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November 2010

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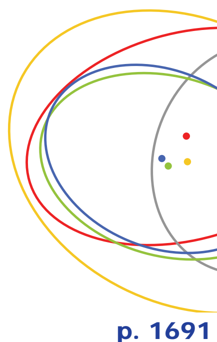

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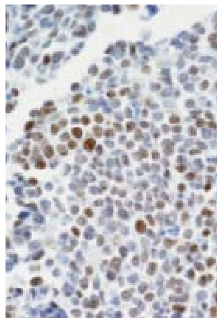

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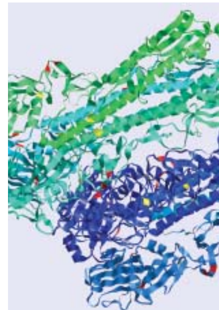

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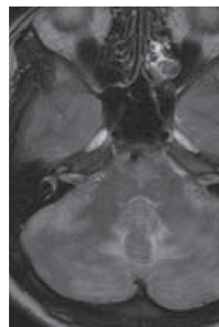

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Regulatory Oversight and Safety of Probiotic Use

Veena Venugopalan, Kimberly A. Shriner, and Annie Wong-Beringer

Depending on intended use of a probiotic (drug vs. dietary supplement), regulatory requirements differ greatly. For dietary supplements, premarketing demonstration of safety and efficacy and approval by the Food and Drug Administration are not required; only premarket notification is required. *Saccharomyces boulardii* is a probiotic regulated as a dietary supplement intended for use by the general healthy population, not as a drug to prevent, treat, or mitigate disease. However, since recent increases in incidence and severity of *Clostridium difficile* infection, probiotics have been used to treat recurrent and/or refractory disease in hospitalized patients. *Saccharomyces* fungemia secondary to use of the probiotic has been described for patients who are critically ill, are receiving nutrition enterally, or have a central venous catheter. Before use of a probiotic is considered for hospitalized patients, careful assessment of risk versus benefit must be made. To ensure patient safety, probiotics should be properly handled during administration.

Probiotics are defined by the Food and Agriculture Organization of the World Health Organization as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (*1*). The term probiotic can be subcategorized to include probiotic drugs, probiotic foods (e.g., foods, food ingredients, and dietary supplements), direct-fed microbials (probiotics for animal use), and designer probiotics (genetically modified probiotics) (*2*). In the United States, probiotic products are marketed to a generally healthy population as foods or dietary supplements (*3*).

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Recent increases in the incidence and severity of *Clostridium difficile* infection (CDI) have led some clinicians to consider use of probiotics as "drugs," either alone or in combination with traditional antimicrobial agents for the prevention and treatment of CDI. Several recent reviews have summarized results from clinical studies evaluating the efficacy of probiotics in diarrheal illness (*4–12*). Our goal is to highlight the current regulatory oversight for probiotics in the United States, identify potential risk situations associated with their administration, and offer suggestions on practical aspects of probiotic administration to ensure patient safety. This review focuses on *Saccharomyces boulardii* (Florastor; Biocodex Pharmaceutical Laboratories, Gentilly, France) as an example of a probiotic product being used as a "drug" to prevent or treat recurrent CDI, particularly in critically ill patients.

Regulatory Oversight

Depending on the intended use of a probiotic, whether as a drug or a dietary supplement, regulatory requirements differ. According to the Food and Drug Administration (FDA) definition, a drug is an article intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease (*13*). If a probiotic is intended for use as a drug, then it must undergo the regulatory process as a drug, which is similar to that of any new therapeutic agent. An Investigational New Drug application must be submitted and authorized by FDA before an investigational or biological product can be administered to humans. The probiotic drug must be proven safe and effective for its intended use before marketing (*14*).

If a probiotic is intended for use as a dietary supplement, it is placed under the umbrella of "foods," and as such is regulated by FDA's Center for Food Safety and Applied Nutrition (*15*). A dietary supplement is defined by the Dietary Supplement Health and Education Act (DSHEA)

of 1994 as a product taken by mouth that contains a “dietary ingredient” intended to supplement the diet. Supplements must contain ≥1 of the following dietary ingredients: a vitamin; a mineral; an herb or other botanical (excluding tobacco); an amino acid; a dietary substance for use by persons to supplement the diet by increasing the total dietary intake; a concentrate, metabolite, constituent, extract; or combination of any of the above (*16*).

In contrast to drugs, dietary supplements do not need FDA approval before being marketed. However, manufacturers need to notify FDA before marketing a product. According to DSHEA, the manufacturer is responsible for determining that the dietary supplements that it manufactures or distributes are safe and that any representations or claims made about them are substantiated by adequate evidence to show that they are not false or misleading; the manufacturers need not provide FDA with evidence that substantiates the safety or purported benefits of their products, either before or after marketing. If a dietary supplement contains a new dietary ingredient that was not sold before October 15, 1994, then the manufacturer is required to notify FDA and demonstrate to FDA before marketing why the ingredient is reasonably expected to be safe for use in a dietary supplement. On June 22, 2007, FDA announced a final rule establishing Current Good Manufacturing Practice requirements for dietary supplements. To ensure the identity, purity, quality, strength, and composition of dietary supplements, those who manufacture, package, or hold dietary supplements must follow these regulations (*17*). Also, since implementation of the Dietary Supplement and Nonprescription Drug Consumer Protection Act in 2006, manufacturers and distributors of dietary supplements have been required to record and forward to FDA any directly received reports of serious adverse events associated with use of their products. MedWatch Form 3500A (www.fda.gov/downloads/Safety/MedWatch/HowToReport/DownloadForms/ucm082728.pdf) must be completed by the manufacturer or distributor and submitted to FDA. FDA encourages voluntary reporting of adverse events by healthcare professionals, consumers, or patients on MedWatch Form 3500 (www.fda.gov/downloads/Safety/MedWatch/HowToReport/DownloadForms/ucm082725.pdf) (*18*).

Claims for Dietary Supplements

The law allows that in addition to nutrient content claims, manufacturers of dietary supplements may make structure/function or health claims for their products. For a structure/function claim, FDA requires that manufacturers’ substantiation is accepted by experts in the field and that the claim is truthful and not misleading. The data substantiating structure/function claims need not be publicly available and need not be disclosed. In general, the level of substantiation and the quality of evidence needed to make a structure/function claim are less than that needed to make a health claim. When a structure/function claim is made, the manufacturer must state in a disclaimer that FDA has not evaluated the claim and that the product is not intended to “diagnose, treat, cure, or prevent any disease”; such a claim can legally be made only with regard to a drug (*19,20*).

According to FDA, “health claims describe a relationship between a food, food component, or dietary supplement ingredient, and reducing risk of a disease or health-related condition.” In contrast, a structure/function claim describes the process by which the dietary supplement, conventional food, or drug maintains normal functioning of the body and does not need FDA approval before marketing. The data substantiation requirements for the claims described above vary greatly. Before a health claim is authorized, a petition containing the scientific evidence supporting the claim is reviewed by FDA. The systematic review process for a health claim involves defining the relationship between probiotic and disease and identifying relevant studies supporting the claim. Clinical studies are then rated on the basis of quality and strength of evidence. Only data obtained from studies conducted in healthy populations are evaluated because health claims are usually directed at the general population or certain subgroups (e.g. elderly patients). The data supporting a health claim must be published and therefore apply to any product meeting the criteria for the claim (*21*).

Global Standards for Evaluation of Probiotics

In 2001, in an attempt to standardize the requirements needed to make health claims regarding probiotic agents, the Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics developed guidelines for evaluating probiotics in food that could lead to the substantiation of health claims (*1*). The proposed guidelines recommend 1) identification of the genus and species of the probiotic strain by using a combination of phenotypic and genotypic tests as clinical evidence suggesting that the health benefits of probiotics may be strain specific, 2) in vitro testing to delineate the mechanism of the probiotic effect, and 3) substantiation of the clinical health benefit of probiotic agents with human trials. Additionally, safety assessment of the probiotic strain should at a minimum determine 1) patterns of antimicrobial drug resistance, 2) metabolic activities, 3) side effects noted in humans during clinical trials and after marketing, 4) toxin production and hemolytic potential if the probiotic strain is known to possess those properties, and 5) lack of infectivity in animal studies.

The Consultation recommends that specific health claims on labeling material on probiotic food items be allowed when sufficient scientific evidence is available and that the product manufacturer take responsibility for ensur-

ing that an independent third party reviews and evaluates the scientific evidence. Since development of these guidelines, only a few manufacturers have conducted small, randomized, controlled studies in humans to prove efficacy and safety of their products. Until more stringent regulations are in place, when assessing therapeutic potential for a probiotic product, clinicians must weigh the available evidence as outlined above. In addition, the manufacturer should take on the responsibility (albeit not required by law) of providing guidance to consumers or clinicians about the type and extent of safety assessments that have been conducted on its products.

S. boulardii as Probiotic

Since the 1950s, *S. boulardii* has been used internationally and extensively as a probiotic (*22*). *S. boulardii* is a live yeast that has been lyophilized and is available in 250-mg capsules for adults. The probiotic may be prescribed as 1–2 capsules to be taken 1–2×/day (*23*). In the United States, *S. boulardii* is marketed as a dietary supplement. The product package displays the following structure/function claims: 1) maintains the balance of the intestinal flora, 2) keeps intestines functioning well, and 3) promotes intestinal health.

Use of *S. boulardii* in Patients with CDI

Studies of *S. boulardii* in populations other than the healthy general public have demonstrated its efficacy for reducing recurrence of CDI when used in combination with standard therapy. A multicenter, double-blind, placebo-controlled trial investigated the effects of *S. boulardii* (1 g/d) for 4 weeks in combination with vancomycin (high dose 2 g/d or low dose 500 mg/d) or metronidazole (1 g/day) to patients with either initial or recurrent CDI (*24*). Recurrence rates were 16.7% for patients receiving *S. boulardii* with high-dose vancomycin compared with 50% for patients receiving high-dose vancomycin and placebo (p = 0.04). Rates for recurrent CDI did not differ when *S. boulardii* was combined with either low-dose vancomycin or metronidazole. According to the 2010 guidelines for management of CDI in adults, published jointly by the Society of Healthcare Epidemiology of America and the Infectious Diseases Society of America, no compelling evidence exists to support routine use of probiotics for prevention or treatment of CDI (*25*).

Infectious Complications after Receipt of *S. boulardii*

Safety of dietary supplements is conducted postmarketing. Therefore, much of the safety data on use of *S. boulardii* as a probiotic "drug" are derived from case reports. *Saccharomyces* fungemia is the most severe complication secondary to administration of the probiotic. *S. cerevisiae* and *S. boulardii* have been referred to in the literature interchangeably and have recently been shown by genetic fingerprinting and gene sequencing to be similar on a genetic level and to possibly share metabolic properties (*26*).

The most comprehensive literature review on incidence of invasive *Saccharomyces* infections was conducted by Enache-Angoulvant et al. (*27*). They identified 91 documented cases of invasive *Saccharomyces* infection in the literature (54 cases of *S. cerevisiae* invasive infections vs. 37 cases of *S. boulardii* fungemia). In particular, patients infected with *S. boulardii* were more likely than patients infected with *S. cerevisiae* to have digestive tract disease (58% vs. 6%; p<0.01), to have intravenous catheters (83% vs. 29%; p<0.0001), and to be hospitalized in an intensive care unit (32% vs. 0.05%, p<0.01). The use of biotherapeutic agents containing *S. boulardii* was associated with 40% of all invasive cases. A previously conducted literature review by Muñoz et al. identified 60 cases of fungemia caused by *S. cerevisiae* (*28*). Of note, 48% of patients with fungemia had received a *S. boulardii* probiotic preparation, and another 8% were near patients who had received these agents. The latter finding suggests that *S. boulardii* administration presents an environmental risk for patients who are not receiving the agents.

When Hennequin et al. investigated air and surface contamination related to the opening of a 500-mg packet of freeze-dried *S. boulardii,* they found that the simple act of opening a packet of *S. boulardii* produced substantial air contamination (*29*). Organisms persisted on the arm of the simulated patient 30 minutes after the product was opened and as long as 2 hours on the surrounding table surface. The hands of the technician who had opened the packet were noted to be highly and persistently contaminated despite vigorous handwashing.

Several factors constitute excessive and undue risk for development of *Saccharomyces* fungemia during probiotic administration. These factors are the patient's immunocompromised state during critical illness, the potential for live yeast spore contamination of healthcare workers' hands during preparation of the probiotic capsule for administration, and introduction of live yeast from contaminated hands to catheter sites (and patient's bloodstream).

Ensuring Patient Safety

Hospitalized patients for whom clinicians may consider use of a probiotic to manage severe and/or recurrent CDI often have many of the above risk factors for development of fungemia, making administration of *S. boulardii* less than desirable and its routine use unsafe. Guideline experts specifically recommend that administration of *S. boulardii* be avoided for persons who are immunocompromised, are critically ill, or have a central venous catheter (*25*). The Florastor package insert even recommends that

patients with a central venous catheter consult a healthcare professional before starting therapy and further mentions that "very rare cases of fungemia have been observed in patients with a central venous catheter."

Institutional guidelines are needed to address the potential safety issues related to *S. boulardii* use. After the decision is made to use probiotics on the basis of careful risk assessment, we suggest that the following measures be taken: 1) healthcare providers should wear gloves during the handling of probiotic agents for administration, then promptly discard the gloves and properly wash their hands with soap and water, 2) drug capsules should not be opened near patients with central venous catheters because aerosolized spores could cross-contaminate sterile sites (i.e., enter blood through catheter site) of patients receiving the probiotic as well as other patients nearby, 3) enteral administration of *S. boulardii* should be avoided because of the risk for environmental contamination and cross-contamination when the seal of the capsule is opened.

Probiotic products contain different genera, different species, or even different strains of the same species. Although the safety concerns noted here for *S. boulardii* may not be extrapolated to other probiotics such as lactobacilli, bifidobacteria, and others, use of any probiotic dietary supplement as a drug in diseased or immunocompromised populations requires specific evaluation of safety in that population.

Conclusions

The recent increase in incidence and severity of disease caused by hypervirulent strains of *C. difficile* has prompted some clinicians to prescribe probiotics as drugs in combination with standard antimicrobial drug therapy for these patients. However, clinicians need to be aware that, unlike drugs, these probiotic dietary supplements are not required by FDA to undergo rigorous premarketing evaluations for efficacy or safety.

Albeit rare, serious complications (i.e., fungemia) in other than healthy populations receiving probiotics have been reported in the literature. Specifically, most complications related to the administration of *S. boulardii* have occurred in immunocompromised or critically ill patients or in those who had central venous catheters serving as a portal of entry of organisms from healthcare workers' contaminated hands to patients' bloodstream during administration. Careful risk assessment for patients and proper handling of the probiotic during administration need to be conducted before using probiotics as drugs in institutional settings. Vigilant reporting of adverse events resulting from probiotic use is necessary to establish the safety profile of these agents when they are used in other than healthy populations.

Dr Venugopalan is assistant professor at the University of Southern California, School of Pharmacy. Her research interests are in the areas of antimicrobial drug resistance and resistance mechanisms associated with *Staphylococcus aureus*.

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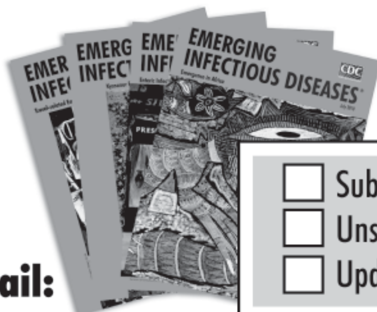

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Sulfadoxine/Pyrimethamine Intermittent Preventive Treatment for Malaria during Pregnancy

Philippe Deloron, Gwladys Bertin, Valérie Briand, Achille Massougbodji, and Michel Cot

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Learning Objectives

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

- Identify pregnant women who are most susceptible to pregnancy-associated malaria and the current recommendations for intermittent preventive treatment in pregnancy for malaria prophylaxis.
- Examine adverse events resulting from malaria during pregnancy and the efficacy of varying chemoprophylactic regimens in prevention.

Editor

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For monitoring efficacy of sulfadoxine/pyrimethamine intermittent preventive treatment for malaria during pregnancy, data obtained from studies of children seemed inadequate. High prevalence of triple and quadruple mutants in the dihydropteroate synthase and dihydrofolate reductase genes of *Plasmodium falciparum* parasites contrasts with the efficacy of sulfadoxine/pyrimethamine in reducing low birthweights and placental infection rates. In light of this discrepancy, emphasis on using molecular markers for monitoring efficacy of intermittent preventive treatment during pregnancy appears questionable. The World Health Organization recently proposed conducting in vivo studies in pregnant women to evaluate molecular markers for detecting resistance precociously. Other possible alternative strategies are considered.

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Malaria during pregnancy is a major cause of anemia and maternal death and one of the main causes of low birthweight (*1*,*2*). Consequently, the World Health Organization (WHO) recommends protection for women during pregnancy. Until recently, prevention consisted of weekly chemoprophylaxis with either chloroquine or sulfadoxine/pyrimethamine. Because of poor patient compliance with prophylaxis and increasing resistance of parasite strains to chloroquine, administration of intermittent preventive treatment in pregnancy (IPTp) with sulfadoxine/pyrimethamine

is now recommended for all pregnant women living in areas with stable malaria transmission (*3*). Sulfadoxine/pyrimethamine is given during antenatal visits at curative doses (1,500 mg sulfadoxine and 75 mg pyrimethamine; i.e., 3× the prophylactic dosage previously used) at least twice during pregnancy, once at the second trimester and once at least 1 month after the first treatment.

