the Philippines. J Parasitol. 1968;54:368–71. DOI: 10.2307/3276953
3. Belizario VY Jr, de Leon WU, Esparar DG, Galang JM, Fantone J, Verdadero C. Compostela Valley: a new endemic focus for *Capillariasis philippinensis.* Southeast Asian J Trop Med Public Health. 2000;31:478–81.
4. Icao C. Capillariasis report. Interagency Conference on Capillariasis in Zamboanga del Norte. 2008 Feb 21. Dipolog City (the Philippines): Zamboanga del Norte Provincial Health Office; 2008.
5. Alipala J. Philippine Daily Inquirer. 70 dead in parasite infection; whole Zambo village afflicted [cited 2008 Feb 27]. http://services.inquirer.net/print/print.php?article_id=103456
6. World Health Organization. Training manual on the diagnosis of intestinal parasites; 2004. Geneva: The Organization [cited 2008 Mar 7]. http://www.who.int/wormcontrol/documents/benchaids/en/trainingmanual_sip98–2.pdf
7. Abramowicz M. Drugs for parasitic infections. The Medical Letter. 2004;46:e1–e12.
8. World Health Organization. WHO model prescribing information: drugs used in parasitic diseases, 2nd ed. Geneva: The Organization; 1995.
9. Katipunan Rural Health Unit. Annual report for 2007. Katipunan, Zamboanga del Norte (the Philippines): The Unit; 2007.

Address for correspondence: Vicente Y. Belizario Jr, University of the Philippines–National Institutes of Health, Pedro Gil St, Ermita 1000 Manila, the Philippines; email: vbelizar@yahoo.com

Buruli Ulcer Lesions in HIV-Positive Patient

To the Editor: *Mycobacterium ulcerans* disease (Buruli ulcer) is a neglected and emerging tropical disease (*1*). It often leads to extensive destruction of skin and soft tissue with the formation of large ulcers (*2*). In 2004, the World Health Organization (WHO) recommended the combination treatment of rifampin/streptomycin for patients with this disease (*3*). According to WHO, development of new antimicrobial drug treatment is one of the major advances since the establishment of the Global Buruli Ulcer Initiative (*1*). Treatment with rifampin/streptomycin for ≥4 weeks can inhibit the growth of *M. ulcerans* in preulcerative lesions (*4*). In other patients, despite 4 weeks of treatment, lesions may deteriorate. Whether this treatment is less efficacious in persons with HIV infection is unknown.

In August 2008, a 35-year-old man was referred to the Medical Centre of the Democratic Republic of Congo for assessment of chronic ulcers. Lesions had appeared 12 months earlier when the patient was living in Kafufu/Luremo, a new focus of Buruli ulcer in Angola (*5*). Tissue specimens were subjected to Ziehl-Neelsen staining, culture, and PCR. All results were positive for *M. ulcerans*. Histopathologic analysis of formalin-fixed tissue confirmed the diagnosis of active Buruli ulcer. We treated the patient with a combination of rifampin (10 mg/kg/day, orally) and streptomycin (15 mg/kg/day, by intramuscular injection). Wound dressings containing an aqueous solution of chloramine/metronidazole/nitrofurantoine were changed daily (*6*). For logistic reasons, surgery (large excision) under general anesthesia was not possible.

Characteristics of the patient are shown in the online Technical Appendix (www.cdc.gov/EID/content/16/4/738-Techapp.pdf). At the start of treatment, the patient had a large ulcer on the right leg and thigh, a nodule 2 cm in diameter on the left thigh, and a plaque 8 cm in diameter on the left thigh. After 2 weeks of treatment, the size of the large ulcer had increased. After 4 weeks, the nodule became an ulcer 6 cm in diameter, and the plaque became a large ulcer 15 cm in diameter with a satellite ulcer 2 cm in diameter. After 8 weeks, we observed enlargement of all lesions and the appearance of an ulcer on the left wrist. Treatment was continued for an additional 4 weeks (total 12 weeks). Radiologic investigation did not disclose any bone destruction.

The patient was positive for HIV by the Determine HIV-1/2 test (Abbott Laboratories, Dainabot Co. Ltd., Tokyo, Japan), the Uni-Gold HIV test (Trinity Biotech PLC, Bray, Ireland), and the Genie II HIV-1/HIV-2 test (Bio-Rad, Marnes-la-Coquette, France). Results of PCR for *M. ulcerans* and Ziehl-Neelsen staining were positive for all specimens obtained during the 8 weeks of initial treatment. The patient died 2 weeks after treatment ended, just when antiretroviral treatment had been scheduled to begin.

Although the patient did not respond clinically to treatment with rifampin/streptomycin, whether the treatment also failed microbiologically is more difficult to prove. Results of PCRs performed during treatment remained positive. However, PCR does not differentiate between living and dead *M. ulcerans* bacteria. Therefore, our positive results suggest, but do not prove, treatment failure. The positive culture after 2 weeks of treatment also suggests treatment failure but cultures obtained at 4 and 8 weeks were contaminated. However, culturing *M. ulcerans* bacteria is difficult, especially if samples must be transported (*7*).

Patients with Buruli ulcer may also be infected with HIV. In a study conducted during January 2002–Au-

gust 2003 that compared HIV prevalence in 426 patients with Buruli ulcer and 613 controls in southern Benin, HIV prevalence among patients with Buruli ulcer was higher (2.6%, 11/426) than among controls (0.3%, 2/613) (odds ratio 8.1) (*8*). However, none of these reported HIV-positive patients with Buruli ulcer were treated with rifampin/streptomycin and antiretroviral therapy (*8*).

A study of 224 patients with Buruli ulcer in Benin that evaluated the WHO-recommended regimen of 8 weeks of treatment with rifampin/streptomycin showed promising results (*9*). Chemotherapy alone was successful in achieving a cure rate of 47% of patients and was effective against ulcers <5 cm in diameter (*9*). However, HIV testing was not performed in this study. In Spain, an HIV-positive patient with aggressive, multifocal Buruli ulcer and osteomyelitis was cured by surgery, broad-spectrum antimicrobial drugs (not rifampin/streptomycin), and antiretroviral drugs (*10*). Relapse was not reported in this study at 6-months follow-up.

For control of Buruli ulcer in HIV-positive patients, patients should be treated with rifampin/streptomycin and antiretroviral therapy to stimulate their immunity. Our report emphasizes the urgent need to evaluate treatment of HIV-positive patients infected with Buruli ulcer with rifampin/streptomycin and antiretroviral drugs.

This study was supported by the Directorate-General for Development and Cooperation (DGDC), Brussels, Belgium. K.K. was supported by a grant from DGDC.

Kapay Kibadi, Robert Colebunders, Jean-Jacques Muyembe-Tamfum, Wayne M. Meyers, and Françoise Portaels

Author affiliations: Institute of Tropical Medicine, Antwerp, Belgium (K. Kibadi, R. Colebunders, F. Portaels); Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of Congo (K. Kibadi, J.-J. Muyembe-Tamfum); University of Kinshasa, Kinshasa (K. Kibadi, J.-J. Muyembe-Tamfum); University of Antwerp, Antwerp (R. Colebunders); and Armed Forces Institute of Pathology, Washington, DC, USA (W.M. Meyers)

DOI: 10.3201/eid1604.091343

References

1. World Health Organization. Buruli ulcer: progress report. Wkly Epidemiol Rec. 2008;83:145–56.
2. Portaels F, Silva MT, Meyers WM. Buruli ulcer. Clin Dermatol. 2009;27:291–305. DOI: 10.1016/j.clindermatol.2008.09.021
3. World Health Organization. Provisional guidance on the role of specific antibiotics in the management of *Mycobacterium ulcerans* disease (Buruli ulcer) WHO/CDS/CPE/GBUI.10; 2004 [cited 2009 Dec 23]. http://www.who.int/buruli/information/antibiotics/en/index.html
4. Etuaful S, Carbonnelle B, Grosset J, Lucas S, Horsfield C, Phillips R, et al. Efficacy of the combination rifampin-streptomycin in preventing growth of *Mycobacterium ulcerans* in early lesions of Buruli ulcer in humans. Antimicrob Agents Chemother. 2005;49:3182–6. DOI: 10.1128/AAC.49.8.3182-3186.2005
5. Kibadi K, Panda M, Tamfum JJ, Fraga AG, Longatto Filho A, Anyo G, et al. New foci of Buruli ulcer, Angola and Democratic Republic of Congo. Emerg Infect Dis. 2008;14:1790–2. DOI: 10.3201/eid1411.071649
6. Kibadi K. *Mycobacterium ulcerans* disease (Buruli ulcer): surgical treatment of 102 cases in the Democratic Republic of Congo [in French]. Med Trop (Mars). 2005;65:444–8.
7. Eddyani M, Debacker M, Martin A, Aguiar J, Johnson CR, Uwizeye C, et al. Primary culture of *Mycobacterium ulcerans* from human tissue specimens after storage in semi-solid transport medium. J Clin Microbiol. 2008;46:69–72. DOI: 10.1128/JCM.00301-07
8. Johnson RC, Nackers F, Glynn JR, de Biurrun Bakedano E, Zinsou C, Aguiar J, et al. Association of HIV infection and *Mycobacterium ulcerans* disease in Benin. AIDS. 2008;22:901–3. DOI: 10.1097/QAD.0b013e3282f7690a
9. Chauty A, Ardant MF, Adeye A, Euverte H, Guédénon A, Johnson C, et al. Promising clinical efficacy of streptomycin-rifampin combination for treatment of Buruli ulcer (*Mycobacterium ulcerans* disease). Antimicrob Agents Chemother. 2007;51:4029–35. DOI: 10.1128/AAC.00175-07
10. Toll A, Gallardo F, Ferran M, Gilaberte M, Iglesias M, Gimeno JL, et al. Aggressive multifocal Buruli ulcer with associated osteomyelitis in an HIV-positive patient. Clin Exp Dermatol. 2005;30:649–51. DOI: 10.1111/j.1365-2230.2005.01892.x

Address for correspondence: Françoise Portaels, Mycobacteriology Unit, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium; email: portaels@itg.be

Hantavirus Pulmonary Syndrome, French Guiana

To the Editor: Hantaviruses are rodent-borne negative-sense RNA viruses belonging to the *Bunyaviridae* family, genus *Hantavirus*. Since the first report of a hantavirus in 1993 in the United States (*1*), different viruses belonging to this genus have been reported in the Americas (*2–5*). These New World viruses are responsible for a disease called hantavirus pulmonary syndrome (HPS), a respiratory illness caused by the inhalation of dust contaminated by rodent feces or urine containing the virus (*6–8*).