IPTp with sulfadoxine/pyrimethamine has proven efficacious in reducing the incidence of pregnancy-associated malaria (*4,5*) and is currently part of the national malaria prevention program in most countries in Africa. However, resistance to sulfadoxine/pyrimethamine is increasing in Africa (*6,7*). In many countries, sulfadoxine/pyrimethamine now demonstrates inadequate therapeutic efficacy in children <5 years of age (*8–10*) and is no longer the drug of choice for treatment, having been replaced by artemisinin combination therapy, according to WHO guidelines. Thus, this drug will soon be compromised, and an urgent need exists to assess alternative drug regimens for IPTp.

Monitoring Drug Efficacy during IPTp with Sulfadoxine/Pyrimethamine

WHO has recently stressed the inadequacy of sulfadoxine/pyrimethamine efficacy data obtained from studies of children <5 years of age with symptomatic malaria as a reliable indicator for pregnant women (*11*). Antimalarial immunity and pregnancy-specific differences in pharmacokinetics explain that in vivo data obtained for these children cannot be extrapolated to adult women (*12,13*). Therapeutic efficacy of sulfadoxine/pyrimethamine in children with clinical *Plasmodium falciparum* malaria largely underestimates its efficacy during IPTp because sulfadoxine/pyrimethamine efficacy in pregnant women may likely depend on their previous immunity. Furthermore, primigravidae, who are the most vulnerable to the effects of pregnancy-associated malaria, are also the least protected among pregnant women who are given sulfadoxine/pyrimethamine in areas where resistance is increasing (*13*).

In Tanzania, 28 days after treatment with sulfadoxine/pyrimethamine, the rate of treatment failure was 16% in pregnant women and 80% in children <5 years of age 2 years earlier (*14*). A recent systematic review indicated that 2 doses of IPTp with sulfadoxine/pyrimethamine retained activity to reduce placental malaria and low birthweight in areas with 19%–26% in vivo resistance in children (*5*). Also, the proportional reduction of peripheral parasitemia at delivery compared with that at enrollment with 2 doses of IPTp with sulfadoxine/pyrimethamine remained >60%, even at in vivo resistance rates ≤39%. In southern Benin, where the in vivo resistance rate to sulfadoxine/pyrimethamine reached 72% in children <5 years of age at day 28 (*9*), IPTp with sulfadoxine/pyrimethamine was still able to reduce the rate of low birthweight by 40% and the proportion of placental infection by 75% compared with the efficacy of chloroquine prophylaxis administered the previous year (*15*). However, a recent study in an area of high drug resistance in Tanzania demonstrated no clinical benefit of IPTp with sulfadoxine/pyrimethamine, in addition to a worse outcome (*16*).

An additional rationale for not extrapolating sulfadoxine/pyrimethamine efficacy data obtained in studies of young children to the efficacy of IPTp is that the primary outcome of interest differs. In children, the main outcome of the in vivo test is parasite clearance. Although parasite clearance is always highly desirable, the main rationale for administering IPTp is to avoid birthweight reduction as a consequence of massive placenta infection. How IPTp achieves such results is unknown. However, parasite clearance may not be required. A high reduction in parasite load in blood is likely to be paralleled in the placenta and may restore transplacental exchanges.

Overall, these findings have led the WHO technical report group to recommend that the protective efficacy of sulfadoxine/pyrimethamine be evaluated in asymptomatic pregnant women instead of in children, in parallel with constant monitoring of the effectiveness of IPTp with sulfadoxine/pyrimethamine at sentinel sites. Another priority identified by the WHO technical group is urgent evaluation of the prevalence of molecular markers associated with drug resistance as a surrogate to the protective efficacy of IPTp (*11*).

Usefulness of Molecular Methods

The WHO technical report group recommended genotyping of *Plasmodium* spp. dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes, a method commonly used in molecular epidemiology, to monitor the protective efficacy of sulfadoxine/pyrimethamine. Numerous molecular epidemiologic studies showed that resistance to pyrimethamine is associated with the acquisition of mutations in *dhfr*; the most common mutations related to pyrimethamine resistance are Ser108Asn, Asn51Ile, Cys59Arg, and Ile164Leu (*17,18*). Similarly, resistance to sulfadoxine is associated with 3 mutations in *dhps*: Ala437Gly, Ser436Phe, and Lys540Glu (*19,20*). Each mutation leads to a decrease in sensitivity to pyrimethamine (*dhfr* gene) and sulfadoxine (*dhps* gene).

Molecular markers are useful for tracking the emergence and spread of drug resistance where resistance is low or moderate. However, even for markers with virtually absolute correlations between genotype and in vitro phenotype (such as those for sulfadoxine/pyrimethamine), other factors (including acquired immunity and pharmacokinetic parameters) contribute to clearance of drug-resistant parasites, thus explaining the poor correlation with in vivo efficacy.

Djimdé et al. proposed a model accounting for immunity by controlling for age to predict treatment failure rates (*21*). In this model, the genotype-failure index (ratio of prevalence of resistant genotypes to rate of treatment failure in a population) was proposed for mapping resistance by using molecular methods. However, the genotype-failure index model is of particular interest where resistance is still low to moderate (*22*). Conversely, the predictive value of the model is limited when the prevalence of the marker is >80%, approaching fixation in the population (*23*), defined by a mutation being present without drug pressure and recovered in subsequent parasite generations. Unfortunately, such a situation is now encountered for sulfadoxine/pyrimethamine resistance markers in most countries in Africa where the prevalence of *dhfr*/*dhps* quadruple mutants is 50% (*24,25*) to 90% (*26,27*).

Only a few studies have investigated molecular markers of drug resistance in the context of pregnant women (*28–31*). The prevalence of sulfadoxine/pyrimethamine–resistant mutant parasites in pregnant women does not seem to differ greatly from the prevalence observed in the overall population. IPTp administration may induce an increase in this prevalence during pregnancy, but this increase seems limited and is not constantly observed (*28–31*). However, recent reports demonstrate that further drug pressure from sulfadoxine/pyrimethamine in an area of high resistance may select for a new triple mutant allele of the *dhps* gene that carries an additional mutation at codon 581 (*10*).

Methods for Monitoring Drug Efficacy

In addition to genotyping of *Plasmodium* spp. *dhfr* and *dhps* genes, traditional methods to survey drug efficacy include in vivo and in vitro tests. The in vivo test consists of administering a curative regimen of an antimalarial drug to an infected person and following the evolution of clinical symptoms and parasite density over a few weeks. When the drug is effective, clinical signs and parasitemia levels rapidly decrease then clear, without reappearing thereafter, which is the so-called adequate clinical and parasitologic response (*32*). According to drug efficacy, parasite density only decreases or disappears but eventually reappears. To monitor drug sensitivity, children <5 years of age are the study population recommended by WHO. However, in our context, the population of choice should be pregnant women, which is consistent with the inadequacy of performing studies in children, as discussed previously.

The current policy of giving IPTp to all pregnant women generates numerous difficulties in identifying infected pregnant women and interpreting results of the tests. The prevalence of *P. falciparum* infection in pregnant women receiving IPTp is low (3% in the trial in Benin) (*33*), whereas it may reach 15%–35% in the absence of IPTp (*34–36*). However, one may take the opportunity to enroll women at the first antenatal care visit, when pregnancy is diagnosed, and IPTp administration has not yet started. Such a low prevalence rate will complicate identification of *P. falciparum*–infected pregnant women and will compromise enrollment of a sufficiently large number of women because this prevalence may require screening several hundred pregnant women. Conversely, treatment with sulfadoxine/pyrimethamine for a woman who has recently received (or will soon receive) a regimen of sulfadoxine/pyrimethamine as part of IPTp may increase the risk for drug hepato-toxicity and severe cutaneous side effects (*37*). In addition, such treatment with a drug that is likely to be ineffective (because parasites have persisted after IPTp with the same drug) obviously constitutes an ethical problem.

The in vitro drug assay involves culturing parasites in the presence of increasing concentrations of antimalarial drugs (in this case sulfadoxine/pyrimethamine) and determining the drug concentration that inhibits parasite maturation. For monitoring IPTp efficacy, in vitro tests are problematic because pregnant women are involved and because of the antifolate nature of the sulfadoxine/pyrimethamine. If all pregnant women are receiving IPTp with sulfadoxine/pyrimethamine, the same limitations for enrolling infected women apply, and any *P. falciparum* parasites encountered in pregnant women are likely to be resistant to sulfadoxine/pyrimethamine. Moreover, given the long half-life of sulfadoxine/pyrimethamine, many women will have residual concentrations of the drug in their blood, which may interfere with drug activity measurement. In addition, in vitro assays for sulfadoxine/pyrimethamine pose a technical challenge because they require a modified culture medium and are only partially successful compared with assays for other drugs (*38*).

What Alternatives Can Be Proposed?

Obviously, no traditional approach is satisfactory for IPTp monitoring and one must search for alternatives. One of these alternatives may be administration of each IPTp dose to achieve a simplified in vivo efficacy test. Because each woman is given a curative dose of sulfadoxine/pyrimethamine, it should be easy to obtain a blood smear at each drug administration and to check whether parasites are cleared. The 2 curative doses of sulfadoxine/pyrimethamine are given ≈1 month apart, which corresponds to the usual follow-up period of such drug efficacy tests. A blood smear may be replaced by rapid diagnostic test or by filter paper blood spot for subsequent PCR detection of parasites. Blood spots will also enable distinguishing true therapeutic failures from reinfections by comparing the banding patterns of PCR amplicons of variable loci (such as genes for merozoite surface protein 1, merozoite surface protein 2, and glutamate-rich protein), before and after sulfadoxine/pyrimethamine administration. As mentioned earlier, the

expected low prevalence of *P. falciparum* infection in this population of women receiving IPTp will explain that a high number of women under survey are likely to be required to generate useful data for public health applications.

An alternative approach involves systematic detection of placental infection at delivery by using blood smear, rapid diagnostic test, or PCR with placental blood. This method is easy to perform and would enable monitoring IPTp efficacy over several years in all centers able to diagnose malaria in an entire country. The advantage is that placental infection is a good proxy of birthweight, the major outcome in terms of public health (*39,40*). This approach will enable a pragmatic measure of IPTp with sulfadoxine/pyrimethamine efficacy and account for the quality of its application. Conversely, placental infection prevalence may change with time because of changes in sulfadoxine/pyrimethamine efficacy (likely to decrease) and quality of IPTp implementation (likely to increase). If the 2 variables evolve simultaneously, the resulting indicator may remain unchanged. Such an approach would also provide baseline data to assess efficacy of all preventive measures against pregnancy-associated malaria, including IPTp and use of insecticide-impregnated bed nets, and will enable assessment of these effects in a specific population. In practice, because these approaches complement each another by monitoring IPTp efficacy at different times during pregnancy, the association of these 2 approaches should be worthwhile.

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Oropharyngeal Cancer Epidemic and Human Papillomavirus

Torbjörn Ramqvist and Tina Dalianis

A growing body of research shows that human papillomavirus (HPV) is a common and increasing cause of oropharyngeal squamous cell carcinoma (OSCC). Thus, the International Agency for Research against Cancer has acknowledged HPV as a risk factor for OSCC, in addition to smoking and alcohol consumption. Recently, in Finland, the United Kingdom, the Netherlands, the United States, and Sweden, incidence of OSCC has increased, and an increase in the proportion of HPV-positive tumors was noted. On the basis of these data and reports indicating that patients with HPV-positive cancer have their first sexual experience at a young age and have multiple partners, we postulate that increased incidence of OSCC in the United States and some countries in northern Europe is because of a new, primarily sexually transmitted HPV epidemic. We also suggest that individualized treatment modalities and preventive vaccination should be further explored.

In many countries, vaccines against some human papillomavirus (HPV) types are now administered to girls and young women with the goal of protecting them against HPV-induced cervical cancer (*1*,*2*). The introduction of HPV vaccines has also drawn more attention to the fact that HPV is associated not only with cervical cancer and genital warts but also with other tumors, such as head neck and anogenital cancers (*3*). We focus on the role of HPV in the increased incidence of oropharyngeal squamous cell carcinoma (OSCC), the head and neck cancer in which HPV is most commonly found (*4*).

Head and neck cancer most commonly is of the squamous cell carcinoma type (HNSCC) and includes cancers of the oral cavity, oropharynx, hypopharynx, larynx, sinonasal tract, and nasopharynx. HNSCC is the sixth most common type of cancer in the world; almost 600,000 cases are reported annually, and of these, ≈10% (or more for some geographic locations) are OSCC (*5*). Globally, the incidence and localization of HNSCC varies widely. It is the most common form of cancer in India, and incidence is higher in countries in Latin America than in the United States and northern Europe. In addition, men are generally more often affected than women. Smoking, alcohol consumption, and betel chewing are traditional risk factors for HNSCC and OSCC (*6*). However, during the past decade several reports have documented HPV in OSCC (*7*–*9*). HPV infection, with dominance of HPV16 infection, has therefore been acknowledged by the International Agency for Research against Cancer as a risk factor for OSCC (*10*). Moreover, there are accumulating reports from many countries that the incidence of OSCC is increasing. We suggest that this increase is caused by a slow epidemic of HPV infection–induced OSCC.

OSCC

Tonsillar cancer is the most common OSCC, followed by base of tongue cancer. Together, these 2 cancers account for 90% of all OSCCs (*6*,*9*). Patients usually do not seek counseling until the tumors are large because small tumors cause little distress and may not be noticed by the patient. Curative treatment implies surgery, radiotherapy, and chemotherapy; the goal is to cause as little functional and cosmetic damage as possible (*6*,*9*). If a cure cannot be obtained, palliative therapy is given to treat pain and discomfort. Similar to HNSCC, in general, survival rates for patients with OSCC are poor. Patients with OSCC have an overall 5-year survival rate of ≈25% (*6*,*9*). Furthermore, even when standardized treatment is used and tumors are

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at the same stage and have similar histologic features, it is difficult to predict the outcome. Several reports now describe the incidence of OSCC as increasing and indicate that HPV-positive OSCC has a better clinical outcome than HPV-negative OSCC (*7–9*,*11–19*). Thus, predictive and prognostic markers would be of clinical value for prevention and treatment of OSCC.

HPV

There are >100 HPV types, some found in skin warts and others in mucous tissues, and the association of different HPV types with cervical, some anogenital, and head and neck cancers is well established (*3*). The 8-kb, double-stranded, circular DNA HPV genome, enclosed in a 52–55 nm viral capsid, codes for the L1 and L2 viral capsid proteins and for the E1–E2 and E4–E7 proteins, which play major roles in gene regulation, replication, pathogenesis, and transformation (*3*). In high-risk HPVs (i.e., those that are more likely to cause lesions that may develop into cancer [www.cancer.gov/cancertopics/factsheet/Risk/HPV]), E6 and E7 deregulate cell cycle control by E6 binding and degradation of p53, and E7 binds and inhibits the function of the retinoblastoma protein (Rb) (*3*). The L1 protein can self-assemble into virus-like particles, which form the basis of both currently approved vaccines against HPV infection (*1–3*).

HPV and Methods for Detection in OSCC

During the past few decades, HPV DNA has been detected in ≈25% of HNSCCs overall, but especially in OSCC, for which 45%–100% cases were reported to be HPV positive (*7–9*,*11–19*). The latter variation may depend on OSCC location, the type of specimens available, the techniques used for testing, and the time period and country from which the sample material was obtained (*7–9*,*11–19*).

Analysis of HPV DNA was (and still is) performed primarily by using formalin-fixed, paraffin-embedded tissue, in which the DNA can be partially degraded. It is now widely accepted that it is easier to detect longer HPV DNA fragments in fresh or fresh-frozen material, although newer techniques are more sensitive. Many early studies during the 1980s were based on Southern blot techniques or in situ hybridization for detection of HPV.

Since the 1990s, virology laboratories used PCR for detection of HPV DNA (*20–23*). Screening for HPV was initially performed by using general PCR primers for HPV, which enabled detection of several HPV types (*21–23*). PCR of a control cellular gene was used to assess the DNA quality of samples. These techniques are robust and are still used but need additional methods for HPV typing. There are now many other methods that directly determine the presence of several different HPV types. The Food and Drug Administration–approved Hybrid Capture II (Digene Corporation, Gaithersburg, MD, USA) detects 5 low-risk and 13 high-risk HPV types and uses the fact that HPV DNA hybridizes with synthetic RNA probes complementary to DNA sequences from specific HPV types (*20*). An assay used in several studies, the Roche (Basel, Switzerland) linear array HPV Genotyping Test, detects 37 HPV types and is based on a method developed by Gravitt et al. (*24*). In this method, HPV PCR products are hybridized to a linear array of type specific probes. Recently, Schmitt et al developed a sensitive bead-based multiplex method, originally set up for 22 different HPV types but later expanded, in which HPV PCR products are coupled to type-specific probes on beads and analyzed by using Luminex (*25*).

To assay for biologic activity of HPV in tumors, analysis of E6 and E7 expression by detecting E6 and E7 mRNA by reverse transcription followed by real-time PCR is also often performed (*13*). In pathology departments, HPV screening is often conducted by in situ hybridization, and in some instances p16 immunohistochemical analysis is used as a substitute to assay for biologically active HPV because there is a correlation between the presence of HPV and overexpression of p16 (*26*,*27*).