Until recently, no information was available concerning the presence of hantaviruses in French Guiana, a French overseas department (administrative unit) in South America. Nevertheless, the description of atypical pneumonia cases not related to any known etiologic agent and the identification of hantavirus reservoirs in neighboring countries led us to con-

duct a serologic study in a selected population of patients with compatible symptoms. The prevalence of immunoglobulin (Ig) G antibodies to hantavirus in this population was 1.42% (*9*). Subsequently, we systematically screened patients who had suggestive pathologies for hantavirus serology, which led us to the characterization of a divergent hantavirus.

On August 4, 2008, a 38-year-old man sought medical attention at the emergency department of Cayenne Hospital. He had had persistent symptoms of fever (≥38.5°C), myalgia, diarrhea with melena, cough for 8 days, recurrent vomiting for 4 days, and dyspnea for 2 days. At consultation, tachypnea (respiratory rate 28/min) and oxygen desaturation (SaO_2 83%) were observed. Chest radiograph showed bilateral diffuse interstitial infiltrates causing respiratory distress; mechanical ventilation was required. The patient was admitted to the intensive care unit for treatment of acute respiratory distress syndrome. Results of laboratory investigations performed when the patient was admitted showed thrombocytopenia (50,000 cells/mm^3), leucocytosis (22,500 cells/mm^3) associated with a high neutrophil count (20,300 cells/mm^3), moderate hepatonephritis (alanine aminotransferase 17 IU/L, aspartate aminotransferase 31 IU/L, gamma-glutanyl transferase 44 IU/L; alkaline phosphatase 44 IU/L; creatinine 192 μmol/L and urea 9.3 mmol/L); and an elevated C-reactive protein concentration (>192 mg/L). Laboratory tests for infectious agents ruled out malaria, dengue, leptospirosis, Chagas disease, Q fever, cytomegalovirus, and HIV, and blood cultures were negative for bacterial growth. The patient remained under respiratory assistance for 25 days in the intensive care unit and was discharged from hospital 47 days after admission with a complete clinical recovery.

With no etiologic agent identified, 2 factors led to the suspicion of hantavirus infection: clinical symptoms compatible with HPS and the patient's exposure to potential reservoirs. Indeed, a month before the onset of symptoms, he had moved to a rural municipality located near agricultural lands and forest.

Retrospective serologic investigations were performed with the 3 available serum samples obtained during the hospitalization. These samples were tested by IgM capture with inactivated Sin Nombre virus antigens and by indirect ELISA with recombinant antigens to detect IgG antibodies to Sin Nombre virus (*10*). IgM to Sin Nombre virus were present in the samples collected 8 and 9 days, respectively, after onset of the disease, confirming hantavirus infection. Furthermore, IgG to Sin Nombre virus were only detected in the convalescent-phase serum samples obtained on day 41 of the disease. These serologic results suggested a recent infection with hantavirus.

Molecular investigations were performed to characterize and identify the virus. Viral RNA was extracted from the 2 acute serum samples. Reverse transcription–PCR was performed with consensus primers targeting the S segment of the hantavirus genome as described in Johnson et al. (*4*). Amplification products of the expected size (434 bp of the nucleoprotein N-encoding region) were obtained from both samples. Cloning and sequencing of these products allowed obtaining a consensus sequence, which was deposited with GenBank (GQ179973). Database searches using BLAST (www.ncbi.nlm.nih.gov/blast) demonstrated that this sequence, although novel, is most similar to Rio Mamore hantavirus strain OM-556 (GenBank accession no. U52136), showing 83% nucleotide identity (393 bp analyzed, excluding the primers). In addition, comparison with representative hantavirus sequences from New World isolates showed that the amplified fragment exhibited from

Table. Comparison of nucleotide and deduced amino acid sequences between Maripa virus and representative New World hantaviruses*

Maripa virus	% Nucleotide sequence identity	% Amino acid sequence identity
RIOM_B	83.0	96.9
RIOM_P	81.9	96.2
ANAJ	81.2	96.2
LAN	81.2	96.2
RIME	77.9	94.7
PARN	78.4	93.9
ARAQ	80.2	93.1
AND	78.6	93.1
ORN	78.4	93.1
MCL	76.8	93.1
CHO	76.1	93.1
PRG	76.6	93.1
LEC	80.2	92.4
BMJ	79.4	92.4
CAD	77.1	92.4
ARAC	77.6	91.6
C_Plata	77.6	90.8
SN	73.5	90.1

*RIOM_B, Rio Mamoré virus strain OM-556 (U52136); RIOM_P, Rio Mamoré virus strain HTN-007 (AF133254); ANAJ, Anajatuba virus isolate Of58 (DQ451829); LAN, Laguna Negra virus strain 510B (AF005727); RIME, Rio Mearim virus isolate Hs85 (DQ451828); PARN, Paranoa virus (EF576661); ARAQ, Araraquara virus (AF307325); AND, Andes virus strain AH-1 (AF324902); ORN, Oran virus strain 22996, (AF482715); MCL, Maciel virus strain 13796 (AF482716); CHO, Choclo virus (DQ 285046); PRGm Pergamino virus strain 14403 (AF482717); LEC, Lechiguanas virus strain 22819 (AF482714); BMJ, Bermejo virus strain Oc22531 (AF482713); CAD, Cano Delgadito virus isolate VHV-574 (AF000140); ARAC, Araucaria virus strain HPR/04-102 (AY740633); CAS, Castelo dos Sonhos virus (AF307324); C_Plata, Central Plata virus strain 714LC (EU564715); SN, Sin Nombre virus strain NM H10 (L25784).

73.5% to 81.9% nucleotide sequence identity and from 90.1% to 96.9% amino acid sequence identity (Table). This level of sequence divergence, as well as the geographic specificity of this hantavirus in French Guiana led us to provisionally name it Maripa virus.

Results of a serologic survey to identify cases of respiratory disease with no evident etiology led us to identify an HPS case-patient in French Guiana who had been infected with a new divergent hantavirus strain. Human hantavirus epidemics are associated with fluctuations of rodent populations caused by climatic, ecologic and environmental changes or with changes in human activities associated with nature or agriculture. Therefore, in this region where 90% of the land is tropical rain forest but in which there is increasing economic development, continuous surveillance for the virus in the human population would be beneficial. Surveys of potential reservoirs may help reduce the risk of viral emergence.

This study was supported in part by the Centre National de Référence des Arbovirus financed by the Institut Pasteur de la Guyane and the Institut de Veille Sanitaire (St-Maurice, France). Grants were provided by the CPER/DocUP 2000–2006 and the FEDER 2007–2013 programs to the Laboratoire des Interactions Virus-Hôtes, Institut Pasteur de la Guyane.

Séverine Matheus, Félix Djossou, David Moua, Anne Marie Bourbigot, Didier Hommel, Vincent Lacoste, Philippe Dussart, and Anne Lavergne

Author affiliations: Institut Pasteur de la Guyane, Cayenne, French Guiana (S. Matheus, D. Moua, V. Lacoste, P. Dussart, A. Lavergne); and Centre Hospitalier Andrée Rosemon, Cayenne (F. Djossou, A.M. Bourbigot, D. Hommel)

DOI: 10.3201/eid1604.090831

References

1. Nichol ST, Spiropoulou CF, Morzunov S, Rollin PE, Ksiazek TG, Feldmann H, et al. Genetic identification of a hantavirus associated with an outbreak of acute respiratory illness. Science. 1993;262:914–7. DOI: 10.1126/science.8235615
2. da Silva MV, Vasconcelos MJ, Hidalgo NT, Veiga AP, Canzian M, Marotto PC, et al. Hantavirus pulmonary syndrome. Report of the first three cases in Sao Paulo, Brazil. Rev Inst Med Trop Sao Paulo. 1997;39:231–4.
3. Lopez N, Padula P, Rossi C, Miguel S, Edelstein A, Ramirez E, et al. Genetic characterization and phylogeny of Andes virus and variants from Argentina and Chile. Virus Res. 1997;50:77–84. DOI: 10.1016/S0168-1702(97)00053-1
4. Johnson AM, Bowen MD, Ksiazek TG, Williams RJ, Bryan RT, Mills JN, et al. *Laguna Negra* virus associated with HPS in western Paraguay and Bolivia. Virology. 1997;238:115–27. DOI: 10.1006/viro.1997.8840
5. Vincent MJ, Quiroz E, Gracia F, Sanchez AJ, Ksiazek TG, Kitsutani PT, et al. Hantavirus pulmonary syndrome in Panama: identification of novel hantaviruses and their likely reservoirs. Virology. 2000;277:14–9. DOI: 10.1006/viro.2000.0563
6. Schmaljohn C, Hjelle B. Hantaviruses: a global disease problem. Emerg Infect Dis. 1997;3:95–104. DOI: 10.3201/eid0302.970202
7. Klein SL, Calisher CH. Emergence and persistence of hantaviruses. Curr Top Microbiol Immunol. 2007;315:217–52. DOI: 10.1007/978-3-540-70962-6_10
8. Bi Z, Formenty PB, Roth CE. Hantavirus infection: a review and global update. J Infect Dev Ctries. 2008;2:3–23. DOI: 10.3855/jidc.317
9. Matheus S, Meynard JB, Rollin P, Maubert B, Morvan J. New World hantavirus in humans, French Guiana. Emerg Infect Dis. 2006;12:1294–5.
10. Ksiazek TG, Peters CJ, Rollin PE, Zaki S, Nichol S, Spiropoulou C, et al. Identification of a new North American hantavirus that causes acute pulmonary insufficiency. Am J Trop Med Hyg. 1995;52:117–23.

Address for correspondence: Séverine Matheus, Laboratoire de virologie, Centre National de Référence des Arbovirus, Institut Pasteur de la Guyane, 23 avenue Pasteur, BP 6010 – 97306 Cayenne CEDEX, French Guiana; email: smatheus@pasteur-cayenne.fr

Fatal Human Case of West Nile Virus Disease, Mexico, 2009

To the Editor: West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) was first recognized in the Western Hemisphere in 1999 during an outbreak of human, equine, and avian encephalitis in New York (*1*). The virus has since spread across the United States and Canada, where it has caused ≈30,000 human infections and ≈1,000 deaths. Serologic evidence has demonstrated that WNV is present throughout Mexico, Central America, South America, and the Caribbean region (*2*–*8*). However, WNV illness in humans and vertebrate animals in these regions has been only sparsely reported. For instance, 7 human cases of WNV infection have occurred in Mexico (excluding the case described here), 3 of which were severe. All patients survived. To our knowledge, no fatal human cases of WNV infection have occurred in Central America, South America, or the Caribbean region.