HPV in OSCC

When HPV in OSCC became more obvious, several studies concentrated on characterizing HPV-positive OSCC (*4*,*7–9*,*11–19*). HPV type 16 was highly prevalent (≈90%) in OSCC in all studies; other HPV types (e.g., HPV-31, -33, -58, -59, -62, and -72) were less common, and HPV was demonstrated to be episomal or integrated into the cellular genome (*14*,*28*).

In several studies, E6 and E7 expression in OSCC were shown, suggesting that HPV was actively involved in the etiology of the tumors (*13*). In addition, the association of p16 overexpression with HPV was a further indicator of active E7 because of E7-induced cell cycle activation and upregulation of p16 by inactivating the Rb pathway (*3*,*26*,*27*). HPV-positive tumors were also less likely to have mutated p53 (*7*) and were more frequently aneuploid and less differentiated than HPV-negative tumors (*29*). Furthermore, comparative genomic hybridization indicated that HPV-positive tonsillar cancer, in contrast to HPV-negative cancer, often showed chromosome 3q amplification similar to that in HPV-positive cervical and vulvar cancer, which further supports the oncogenic role of HPV in OSCC (*30*).

It was also observed that patients with HPV-positive OSCC were younger and lacked the traditional risk factors of smoking and alcohol consumption (*7–9*,*13*,*17*). Moreover, a major feature, noted in several studies, was that HPV was a favorable prognostic factor for clinical outcome of OSCC, as demonstrated in Figure 1 (*7–9*,*11–19*). This finding was independent of tumor stage, age, gender, grade

of differentiation, p53 immunohistochemical results, or DNA ploidy (*7–9*). However, Lindquist et al. (*13*) observed that patients with HPV-positive tonsillar cancer who had never smoked had a better prognosis than those who were smokers, and this observation was recently confirmed by Ang et al. (*16*). The reasons for this finding are most likely complex and should be investigated further. One could speculate, for example, that HPV induces an immune response and that smoking abrogates this response. A different option is that smoking and HPV in combination induce a different category of tumors and that smoking induces additional genetic alterations in these tumors, as was also suggested by Ang et al. (*16*).

In general, the prognostic value of HPV status is for OSCC and not for HNSCC. In some studies with conflicting findings, the prognostic value of HPV was determined for all HNSCC anatomic sites (*9*). However, because there are differences in survival rates and presence of HPV at different locations, even for OSCC, studies should be performed per location.

In summary, the accumulated data suggest different entities of OSCC, where some primarily depend on smoking and alcohol and others on HPV infection. It is also likely that there are combined etiologies. Nevertheless, patients with HPV-positive OSCC consistently have a better prognosis (*7–9*,*11–19*).

An HPV-induced Epidemic of OSCC

We suggest the increased incidence of OSCC depends on HPV infection and results in an increased proportion of HPV-positive OSCCs. During the past decades, studies from the United States, Finland, Sweden, the Netherlands, the United Kingdom, and Scotland showed an increase in the incidence of OSCC, tonsillar cancer, and base of tongue cancer (*31–36*). In addition, during the past 10 years, an increase in the proportion of HPV-positive OSCC has been reported, and we speculate that this is not caused simply by use of more sensitive diagnostic techniques. In many of these studies the same assay was used when studying OSCC over time (*12*,*18*,*19*). Furthermore, the general PCR amplifiability of the DNA from the older tested samples was also validated.

Using the Swedish Cancer Registry, which covers basically all cancer cases in Sweden for 1970–2002, we disclosed a 2.8-fold increase (2.6-fold for men and 3.5-fold for women) in the incidence of tonsillar cancer in the Stockholm area (*12*), where 25%–30% of all patients in Sweden with tonsillar cancer are treated. In parallel, we examined all 237 available samples from the 515 patients with tonsillar cancer in Stockholm during the same period and found a 2.9-fold increase in the proportion of HPV-positive tonsillar cancer from 23% to 68% (*12*). We thus suggested HPV infection played a role in the increase of this disease (*12*).

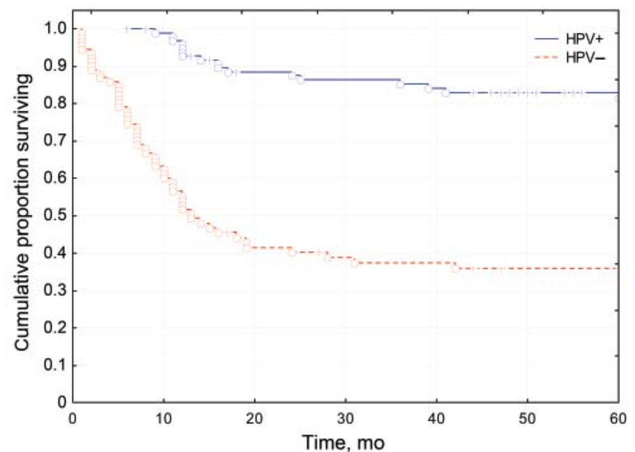

Figure 1. Survival rates for patients with human papillomavirus (HPV)–positive tonsillar cancer compared with those for patients with HPV-negative cancer. Circles indicate patients who died of tonsillar cancer during the follow-up period; plus signs indicate patients who were removed from the study for other reasons (e.g., died from a cause other than tonsillar cancer, left the country); p<0.0005. Data from Lindquist et al. (*13*), with permission of Elsevier (www.elsevier.com).

In continuation of the above study, we followed the incidence of tonsillar and base of tongue cancer in Stockholm in the Swedish Cancer Registry and demonstrated a substantial increase for both tumor types during 1970–2006, as shown in Figure 2 (*18*,*19*,*34*). We then performed a follow-up study in the Stockholm area of the prevalence of HPV in tonsillar cancer during 2003–2007; using the Swedish Cancer Registry, we identified 120 patients (*18*). Using the same methods as in the first study, we found that the proportion of HPV-positive cancers in the 98 available pretreatment biopsy specimens had significantly increased both from 1970 through 2007 (p<0.0001) and from 2000 through 2007 (p<0.01). During the last 2 years of the study (2006–2007), 93% of all tonsillar cancer was HPV positive. Moreover, the incidence of HPV-positive tumors almost doubled each decade during 1970–2007, indicating a 7-fold increase over the whole period; in parallel, a decline of HPV-negative tumors was observed (Figure 3).

Shortly afterwards, we demonstrated that the prevalence of HPV-positive base of tongue cancer also had increased in the Stockholm area during 1998–2007 (*19*). When we analyzed 95 pretreatment biopsy specimens from base of tongue SCC from the 109 patients reported during 1998–2007 in the Swedish Cancer Registry in a similar way as above, we found an increase in the proportion of HPV-positive tumors from 54% in 1998–1999 to 84% in 2006–2007.

The strength of the above studies is that the Swedish Cancer Registry covers all cancer cases in Sweden and that we have analyzed all available pretreatment diagnostic bi-

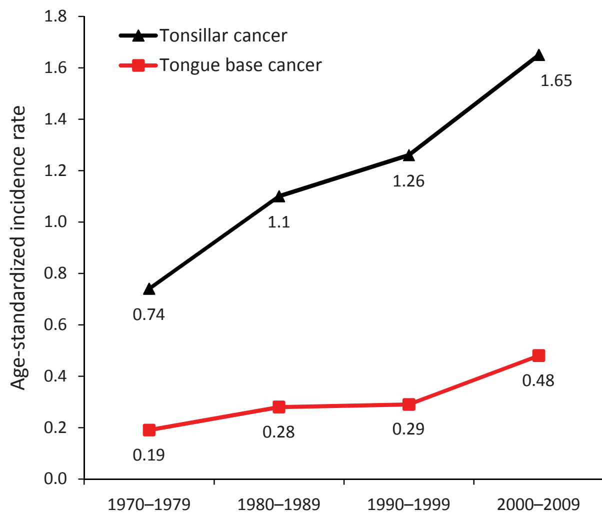

Figure 2. Age-standardized incidence of tonsillar and base of tongue cancers, Stockholm, Sweden, 1970–2006.

opsy specimens for HPV. The limitation of our study is that Sweden is a small country with only 9 million inhabitants.

Covering almost the same time period as above, Chaturvedi et al. reported an increase in the proportion of potentially HPV-related OSCC in the United States (*35*). However, the previous year, Sturgis and Cinciripini had already proposed a possible emerging epidemic of HPV-associated cancers (*11*). More recently, Marur et al. reviewed many studies, further supporting an increase in OSCC (*17*). Notably, it was also suggested that the increase in OSCC occurs mainly in men (*17*). However, using the Swedish Cancer Registry, in the Stockholm area, Hammarstedt et al. observed also an increase of OSCC in women (*12*). It is necessary to acknowledge that the numbers of women with OSCC are more limited and that it could be more difficult to identify major changes in this group.

The possible causes for this increase have been discussed extensively and have focused on changes in sexual patterns, such as increased oral sex or increasing numbers of sex partners. A significant association has been shown between HPV-positive tonsillar cancer and early initial sex or number of oral or vaginal sex partners (*37*).

Furthermore, in a recent study by D'Souza et al., it was shown that the risk of developing oral HPV infection increased with increases in lifetime oral or vaginal sex partners (*38*). It has also been reported that not only oral sex, but also open-mouthed kissing, was associated to the development of oral HPV infection (*38*). In this study, 2 study populations were included, one (332 patients) consisted of a control patient group >18 years of age from the Johns Hopkins outpatient otolaryngology clinic (2000–2006) enrolled in 2 case–control studies within a prospective cohort with HNSCC. The other (210 students) consisted of students >17 years of age recruited from the campuses of Towson University and the University of Maryland in 2007. The latter study may have had some limitations because it was not population based, and data for open-mouthed kissing for control patients and smoking for college students were absent. Nevertheless, this study suggests that oral-to-oral contact may play a role for oral HPV transmission and could play a major role in timing of prophylactic vaccination of children.

Several reports show an increase in OSCC and the proportion of HPV-positive OSCC and an association of the latter to early sex debut and many partners. Thus, we suggest that we are encountering a slow epidemic of mainly sexually transmitted HPV-induced OSCC.

HPV in OSCC and Consequences for Treatment and Prevention

The possibility that we are dealing with an HPV-induced epidemic of OSCC warrants special attention. For example, in Stockholm, the incidence of HPV-positive tonsillar cancer has increased 7-fold over 30 years (*18*). OSCC now accounts for approximately one third of all HNSCC cases annually in Sweden. The fact that HNSCC in general is decreasing, and OSCC is increasing, may in 10 years result in OSCC accounting for half of all HNSCCs in Sweden, and similar trends are likely elsewhere, e.g., the United States, United Kingdom, the Netherlands, and Finland. It is also known that patients with HPV-positive OSCC are younger and have a better prognosis than HNSCC patients and patients with HPV-negative OSCC (*7*,*8*). In contrast, because of the poor prognosis for HNSCC and, in the past for OSCC, therapeutic measures have recently been intensified with induction chemotherapy, hyperfractionated radiotherapy, surgery, and occasional use of epidermal growth factor receptor inhibi-

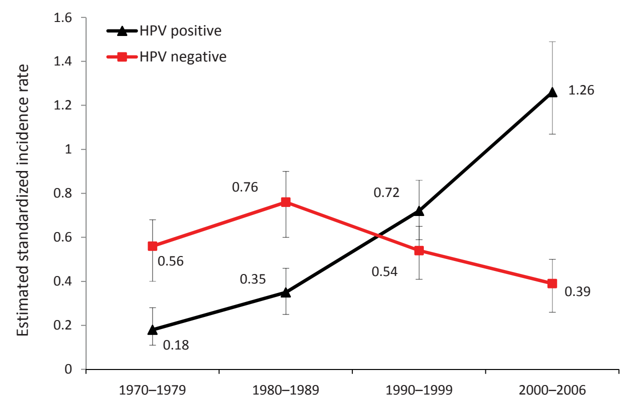

Figure 3. Estimated age-standardized incidence of human papillomavirus (HPV)–positive and HPV-negative tonsillar cancer squamous cell carcinoma cases per 100,000 person-years, Stockholm, Sweden, 1970–2006. Error bars indicate 95% confidence intervals. Data from Näsman et al. (*18*), with permission of John Wiley and Sons (www.interscience.wiley.com).

tors. This intensified therapy results in more severe acute and chronic side effects, such as difficulties in swallowing or talking, dry mouth, and necrosis of the jawbone, and is also more expensive for society. Accordingly, it is possible that increasing numbers of OSCC patients with a better prognosis are being treated with intensified therapy. As a result, many patients have substantial chronic unnecessary side effects. It is therefore necessary to identify which patients need and which do not need intensified treatment, both to increase patient survival times and quality of life and for the socioeconomic benefit of society.

Several reports have been published and other studies are ongoing to assess which molecular factors, such as p16, p53, and others, besides the presence of HPV in OSCC, can best predict clinical outcome and which treatments are optimal according to the same predictive markers (*7–9*,*17*,*26*). Some retrospective reports have suggested that persons with HPV-positive OSCC have higher response rates to chemotherapy and radiation; however, in other reports this has not been confirmed (*9*,*17*). A recent study also observed that tumor HPV status is a strong independent prognostic factor for survival among patients with HPV-positive OSCC irrespective of treatment (*16*). However, in the same study, among patients with HPV-positive tumors, the risk for death significantly increased with each additional pack-year of smoking, independent of treatment modality (*16*), a result similar to that found by Lindquist et al. (*13*).

It has been shown in an experimental setting that HPV-positive tumors were not more curable on the basis of increased epithelial sensitivity to cisplatin or radiation therapy (*39*). Instead, Spanos et al. demonstrated that radiation and cisplatin induced an immune response to this antigenic type of cancer. This finding could suggest that the presence of HPV in a tumor induced by smoking could be of benefit, but it is possible that smoking also may abrogate the immune response. As mentioned, the relationship between smoking and HPV and their roles in OSCC is most likely complex. In future studies, it would therefore be valuable to obtain more molecular and immunologic information and to determine if it is a survival benefit to stop smoking during and after therapy.

Summarizing treatment of OSCC patients, it is obvious that additional information will be required before it will be possible to guide treatment decisions for the individual patient on the basis of HPV status. Nevertheless, there is accumulating evidence that HPV status and overexpression of p16, and having never smoked, is of benefit. Future prospective clinical studies, including diagnostics of HPV, molecular and immunologic profiles, history of smoking, cessation of smoking during therapy, and effects of different treatment modalities and their side effects on quality of life, will be of benefit for personalized treatment.

Finally, it is also essential to keep in mind that we now have vaccines directed against HPV16, which accounts for ≈80%–90% of all HPV-positive OSCC, at least in Europe and the United States (*7–9,11–9*). Although it will likely take several decades before the effects of HPV vaccination on cancer incidence will be detected, it is crucial to monitor the effects of the present HPV vaccination, not only on the incidence of cervical cancer but also on the incidence of OSCC.

Few if any of other studies have focused on performing isolated health economic analysis of the effect of HPV on OSCC. However, a recent study pointed out that there is an improvement of the present cost-effectiveness of HPV vaccines when the effects on other HPV-associated tumors cancers are included (*40*). Furthermore, in countries with effective cervical cancer screening programs, other HPV-associated noncervical cancers represent a relatively high proportion of HPV-positive cancers (*15*). Considering that OSCC is the second most common HPV-associated cancer and its incidence is increasing, the effect of the HPV vaccine on this tumor deserves attention, and we need to know if future vaccination against HPV infection should include both women and men.