We describe a fatal case of WNV infection in a human in Central America. The patient, a man 40 years of age, lived in Monterrey, Nuevo León State, in northern Mexico. He had not traveled outside of the metropolitan area in the 6 months before illness onset. On June 11, 2009, influenza-like signs and symptoms (i.e., fever, malaise, fatigue, arthralgia, headache, and dizziness) developed in the patient. On June 26, the signs and symptoms had not resolved, and the man was admitted to University Hospital "Dr. José E. Gonzalez" at the Universidad Autonoma de Nuevo León (UANL). At the time of admission, cerebrospinal fluid (CSF) was collected, and laboratory analysis indicated a markedly elevated leukocyte count (182 cells/mm^3; reference range 0–5 cells/mm^3) and slightly elevated protein and glucose levels.

Several days later, serious neurologic signs that included loss of consciousness developed in the patient. On July 6, he lapsed into a coma and was transferred to the intensive care unit and treated for intracranial hypertension. Another CSF specimen was collected, and laboratory findings demonstrated that the leukocyte count had increased to 495 cells/mm^3. CSF cytologic examination showed atypical lymphocytes, some of which resembled plasma cells. Brain magnetic resonance imaging showed hydrocephalus with no brain parenchymal lesions. Because the patient was suspected to have a herpes simplex virus infection, intravenous acyclovir was administered. Several days later, the patient showed signs of improvement; on July 15, he was discharged. Eleven days later, he experienced severe headaches and, on July 29, was readmitted to the UANL Hospital for intracranial hypertension. On July 30, a ventriculoperitoneal shunt was implanted; however, the patient's condition continued to decline, and he died on August 1.

Personnel in the Laboratory of Molecular Infectology at the UANL were informed of the patient and were provided with the remainder of the second CSF specimen several days before his death. Total RNA and DNA were extracted from the CSF by using the QIAamp viral RNA extraction kit (QIAGEN, Valencia, CA, USA) and DNAzol (Invitrogen, Carlsbad, CA, USA) and tested for nucleic acid to various pathogens associated with human central nervous system infections, specifically herpes simplex virus types 1 and 2, human enterovirus A–D, dengue virus types 1–4, WNV, and *Mycobacterium tuberculosis*. Complementary DNA samples were generated by using Superscript III reverse transcription (Invitrogen), and PCRs were performed by using *Taq* polymerase (Invitrogen) in accordance with the manufacturer's instructions. PCR amplifications were conducted by using the following reaction conditions: 94°C for 3 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; followed by a final extension at 72°C for 8 min. Reverse transcription–PCRs performed with diethyl pyrocarbonate–treated distilled water in place of nucleic acid were included as negative controls. All test and control reactions were performed in duplicate. PCR products were examined by 2% agarose gel electrophoresis and visualized with ethidium bromide. A PCR product of the expected size was observed when the WNV-specific primers WN-*cap*-F (5′-CAGT GCTGGATCGATGGAGAG-3′) and WN-*cap*-R (5′-CCGCCGATTG ATAGCACT GGT-3′) were used. These primers amplify a 104-nt region of the capsid gene. All other assay results were negative. Subsequent reactions were performed by using a second set of WNV-specific primers, WN-*env*-F (5′-GATGTGAAG ATGATGAATATGG-3′) and WN-*env*-R (5′-AATGCTTCCTTTGCCAA ATAG-3′), which amplify a 216-nt region of the envelope gene. A PCR product of the expected size was again observed. PCR products were purified by using the Purelink Gel Extraction Kit (Invitrogen) and sequenced by using a 3730×1 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Because of the small volume of CSF obtained, a comprehensive laboratory analysis (virus isolation, plaque reduction neutralization test) could not be performed. Nevertheless, detection of WNV in the CSF of a patient with encephalitis meets the Centers for Disease Control and Prevention established criteria for a case of West Nile neuroinvasive disease (*9*). Our findings highlight the fact that the low number of WNV cases in Mexico and elsewhere in Latin America should not deter healthcare personnel from performing WNV diagnostic testing and the public from using personal protective measures in these regions.

Acknowledgments

We thank the staff at the Department of Preventive Medicine of the Hospital Universitario "Dr. José E. Gonzalez" at UANL for their support and technical advice.

This work was supported in part by grant FOMIX NL-2003-CO4-12553 to A.M.R.-E.

Clara Rios-Ibarra, Bradley J. Blitvich, Jose Farfan-Ale, Javier Ramos-Jimenez, Sissy Muro-Escobedo, Hector R. Martínez-Rodriguez, Rocío OrtizLópez, Ernesto Torres-López, and Ana María Rivas-Estilla

Author affiliations: Universidad Autónoma de Nuevo León, Monterrey, México (C. Rios-Ibarra, J. Ramos-Jimenez, S. Muro-Escobedo, H.R. Martínez-Rodriguez, R. OrtizLópez, E. Torres-López, A.M. Rivas-Estilla); Iowa State University, Ames, Iowa, USA (B.J. Blitvich); and Universidad Autónoma de Yucatán, Mérida, México (J. Farfan-Ale)

DOI: 10.3201/eid1604.091614

References

1. Nash D, Mostashari F, Fine A, Miller J, O'Leary D, Murray K, et al. The outbreak of West Nile virus infection in the New York City area in 1999. N Engl J Med. 2001;344:1807–14.
2. Blitvich BJ, Fernandez-Salas I, Contreras-Cordero JF, Marlenee NL, Gonzalez-Rojas JI, Komar N, et al. Serologic evidence of West Nile virus infection in horses, Coahuila State, Mexico. Emerg Infect Dis. 2003;9:853–6.
3. Cruz L, Cardenas VM, Abarca M, Rodriguez T, Reyna RF, Serpas MV, et al. Short report: serological evidence of West Nile virus activity in El Salvador. Am J Trop Med Hyg. 2005;72:612–5.
4. de Lourdes Garza Rodriguez M, Rodriguez Rodriguez DR, Blitvich BJ, Lopez MA, Fernandez-Salas I, Jimenez JR, et al. Serologic surveillance for West Nile virus and other flaviviruses in febrile patients, encephalitic patients, and asymptomatic blood donors in northern Mexico. Vector Borne Zoonotic Dis. 2009 Jun 3; [Epub ahead of print].

5. Dupuis AP II, Marra PP, Kramer LD. Serologic evidence of West Nile virus transmission, Jamaica, West Indies. Emerg Infect Dis. 2003;9:860–3.
6. Estrada-Franco JG, Navarro-Lopez R, Beasley DW, Coffey L, Carrara AS, Travassos da Rosa A, et al. West Nile virus in Mexico: evidence of widespread circulation since July 2002. Emerg Infect Dis. 2003;9:1604–7.
7. Mattar S, Edwards E, Laguado J, Gonzalez M, Alvarez J, Komar N. West Nile virus antibodies in Colombian horses. Emerg Infect Dis. 2005;11:1497–8.
8. Elizondo-Quiroga D, Davis CT, Fernandez-Salas I, Escobar-Lopez R, Velasco Olmos D, Soto Gastalum LC, et al. West Nile virus isolation in human and mosquitoes, Mexico. Emerg Infect Dis. 2005;11: 1449–52.
9. Centers for Disease Control and Prevention. Neuroinvasive and non-neuroinvasive domestic arboviral diseases [cited 2010 Jan 12]. http://www.cdc.gov/epo/dphsi/casedef/arboviral_current.htm

Address for correspondence: Ana María Rivas-Estilla, Department of Biochemistry and Molecular Medicine, Faculty of Medicine, Universidad Autónoma de Nuevo León Av. Francisco I. Madero y Eduardo Aguirre Pequeño s/n Col. Mitras Centro CP 64460, Monterrey, Nuevo León, México; email: amrivas1@yahoo.ca

Bartonella spp. Infections, Thailand

To the Editor: *Bartonella* are fastidious hemotropic gram-negative bacteria with a worldwide distribution. In Thailand, *Bartonella* species have been demonstrated in mammalian hosts, including rodents, cats and dogs, and in potential vectors, including fleas (*1*–*4*). However, data on human infection have been limited to case reports (*5*,*6*) and 1 seroprevalence survey, which found a 5.5% prevalence of past *B. henselae* infection (*7*). No studies have systematically assessed the frequency, clinical characteristics, or epidemiology of human *Bartonella* infections in Thailand.

We conducted a prospective study to determine causes of acute febrile illness in 4 community hospitals, 2 in Chiang Rai (northern Thailand) and 2 in Khon Kaen (northeastern Thailand). We enrolled patients ≥7 years of age with a temperature >38°C who were brought to study hospitals for treatment from February 4, 2002, through March 28, 2003. Patients were excluded if they had a history of fever for ≥2 weeks or an infection that could be diagnosed clinically. Acute-phase serum samples were collected at the time of enrollment and convalescent-phase serum samples 3–5 weeks later. We enrolled nonfebrile control patients ≥14 years of age who had noninfectious conditions; acute-phase serum samples were collected. Clinical information was abstracted from patient charts. Nurses conducted physical examinations and personal interviews to collect information on patients' demographic characteristics, exposures to animals, and outdoor activities.

Serum samples were tested for immunoglobulin (Ig) G antibodies to *Bartonella* spp. by immunofluorescent antibody assay at the Bartonella Laboratory of the Centers for Disease Control and Prevention, Fort Collins, CO, USA. Strains used for antigen production were: *B. elizabethae* (F9251), *B. henselae* (Houston-1), *B. quintana* (Fuller), and *B. vinsonii* subsp. *vinsonii* (Baker). Homologous hyperimmune serum specimens were produced in BALB/c mice as previously described (*8*). *Bartonella* infection was considered confirmed in febrile patients who had a ≥4-fold rise in IgG antibody titers and a convalescent-phase titer >64. Probable infection was defined as 1) a 4-fold antibody titer rise but convalescent-phase titers of 64, or 2) high and stable titers (≥512 in acute-phase and convalescent-phase serum samples), or 3) acute-phase titer ≥512 with a ≥4-fold titer fall. Paired serum samples from febrile patients were also tested for serologic evidence of other common causes of febrile illness in Southeast Asia.

Febrile patients with acute-phase and convalescent-phase IgG antibody titers <128 were considered not to have *Bartonella* infection; we compared demographic and clinical characteristics of these patients to *Bartonella*-infected patients. To evaluate potential risk factors, we compared *Bartonella*-infected case-patients ≥14 years of age without serologic evidence of other infections (n = 20) to nonfebrile controls with IgG to *Bartonella* <128 (n = 70). Age adjusted odds ratios (AORs) with 95% confidence intervals (CIs) were calculated.