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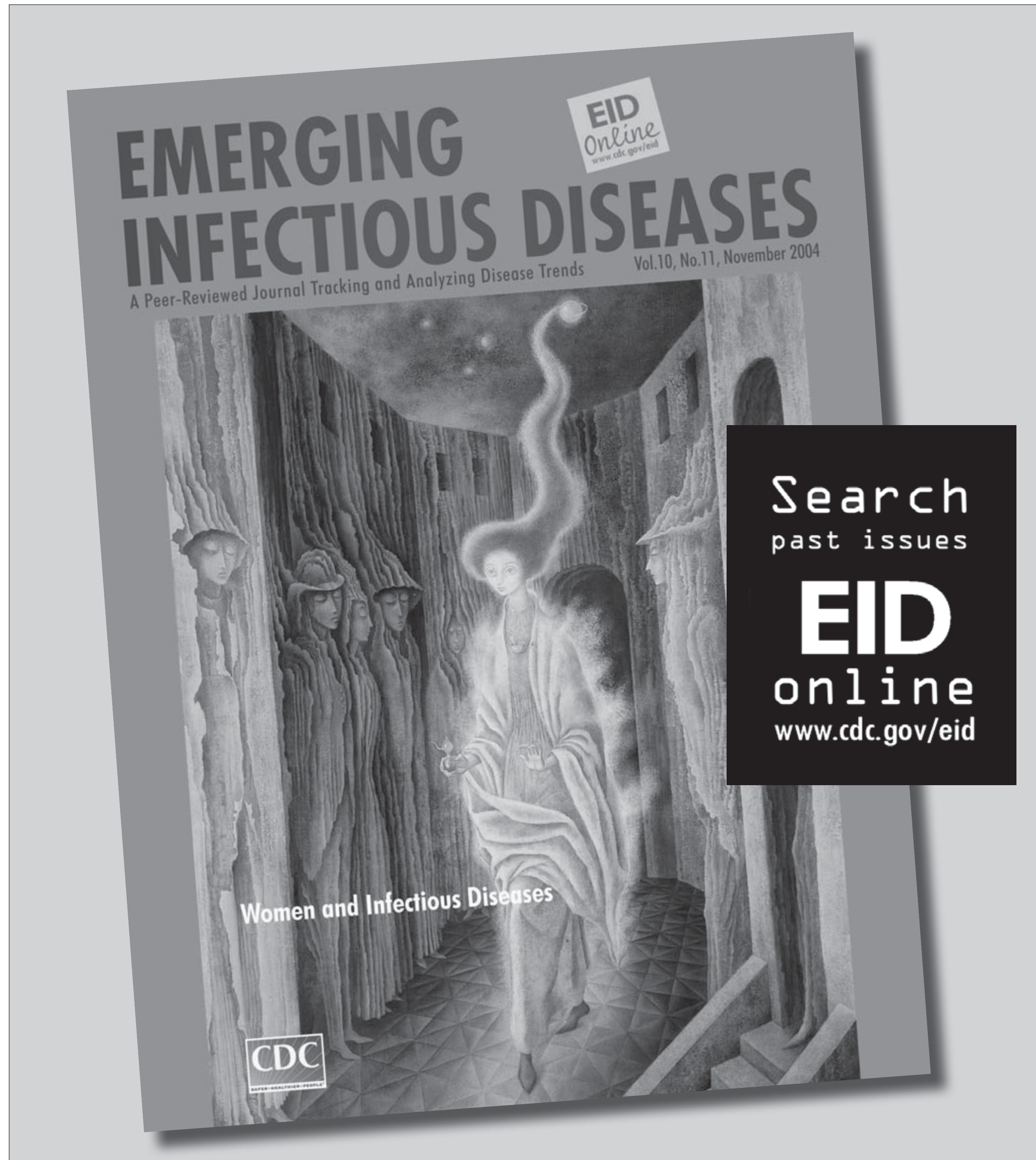

Salmonella enterica Pulsed-Field Gel Electrophoresis Clusters, Minnesota, USA, 2001–2007

Joshua M. Rounds, Craig W. Hedberg, Stephanie Meyer, David J. Boxrud, and Kirk E. Smith

We determined characteristics of *Salmonella enterica* pulsed-field gel electrophoresis clusters that predict their being solved (i.e., that result in identification of a confirmed outbreak). Clusters were investigated by the Minnesota Department of Health by using a dynamic iterative model. During 2001–2007, a total of 43 (12.5%) of 344 clusters were solved. Clusters of ≥4 isolates were more likely to be solved than clusters of 2 isolates. Clusters in which the first 3 case isolates were received at the Minnesota Department of Health within 7 days were more likely to be solved than were clusters in which the first 3 case isolates were received over a period >14 days. If resources do not permit investigation of all *S. enterica* pulsed-field gel electrophoresis clusters, investigation of clusters of ≥4 cases and clusters in which the first 3 case isolates were received at a public health laboratory within 7 days may improve outbreak investigations.

Salmonellosis is a major foodborne illness that results in ≈1.4 million infections, 15,000 hospitalizations, and 400 deaths each year in the United States (*1,2*). *Salmonella* infections are primarily of foodborne origin but can also occur through contact with infected animals, humans, or their feces (*3*). The epidemiology of salmonellosis is complex largely because there are >2,500 distinct serotypes (serovars) with different reservoirs and diverse geographic incidences (*4*). Changes in food consumption, production, and distribution have led to an increasing frequency of multistate outbreaks associated with fresh produce and processed foods (*5*).

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The development of molecular subtyping by pulsed-field gel electrophoresis (PFGE) has revolutionized *Salmonella* spp. surveillance. The National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet) provides state and local public health department laboratories with standardized methods to subtype *Salmonella* serovars and normalize PFGE patterns against a global reference standard provided by the Centers for Disease Control and Prevention (CDC) (*6,7*). Molecular subtyping enhances case definition specificity, enabling outbreaks to be detected and controlled at an earlier stage, and enabling detection of geographically dispersed outbreaks (*8–10*).

Although the benefits of molecular subtyping, specifically by PFGE, in foodborne disease outbreak detection and investigation have been well established, there is no consensus about when a PFGE cluster warrants further investigation and almost no quantitative analysis about characteristics of PFGE clusters that indicate a common source will be identified (*11–15*). Cluster size and the number of days from receipt of the first cluster case isolate to the third case isolate received by the public health laboratory were predictors of a source of infection being identified for *Listeria monocytogenes* clusters in France (*16*). The objective of this study was to determine characteristics of *Salmonella* PFGE clusters that could serve as useful predictors for their being solved (i.e., result in identification of a confirmed outbreak). This information could help public health agencies with limited resources prioritize investigation of *Salmonella* PFGE clusters.

Materials and Methods

Salmonella infections are reportable to the Minnesota Department of Health (MDH) by state law (*17*). Clinical laboratories are required to forward all *Salmonella* isolates to the MDH Public Health Laboratory (PHL). PFGE sub-

typing after digestion with *XbaI* is conducted on all isolates as soon as they are received according to PulseNet protocols (*18*). PFGE subtypes are uploaded into the national PulseNet database (*6*). All Minnesota residents with a culture-confirmed *Salmonella* infection are routinely interviewed as soon as possible by MDH staff with a standard questionnaire about symptom history, food consumption, and other potential exposures occurring in the 7 days before onset of illness. The questionnaire contains detailed food exposure questions, including open-ended food histories and objective yes/no questions about numerous specific food items, as well as brand names and purchase locations. Clusters are investigated by using an iterative model in which suspicious exposures identified during initial case-patient interviews are added to the standard interview for subsequent cases (*19–21*). Similarly, initial cluster case-patients may be reinterviewed to ensure uniform ascertainment of the suspicious exposures. This iterative approach is used to identify exposures for further evaluation with formal hypothesis testing, product sampling, or product tracing (*19*).

A cluster was defined as ≥2 cases of salmonellosis in different households with isolates of the same serovar and PFGE subtype and with specimen collection dates within 2 weeks (*22*). Thus, a single cluster would be ongoing as long as a new isolate was collected within 2 weeks after the most recent isolate in the cluster. A cluster was considered solved if the epidemiologic evaluation of that cluster resulted in the identification of a common source of infection for those cases and consequently the documentation of a confirmed outbreak. Therefore, the terms solved cluster and confirmed outbreak are equivalent and used interchangeably.

Inclusion and Exclusion Criteria

Laboratory-confirmed cases of nontyphoidal *Salmonella enterica* infection among Minnesota residents with specimen collection dates from January 1, 2001, through December 31, 2007, for which isolates were received and subtyped by MDH PHL were included in the study. Isolates not received through routine surveillance (i.e., testing was requested or conducted by MDH as a part of an ongoing investigation) were excluded from the analysis.

Solved clusters were included if they were detected and identified solely on the basis of investigation of cases identified through submission of isolates to MDH for routine laboratory surveillance. Solved clusters for which a call to the MDH foodborne disease hotline (www.health.state.mn.us/divs/idepc/dtopics/foodborne/reporting.html) (e.g., from the public or a healthcare provider) directly contributed to the identification of an outbreak were excluded from analysis. Secondary clusters, defined as clusters in which the cases were part of a confirmed outbreak that had been previously identified, were also excluded from analysis. Clusters that were part of a probable outbreak (an epidemiologic evaluation suggested, but did not confirm, a common source of infection) were also excluded.

Study Variables

Variables incorporated into the analysis were cluster year, cluster size, cluster case density, cluster serovar, cluster subtype, and cluster serovar diversity. Cluster size was defined as the number of cases in each cluster and was categorized into cluster sizes of 2, 3, 4, and ≥5. For clusters in which a common source was identified, only cases received before the cluster was solved were included. Cluster case density was defined as the number of days from receipt date of the first cluster isolate at MDH PHL to the receipt date of the third cluster isolate and was categorized into cluster case densities of 0, 1–7, 8–14, and >14 days (*16*).

Cluster serovar was coded as a categorical variable on the basis of serovar frequency. Serovars representing >20% of all isolates (Typhimurium and Enteritidis) were categorized as very common, those representing 3%–20% (Newport, Heidelberg, and Montevideo) as common, and those representing <3% (all other serovars) as uncommon. The relationship between common and uncommon PFGE subtypes and solving a cluster was examined for serovars Typhimurium and Enteritidis. For serovar Typhimurium, clusters with CDC PFGE subtype designations JPXX01.0003, JPXX01.0410, and JPXX01.0111 (each representing >8% of all Typhimurium isolates) were categorized as common, and all other subtypes were categorized as uncommon. For serovar Enteritidis, clusters with CDC PFGE subtype designations JEGX01.0004 and JEGX01.0030 (each representing >20% of all Enteritidis isolates) were categorized as common, and all other subtypes were categorized as uncommon.

Cluster serovar diversity was examined by categorizing the 17 most frequent serovars into highly clonal or low clonality serovars on the basis of the Simpson diversity index (*23*). Serovars with a Simpson index score <0.90 were considered highly clonal, and serovars with a Simpson index score ≥0.90 were considered to have low clonality. Cluster investigation thresholds were examined by comparing the percentage of outbreak clusters meeting a threshold, cluster investigation positive predictive value, and estimated interview burden in hours per year for various investigational thresholds. The time required to interview each patient with a *Salmonella* infection by using the MDH standard questionnaire was recorded for a 6-month period in 2008, and the median interview time was calculated.

Statistical Analysis

A descriptive analysis was conducted to characterize the frequency of *Salmonella* serovars and subtypes. Mantel-Haenszel χ^2 test for trend was used to characterize tem-

poral trends in the number of *Salmonella* clusters that were solved. Two-sided Wilcoxon rank-sum tests were used to compare the median cluster size and cluster density of point source and non–point source outbreaks. Univariate analysis was performed to calculate odds ratios (ORs) and 95% confidence intervals (CIs) characterizing the crude associations between *Salmonella* cluster serovar, cluster PFGE subtype, cluster serovar diversity, cluster size, and cluster case density and a cluster being solved. Mantel-Haenszel χ^2 tests for trend and interaction terms were used to investigate the linear nature of the relationship between cluster size, cluster case density, and the outcome. SAS software version 9.1 (SAS Institute, Cary, NC, USA) was used for descriptive and univariate analysis. An α value ≤ 0.05 was considered significant.

Results

During 2001–2007, a total of 4,154 nontyphoidal *Salmonella* isolates from Minnesota residents were received at MDH through routine surveillance; they represented 98% of reported *Salmonella* cases (n = 4,235, incidence 11.78 cases/100,000 person-years). PFGE subtyping was performed for 4,018 (97%) isolates, which were included in the study. Among these isolates, 194 *Salmonella* serovars were observed. The 6 most common *S. enterica* serovars were Typhimurium, 1,004 (25%); Enteritidis, 822 (20.5%); Newport, 314 (7.8%); Heidelberg, 223 (5.6%); Montevideo, 121 (3.0%); and Saintpaul, 81 (2.0%) (Figure 1).

The frequency of PFGE subtypes was examined in detail for serovars Typhimurium and Enteritidis. The 3 most common subtypes of serovar Typhimurium were JPXX01.0003, 107 (11%); JPXX01.0410, 87 (9%); and JPXX01.0111, 85 (8%). The 3 most common subtypes of serovar Enteritidis were JEGX01.0004, 309 (38%); JEGX01.0030, 181(22%); and JEGX01.0005, 106 (13%).

Serovar diversity was examined by comparing Simpson diversity indices for the 17 most frequent serovars (Table 1). Javiana, Newport, Agona, Infantis, and Typhimurium were low clonality serovars. Heidelberg, Hadar, Enteritidis, Thompson, and I 4,5,12:I:– were highly clonal serovars.

Cluster and Outbreak Characteristics

During 2001–2007, a total of 376 *Salmonella* PFGE clusters were detected; they represented 1,399 (35%) isolates. Thirty-two (8.5%) clusters were excluded from analysis (21 secondary clusters, 7 clusters in which a hotline call directly contributed to identification of an outbreak, and 4 probable outbreak clusters). Forty-three (12.5%) of the 344 clusters included in the analysis were solved.

During 2001–2007, a total of 65 confirmed *Salmonella* outbreaks involving Minnesota cases were identified; these represented 502 (12.5%) isolates. Twenty-two (34%) outbreaks were excluded from analysis (6 were multistate outbreaks in which only 1 case was identified in Minnesota; in 7 outbreaks, a hotline call contributed to identification of the outbreak; 1 was an outbreak was not detected by PFGE; 4 were outbreaks that did not have cases that met the cluster definition; and 4 outbreaks were considered probable). The remaining 43 outbreaks, representing 287 (7%) isolates, were included in the analysis and were composed of 35 foodborne, 6 person-to-person, and 2 animal contact outbreaks. Of these 43 outbreaks, 30 (70%) involved 1 facility (restaurant, daycare center, school) or event and therefore were classified as point source. Thirteen (30%) involved commercially distributed food items at multiple points of sale (grocery stores, restaurants) and therefore were classified as non–point source. The median cluster size of point source outbreaks was 3 cases, and the median cluster size of non-point source outbreaks was 5 cases (p<0.01, by Wilcoxon rank-sum test). The median cluster density was 6 days for point source and non-point source outbreaks (p = 0.74 by Wilcoxon rank-sum test).

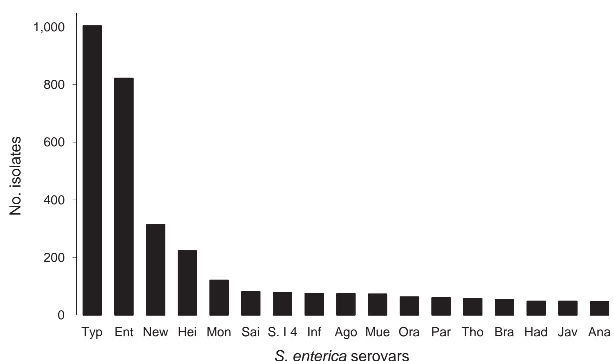

Figure 1. Frequency of the 17 most common *Salmonella enterica* serovars among clinical case isolates submitted to the Minnesota Department of Health, 2001–2007. Typ, Typhimurium; Ent, Enteritidis; New, Newport; Hei, Heidelberg; Mon, Montevideo; Sai, Saintpaul; S.I4, S.I 4,5,12:I:–; Inf, Infantis, Ago, Agona; Mue, Muenchen; Ora, Oranienburo; Par, Paratyphi B var. L; Tho, Thompson; Bra, Braenderup; Had, Hadar; Jav, Javiana; Ana, Anatum.

Temporal Trends

During the study period, the median number of *Salmonella* isolates subtyped per year was 567 (range 507–662 isolates). The median number of *Salmonella* clusters per year was 50 (range 44–57 clusters). The median number of confirmed *Salmonella* outbreaks per year was 6 (range 4–8 outbreaks). There were no statistically significant trends in the proportion of *Salmonella* clusters that resulted in identification of a confirmed outbreak (p = 0.20) (Figure 2).

Table 1. *Salmonella enterica* serovar diversity identified by pulsed-field gel electrophoresis among case isolates submitted to the Minnesota Department of Health, 2001–2007*

Serovar	No. isolates	No. PFGE subtypes observed	Serovar isolates represented by most common subtype, %	Serovar isolates represented by 2 most common subtypes, %	Serovar isolates represented by 3 most common subtypes, %	Simpson index†
Heidelberg	223	46	57	62	66	0.67
Hadar	48	20	48	54	58	0.77
Enteritidis	822	80	38	60	73	0.79
Thompson	57	23	42	53	58	0.81
I 4,5,12:I:–	78	25	31	50	60	0.86
Braenderup	53	30	26	36	43	0.92
Oranienburg	63	26	21	32	41	0.93
Anatum	46	22	17	33	46	0.93
Paratyphi B var. L	60	35	22	37	43	0.93
Montevideo	121	59	22	30	36	0.94
Muenchen	73	50	21	25	27	0.96
Saintpaul	81	44	17	26	32	0.96
Typhimurium	1,004	285	11	20	28	0.96
Infantis	75	43	9	17	24	0.97
Agona	74	48	10	16	22	0.98
Newport	314	143	10	15	19	0.98
Javiana	48	41	6	11	15	0.99

*PFGE, pulsed-field gel electrophoresis.
†Calculated as $1 - D = (\Sigma n(n-1))/(N(N-1))$, where n is number of isolates of each subtype and N is total number of isolates of a serovar. A value of 1 indicates infinite diversity, and a value of 0 indicates no diversity.

Cluster Serovar and Cluster Serovar Diversity

Clusters of the common *Salmonella* serovars Newport, Heidelberg, and Montevideo had 2.7× higher odds of being solved than did clusters of the very common serovars Enteritidis and Typhimurium (Table 2). The proportion of uncommon serovar clusters that were solved did not differ significantly from the proportion of very common or common serovar clusters that were solved (Table 2). Low clonality serovar clusters were not significantly more likely to be solved than highly clonal serovar clusters (OR 1.6, 95% CI 0.8–3.1).

Cluster Subtype

No significant associations between the subtype frequency of a cluster and a cluster being solved were observed. Uncommon serovar Enteritidis subtype clusters were not significantly more likely to be solved than were common clusters (OR 1.4, 95% CI 0.4–5.1). Uncommon serovar Typhimurium subtype clusters were not significantly more likely to be solved than were common clusters (OR 0.9, 95% CI 0.3–3.2).