Serologic testing was completed on paired serum samples for 336 (46%) of 732 febrile patients enrolled; 92 (27%) had serologically confirmed (50) or probable (42) *Bartonella* infections. Thirty-five (38%) of these 92 had serologic evidence of infection with another pathogen. The remaining 57 *Bartonella*-infected case-patients (34 confirmed, 23 probable) had a median age of 19 years (range 7–72 years); 65% were males, 47% were students, and 35% were rice farmers. Common clinical characteristics of *Bartonella*-infected patients included myalgias (83%), chills (79%), and headache (77%). Thirty (60%) patients had anemia (hemoglobin level <13 mg/dL); 18 (32%) had a hemoglobin level <12 mg/dL, and 4 (7%) had <11 mg/dL. When compared with 193 febrile patients without *Bartonella* infection, the 57 *Bartonella*-infected patients were similar in age and sex but were more likely to be rice farmers and were more likely to have leukocytosis (Table). Compared with the 70 nonfebrile controls, *Bartonella*-infected case-patients were more likely to report tick exposure (32% vs. 7.9%; AOR = 5.6, 95% CI 1.5–21) and outdoor activities (55% vs. 31%; AOR = 2.7, 95% CI 1.0–7.4) during the 2 weeks before

illness onset. Prevalence of reported rat exposure and animal ownership (cats, dogs, pigs, cows, or buffaloes) was similar among case-patients and controls.

We describe the frequency and clinical characteristics of acute *Bartonella* infection among febrile patients in Thailand. Over 25% of patients with undifferentiated febrile illness had serologic evidence of *Bartonella* infection (including 15% serologically confirmed). Our findings indicate that *Bartonella* infections may be common and underrecognized causes of acute febrile illness in rural Thailand. Although our results are limited by lack of culture confirmation, we used conservative case definitions for serologic diagnosis and therefore believe that most cases represent true *Bartonella* infections. The common clinical features of anemia and leukocytosis and the frequent tick exposure and outdoor activity are consistent with known features of *Bartonella* infections and lend support to serologic findings. Because of the potential for serologic cross-reactivity between *Bartonella* species, we did not attempt species identification. The case-control study was therefore limited by grouping case-patients that were likely infected with different *Bartonella* species for which risk factors may differ. Such studies could lead to meaningful recommendations for prevention and control of *Bartonella* infections. Additional epidemiologic and transmission studies are needed to improve understanding of risk factors, identify key animal reservoirs and vectors, and ascertain transmission dynamics.

Table. Demographic and clinical characteristics of febrile patients with *Bartonella* infection compared with febrile patients who had no evidence of *Bartonella* infection, Thailand, 2002–2003*

Variables	No. (%) *Bartonella*-infected case-patients,† n = 57	No. (%) non–case-patients,‡ n = 193	p value
Median age, y (range)	19 (7–72)	20 (7–79)	0.9
Male sex	37 (64.9)	113 (58.5)	0.4
Occupation			
Student	27 (47.4)	84 (43.5)	0.6
Rice farmer	20 (35.1)	40 (20.7)	0.03
Business	3 (5.3)	13 (6.7)	0.7
Other§	7 (12.3)	56 (29.0)	0.01
Signs and symptoms			
Headache	44 (77.2)	161 (83.4)	0.3
Eye pain	17 (29.8)	58 (30.1)	1.0
Myalgias	47 (82.5)	141 (73.1)	0.1
Extremity pain	39 (68.4)	115 (59.6)	0.2
Joint pain	26 (45.6)	74 (38.3)	0.3
Vomiting	22 (38.6)	80 (41.5)	0.7
Abdominal pain	12 (21.1)	64 (33.2)	0.08
Rash	7 (12.3)	17 (8.8)	0.6
Lymphadenopathy	9 (15.8)	24 (12.4)	0.5
Laboratory results			
Anemia (Hb <13 mg/dL)	30 (60.0)	93 (50.3)	0.2
Thrombocyte count <100,000/mm^3	5 (8.9)	14 (7.4)	0.7
Leukopenia (leukocytes ≤5,000/mm^3)	10 (17.5)	63 (32.6)	0.03
Leukocytosis (leukocytes ≥11,000/mm^3)	20 (35.1)	33 (17.1)	<0.01
Creatinine ≥1.5 mg/dL	5 (8.9)	12 (6.2)	0.5
Bilirubin ≥1.3 mg/dL	6 (10.7)	19 (9.8)	0.8
Alkaline phosphatase ≥121 IU/L	36 (64.3)	132 (68.8)	0.5
AST ≥36 IU/dL	18 (32.1)	88 (45.6)	0.07
ALT ≥36 IU/dL	9 (16.1)	50 (25.9)	0.1

*Hb, hemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
†Excluded *Bartonella*-infected patients with serologic evidence of infection with other common pathogens (dengue virus, *Leptospira*, or *Burkholderia pseudomallei*; n = 35).
‡Febrile patients with acute-phase and convalescent-phase immunoglobulin G titer against *Bartonella* species <128.
§Other occupation includes housewife, government officer, day laborer, or construction worker.

Acknowledgments

We are grateful for the contributions of the many study collaborators, especially K. Limpakarnjanarat, S. Thamthitiwat, P. Mock, U. Siangphoe, P. Srisaengchai, P. Sawatwong, and A. Nisalak; the dedicated study staff; and all volunteer study participants.

Saithip Bhengsri, Henry C. Baggett, Leonard F. Peruski Jr, Christina Morway, Ying Bai, Tamara L. Fisk, Anussorn Sitdhirasdr, Susan A. Maloney, Scott F. Dowell, and Michael Kosoy

Author affiliations: Thailand Minstry of Public Health–US Centers for Disease Control and Prevention Collaboration, Nonthaburi, Thailand (S. Bhengsri, H.C. Baggett, L.F. Peruski Jr, T.L. Fisk, S.A. Maloney); Thailand Ministry of Public Health, Nonthaburi (A. Sitdhirasdr); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (S.F. Dowell); and Centers for Disease Control and Prevention, Fort Collins, Colorado, USA (C. Morway, Y. Bai, M. Kosoy).

DOI: 10.3201/eid1604.090699

References

1. Maruyama S, Sakai T, Morita Y, Tanaka S, Kabeya H, Boonmar S, et al. Prevalence of *Bartonella* species and 16s rRNA gene types of *Bartonella henselae* from domestic cats in Thailand. Am J Trop Med Hyg. 2001;65:783–7.
2. Parola P, Sanogo OY, Lerdthusnee K, Zeaiter Z, Chauvancy G, Gonzalez JP, et al. Identification of *Rickettsia* spp. and *Bartonella* spp. in fleas from the Thai-Myanmar border. Ann N Y Acad Sci. 2003;990:173–81. DOI: 10.1111/j.1749-6632.2003.tb07359.x

3. Castle KT, Kosoy M, Lerdthusnee K, Phelan L, Bai Y, Gage KL, et al. Prevalence and diversity of *Bartonella* in rodents of northern Thailand: a comparison with *Bartonella* in rodents from southern China. Am J Trop Med Hyg. 2004;70: 429–33.
4. Suksawat J, Xuejie Y, Hancock SI, Hegarty BC, Nilkumhang P, Breitschwerdt EB. Serologic and molecular evidence of coinfection with multiple vector-borne pathogens in dogs from Thailand. J Vet Intern Med. 2001;15:453–62. DOI: 10.1892/0891-6640(2001)015<0453:SAMEOC>2.3.CO;2
5. Kosoy M, Morway C, Sheff KW, Bai Y, Colborn J, Chalcraft L, et al. *Bartonella tamiae* sp. nov., a newly recognized pathogen isolated from three human patients from Thailand. J Clin Microbiol. 2008;46:772–5. DOI: 10.1128/JCM.02120-07
6. Paitoonpong L, Chitsomkasem A, Chantrakooptungool S, Kanjanahareutai S, Tribuddharat C, Srifuengfung S. *Bartonella henselae*: first reported isolate in a human in Thailand. Southeast Asian J Trop Med Public Health. 2008;39:123–9.
7. Maruyama S, Boonmar S, Morita Y, Sakai T, Tanaka S, Yamaguchi F, et al. Seroprevalence of *Bartonella henselae* and *Toxoplasma gondii* among healthy individuals in Thailand. J Vet Med Sci. 2000;62:635–7. DOI: 10.1292/jvms.62.635
8. Kosoy MY, Regnery RL, Tzianabos T, Marston EL, Jones DC, Green D, et al. Distribution, diversity, and host specificity of *Bartonella* in rodents from the southeastern United States. Am J Trop Med Hyg. 1997;57:578–88.

Address for correspondence: Saithip Bhengsri, International Emerging Infections Program, Thailand Ministry of Public Health–US Centers for Disease Control and Prevention Collaboration, 3rd Floor, Bldg 7, Department of Disease Control, Ministry of Public Health, Nonthaburi, 11000, Thailand; email: saithipb@th.cdc.gov

Cholera Outbreak, Laos, 2007

To the Editor: Cholera is a major public health problem in countries where access to safe water and adequate sanitation cannot be guaranteed for all. *Vibrio cholerae* serogroups O1 and O139 are the causative agents of cholera (*1*). One of the most powerful virulence factors in this organism is cholera toxin encoded by the *ctxAB* gene, located on the CTX prophage. *V. cholerae* O1 is classified into 2 biotypes, classical and El Tor. The El Tor type of *V. cholerae* O1 is responsible for the ongoing seventh worldwide pandemic of cholera (*2*). The sequence of *ctxB* of a certain strain has been believed to correspond to its biotype; that is, a biotype classical strain has classical type *ctxB*, and a biotype El Tor strain has El Tor type *ctxB*. However, recent research studies suggest that novel types of *V. cholerae* O1, hybrid strains, and altered El Tor or El Tor variant strains (*1*,*3*) are emerging. Altered El Tor or El Tor variant strains are biotype El Tor but produce classical cholera toxin (*3*,*4*). Recent reports suggest that this type of *V. cholerae* O1 is spreading to many areas of the world (*5*).

In December 2007–January 2008, a cholera outbreak occurred in Xekong Province in southeastern Laos, in the Mekong Basin. The first case of the outbreak was detected on December 23, 2007. The outbreak spread to 10 villages and lasted through January 2008. Specifically, in the Thateng District, 117 cases occurred and 2 deaths were reported. The sources of the outbreak were suspected to be regularly used water. In October 2007, 2 months before the outbreak, 3 sporadic cases of *V. cholerae* infection had been identified in Vientiane (the capital city) and Xaignabouri Province in north-central and northwestern Laos, respectively. The outbreak investigation in the Xekong Province identified no linkage between these sporadic cases and the outbreak cases.

In this study, we analyzed 18 *V. cholerae* isolates obtained in 2007: 3 were from patients with sporadic cases, and 15 were from the Xekong outbreak (13 from patients and 2 from water samples). All the isolates were serotype O1 Ogawa and biotype El Tor, but their *ctxB* types were classical, according to the method previously described (*6*). This finding indicates that they were the type of altered El Tor.

We used pulsed-field gel electrophoresis (PFGE) to investigate relationships between the isolates according to the PulseNet protocol (*7*). All 18 isolates from the sporadic cases and the outbreak in 2007 displayed profiles indistinguishable from each other (Figure). We also compared 2 additional *V. cholerae* O1 isolates, 1 from a patient in Vientiane in 1998 and another from a patient in Louangphabang in 2000 (Figure). The profiles of the isolates obtained in 1998 and 2000 clearly differed from those obtained in 2007. These results indicate that all isolates from sporadic and outbreak cases in 2007 were likely from the same source of contamination, although extensive epidemiologic investigation did not identify any common source.

Nguyen et al. characterized the isolates from a cholera outbreak in Vietnam from late 2007 to early 2008 (*8*). Their report suggests that the isolates from the outbreaks in Vietnam and Laos shared the same elements of the CTX prophage. Our study suggests a common source for the strains of sporadic cases in Vientiane and Xaignabouri Province in October 2007 and those of the outbreak in Xekong Province in December 2007. Molecular typing suggests that a novel clone of *V. cholerae* O1 is being disseminated along the Mekong Basin. However, no epidemiologic association has been identified so far. Thus, a more extensive regionwide surveillance system is needed to identify and control *V. cholerae* infection in Laos and neighboring countries.

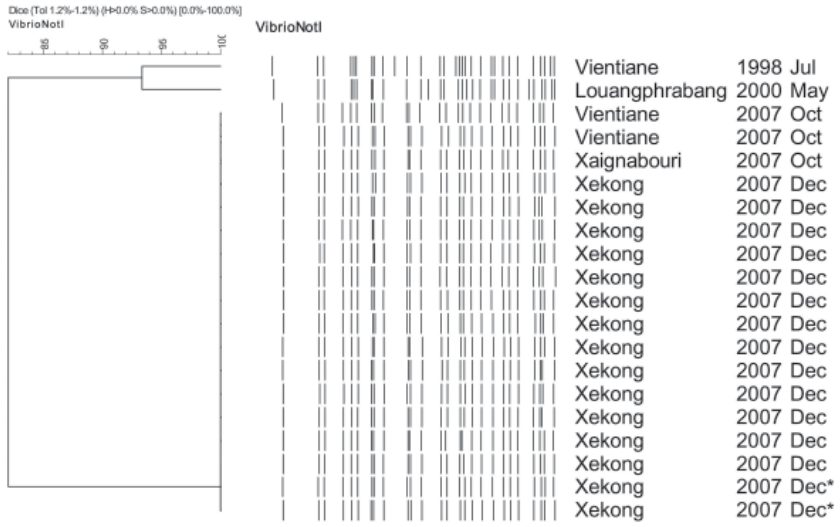


Figure. Dendrogram for *Not*I-digested pulsed-field gel electrophoresis profiles of *Vibrio cholerae* isolates, Laos, December 2007–January 2008. Origin of each isolate is shown on the right. *Water sample.

Acknowledgments

We thank Khambien Yanphichit, the Xekong Province team, and the epidemiologic team of the National Center for Laboratory and Epidemology, Vientiane, Laos.

This study was partly supported by grants-in-aid from the Ministry of Health, Labour and Welfare of Japan (H18-Shokuhin-Ippan-003, H20-Shinko-Ippan-013, H20-Shinko-Ippan-015, and International Health Cooperation Research 18C-5), and from Reiko Tsuyuoka and her team, CSR, World Health Organization, Laos.

Noikaseumsy Sithivong, Hidemasa Izumiya, Khampheuy Munnalath, Traykhouane Phouthavane, Khampheng Chomlasak, Lay Sisavath, Arounnapha Vongdouangchanh, Phengta Vongprachanh, Haruo Watanabe, and Makoto Ohinishi

Author affiliations: National Center for Laboratory and Epidemiology, Vientiane, Laos (N. Sithivong, K. Munnalath, T. Phouthavane, K. Chomlasak, L. Sisavath, A. Vongdouangchanh, P. Vongprachanh); and National Institute of Infectious Diseases, Tokyo, Japan (H. Izumiya, H. Watanabe, M. Ohinishi)

DOI: 10.3201/eid1604.091493

References

1. World Health Organization. Cholera 2007. Wkly Epidemiol Rec. 2008;83:269–83.
2. Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. Lancet. 2004;363:223–33. DOI: 10.1016/S0140-6736(03)15328-7
3. Raychoudhuri A, Mukhopadhyay AK, Ramamurthy T, Nandy RK, Takeda Y, Nair GB. Biotyping of *Vibrio cholerae* O1: time to redefine the scheme. Indian J Med Res. 2008;128:695–8.
4. Nair GB, Qadri F, Holmgren J, Svennerholm AM, Safa A, Bhuiyan NA, et al. Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. J Clin Microbiol. 2006;44:4211–3. DOI: 10.1128/JCM.01304-06
5. Safa A, Sultana J, Dac Cam P, Mwansa JC, Kong RY. *Vibrio cholerae* O1 hybrid El Tor strains, Asia and Africa. Emerg Infect Dis. 2008;14:987–8. DOI: 10.3201/eid1406.080129
6. Morita M, Ohnishi M, Arakawa E, Bhuiyan NA, Nusrin S, Alam M, et al. Development and validation of a mismatch amplification mutation PCR assay to monitor the dissemination of an emerging variant of *Vibrio cholerae* O1 biotype El Tor. Microbiol Immunol. 2008;52:314–7. DOI: 10.1111/j.1348-0421.2008.00041.x
7. Cooper KL, Luey CK, Bird M, Terajima J, Nair GB, Kam KM, et al. Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae.* Foodborne Pathog Dis. 2006;3:51–8. DOI: 10.1089/fpd.2006.3.51
8. Nguyen BM, Lee JH, Cuong NT, Choi SY, Hien NT, Anh DD, et al. Cholera outbreaks caused by an altered *Vibrio cholerae* O1 El Tor biotype strain producing classical cholera toxin B in Vietnam in 2007 to 2008. J Clin Microbiol. 2009;47:1568–71. DOI: 10.1128/JCM.02040-08

Address for correspondence: Hidemasa Izumiya, Department of Bacteriology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan; email: izumiya@nih.go.jp

Buruli Ulcer, Central African Republic

To the Editor: Buruli ulcer, the third most common mycobacterial disease of humans after tuberculosis and leprosy, is an important disfiguring and disabling cutaneous infection disease caused by *Mycobacterium ulcerans*. Buruli ulcer was declared an emerging skin disease of public health concern by the World Health Organization (WHO) in 1998. Although the disease is known to be associated with swampy areas and environmental changes, the mode of transmission is not yet clearly understood. A possible role for water bugs in the transmission has been postulated in the last 10 years. In this direction, several researchers have proposed that biting water bugs could be vectors for *M. ulcerans* (*1*). *M. ulcerans* produces a potent toxin known as mycolactone (*2*), which lyses dermal cells, leading to the development of continuously expanding ulcers with undermined edges. Surgery is the only treatment for late lesions, which involves excision of necrotic tissues, followed by skin grafting. After such treatment, patients suffer from functional limitations, social stigmatization, and the loss of livelihood (*3*).

Antimicrobial drug treatment is available (a combination of rifampin and streptomycin), but it is effective only for early lesions (*4*).

The disease is endemic in rural wetlands of tropical countries of Africa, the Americas, and Asia. Over the past decade, the prevalence of Buruli ulcer was highest in western Africa (*3*,*5*), with an alarming increase in detected cases. In central Africa, foci of Buruli ulcer have been reported in Gabon, Equatorial Guinea, Cameroon, Congo, the Democratic Republic of Congo, and Sudan (*6*), which are all neighboring countries of the Central African Republic (CAR). Surprisingly, in CAR, no cases of Buruli ulcer have been reported so far, even though its presence in this country was suspected in 2006, although not confirmed. This situation motivated us to begin a passive survey in the hospitals of Bangui, the capital of CAR. We report here 2 confirmed cases of Buruli ulcer that were found through this survey. The 2 patients were admitted in April 2007 to Hôpital de l'Amitié, Bangui, CAR, with extensive skin ulcers, which might correspond to Buruli ulcer according to WHO guidelines (*7*). Both patients were farmers from the Ombella M'poko region. They lived on the border of the M'poko River and carried out daily activities in an aquatic environment.

The first patient was a 62-year-old man who had a large ulceration of the right limb (Figure, panel A). Differential diagnosis eliminated other ulcerative diseases such as drepanocytosis, and the patient was HIV negative. For bacteriologic diagnosis, 4 samples were taken with sterile cotton swabs from beneath the undermined edges of the ulcer. *Proteus mirabilis* was isolated from the lesion, and a few acid-fast bacilli were shown by Ziehl-Neelsen (ZN) staining. Unfortunately, 1 week later, the patient died of an unknown cause.

The second patient was a man of the same age who had an ulceration 6.5 cm in diameter on the left ankle (Figure, panel B). His condition had been treated with various antimicrobial agents without any result. Blood testing showed minor anemia (hemoglobin 12.4 g/dL) and that the patient was HIV negative. Bacteriologic analysis found no gram-positive and gram-negative bacteria, and ZN staining showed the presence of acid-fast bacilli. He received the specific recommended treatment for *M. ulcerans* infection (antimicrobial drug regimen: rifampin, 10 mg/kg, and streptomycin, 15 mg/kg), and the lesions had receded 2 months later (Figure, panel C).

The identification of *M. ulcerans* was confirmed by PCR on the basis of the IS*2404* repeated insertion sequences of *M. ulcerans* as described by Stinear et al. (*8*). The positive results were confirmed by quantitative real-time PCR, in the Laboratory of Bacteriology at Central Hospitalier universistaire, Angers, France, on 2 specific sequences: IS*2404* sequence and ketoreductase B domain of the mycolactone polyketide synthase gene from the plasmid pMUM001 (*9*).