Cluster Size

The probability of a cluster being solved increased significantly as the number of cluster cases increased (Mantel-Haenszel χ^2 for trend 13.7, $p<0.001$) (Table 2). The odds of solving a cluster of ≥5 cases were 3.8× higher than the odds of solving a cluster of 2 cases. Clusters of 4 cases were 3.9× more likely to be solved than were clusters of 2 cases. Twenty-four percent of clusters with ≥4 cases were solved (Table 2). Clusters of 3 cases were 2.1× more likely to be solved than clusters of 2 cases, but the difference was not statistically significant. There was statistical evidence of a nonlinear relationship between cluster size and solving the cluster (Wald χ^2 for interaction 5.0, $p = 0.03$). The dose response between cluster size and solving a cluster plateaued after a cluster size of 4.

Cluster Case Density

The proportion of clusters solved increased significantly as the density of cluster cases increased (Mantel-Haenszel χ^2 for trend, 12.7, $p<0.001$) (Table 2). The odds of solving a cluster if the first 3 case isolates were received on the same day were 25.8× higher than the odds of solving a cluster in which the first 3 case isolates were received during a period >14 days (Table 2). The odds of solving a cluster if the first 3 case isolates were received within 1–7 days were 5.0× higher than the odds of solving a cluster in which the first 3 case isolates were received during a period >14 days. Clusters in which the first 3 case isolates were received within 8–14 days were 2.8× more likely to be solved than clusters in which the first 3 case isolates were received during a period >14 days, but the difference was not statistically significant (Table 2). There was statistical evidence of a nonlinear relationship between cluster case density and solving the cluster (Wald χ^2 for interaction, 6.96, $p<0.01$).

Cluster Investigation Threshold

During June–December 2008, 10 MDH staff interviewed 214 persons with *Salmonella* infections and recorded the time required to complete the MDH standard

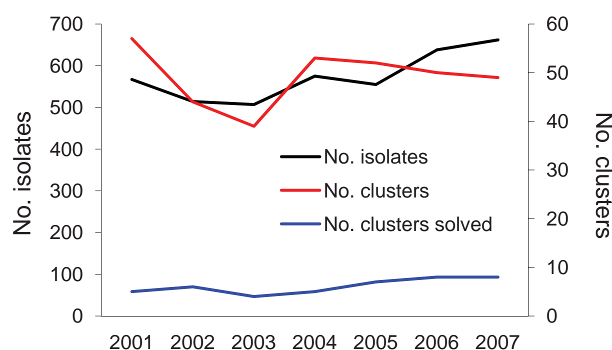

Figure 2. Temporal trends in number of *Salmonella enterica* isolates, number of clusters, and number of clusters solved (i.e., result in identification of a confirmed outbreak), Minnesota, USA, 2001–2007.

questionnaire. Interview times did not vary between interviewers. The median interview time was 27 minutes (range 13–56 minutes). Therefore, conducting standard interviews of all cases in the 344 clusters of ≥2 cases (n = 1,182 [31%] cases) required an estimated 76 interview hours/year. This threshold detected all 43 outbreaks identified through routine laboratory surveillance during the study period and resulted in a cluster investigation positive predictive value (percentage of clusters investigated that were solved) of 13% (Table 3). Other cluster investigation thresholds had outbreak detection sensitivities of 53%–81% and positive predictive values of 23%–28% (Table 3).

Discussion

During the study period, 344 *Salmonella* PFGE clusters were identified and 43 (13%) were solved. Cluster size and cluster case density were the most useful predictors of a cluster being solved. The proportion of clusters that were solved increased as the number of cases in the cluster increased (up to 4 cases). The association was not linear and the percentage solved did not increase further for clusters with ≥5 cases. The observed association is logical because as the number of cluster cases increases, the amount of epidemiologic data available for evaluation also increases. Our results suggest that public health officials should not wait to investigate *Salmonella* clusters if ≥4 cluster cases have been received.

The ability to solve a cluster of cases of *Salmonella* infection was also strongly associated with the density of the cluster cases. The proportion of clusters that were solved increased as the density of the cluster cases increased, but this relationship was not linear. This association is also logical. Dense clusters increase the likelihood that the cluster cases are epidemiologically linked rather than unrelated sporadic cases. In addition, dense clusters also likely signal larger outbreaks. Our results demonstrated a clear increase in the success of solving clusters in which the first 3 case isolates were received within 7 days.

In theory, PFGE subtyping is less useful for recognizing clusters of unusual serovars worth investigating. In the current study, clusters of the common serovars Newport, Montevideo, and Heidelberg were statistically

Table 2. Univariate association between *Salmonella enterica* serovar frequency, cluster size, cluster density, and cluster being solved, Minnesota, USA, 2001–2007*

Characteristic	No. (%) solved clusters	No. unsolved clusters	Odds ratio (95% confidence interval)
Serovar			
Very common†	22 (10)	203	Referent
Common‡	11 (23)	37	2.74 (1.23–6.13)
Uncommon§	10 (14)	61	1.51 (0.68–3.37)
Total	43 (13)	301	
Cluster size¶			
2	16 (8)	194	Referent
3	8 (15)	47	2.06 (0.83–5.11)
4	7 (24)	22	3.86# (1.43–10.40)
≥5	12 (24)	38	3.83 (1.68–8.74)
Total	43 (13)	301	
Cluster density, d**			
0	5 (71)	2	25.8 (3.42–195.37)
1–7	16 (33)	33	5.01 (1.33–18.89)
8–14	11 (22)	40	2.84 (0.73–11.07)
≥15	3 (9)	31	Referent
Total	35 (25)	106	

*A solved cluster is one that results in identification of a confirmed outbreak.
†*S. enterica* serovars Typhimurium and Enteritidis.
‡*S. enterica* serovars Newport, Heidelberg, and Montevideo.
§All other serovars.
¶Significant Mantel-Haenszel χ^2 test result for trend (p<0.001).
#Clusters of 4 cases compared with clusters of 3 cases odds ratio 1.87, 95% confidence interval 0.52–6.66.
**Cluster density measured as the number of days from receipt of first cluster case to third case received at the Minnesota Department of Health Public Health Laboratory.

Table 3. Comparison of *Salmonella enterica* cluster investigation thresholds, Minnesota, USA, 2001–2007*

Cluster investigation threshold	No. isolates represented in clusters	All *Salmonella* isolates, % (n = 3,803†)	Estimated interview time, h/y‡	No. (%) outbreak clusters meeting threshold	Cluster investigation PPV
All clusters (n = 344)	1,182	31	76	43 (100)	13
Clusters ≥3 cases (n = 152)	778	20	50	35 (81)	23
Clusters ≥4 cases (n = 83)	601	16	39	23 (53)	28
Clusters with a density of 0–14 d§ (n = 119)	633	17	41	32 (74)	27
Clusters ≥4 cases or with a density of 0–7 d§ (n = 100)	652	17	42	28 (65)	28

*PPV, positive predictive value.
†A total of 215 isolates associated with excluded clusters were removed from study isolate total (n = 4,018).
‡Based on a 27-min median interview time per case-patient.
§Density defined as the number of days from receipt of first cluster case isolate to third case isolate received at the Minnesota Department of Health Public Health Laboratory.

more likely to be solved than clusters of the very common serovars Enteritidis and Typhimurium. However, clusters of uncommon serovars were not more likely to be solved than were clusters of common or very common serovars. It has been suggested that uncommon serovar clusters may be associated with uncommon food vehicles, which makes them more difficult to solve by using standard methods (*24*). The relationship between serovar frequency and the likelihood of solving a cluster is unclear and warrants further study.

The limited number of solved clusters prevented multivariate analysis from being used to characterize the independent effect of predictors and possible effect modification between predictors. However, comparing the magnitude of the estimated effect of cluster size and cluster case density suggests that cluster case density may be a more useful predictor of a cluster being solved.

The 22 confirmed outbreaks that were excluded from the analysis demonstrate the value for national collaboration such as PulseNet and use of outbreak detection methods in addition to PFGE clustering within a given state. Six outbreaks were solved in which Minnesota only had 1 case, which demonstrated the utility of molecular subtyping in detecting geographically dispersed outbreaks. For 7 confirmed outbreaks, a call placed to the MDH foodborne illness hotline contributed to identification of the outbreak and demonstrated the utility of complaint systems in detecting outbreaks.

Interviewing all persons with *Salmonella* infection required a median of 27 minutes per person with *Salmonella* infection when the MDH standard questionnaire was used. By extrapolation, MDH staff spent ≈244 hours/year conducting routine interviews of persons with *Salmonella* infections. This figure does not include time spent attempting to reach persons, gathering demographic information from clinicians, or reinterviewing persons for cluster investigations. We recommend interviewing all persons with *Salmonella* infection and investigating all PFGE clusters to identify as many outbreaks as possible. However, many health departments do not have the resources to interview all persons with *Salmonella* infection or investigate all small clusters. Rather, they must balance the time required for these efforts and the ability to detect outbreaks (*25*).

Incorporating a cluster investigation threshold on the basis of cluster size and cluster case density can decrease the number of unsuccessful cluster investigations and conserve public health resources. However, this approach would also reduce the number of outbreaks that would be identified. One reason for this finding is that outbreaks that are manifested as smaller, less dense clusters would not be investigated. Another potential disadvantage of a cluster threshold approach is that delay of interviews until a cluster is solved can decrease the quality of exposure information obtained and therefore the likelihood that the cluster will be solved (*12*).

Four confirmed outbreaks during the study did not meet the cluster definition, and many confirmed outbreaks had cases that were outside the cluster definition. This finding is an important reminder that lack of temporal clustering does not eliminate the possibility of an outbreak. Increasing the period covered by a cluster definition will yield the benefit of solving more outbreaks. However, more resources will be expended conducting unsuccessful cluster investigations. The results of this study suggest that the use of a 2-week cluster window is sufficiently sensitive to detect most outbreaks. However, in practice, MDH epidemiologists do not use a strict 2-week cluster window when investigating clusters. Instead, all persons with *Salmonella* infection are interviewed and cases with matching PFGE patterns are often compared even if the second case is received >2 weeks after the first case.

The potential utility of the cluster investigation thresholds reported is based on the characteristics of the population of Minnesota and MDH surveillance methods: conducting real-time PFGE subtyping of all *Salmonella* isolates, interviewing all case-patients in real time by using a detailed exposure questionnaire from a central location for the entire state, and investigating clusters by using an iterative model (*19–21*). These factors aid in the timeliness of outbreak detection and investigation in Minnesota. These re-

sults may not be applicable in jurisdictions in which PFGE is not conducted in real time or batching of PFGE isolates occurs. Additional studies at the national level and in other states are needed to understand surveillance characteristics in other states and determine useful predictors of multistate clusters being solved.

Although successful cluster investigations will depend on the experience and ability of public health staff involved, this study demonstrates the increased probability of a cluster being solved as the number of cases in a cluster increases and as the cluster density increases. Specifically, investigation of PFGE clusters of ≥4 *Salmonella* case isolates and clusters in which the first 3 cases were received at the MDH PHL within 1 week yielded a major benefit in terms of outbreak identification. These results establish a benchmark for surveillance of *Salmonella* infections, and may provide a basis for investigating clusters of *Salmonella* cases for public health agencies with limited resources.

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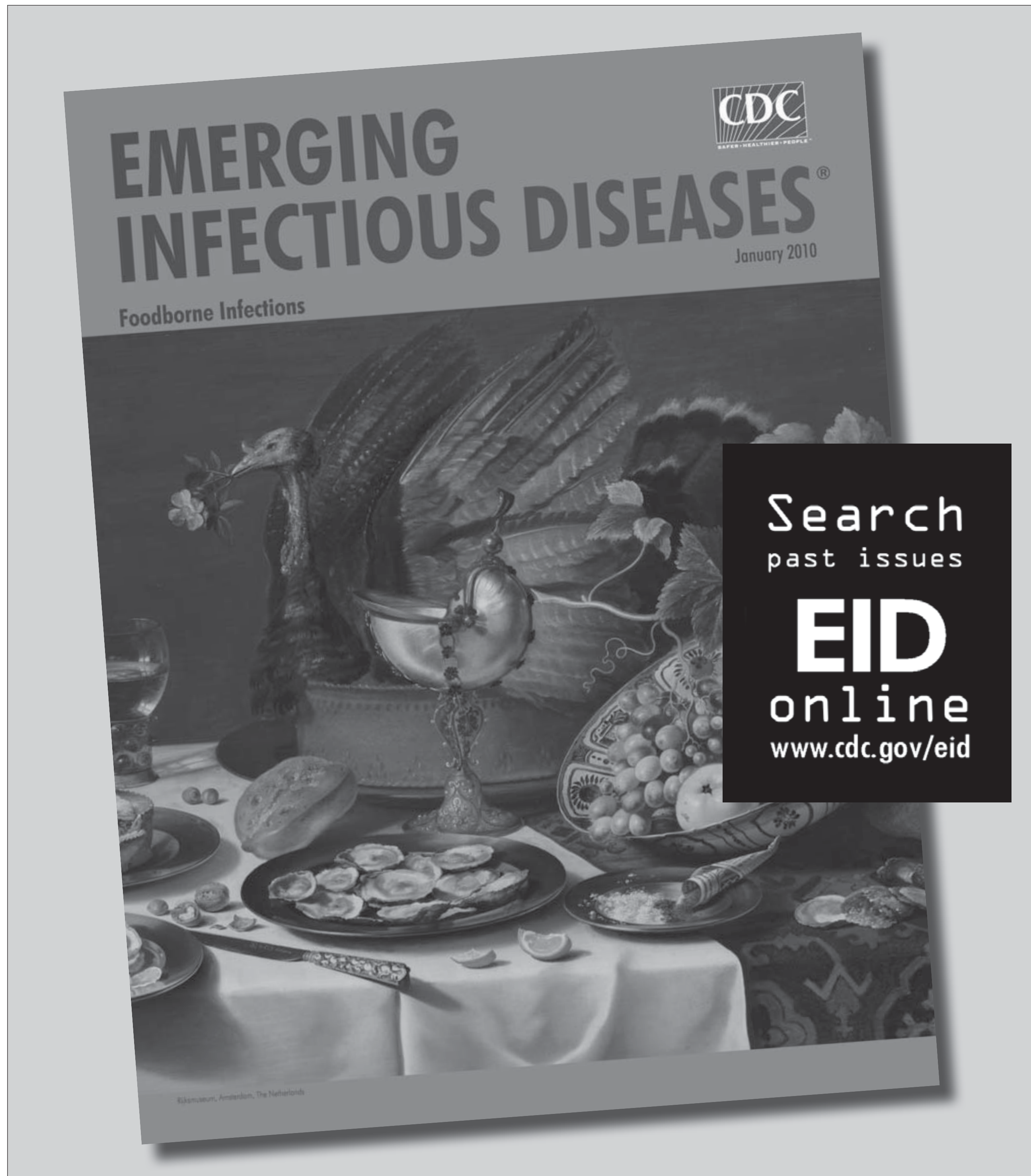

Genetic Structure of *Plasmodium falciparum* and Elimination of Malaria, Comoros Archipelago

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The efficacy of malaria control and elimination on islands may depend on the intensity of new parasite inflow. On the Comoros archipelago, where falciparum malaria remains a major public health problem because of spread of drug resistance and insufficient malaria control, recent interventions for malaria elimination were planned on Moheli, 1 of 4 islands in the Comoros archipelago. To assess the relevance of such a local strategy, we performed a population genetics analysis by using multilocus microsatellite and resistance genotyping of *Plasmodium falciparum* sampled from each island of the archipelago. We found a contrasted population genetic structure explained by geographic isolation, human migration, malaria transmission, and drug selective pressure. Our findings suggest that malaria elimination interventions should be implemented simultaneously on the entire archipelago rather than restricted to 1 island and demonstrate the necessity for specific chemoresistance surveillance on each of the 4 Comorian islands.

Plasmodium falciparum causes malaria worldwide; 250 million cases and ≈1 million deaths occur annually, mostly in sub-Saharan Africa. However, recently increased international financial commitment has revived hope for malaria elimination from selected areas to which it is endemic, and the feasibility of elimination has become a topic of research (*1*). The successful elimination of malaria from several Caribbean islands, Cyprus, Reunion, Mauritius, Maldives, Taiwan, and Singapore in the context of the Global Malaria Eradication Program (1955–1968) (*2*) suggests that islands are prime targets for elimination interventions. Because most parasites among neighboring areas are exchanged through human migrations, the geographic isolation of islands can limit malaria importation and may make control easier (*3*,*4*).

Several molecular epidemiologic studies have shown that *P. falciparum* populations are more or less homogeneous within malaria-endemic areas and may exhibit genetic structure patterns shaped by various transmission rates and geographic isolation levels (*4*–*6*). Although geographic isolation may be more relevant on islands than within continents, the role of parasite genetic structure in malaria-endemic archipelagos or among malaria-endemic islands and the nearest continent remains unknown. Past failures of malaria elimination in Zanzibar off the coast of mainland Tanzania; in Sri Lanka (*1*); or in Mayotte, a France-administered island of the Comoros archipelago (*7*), raise the question of the minimal geographic isolation level and the optimal size of intervention area required for malaria elimination success. Analysis of malaria epidemiology in Comoros archipelago, where a limited malaria elimination program is ongoing, may help to answer this question.