According to WHO criteria, 2 confirmative test results should be obtained of 4 laboratory tests (ZN staining, positive culture of *M.ulcerans*, specific gene amplification, pathognomonic histopathologic features) to establish a definitive diagnosis (*7*). Concerning the 2 patients in this study, results of ZN staining and PCR were positive, thus confirming the diagnosis of Buruli ulcer. Samples were inoculated on Löwenstein-Jensen (LJ) media and incubated at 30°C for 2 months, but the culture did not grow the organism. This result could be accounted for by the paucity of bacilli in the samples. In conclusion, our study confirms that, although infrequently diagnosed, Buruli ulcer is an endemic disease in CAR.

Identification and control of Buruli ulcer remain difficult in CAR, where this disease is often not considered. Even with evocative clinical signs, confirmation of diagnosis by biological analysis is still not easy. It

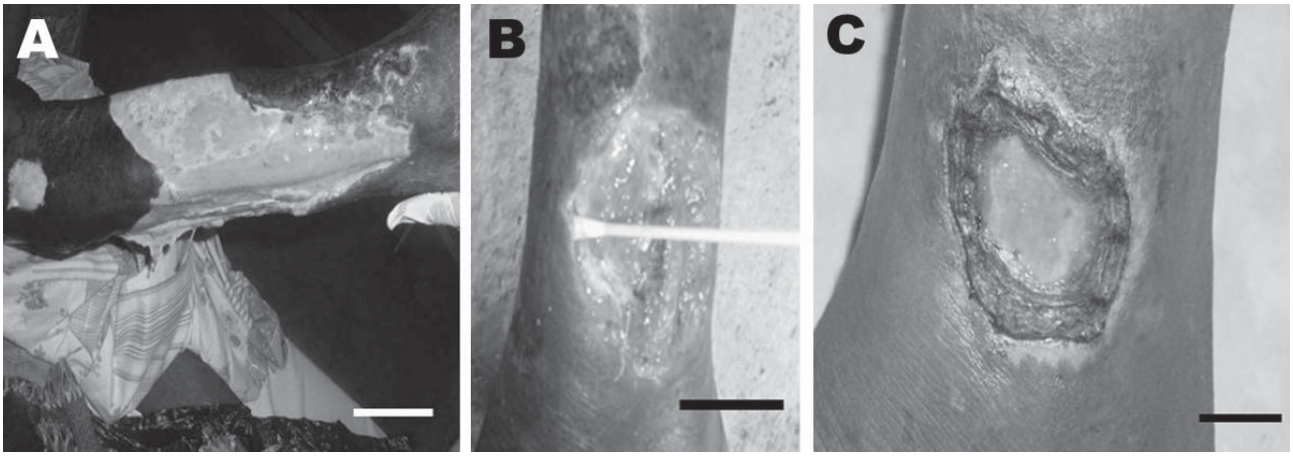


Figure. Patient 1: extensive ulcer of the right limb (A). Patient 2: ulcer of the left ankle before treatment (B) and 8 weeks after specific antimicrobial drug therapy (C). Scale bars = 12 cm (A), 5 cm (B), and 2 cm (C).

is therefore of high importance that the public health authorities are fully informed and properly trained to identify this neglected disease in the early stages so patients can be cured before the onset of functional impairment and the appearance of extensive lesions. Further investigation to isolate strains present in CAR is also essential.

Acknowledgments

We thank N. Komas, E. Nakoune, and M. Huerre for their technical assistance. Also, we are grateful to those involved in the recruitment of patients.

This work was supported by Pasteur Institute, Paris, Association Aide aux Lépreux Emmaüs Switzerland, the Fondation Française Raoul Follereau, the Institut National de la Santé et de la Recherche Médicale, and WHO.

Fanny Minime-Lingoupou, Narcisse Beyam, Germain Zandanga, Alexandre Manirakiza, Alain N'Domackrah, Siméon Njuimo, Sara Eyangoh, Jane Cottin, Laurent Marsollier, Estelle Marion, Francoise Portaels, Alain Le Faou, and Raymond Bercion

Author affiliations: Pasteur Institute, Bangui, Central African Republic (F. Minime-Lingoupou, N. Beyam, G. Zandanga, A. Manirakiza, A. N'Domackrah, S. Njuimo, A. Le Faou, R. Bercion); Pasteur Center, Yaounde, Cameroon (S. Eyangoh); Central Hospitalier universitaire, Angers, France (J. Cottin, E. Marion); Université d'Angers, Angers (J. Cottin, L. Marsollier, E. Marion); and Institute for Tropical Medicine, Anvers, Belgium (F. Portaels)

DOI: 10.3201/eid1604.090195

References

1. Marsollier L, Deniaux E, Brodin P, Marot A, Wondje CM, Saint-André JP, et al. Protection against *Mycobacterium ulcerans* lesion development by exposure to aquatic insect saliva. PLoS Med. 2007;4:e64. DOI: 10.1371/journal.pmed.0040064
2. George KM, Chatterjee D, Gunawardana G, Welty D, Hayman J, Lee R, et al. Mycolactone: a polyketide toxin from *Mycobacterium ulcerans* required for virulence. Science. 1999;283:854–7. DOI: 10.1126/science.283.5403.854
3. Van der Werf TS, Stienstra Y, Johnson RC, Phillips R, Adjei O, Fleischer B, et al. *Mycobacterium ulcerans* disease. Bull World Health Organ. 2005;83:785–91.
4. Chauty A, Ardant MF, Adeye A, Euverte H, Guédénon A, Johnson C, et al. Promising clinical efficacy of streptomycin-rifampin combination for treatment of Buruli ulcer (*Mycobacterium ulcerans* disease). Antimicrob Agents Chemother. 2007;51:4029–35. DOI: 10.1128/AAC.00175-07
5. Van der Werf TS, Van der Graaf WT. Buruli ulcer in West Africa [letter]. Lancet. 1990;336:1440. DOI: 10.1016/0140-6736(90)93133-A
6. Johnson PD, Stinear T, Small PL, Pluschke G, Merritt RW, et al. Buruli ulcer (*M. ulcerans* infection): new insights, new hope for disease control. PLoS Med. 2005;2:e108. DOI: 10.1371/journal.pmed.0020108
7. World Health Organization. Portaels F, Johnson P, Meyers WM, editors. Buruli ulcer. Diagnosis of *Mycobacterium ulcerans* disease. A manual for health care providers. Geneva: The Organization; 2001 [cited 2009 Jan 15]. http://whqlibdoc.who.int/hq/2001/WHO_CDS_CPE_GBUI_2001.4.pdf
8. Stinear T, Ross BC, Davies JK, Marino L, Robins-Browne RM, Oppedisano F, et al. Identification and characterization of IS*2404* and IS*2606*: two distinct repeated sequences for detection of *Mycobacterium ulcerans* by PCR. J Clin Microbiol. 1999;37:1018–23.
9. Rondini S, Mensah-Quainoo E, Troll H, Bodmer T, Pluschke G. Development and application of real-time PCR assay for quantification of *Mycobacterium ulcerans* DNA. J Clin Microbiol. 2003;41:4231–7. DOI: 10.1128/JCM.41.9.4231-4237.2003

Address for correspondence: Fanny Minime-Lingoupou, Laboratory of Mycobacteria, Pasteur Institute, Bangui, Central African Republic; email: flingoupou@yahoo.fr

Infectious Disease Surveillance

N.M. M'ikanatha, R. Lynfield, C.A. Van Beneden, H. de Valk, editors

Blackwell Publishing, Malden, MA, USA, 2007
ISBN-13: 978-1-4051-4266-3
Pages: 560; Price: US $195.00

This book devotes chapters to the usual infectious disease suspects and surveillance concepts and systems. I will not go into details of its contents and glowing attributes; the publisher (www.blackwellpublishing.com/book.asp?ref=9781405142663) and reviewers (*1*,*2*) have done a good job on this. Instead, I conducted a simple review to try and answer the following question: compared with a free Internet search, is purchase of a $195.00 book worth it? My hypothesis was that by entering the title of a chapter from the book in a search engine, one could find comparable content within the first 20 results (links) returned.

Methods

After selecting the chapter that corresponded with every 50th page of the book, I visually scanned that chapter for scope and content. Then I performed a Google search using the chapter title and ranked the level of congruence between the topics in the book chapter and the topics at the Internet links as follows: fully comparable, ≥1 links covered most (≈%75) topics in the chapter; partially comparable, ≥1 links covered many (≈50%) of the topics; and not comparable, all links reviewed (up to 20) covered <50% of the topics. Each search result link was reviewed in successive order up to 20 results until ≥1 enabled a ranking of fully comparable. Links that led to other links would be followed only 1 level deep. Links that required log-in or payment or that reproduced a complete chapter were excluded.

Results

Of the 40 chapters, 10 were reviewed. Of these, 4 were ranked as fully comparable, 4 as partially comparable, and 2 as not comparable.

For example, a rating of fully comparable was given for the chapter "Public Health Surveillance for Vaccine Adverse Events." After 6 links were reviewed, 2 were found to provide similar information to that of the book chapter. A rating of partially comparable was given for "The Netherlands' Infectious Diseases Surveillance Information System." Of 20 links reviewed, several contained related information, but none were as complete as the book chapter and some provided a more global view. A rating of not comparable was given for "Use of Molecular Epidemiology in Infectious Disease Surveillance" after a review of all 20 links. Although several links provided information on the topic, none provided as organized and clear a summary as did the book chapter.

Discussion

Extrapolation of these findings to all 40 chapters is difficult because of variation in topics and titles, and extrapolation to other research areas may depend on the proportion of information published without restrictions, i.e., without requesting registration, passwords, or money. Overall, a considerable amount of Internet information was obtained for most chapters evaluated, although usually >1 link had to be reviewed. The convenience of being able to read at one's own pace a book containing the full gamut and organized presentation of such subject matter must be weighed against the timeliness and cost differential of an Internet search.

Conclusions

This book is a good value for public health students and professionals frequently involved in surveillance and surveillance issues. It is not necessarily a good value for those with only infrequent needs to find information quickly or to find up-to-date information on several surveillance topics.

Marc A. Strassburg
Author affiliations: Los Angeles County Department of Public Health, Los Angeles, California, USA; University of California, Los Angeles, California, USA; and TUI University, Cypress, California, USA

DOI: 10.3201/eid1604.090584

References

1. DeMaria A Jr. Infectious disease surveillance. N Engl J Med. 2008;358:1413–4
2. Grassly NC. Infectious disease surveillance. JAMA. 2008;300:1591–2.

Address for correspondence: Marc A. Strassburg, Los Angeles County Web Informatics, Los Angeles County Department of Public Health, 313 N. Figueroa St, Rm 127, Los Angeles, CA 90012, USA; email: mstrassburg@ph.lacounty.gov

Denialism: How Irrational Thinking Hinders Scientific Progress, Harms the Planet, and Threatens Our Lives

Michael Specter

Penguin Press, New York, NY, USA, 2009
ISBN: 1594202303
Pages: 304; Price: US $27.95

Vaccines remain one of our most critical public health interventions, yet an increasingly vocal group of antivaccinationists call vaccines toxic and attribute numerous conditions, such as autism, to vaccination despite

evidence to the contrary. Former Playboy model Jenny McCarthy is viewed as a respected source of information, whereas pediatric infectious diseases researcher Paul Offit receives death threats for his scientific contributions. Journalist Michael Specter describes this phenomenon in Denialism: How Irrational Thinking Hinders Scientific Progress, Harms the Planet, and Threatens Our Lives.

Spector defines denialism as "denial writ large—when an entire segment of society, often struggling with the trauma of change, turns away from reality in favor of a more comfortable lie." Denialism describes this rejection of fact-based reality in 6 different areas related to health and medicine, beginning with the mistrust of pharmaceutical companies and their products. He also examines the dismissal of racial differences in medical research, the organic food movement as a form of denialism, and the obsession with vitamins and complementary alternative medicine despite evidence that these modalities do not work or may even be harmful. He ends with a look to the future: creation of life itself and why we must overcome denialism and embrace change to continue advancing as a society or risk the survival of our species.

This book is sure to cause some controversy. In many circles, the term denialism is still linked to rejection of the Holocaust, and any implication that parents' refusal of vaccination or the philosophy of choosing organic-only food is akin to dismissal of one of humanity's great atrocities surely will make some readers dismiss Specter's arguments. Likewise, although Specter supports industry, he is pragmatic and does not put himself in a cheerleader role, instead acknowledging corporate culpability in driving and perpetuating denialism: "Corporations, wrapping themselves in the mantle of progress but all too often propelled by greed, have done more than religion or even Luddism to inflame denialists and raise doubts about the objectivity of science." Specter realizes that industry, and scientists, have lost the public's trust, and this broken relationship needs to be fixed through additional communication to the public by scientists and science writers, open debates about the future of scientific progress and implications of emerging technologies, and improvements in education.

Persons picking up this book may be surprised by the lack of discussion about some prominent topics of science denialism, including evolution and global warming. Likewise, Specter does not discuss HIV/AIDS denial, a topic he has covered previously in The New Yorker (*1*). Referring to the latter, Specter notes that Holocaust and HIV/AIDS denialists are "… intensively destructive—even homicidal—but they don't represent conventional thought and they never will. This new kind of denialism is less sinister but more pervasive than that." Furthermore, the types of denialism Specter describes cut across political and religious divisions to combine fear and uncertainty in a manner that makes them contagious. Indeed, a central tenet of denialism is that "fear is more infectious than any virus" and, like infection, needs to be addressed and, ideally, prevented through healthy skepticism.

Spector notes that "Denialism must be defeated. There is simply too much at stake to accept any other outcome." Its defeat is a tall order, but an imperative one if science is truly to be restored to its rightful place. Specter's book is a good starting place for any scientist or layman interested in delving into this phenomenon.

Tara C. Smith

Author affiliation: University of Iowa, Iowa City, Iowa, USA

DOI: 10.3201/eid1604.091710

Reference

1. Specter M. Annals of science: the denialists. The New Yorker. 2007 Mar 12:32.

Address for correspondence: Tara C. Smith, Department of Epidemiology, Center for Emerging Infectious Diseases, University of Iowa, 200 Hawkins Dr, C21F GH, Iowa City, IA 52242, USA; email: tara-smith@uiowa.edu

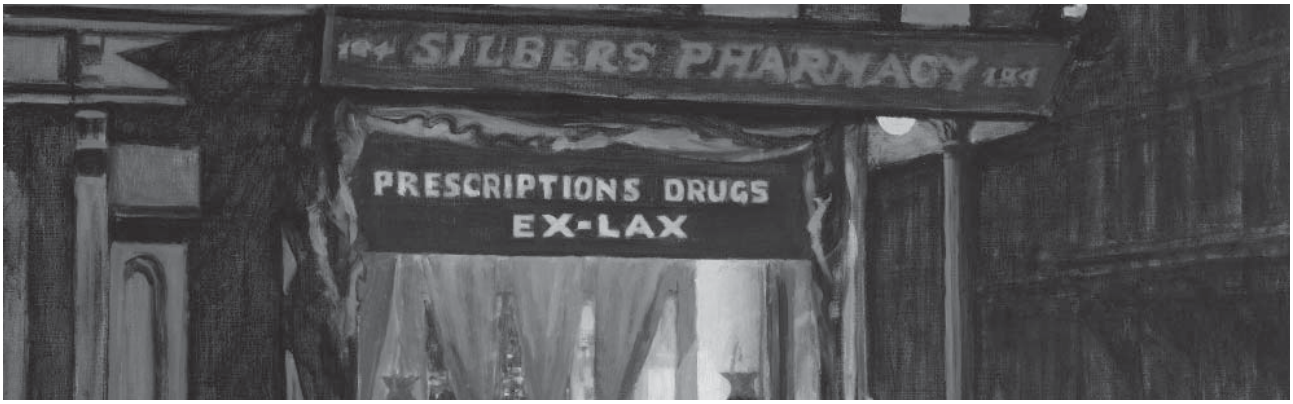
Errata—Vol. 16, No. 2

The article Hendra Virus Outbreak with Novel Clinical Features, Australia (H. Field et al.) contained several errors related to specific case descriptions and spillover events. The article has been corrected online (www.cdc.gov/eid/content/16/2/238.htm).

The author list for the article Epidemiology of *Cryptoccocus gattii,* British Colombia, Canada, 1999–2007 (E. Galanis et al.) was incomplete. Authors were Eleni Galanis, Laura MacDougall, Sarah Kidd, Mohammad Morshed, and the British Columbia *Cryptococcus gattii* Working Group. Working Group members involved in this study were Patrick Doyle, John Galbraith, Linda Hoang, Pamela Kibsey, Min-Kuang Lee, Sultana Mithani, Marc Romney, and Diane Roscoe. The article has been corrected online (www.cdc.gov/eid/content/16/2/251.htm).

Erratum—Vol. 16, No. 3

The author list for the article Use of Avian Bornavirus Isolates to Induce Proventricular Dilatation Disease in Conures (P. Gray et al.) omitted W. Ian Lipkin. The article has been corrected online (www.cdc.gov/eid/content/16/3/473.htm).



Edward Hopper (1882–1967). Drug Store (1927) (detail). Oil on canvas. (73.6 cm × 101.9 cm). The Museum of Fine Arts, Boston, Massachusetts, USA. Bequest of John T. Spaulding, 48.564

A Clean, Well-Lighted Place[1]

Polyxeni Potter

"The man's the work. Something doesn't come out of nothing," Edward Hopper once said. This private and introspective man, known for his dry wit and "monumental silences" and for expressing himself "tersely but with weighted exactness in a slow reluctant monotone," was offering a glimpse into the creative process as it applied to him. Much was made of the sense of isolation and despair in his work and their connection with modern life. But "The loneliness thing is overdone," he noted. "My aim in painting is always, using nature as the medium, to try to project upon canvas my most intimate reaction to the subject as it appears when I like it most; when the facts are given unity by my interest and prejudices."

"Hudson River Dutch" is how Hopper described his ancestry in Nyack, New York. The son of a dry goods merchant, he was not discouraged in his artistic ambitions, though his family did steer him toward commercial illustration for its earning potential. The skill stood him in good stead during the lean years. He attended the New York School of Art and studied under Robert Henri, one of the fathers of American Realism, "the most influential teacher I had." He visited Europe several times. "Paris had no great or immediate impact on me." But when he returned to the United States, his work reflected what he had seen abroad. "It took me ten years to get over Europe."

Hopper settled in New York, where he would make his primary residence. He moved into a 74-step, cold-water walk-up with a sky-lit studio in Greenwich Village. He had to haul coal for the furnace up four flights of stairs, but the space suited his self-sufficient and frugal nature. He painted many major works there and, despite a reasonable measure of success during his lifetime, never pursued more plush quarters.

During a career that spanned 60 years and saw the heyday of abstract expressionism, as well as a resurgence of realism in the United States, he made a unique contribution. In a 1932 exhibition at the Museum of Modern Art, his work was described as "part of a new international progressive trend emerging within modernism, represented by a balance between 'form' and 'content' in its work." Hopper's approach, which appealed to his colleagues from all factions, explored natural and artificial light on surfaces, particularly on the vernacular architecture of American cities: motels, gas stations, storefronts, diners, apartments. "What I wanted to do was to paint sunlight on the side of a house." His work employed classical elements and formal discipline in the dispassionate presentation of everyday scenes.

Hopper captured what he called "our native architecture with its hideous beauty, its fantastic rooms, pseudo-gothic, French Mansard, Colonial, mongrel or what not, with eye searing color or delicate harmonies of faded paint, shouldering one another along interminable streets that taper off into swamps or dump heaps," and by capturing it, he defined it. His images, often described as theatrical or cinematic, went on to inspire the cinema and its greats, among them John Huston, Elia Kazan, John Cassavetes. Alfred Hitchcock credited Hopper for influencing his films *Rear Window* and *Psycho.*