Falciparum malaria remains a major public health problem on the 4 islands of the Comoros archipelago (Grande Comore, Moheli, Anjouan, and Mayotte) (Table 1) in the Indian Ocean between Madagascar and the eastern coast of Africa. Malaria control has been hampered by the emergence of *P. falciparum* resistance to chloroquine and to pyrimethamine/sulfadoxine in the early 1980s (*7*,*10*) and of *Anopheles* mosquitoes resistance to DDT. Malaria con-

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Table 1. Epidemiologic and sampling characteristics of 5 sites studied for *Plasmodium falciparum* malaria, Comoros archipelago and Marseille, France

Characteristic	Grande Comore	Moheli	Anjouan	Mayotte	Marseille
Area, km^2	1,148	290	424	374	
Total population	330,000	40,000	280,000	190,000	50,000–80,000
No. bites by infected mosquitoes/person*	10–20/y (up to 200/y)	10/y (up to 1/night)	No data	Low	None
Endemicity*†	Mesoendemic to hyperendemic	Mesoendemic to hyperendemic	Mesoendemic to hyperendemic	Hypoendemic	None
Total no. reported cases (% confirmed cases), 2006*	51,148 (34)	7,866 (27)	15,408 (19)	496 (100)	84 (100)
Incidence/1,000 inhabitants, 2006*	150	150	50	3	Null
% *P. falciparum* malaria cases,* 2006	96	96	96	90	97
Period of sampling, 2007	Apr–May	Apr–May	Apr–May	Entire year	Entire year
No. patients sampled, 2007	62	61	63	227	111
Median age of sampled patients, y (IQR) ‡	4 (2–14.8)	7.5 (2.6–21)	7 (3.3–18)	19 (15–25)	33 (9.8–40)
No. sampled patients in site (A) with history of recent arrival from another site (B)	7	17	6	13	111§
Anjouan	6	9	–	7	
Grande Comore	–	8	5	6	
Mayotte	1	1	0	–	
Moheli	0	–	2	0	
No. randomly genotyped isolates	36	36	36	36	36

*Data from World Malaria Report 2008 (*8*) and from various Comorian and French official reports and references published in French, all reviewed by the first author in a recent, unpublished thesis (Rebaudet S. Molecular epidemiology and population genetics study of *Plasmodium falciparum* in Comoros archipelago. Impacts on malaria control [thesis] [in French]. Marseille (France): Université de la Méditerranée; 2009). Cases for Marseille represent those in persons with history of recent arrival from one of the 4 islands.
†Malaria endemicity levels based on 2–9 years of available parasite prevalence data, according to the World Health Organization classification: hypoendemic, 0–10%; mesoendemic, 11%–50%; hyperendemic, 51%–75%; and holoendemic, >75% (*9*).
‡IQR, interquartile range.
§Mainly from Grande Comore (S. Rebaudet, pers. comm.).

trol also has had recurrent political, economic, and structural weaknesses in the Union of the Comoros (the state comprising Grande Comore, Moheli, and Anjouan islands). Under stable political and economic conditions, notable efforts in case management and vector control in Mayotte failed to eliminate falciparum malaria and to prevent recurrent epidemics (Table 1). During the past 6 years Since 2004, health authorities in Grande Comore and France have introduced an artemisinin-based combined therapy (artemether plus lumefantrin) as first-line treatment for uncomplicated falciparum malaria (*7*,*10*). Large-scale distribution of insecticide-treated mosquito nets also has been gradually implemented on Grande Comore, Moheli, and Anjouan (*8*), with the goal of reaching up to 89.1% and 46.3% of the households with at least 1 mosquito net and 1 insecticide-impregnated mosquito net, respectively, among 1,620 households from the 3 islands (Comoran National Malaria Control Program, unpub. data, 2007).

In Mayotte, anti–*Anopheles* spp. mosquito larvae measures have been strengthened. Finally, by late 2007, a controversial malaria elimination project was launched on the sole island of Moheli with assistance from China. Mass treatment of the residing and disembarking population with artemisinin plus piperaquine (Artequick; Artepharm Co., Guangzhou, People's Republic of China) and primaquine was initiated without enhancement of vector control. Because of continual human travel across the archipelago, the long-term success of such a spatially limited elimination attempt is questionable.

In addition, surveillance of *P. falciparum* chemosusceptibility has been chaotic and unequal among the islands of the archipelago, and results of the few available therapeutic efficacy tests and in vitro and molecular resistance studies often have been discordant. A more rational and efficient surveillance system is urgently needed. Because Marseille, France, houses a Comorian community of 50,000–80,000 persons who annually import several hundred malaria cases, the city was proposed as a relevant surveillance site for chemosusceptibility of *P. falciparum* imported from Comoros (*11*). However, extrapolating these chemoresistance data to the entire archipelago remains difficult.

As already proposed for Borneo (*12*) and the Philippines (*13*), our main objective was to analyze the genetic structure of *P. falciparum* on the Comoros islands to 1) forecast the chances of middle-term and long-term success for the current elimination program focalized in Moheli, 2) guide future malaria elimination programs on the archipelago, and 3) adjust its chemoresistance monitoring and treatment policies. Study results also would provide a pertinent model for determining which other malaria-endemic areas might be eligible for malaria elimination. A secondary objective was to assess whether the diversity of the *P.*

falciparum strains imported into Marseille were representative of the *P. falciparum* populations from Comoros so we could evaluate the relevance of distant chemoresistance surveillance from Marseille.

We characterized *P. falciparum* populations from each of the 4 islands and from Marseille (imported from the archipelago) by multilocus microsatellite genotyping. The genetic polymorphism of 3 genes involved in *P. falciparum* resistance to chloroquine, pyrimethamine and cycloguanil, or sulfadoxine was also investigated.

Materials and Methods

P. falciparum Isolates

The study was conducted in 2007 (before the malaria elimination program was launched in Moheli) in each of the 4 islands of the Comoros archipelago and in Marseille. The protocol was approved by the ethics committee of the university hospitals of Marseille and by the Comorian Ministry of Health. Blood samples were obtained after informed consent from patients seeking care for symptomatic falciparum malaria at healthcare centers of the archipelago or at emergency departments of hospitals in Marseille.

Blood samples were absorbed onto Whatman FTA Elute absorbent filter paper in Grande Comore, Moheli, and Anjouan islands, on Whatman 903 Protein Saver filter paper (Whatman Inc., Florham Park, NJ, USA) in Mayotte, and collected into Vacutainer tubes (Becton Dickinson, Le Pont-De-Claix, France) in Marseille. All samples were frozen and kept at –20°C. After eliminating samples with missing data or the lowest parasitaemia levels (<0.01%), 36 isolates per site were randomly chosen for genotyping, a sample size considered adequate for the planned population genetics analyses.

Collection of Epidemiologic Data

Patient's age, sex, history of travel across or outside the archipelago (during the past year for Grande Comore, Moheli, and Anjouan; during the past 3 weeks for Mayotte) and history of recent clinical malaria episodes and intake of antimalarial drugs (during the previous month) were collected by oral questioning. Distances between each island were measured by using Google Earth software.

Genotyping Procedures

DNA was extracted from filter papers according to the manufacturer's recommendations (Whatman Inc.) and from whole blood from Vacutainer tubes by using the EZNA Blood DNA Kit (Biofidal, Vaulx-en-Velin, France). Next, the entire genome was amplified by using the Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Little Chalfont, UK).

Molecular Markers

Length polymorphism was analyzed for 6 complex and putatively neutral microsatellite loci previously described (*4*): *Pf2689*, *7A11*, *C4M79*, *Pf2802*, *TRAP*, and *C4M69* (online Appendix Table, www.cdc.gov/EID/content/16/11/1686-appT.htm). The studied chemoresistance markers were the K76 point mutation of the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene (associated with *P. falciparum* resistance to chloroquine) (*14*); point mutations of *P. falciparum* dihydrofolate reductase (*Pfdhfr*) gene codons 108, 16, 51, 59, and 164 (associated with *P. falciparum* resistance to pyrimethamine and cycloguanil, i.e., proguanil metabolite) (*15*); and *P. falciparum* dihydropteroate synthase (*Pfdhps*) gene codons 436, 437, 540, 581, and 613 (associated with *P. falciparum* resistance to sulfadoxine) (*15*) (online Appendix Table).

Genotyping by PCR

Microsatellite loci were amplified by nested PCR with fluorescent end-labeled primers before electrophoresis on polyacrylamide gels with Genescan-500 LIZ labeled size standards on an ABI 3130XL capillary sequencer (Applied Biosystems, Warrington, UK) (online Appendix Table). Their length was then analyzed by using GENESCAN software (Applied Biosystems, Carlsbad, CA, USA), as described (*4*). The *Pfcrt* gene was amplified by seminested PCR, and the codon 76 mutation was genotyped by using a simple PCR-restriction fragment digest assay and fluorescent detection of products on an ABI 3130XL capillary sequencer, as described (*16*). The *Pfdhps* and *Pfdhfr* genes were amplified by nested PCR, and their mutations were genotyped by using a primer extension method, as described (*17*) and electrophoresis on the ABI 3130XL capillary sequencer.

Statistical Analysis

The multiplicity of infection (MOI, i.e., the number of parasites genetically distinguishable by different alleles) with *P. falciparum* was estimated for each isolate from the microsatellite locus that exhibited the highest number of alleles. The mean MOI for each *P. falciparum* population (Grande Comore, Moheli, Anjouan, Mayotte, and Marseille) was then calculated. Each pair of sites was compared for MOI by using the Mann-Whitney U test.

For parasites with multiple infection, i.e., >1 allele at each locus, we conducted separate subsequent analysis considering the following: 1) complete dataset, 2) curtailed dataset with single or main clones after elimination of isolates unsuccessfully genotyped at all 6 microsatellite loci, or 3) reconstructed multilocus genotypes after elimination of samples with impossible reconstruction (>1 allele at >1 locus with equivocal peak intensities) and elimination of unsuccessfully genotyped isolates (Table 1).

Genetic Diversity

Genetic diversity of the 5 *P. falciparum* populations was assessed by the number of alleles per locus and by the Nei unbiased expected heterozygosity index (H_e) calculated from allelic frequencies on the 6 microsatellites for complete datasets by using GENETIX software version 4.05 (*18*,*19*). Comparison between H_e of the 5 different populations was performed on FSTAT software version 2.9.4 with a 1,000 permutations bilateral comparison test (*20*).

Population Genetic Structure

Population genetic structure was investigated by using the Wright F statistic (F_{ST}) (*21*). The F_{ST} index was computed for the 6 microsatellite markers and 5 populations on FSTAT software version 2.9.4 (*20*,*22*) and by using the Slatkin index on ARLEQUIN software (*23*). A canonical correspondence analysis of the reconstructed multilocus genotypes set was conducted to illustrate measures of population structure (*24*) by using CANOCO software (*25*), and its graphic representation was performed by using R software. A Monte Carlo procedure permuting genotypes among the populations was used to test the significance of the canonical axes and estimate the 95% confidence intervals of the centroid of each population (*25*).

Frequency of Mutations Associated with Chemoresistance

We estimated the frequency of point mutations on the *Pfcrt* (K76T mutation), *Pfdhfr* (108 + 59 + 51 triple mutation), and *Pfdhps* (437 + 540 double mutation) genes. Differences among sites were tested by using the Fisher exact test.

Associations between F_{ST} and Estimations of Parasite Flux

The association between genetic distance (transformed as $F_{ST}/[1 - F_{ST}]$) and the natural log of the geographic distance in kilometers was investigated for each pair of islands according to the isolation-by-distance model (*5*,*12*,*26*). When we considered the number of patients in each sampled island (A) with history of recent arrival from each of the neighboring islands (B) and thus possibly with imported malaria (Table 1), the relationship between F_{ST} and the mean proportion of these travelers among patients, calculated as $([N_{B\rightarrow A}/N_A] + [N_{A\rightarrow B}/N_B])/2$, was investigated for each pair of islands.

Results

P. falciparum was detected by PCR in each of the 36 genotyped blood samples from all 5 sites. Microsatellite genotyping was complete for 149 (83%) of the 180 samples (Table 2).

Mean MOIs

Of the 180 samples, 59 isolates were multi-infected; the proportion of multi-infection among islands differed substantially (Table 2). The mean MOI ranged from 1.22 in Mayotte to 2.11 in Anjouan (Table 3). It was significantly higher in Anjouan than in Grande Comore (p = 0.0015), Moheli (p = 0.0051), and Mayotte (p = 0.0001) and higher in Marseille than in Mayotte (p = 0.0093).

Genetic Diversity

Genetic diversity (H_e) of each population, estimated by unbiased expected heterozygosity based on allelic frequencies of the 6 microsatellites and the complete dataset, is shown in Table 4. The highest diversity was observed for Anjouan and Moheli (each H_e = 0.71) and the lowest diversity for Mayotte (H_e = 0.63). The mean H_e was significantly lower for Mayotte than for Marseille (p = 0.04) and lower than for the other sites combined (p = 0.001).

Genetic Differentiation among Islands and Population Structure

Figure 1 shows the centroid of each falciparum population surrounded by its 95% confidence interval, and both axes were significant (p = 0.0001 and p = 0.0004 for 1,000 permutations, respectively). Grande Comore, Moheli, and Anjouan nearby centroids suggest closely related populations. The detached Mayotte centroid suggests a marked differentiation from all the other populations.

Figure 2 shows the pairwise differentiation coefficients (F_{ST}) estimated for the 5 parasite populations according to the 6 microsatellite loci and the complete dataset (n = 281). The number of *P. falciparum* clones used to calculate F_{ST} between sites was 50, 53, 76, 44, and 58 in Grande Comore, Moheli, Anjouan, Mayotte, and Marseille, respectively. The Moheli parasite population did not differ significantly from the Grande Comore and Anjouan populations. Conversely, the Mayotte population differed significantly from the populations of the 4 other sites. Marseille parasite populations differed significantly from those from all sites except Grande Comore. Similar differentiation index were

Table 2. Genotyping results of the 5 sites studied for *Plasmodium falciparum* malaria, Comoros archipelago and Marseille, France

Characteristic	Grande Comore	Moheli	Anjouan	Mayotte	Marseille	Total
No. randomly genotyped isolates	36	36	36	36	36	180
No. detected parasites	50	53	76	44	58	281
No. single or main clones successfully genotyped	28	20	29	36	36	149
No. reconstructed multilocus genotypes	30	24	40	37	39	170
No. multi-infected isolates	9	10	22	4	14	59

Table 3. MOIs of *Plasmodium falciparum* infections for the 5 sites studied, Comoros archipelago and Marseille, France*

	Grande Comore		Moheli		Anjouan		Mayotte		Marseille	
Locus	No.	MOI	No.	MOI	No.	MOI	No.	MOI	No.	MOI
All 6 loci	36	1.39	36	1.47	36	2.11	36	1.22	36	1.64
Pf2689	34	1.03	27	1.07	36	1.19	36	1.08	36	1.22
7A11	34	1.12	32	1.19	35	1.37	36	1.14	36	1.39
C4M79	34	1.21	25	1.32	33	1.55	36	1.11	36	1.42
Pf2802	31	1.00	23	1.00	32	1.00	36	1.00	36	1.00
TRAP	35	1.42	29	1.14	36	1.67	36	1.08	36	1.19
C4M69	32	1.06	21	1.19	34	1.12	36	1.03	36	1.11
Mean no. alleles per locus	8.5		8		8.5		4.5		7.8	

*Based on no. multi-infected isolates as shown in Table 2. MOI, multiplicity of infection (no. multi-infected isolates/no. randomly genotyped isolates); no., no. randomly genotyped isolates;

obtained by using a curtailed dataset (n = 149) or reconstructed multilocus genotypes (n = 170) and by using the Slatkin index (data not shown).

Relations between F_{ST} and Estimations of Parasite Flux

Association between genetic and geographic distances for each pair of islands is shown in Figure 3. No association was significant. The Anjouan–Mayotte pair exhibited a large F_{ST} despite the close proximity of the 2 islands.

Of the 414 patients sampled in the archipelago, 35 reported recent travel to neighboring islands (Table 1). Figure 3 suggests a negative relationship between the mean percentage of travelers among patients and the corresponding F_{ST}.

Frequency of Point Mutations associated with Chemoresistance

Prevalence of *Pfcrt*, *Pfdhfr*, and *Pfdhps* mutations in the 5 *P. falciparum* study populations are presented in Table 5. Prevalence of the *Pfcrt* mutation (i.e., isolates with the 76T allele or with the 76 K, and T alleles) was significantly lower in Anjouan than in the other parasite populations ($p \leq 0.002$). Prevalence of the *Pfcrt* mutation was significantly higher in Mayotte than in any other population ($p \leq 0.0001$). The prevalences of the *Pfcrt* mutation in Grande Comore, Moheli, and Marseille did not differ significantly.

When mutated, the *Pfdhfr* gene frequently exhibited the association of the 3 mutations 108N + 59R + 51I. Prevalence of this triple mutation was significantly lower in the Anjouan population than in the Grande Comore (p = 0.04), Moheli (p = 0.003), or Marseille populations (p = 0.0004). Its prevalence also was significantly higher in Marseille than in Mayotte (p = 0.02). The prevalence of *Pfdhps* gene mutations appeared low in the 5 populations.

Multi-infected isolates with genotype ambiguities and impossible distinction between associated clones were rare. However, prevalence of these mutations varied little, regardless whether these ambiguous multi-infected isolates were considered.

Discussion

The mean MOIs remained low for the Comoros archipelago in comparison with African areas, where malaria is highly endemic (*4*), most likely because of moderate levels of malaria transmission (*4*,*5*). Likewise, the significantly higher MOI in Anjouan may reflect a higher level of malaria transmission in the rainy and swampy sampled areas, where vector control has for a long time been impaired by recurrent island-specific political crises. The genetic diversities appeared lower on the archipelago than on most of the African continent (*4*,*5*,*27*–*30*), probably because of the geographic isolation of the islands and their lower malaria transmission levels that could limit effective parasite population sizes and outbreeding. However, genetic diversities remained higher than in Asia (*5*,*6*,*12*,*13*) and South America (*5*,*31*).

The genetic differentiation index (F_{ST}) exhibited a contrasted genetic structure between the studied *P. falciparum* populations. Genetic distances were low among parasites

Table 4. Genetic diversity of *Plasmodium falciparum* at the 5 sites studied, Comoros Archipelago and Marseille, France*

	Grande Comore		Moheli		Anjouan		Mayotte		Marseille	
Locus	No.	H_e	No.	H_e	No.	H_e	No.	H_e	No.	H_e
All 6 loci	50	0.63	53	0.71	76	0.71	44	0.51	58	0.63
Pf2689	35	0.47	29	0.44	43	0.52	39	0.15	42	0.53
7A11	38	0.81	38	0.85	48	0.82	41	0.41	50	0.87
C4M79	41	0.86	33	0.85	51	0.83	40	0.74	51	0.87
Pf2802	31	0.00	23	0.48	32	0.43	36	0.48	36	0.20
TRAP	39	0.79	33	0.80	57	0.85	39	0.60	43	0.56
C4M69	34	0.87	25	0.82	38	0.84	37	0.68	40	0.77

*No., no. detected parasites. H_e, Nei unbiased expected heterozygosity index.

on Grande Comore, Moheli, and Anjouan islands. However, F_{ST}s among these 3 populations and Mayotte were as significant as between *P. falciparum* populations of Senegal and Djibouti when the same microsatellite loci were used (*4*) or as between populations of Africa and Southeast Asia when other microsatellite loci were used (*5*). In addition, the genetic distances between falciparum populations across the archipelago seemed associated with the parasite flows among islands, estimated from the proportion of travelers among sampled patients, in particular for Moheli, Grande Comore, and Anjouan.

Our results strongly suggest that despite the insular geographic isolation of Moheli and the malaria elimination program launched in late 2007 on this island only, the mass treatment without enhanced vector control may soon be impaired by the continuous importation of new parasites through intense human migrations. In addition to flights and ferries regularly traveling across the archipelago, humans in Comoros move mainly by small fishing boats, especially from and toward Moheli (S. Rebaudet, pers. comm.). Their ubiquitous and informal traffic makes human flux estimations unreliable (probably several tens of thousands of persons each way annually [S. Rebaudet, pers. comm.]) and their control difficult. These factors might explain why, despite the substantial resources that France has allocated in Mayotte to malaria control since the mid-1970s, malaria importation to this island could not be stopped and autochthonous falciparum malaria could not be eliminated. The disease persists in Mayotte with a hypoendemo-epidemic setting, genetically characterized by low MOI, low H_e, significant linkage disequilibrium (data not shown), and high F_{ST}s, artificially overestimated by the sampling of multiple repeated genotypes (data not shown).

The persistent efforts for malaria elimination in Moheli can be hypothesized to create a Mayotte-like setting requiring efficient vector control to prevent epidemics in a Mohelian population that is losing its immunity. In the Union of the Comoros, the extension of the elimination program based on artemisinin-based combined therapy mass treatment to Grande Comore and Anjouan is being considered by Comorian health authorities and their Chinese interlocutors (R. Silaï, pers. comm.). Its success and the prevention of epidemics will depend on the rapid and large implementation of the preventive, diagnostic, and therapeutic measures planned with the funds granted in 2010 by Round 8 of the Global Fund (http://portfolio.theglobalfund.org/Grant/Index/COM-810-G03-M?lang=en).

Isolation of a specific *P. falciparum* population before planning its elimination needs to be appropriately evaluated. Results from the present Comorian epidemiologic study illustrate how it could be evaluated by a population genetics approach. In that type of geographic setting, population genetics studies provide a probably more direct and reliable estimation of parasite flows and risk for re-introduction than does the evaluation of human population movements by sociodemographic methods. Therefore, the relevance of parasite inflow from Africa (mostly the Tanzania coast, Madagascar, or other malaria-endemic areas) should be evaluated before the elimination project is extended to the rest of the Comoros archipelago. Similar data would also be useful for Sri Lanka, Malaysia, Indonesia, the Philippines, Solomon islands, or Vanuatu, several islands where national or localized malaria elimination projects are being implemented (*2*).

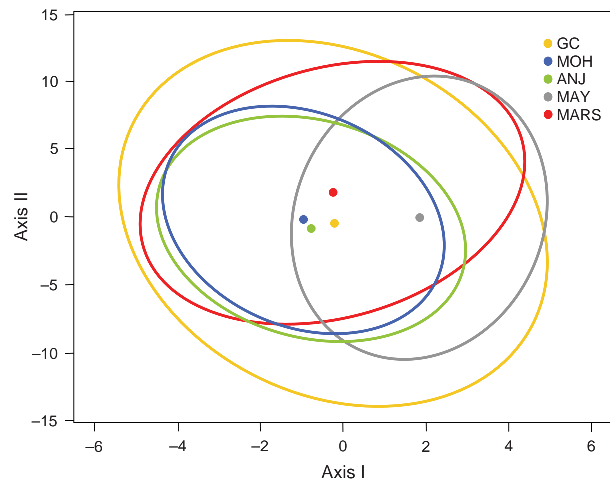

Figure 1. Results of canonical correspondence analysis (CCA) of *Plasmodium falciparum* populations from the islands of Grande Comore (GC), Moheli (MOH), Anjouan (ANJ), and Mayotte (MAY) and from Marseille, France (MARS), according to 6 microsatellite loci. CCA is used as a 2-dimensional representation of genetic distance between plasmodial populations assessed from 6 microsatellite loci (*Pf2689, C4M79, Pf2802, 7A11, TRAP*, and *C4M69*). This representation requires the projection of data from 6-dimensional space to 2-dimensional space. Canonical axes I and II of the new 2-dimensional space are calculated to conserve the highest genetic variance between populations after projection of data, and their significance was tested by Monte Carlo permutation that also enabled estimation of the 95% confidence intervals (ellipses) of the centroid (dots) of each population.

According to the genetic structure of *P. falciparum* populations in Comoros demonstrated by microsatellite genotyping, resistance levels would be expected to be fairly similar across the archipelago, except for Mayotte. However, the study of *Pfcrt* and *Pfdhfr* resistance–associated mutations differed markedly, explainable only by contrasting levels of drug selective pressure among islands. Indeed, the prevalence of the K76T mutation on the *Pfcrt* gene was high in both Grande Comore and Moheli as found in previous studies (*32*,*33*) but substantively lower in Anjouan and significantly higher in Mayotte where chloroquine use was massive during 1975–2007

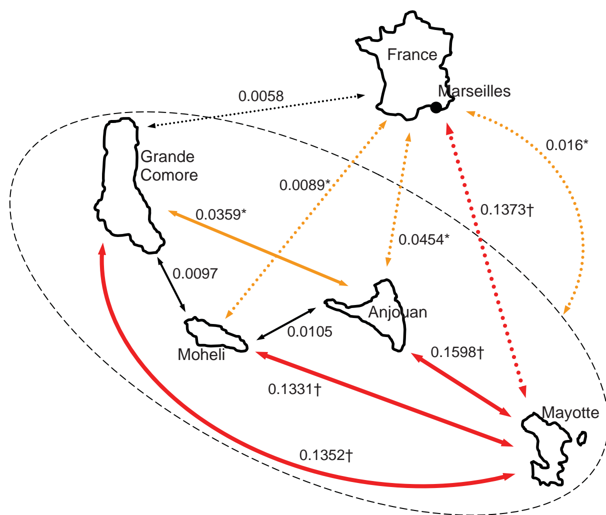

Figure 2. Genetic differentiation (*Fst*) between *Plasmodium falciparum* populations from the islands of Grande Comore (GC), Moheli (MOH), Anjouan (ANJ), and Mayotte (MAY) and from Marseille, France (MARS), according to 6 microsatellite loci. Pairwise comparison among sites that used complete dataset (n = 281) and 6 microsatellite loci (*Pf2689*, *C4M79*, *Pf2802*, *7A11*, *TRAP*, and *C4M69*). Departure of F_{ST} from 0 tested after 10,000 bootstrap simulations and by using Bonferroni corrected p values obtained after 200 permutations. Difference is significant if adjusted p<0.005. Black arrows indicate negligible (F_{ST}<0.01) and nonsignificant differentiation. Asterisks (*) and orange arrows indicate moderate (0.01≤F_{ST}<0.1) and/or statistically significant differentiation. Daggers (†) and red arrows indicate important (F_{ST} ≥0.1) and significant differentiation. Plain arrows indicate genetic differentiation between the parasite populations of the Comoros islands. Dotted arrows indicate genetic differentiation between the parasite population imported in Marseille (from Comoros) and either the overall parasite population of the entire Comoros archipelago (dotted oval and extreme right arrow) or the parasite populations of each of the 4 islands.

(*7*,*34*). Similarly, the prevalence of *Pfdhfr* triple mutants was higher in Moheli than in Anjouan and the prevalence of *Pfdhfr* double or triple mutants higher in Marseille than in Grande Comore.

Although no reliable estimation of past use of antimalarial drugs in Comoros is available, these differences may be explained by a greater use in Moheli of pyrimethamine (in the sulfadoxine/pyrimethamine combination for malaria treatment) and trimethoprim (in cotrimoxazole compound, which is widely prescribed in this island as an antimicrobial drug) and in Marseille of proguanil (in association with chloroquine or atovaquone, used as malaria chemoprophylaxis by travelers to the archipelago) (S. Rebaudet, pers. comm.). Trimethoprim and proguanil are 2 antifolate drugs whose cross-resistance with pyrimethamine has been suspected (*35*,*36*) and that may have selected these *Pfdhfr* mutations. Because of the contrasting resistance levels among islands, the risk for rapid propagation of resistant *P. falciparum* strains across the archipelago suggested by the low F_{ST}s among Grande Comore, Moheli, and Anjouan (*4*,*5*), and the easier selection of multigenic resistance and multiresistance from low MOIs limiting the possibilities of genetic recombinations that could break apart allele combinations (*5*,*37*,*38*), French and Comorian health authorities should organize surveillance of chemoresistance, both regular and separated for each island.

Finally, microsatellite genotypes of the *P. falciparum* population in Marseille substantially differed from those populations on all islands except Grande Comore. Because most of the Comorian inhabitants living in Marseille originated from Grande Comore, malaria is imported mainly from this particular island (S. Rebaudet, pers. comm.). Therefore, if we consider that the *P. falciparum* population in Marseille may be representative only of the Grande Comore population and the distinct levels of drug pressure between Marseille and the other populations, the relevance of distant chemosusceptibility surveillance from Marseille is likely to be limited.

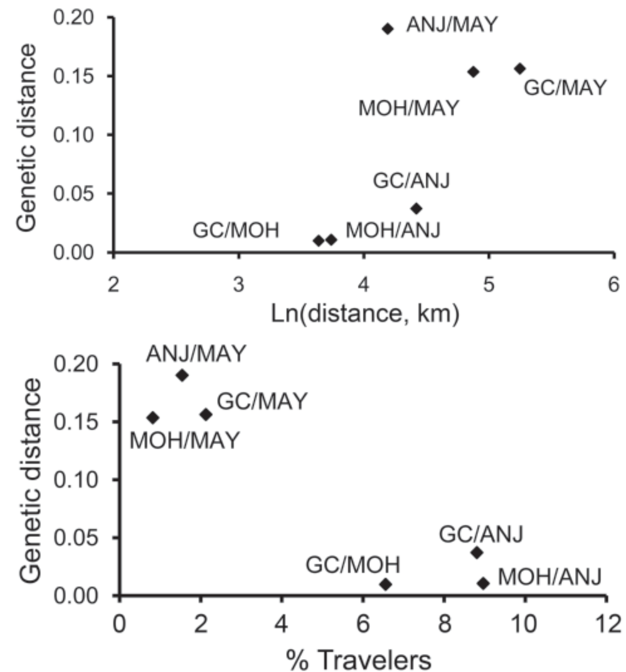

Figure 3. Relationship between geographic and genetic distances for each pair of Comoros islands (top) and between mean percentage of travelers among sampled patients and genetic distance for each pair of Comoros islands (bottom). Genetic distances were calculated as (F_{ST}/1 – F_{ST}), where F_{ST} is the Wright F statistic. Mean percentage of travelers was calculated from the total number of sampled patients in one site (N_A) with history of recent arrival from another site (N_B) by using the equation ($[N_{B \to A}/N_A] + [N_{A \to B}/N_B]$)/2; data in Table 1. GC, Grande Comore; MOH, Moheli; ANJ, Anjouan; MAY, Mayotte; Ln, logarithmically transformed.

Table 5. Frequency of chemoresistance-associated point mutations of 5 sites studied for *Plasmodium falciparum* malaria, Comoros archipelago and Marseille, France*

Locus	No. isolates (% mutations)				
	Grande Comore	Moheli	Anjouan	Mayotte	Marseille
Pfcrt					
76T	33 (45.5)	33 (45.5)	36 (13.9)	36 (91.7)	36 (52.8)
76 T and K	33 (9.1)	33 (6.1)	36 (5.6)	36 (2.8)	36 (0)
K76 (Wt)	33 (45.5)	33 (48.5)	36 (80.6)	36 (5.6)	36 (47.2)
Pfdhfr					
108N	26 (50.0)	19 (84.2)	34 (38.2)	36 (50.0)	36 (80.6)
108 N and S	26 (15.4)	19 (5.3)	34 (11.8)	36 (0)	36 (2.8)
S108 (Wt)	26 (34.6)	19 (10.5)	34 (50.0)	36 (50.0)	36 (16.7)
59R	26 (50.0)	19 (78.9)	34 (26.5)	36 (44.4)	36 (77.8)
59 R and C	26 (7.7)	19 (10.5)	34 (11.8)	36 (0)	36 (2.8)
C59 (Wt)	26 (42.3)	19 (10.5)	34 (61.8)	36 (55.6)	36 (19.4)
51I	26 (38.5)	19 (63.2)	34 (23.5)	36 (44.4)	35 (65.7)
51 I and N	26 (11.5)	19 (5.3)	34 (2.9)	36 (0)	35 (2.9)
N51 (Wt)	26 (50.0)	19 (31.6)	34 (73.5)	36 (55.6)	35 (31.4)
108N and 59R	26 (57.7)	19 (89.5)	34 (38.2)	36 (44.4)	36 (80.6)
108N and 59R and 51I	26 (50.0)	19 (68.4)	34 (26.5)	36 (44.4)	35 (68.6)
Pfdhps					
437G	25 (4.0)	24 (20.8)	31 (0)	36 (0)	36 (8.3)
437 G and A	25 (0)	24 (4.2)	31 (0)	36 (0)	36 (5.6)
A437 (Wt)	25 (96.0)	24 (75.0)	31 (100.0)	36 (100.0)	36 (86.1)
540E	25 (0)	24 (4.2)	31 (0)	36 (0)	36 (0)
540 E and K	25 (4.0)	24 (0)	31 (0)	36 (0)	36 (0)
K540 (Wt)	25 (96.0)	24 (95.8)	31 (100.0)	36 (100.0)	36 (100.0)
437G and 540E	25 (0)	24 (0)	31 (0)	36 (0)	36 (0)

**Pfcrt, P. falciparum* chloroquine resistance transporter; *Pfdhpr*, *P. falciparum* dihydrofolate reductase; *Pfdhps*, *P. falciparum* dihydropteroate synthase.

Acknowledgments

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Effect of Vaccination on *Bordetella pertussis* Strains, China

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Whole-cell pertussis vaccine was introduced in China in the early 1960s. We used standard typing methods to compare 96 *Bordetella pertussis* isolates collected before and after introduction of vaccination, during 1953–2005. The following vaccine-type alleles of the pertussis toxin (*ptx*) gene were characteristic for all prevaccination strains: *ptxA2*, *ptxA3,* and *ptxA4.* The shift to *ptxA1* occurred since 1963. All isolates collected since 1983 contained *ptxA1*. Pertactin (*prn*) allele 1, *prn1,* was predominant, although *prn2* and *prn3* have been detected since 2000. Serotypes fimbriae (Fim) 2 and Fim2,3 were found in all isolates collected before 1986. During 1997–2005, Fim3 became prevalent. Although changes in electrophoresis profiles over time were observed, the predominant profiles during 1997–2005 resembled those during the prevaccine era and those found in Europe before the 1990s. *B. pertussis* strains in China may differ from those in countries that have a long history of high vaccine coverage.

Whooping cough (pertussis) is an acute respiratory infectious disease caused by the bacterium *Bordetella pertussis*. After the whole-cell pertussis (Pw) vaccines were introduced in many countries during 1940–1960, illness and death rates from pertussis have decreased dramatically (*1,2*). However, pertussis remains a leading cause of vaccine-preventable deaths worldwide (*1*). A resurgence of pertussis has been observed in developed countries despite high vaccination coverage (*3–9*).