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

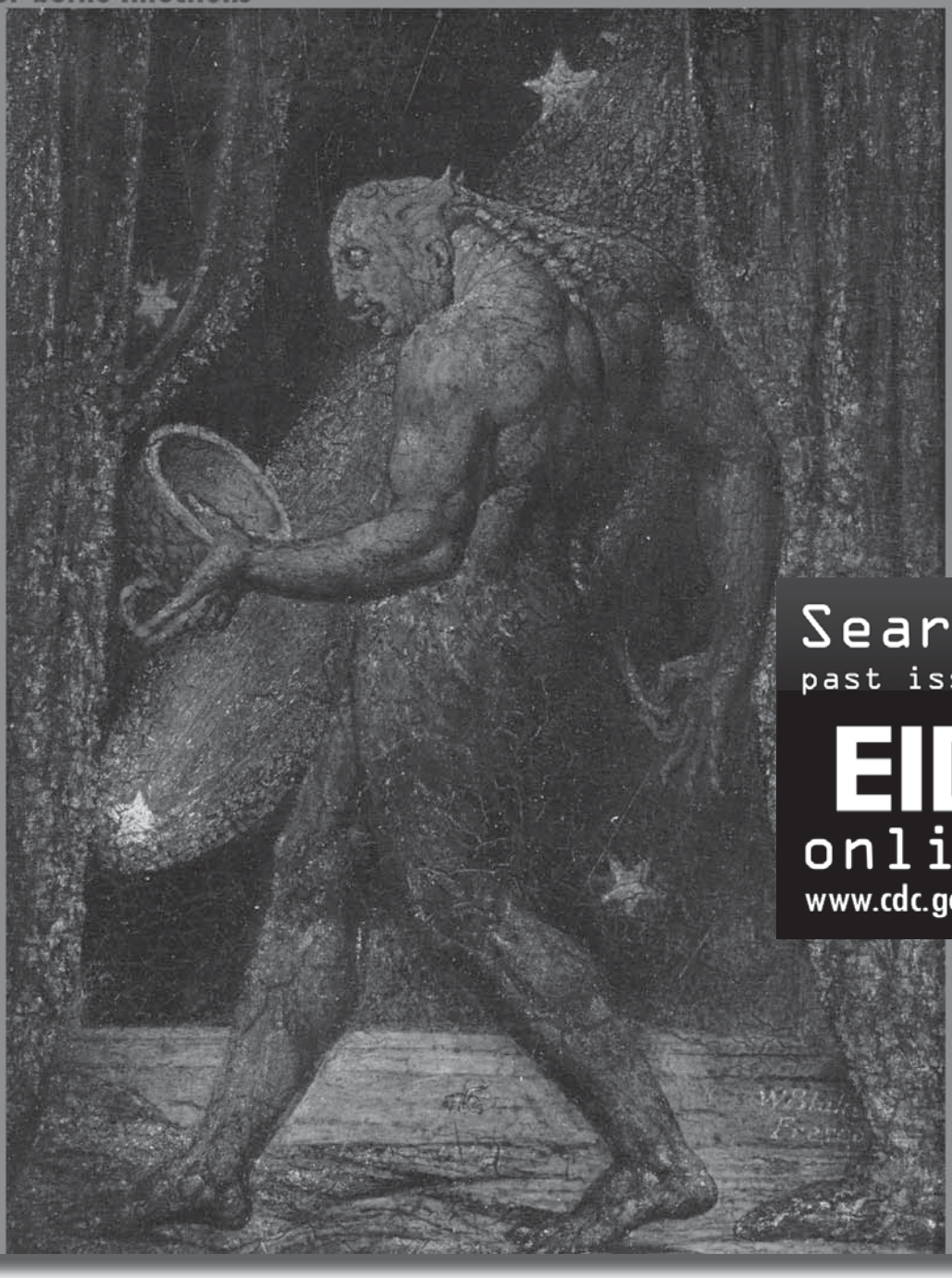
DOI: 10.3201/eid1604.000000

[1]Title of short story by Ernest Hemingway

EMERGING INFECTIOUS DISEASES®

March 2010

Vector-borne Infections



Tate, London, England

"The whole answer is there on the canvas," Hopper said in lieu of explaining his paintings. "I hope it will not tell any obvious anecdotes since none are intended." Just like the content of his paintings, the form was stripped of extraneous details. He sought perfection in perspective, geometric structures, and two-dimensional space, as well as in the use of color and light. "As a child I felt that the light on the upper part of a home was different from that on the lower part. There is a sort of elation about sunlight on the upper part of a house." He described his style as "an amalgam of many races" and refused to belong to any school.

"Hopper is always on the verge of telling a story," observed novelist and art critic John Updike, referring to scenes whose very stillness suggests that something is about to happen. A room seems either recently vacated or soon to be occupied. If inhabited, it is always sparsely, and that also goes for public places—theaters, restaurants, offices. Any occupant has either just arrived or is ready to leave, psychologically absent, or lost in thought. Human presence is not required, although the viewer is always allowed in, either through an open window or some other vista. And the night is just as paintable as the day, or even more so because of its mysterious, even ominous, contrasts and shadows.

Drug Store, on this month's cover, is one of Hopper's nocturnal works. During one of his visits to Europe, he saw Rembrandt's *Night Watch*, "The most wonderful thing…. It almost amounts to deception." In his own work, he came to view the night as an opportunity to scrub a scene from the hustle and bustle. In the darkness, it seems, he could focus on the unexceptional and familiar elements of the uninhabited streets and capture the essence of places.

In *Drug Store*, drama resides strictly on the weight of darkness in back of the setting against the brightly lit establishment forward. The lamp above the entrance lights the window, which with the awning pulled up all the way, is unabashedly exposed. Emotion is rendered in place and time, not human terms. In painting this street icon, Hopper as always, sought the "most exact transcription possible." But what might have been a most sterile, even disturbing, presentation is rendered here with softness and calm. The storefront glows against the surroundings, its curtains and colorful vessels inviting and homelike but for the commercial signage. The wedgelike positioning foreshadows another one of Hopper's famous corner establishments, *Nighthawks*.

Silbers Pharmacy was typical of the profession in the early part of the 20th century. The move away from artisan plasters, powders, and carbonated waters saw increasingly flamboyant advertising of medications based on better understanding of disease etiology and the mechanisms of drug action.

These new drugs were a mixed bag of cure and trouble, just like today's offerings. In this journal issue alone, countless examples underline unintended consequences in the use of otherwise effective medications, among them antimicrobial drugs. Commonly used to treat infections, these drugs also may change intestinal microflora and make patients vulnerable to other infections, as with *Clostridium difficile*. In addition to increased *C. difficile*–associated disease, severity is also increasing from a new strain. And while clinical illness used to occur almost exclusively among the elderly in healthcare facilities, it is now seen in the community, among the young, and apart from antimicrobial drug treatment. Some retail meats contain the pathogen, though its role there is unknown. Changes in disease setting alter who is at risk and what the risk factors are. The clean, well-lighted healthcare setting where antimicrobial drug use leads to pseudomembranous colitis does not describe the risk for or characteristics of community-acquired infections with *C. difficile*.

"I was never able to paint what I set out to paint," Hopper said. During the unpredictable course of the creative process, inspiration injects itself unawares, the miscellaneous and extraneous intercede, color dictates, or thought transforms the initial intention to an unrecognizable final result. The artist's discerning eye must complete the process by honing in on the essential. Public health scientists at work to stay ahead of pathogens also come up against unpredictability in the creative process. Much like Hopper, they must have an eye for the setting. One is not like another, and light makes all the difference. And while the whole answer may indeed be there on the canvas, for Hopper the essence was found in the light, whereas for the scientist trying to capture and define risk factors for infection, the essence may still be lurking in the shadows.

Bibliography

1. Barr A. Edward Hopper: retrospective exhibition. New York: Museum of Modern Art; 1933.
2. Hobbs R. Edward Hopper. New York: Harry N. Abrams, Inc.; 1987.
3. Levin G. Edward Hopper: an intimate biography. New York: Alfred A. Knopf; 1995.
4. Lucie-Smith E. Lives of the great 20th-century artists. New York: Thames and Hudson; 1999.
5. Mulvey MR, Boyd DA, Gravel D, Hutchinson J, Kelly S, McGeer A, et al. Hypervirulent *Clostridium difficile* strains in hospitalized patients, Canada. Emerg Infect Dis. 2010;16:678–81.
6. Zaiß NH, Witte W, Nübel U. Fluoroquinolone resistance and *Clostridium difficile*, Germany. Emerg Infect Dis. 2010;16:675–7.
7. Wagstaff S, ed. Edward Hopper. London: Tate Publishing; 2004.
8. Zilberberg MD, Tillotson GS, McDonald LC. *Clostridium difficile* infections among hospitalized children, United States, 1997–2006. Emerg Infect Dis. 2010;16:604–8.

Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; email: PMP1@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Spread of Adenovirus between Military Installations

Methicillin-Resistant *Staphylococcus aureus,* Southwestern Alaska

Tropheryma whipplei in Children with Gastroenteritis

Influenza Outbreaks during a Mass Gathering and Community Seasonal Influenza Activity

Virus Replication and Shedding after Onset of Pandemic (H1N1) 2009 Illness

Public Health Surveillance in the United States

Anaplasma phagocytophilum in Rodents and Sheep, China

Latent Tuberculosis among Persons at Risk for Infection with HIV, Tijuana, Mexico

Historical Distribution and Molecular Diversity of *Bacillus anthracis*, Kazakhstan

Effect of Pneumococcal Conjugate Vaccination Program, the Netherlands

Adenovirus 36 DNA in Adipose Tissue of Patient with Unusual Visceral Obesity

Extensively Drug-Resistant Tuberculosis, Burkina Faso

Vitamin D Deficiency and Tuberculosis Disease Progression

Unusual Segments in 2 Rare Human Rotavirus Genomes

Complete list of articles in the May issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

April 27–29, 2009
Twelfth Annual Conference
on Vaccine Research
Baltimore Marriott Waterfront Hotel
Baltimore, MD, USA
http://www.nfid.org/conferences/vaccine09

May 20–22, 2010
VIII International Conference on HFRS, HPS and Hantaviruses
Athens, Greece
http://www.hantavirusconference2010.gr

July 11–14, 2010
International Conference on Emerging Infectious Diseases 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.iceid.org

November 11–13, 2010
European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)
Lisbon, Portugal
http://www.escaide.eu

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **www.medscapecme.com/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Community-associated Methicillin-Resistant *Staphylococcus aureus* Strains in Pediatric Intensive Care Unit

CME Questions

1. Patients in the study cohort found to be colonized with community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) were more likely to:

A. Be adolescent-age boys
B. Have been admitted to a general ward in a hospital in the prior 12 months
C. Be white girls
D. Have been admitted to an intensive care unit in the prior 12 months

2. Failure to screen a high-risk group, such as children, for MRSA colonization upon admission to a hospital may result in:

A. Transmission of CA-MRSA strains among hospitalized children
B. Transmission of hospital-acquired (HA)-MRSA strains among children in a community setting
C. Transmission of CA-MRSA strains among children in a community setting
D. Transmission of HA-MRSA strains among hospitalized children

3. Surveillance cultures done on admission in the study cohort detected:

A. A large proportion of children colonized with HA-MRSA
B. A small proportion of children infected with CA-MRSA
C. A large proportion of children colonized with CA-MRSA
D. A small proportion of children infected with HA-MRSA

4. The value of screening patients for MRSA on admission to a hospital is:

A. Notification of family members to initiate home decolonization regimen
B. Initiation of isolation and contact precautions
C. Early treatment of infection
D. Early initiation of a decolonization regimen

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

Manuscript Submission. Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid).

Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.