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In the People's Republic of China, vaccination against pertussis was started in the early 1960s, when 3 doses of Pw vaccine combined with diphtheria and tetanus toxoids were given at 3, 4, and 5 months of age. In 1982, a booster dose at 18 months of age was added (*10,11*). Pw vaccines are free of charge in China. Since 1995, acellular pertussis (Pa) vaccines containing purified pertussis toxin (Ptx) and filamentous hemagglutinin have been also introduced. However, because Pa vaccines are offered at the patient's expense, use of these vaccines has been minimal, especially in some resource-limited areas. Although since 2007 Pa vaccines have been included in the national expanded program on immunization, Pa and Pw vaccines are still used in most provinces because of limited availability and cost of Pa vaccines. Although the reported vaccination coverage for the primary 3 doses increased with time, before the 1980s it was low. From 1983 through 2008, coverage ranged from 58% to 99% (median 93%) (Figure 1) (*12*).

In China, pertussis is a reportable infectious disease, and the number of reported cases has been decreasing (Figure 1). Since the 1990s, incidence has been <1 case/100,000 population (*12*). In China, pertussis is clinically diagnosed by physicians; laboratory methods such as culture, PCR, and serologic analysis are not used for diagnosis of pertussis. Therefore, the reported low incidence may be related to the diagnostic criteria used, suggesting substantial underreporting. Pertussis remains endemic to China (*10–12*), and a local outbreak was reported in April 1997.

In many countries, divergence in major antigens Ptx, pertactin (Prn), fimbriae (Fim) 2, Fim3, and tracheal colonization factor (TcfA) between the vaccine strains and circulating isolates has been reported (*3,4,7,9,13–16*). Furthermore, marked changes in *B. pertussis* strains have been

[1]These authors contributed equally to this article.

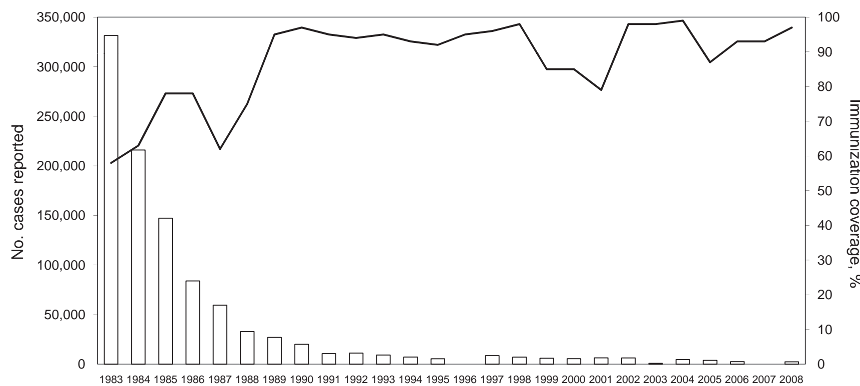

Figure 1. Number of reported pertussis cases and pertussis vaccination coverage in China, 1983–2008 (*12*). Although vaccination coverage increased with time, it was low before the 1980s and only 58% in 1983.

found in these countries after introduction of vaccination. To learn more about the *B. pertussis* strains circulating in China, we used standardized typing methods to analyze and compare *B. pertussis* isolates collected before and after the introduction of vaccination (*17*).

Materials and Methods

Bacterial Strains and Culture

We tested 3 vaccine strains and 96 clinical isolates: 25 isolates from 1953–1958, 52 from 1963–1985, and 19 from 1997–2005. These 3 periods were chosen according to when Pw vaccines were introduced (1960) and when Pa vaccines were added to the vaccination program (1995). Information on vaccine strains and clinical isolates is shown in online Appendix Figure 1, www.cdc.gov/EID/content/16/11/1695-appF1.htm) and in the online Technical Appendix (www.cdc.gov/EID/content/16/11/1695-Techapp.htm). Clinical information for patients from whom *B. pertussis* was isolated was not available. Vaccine strains P3s10 and CS were isolated in Beijing in 1951 (online Appendix Figure 1). Vaccine strain 18530 originated in the United States. Strains P3s10 and 18530 have been used to produce Pw vaccines since the early 1960s, and strain CS has been used to produce Pa vaccines since 1995. All *B. pertussis* isolates were confirmed by slide agglutination with specific antiserum to *B. pertussis* and *B. parapertussis* (Murex Diagnostics, Dartford, UK) and by PCR according to insertion sequence *IS481* (*18*). *B. pertussis* strains were grown on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood, incubated at 37°C for 4–5 days.

Serotyping

Serotyping was performed by a microtiter plate–based monoclonal agglutination assay as described (*17*). Monoclonal antibodies against Fim2 (NIBSC 04/154) or Fim3 (NIBSC 04/156) were used.

DNA Sequencing

PCR-based sequencing of 5 genes (*prn, ptxA, tcfA, fim2*, and *fim3*) was performed as described (*17,19,20*) with minor modifications. Nucleotide sequences were determined for the complete opening read frames of *ptxA*, *fim2*, *fim3*, and *tcfA,* and for part of *prn*. The same primers were used for amplification and sequencing (Table). The sequencing assays were performed with an ABI Prism 3100 DNA sequencing system (Applied Biosystems, Foster City, CA, USA), and data analysis was conducted with DNASTAR (Madison, WI, USA) software.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as recommended (*17*) with minor modifications. Briefly, after the DNA plugs were treated with 50 U of *Xba*I (New England Biolabs, Ipswich, MA, USA), the cleaved DNA fragments were separated by electrophoresis on a 1% agarose gel by using a Chef Mapper (Bio-Rad, Hercules, CA, USA) with pulse times of 2.16–44.69 s for 24 h. The band patterns were analyzed with BioNumerics program version 4.0 (Applied-Maths, Kortrijk, Belgium). The clustering method used was the unweighted pair group method with arithmetic mean. The Pertussis Reference Laboratory of the National Institute for Health and Welfare, Turku, Finland, used PFGE to retest 53 clinical isolates, 3 vaccine strains, and international reference strain 18323 (*7*).

The selection criteria for the 53 isolates included at least 1 strain for 1 unique profile. If 2 strains had identical profiles, both were retested. If multiple strains represented the same profile, the strains isolated in different years or different regions were included. For PFGE, 8 international reference strains were included. The nomenclature was based on the profiles already defined for Finland (BpFINR) and Sweden (BpSR) (*7,21*). Profiles assigned as BpCHR have been found only among the analyzed isolates from China. A 2-sided Fisher exact test was used to compare frequencies of strain serotypes and genotypes from the 3 periods.

Table. Oligonucleotide primers designed and used for PCR amplification and sequencing of *Bordetella pertussis*, China*

Primer	Sequence, 5′ → 3′	Gene	Position
prn-1F	ATGTCTCTGTCACGCATTGTCA	*prn*	151–172
prn-1R	GTCCTGCATGACGACCAGCTTG	*prn*	1653–1632
prn-2F	CTCGAACGTCGGTGCGCTAC	*prn*	1479–1498
prn-2R	TCGCGTCCAGGTAGAAACCG	*prn*	2347–2328
ptxA-F	GGCACCATCAAAACGCAGAGGGG	*ptxA*	476–498
ptxA-R	ATTACCGGAGTTGGGCGGGGCTG	*ptxA*	1347–1325
fim2-F	ACCCATGCAAATCCCTTTCCAACGC	*fim2*	177–201
fim2-R	GGGGGTTGGCGATTTCCAGTTTCTC	*fim2*	877–853
fim3-F	ATGTCCAAGTTTTCATACCCTGCCT	*fim3*	336–360
fim3-R	TTCGTCTCCTGACGCCGCGTAGCGG	*fim3*	1033–1009
tcfA-1F	CCACATTGATTCAGGCCGCT	*tcfA*	251–270
tcfA-1R	CGTCCGCAGGAGACTTGGAA	*tcfA*	1096–1077
tcfA-2F	GACTCCGGTATGTCCGATTC	*tcfA*	976–995
tcfA-2R	CTACCAGGCGTAGCGATAGC	*tcfA*	2346–2327

*Primer positions are listed according to the numbering of the sequences of the following GenBank accession nos.: *ptxA*, M13223; *prn*, J04560; *fim2*, Y00527; *fim3*, X51543; and *tcfA*, U16754. prn, pertactin; ptx, pertussis toxin; fim, fimbriae; tcf, tracheal colonization factor.

Results

Fimbrial Serotypes

Vaccine strains P3s10 and CS were serotype Fim2,3, and vaccine strain 18530 was serotype Fim3. Among the clinical isolates, all 3 serotypes were found (Figure 2, panel A). Significantly more isolates collected during 1963–1986 were serotype Fim2 than were isolates collected during 1953–1958 (p<0.001). Of the 19 isolates collected during 1997–2005, we found that 15 (79%) were Fim3, 3 (16%) were Fim2,3, and 1 (5%) was Fim2. Significantly more isolates collected during 1997–2005 were serotype Fim3 than were isolates collected during 1953–1958 and 1963–1986 (p<0.001 for each).

Alleles of *ptxA, prn, tcfA, fim2*, and *fim3*

Vaccine strains P3s10 and CS contained *ptxA2*/*prn1*/*tcfA2*/*fim2–1*/*fim3–1*, whereas vaccine strain 18530 contained *ptxA3*/*prn1*/*tcfA2*/*fim2–2*/*fim3–1*. Among the clinical isolates, all 4 *ptxA* alleles (*ptxA1*, *ptxA2*, *ptxA3*, and *ptxA4*) were found (Figure 2, panel B). However, the frequency of each allele changed over time. In the prevaccine era, 64% (n = 16), 24% (n = 6), and 12% (n = 3) of isolates contained *ptxA2*, *ptxA3*, and *ptxA4*, respectively. The allele *ptxA1* appeared in 1963 and has become predominant since then. After the 1980s, all isolates contained *ptxA1*.

Altogether 6 *prn* alleles (*prn1, prn2, prn3, prn7, prn10*, and *prn11*) were detected. The amino acid sequences in variable regions of the 6 alleles are shown in online Appendix Figure 2 (www.cdc.gov/EID/content/16/11/1695-appF2.htm). Allele *prn1* remained predominant at 81% (78/96) during the study period (Figure 2, panel C). No significant difference was found in the frequency of finding *prn2* and *prn3* in isolates collected during 1997–2005 than in those collected during 1953–1958 (p = 0.749) and 1963–1986 (p = 0.0513). All 7 isolates with *prn7* contained *ptxA3*, whereas all 3 isolates with *prn10* contained *ptxA4*. Two isolates with *prn11* contained *ptxA2*. All 5 isolates with *prn2* or *prn3* contained *ptxA1*.

Four *tcfA* alleles (*tcfA1, tcfA2, tcfA5*, and *tcfA9*) were identified. Allele *tcfA2* was most common, representing 94% (n = 90) of the isolates (online Technical Appendix). Also detected were 2 *fim2* (*fim2–1* and *fim2–2*) and 3 *fim3* (*fim3–1*, *fim3–2*, and *fim3–4*) alleles. Of the 96 isolates, 90 (94%) contained *fim2–1*. All 6 isolates with *fim2–2* were recovered during 1953–1958. Five of the 6 isolates with *fim2–2* contained *prn7*. For the *fim3* alleles, 96% of isolates were *fim3–1*. All 3 isolates with *fim3–2* and 1 isolate with *fim3–4* were recovered in 1997–2005. Identical amino acid sequences were found for *fim3–1* and *fim3–4*, but a silent mutation at nt 87 was found for *fim3–4*. All 3 isolates harboring *fim3–2* contained *prn2*.

PFGE Profiles

Vaccine strains P3s10 and CS had an identical profile, BpCHR6, whereas vaccine strain 18530 represented BpFINR13 (Figure 3). The 96 isolates produced 27 distinct profiles, 4 of which (BpFINR9, BpSR127, BpSR23, and BpSR11) have been reported in Europe. The PFGE profiles obtained from vaccine strain 18530 (BpFINR13) and international reference strain 18323 were not detected among the isolates from China. The 6 common profiles represented 70% of isolates (41 isolates with BpCHR15, 7 with BpCHR6, 6 with BpCHR2, 5 with BpSR127, 5 with BpSR23, and 3 with BpCHR20). The PFGE profiles changed over time. Dendrogram analysis of the 27 profiles indicated that they belonged to 8 clusters. Among these clusters, 5 (clusters I–IV and VII) have been reported in Europe (*5,22,23*).

Previous studies have shown that cluster I includes international reference strain 18323 and 1 clinical isolate (*21,22*). Clusters II and III include the vaccine strains and most strains that were circulating before the 1990s. Cluster

IV contains most strains currently circulating in Europe. Cluster VII, a new group, consists of some isolates collected from Finland in 2004 (*23*). Of the 3 clusters identified in our study, 1 consisted of 4 profiles (BpCHR1–3 and BpFIN13). Profile BpFINR13 was found only in pertussis vaccine strain 18530 (*7*). The strain was obtained from the United States and used as a vaccine strain in Finland and China. The second cluster contained profiles BpCHR14 and BpCHR19, and the third contained profiles BpCHR20 and BpCHR22.

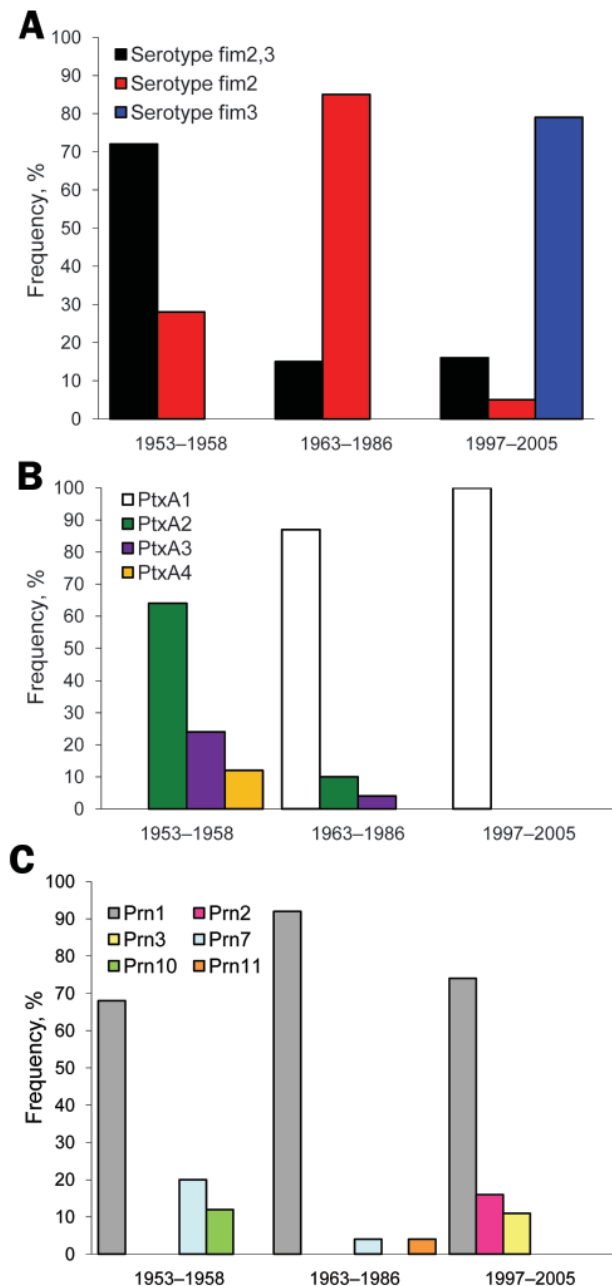

Figure 2. Frequencies of A) fimbrial (*fim*) serotypes, B) pertussis toxin (*ptx*) A alleles, and C) pertactin (*prn)* alleles in *Bordetella pertussis* isolates collected in China during 1953–1958, 1963–1985, and 1997–2005.

In this study, 56 isolates tested belonged to cluster III (Figure 3). Cluster III contained 6 profiles (BpCHR8, BpCHR15, BpCHR17, BpFINR9, BpSR23, and BpSR127) with a minimum of 79% overall relatedness. All isolates belonging to the cluster were collected during 1963–1986 and 1997–2005. Of the 96 clinical isolates, only 2 isolates that belonged to cluster IV (IV-β) were identified; these 2 isolates had PFGE profile BpSR11. Strains with BpSR11 were first detected in France in 1996 (*22*) and have been prevalent in Europe since then (*13*). The 2 isolates with BpSR11 were recovered in 2000 and 2001 and contained *prn2*.

Discussion

Few *B. pertussis* isolates from China contained nonvaccine type alleles *prn2* or *prn3*; those that did were found later. In many countries, the *prn1* allele is found in most vaccine strains and predominated before introduction of vaccination. However, the vaccine type strains were gradually replaced by nonvaccine type strains, mainly with allele *prn2*, after the introduction of vaccination. The most prevalent type in modern isolates is *prn2* (*7,14,20,24*). In Taiwan, Pw vaccines have been offered since 1954 (*25*). When 168 clinical isolates collected in Taiwan during 1993–2004 were analyzed, *prn2* strains accounted for 39% in 1993–1996 and increased to 90% in 1998–2004. In contrast to findings for many countries with long histories of high vaccination coverage, *prn2* was first found in China in 2000. The exact reasons for the low frequency of strains with *prn2* and their relatively late emergence in China are not known. One explanation might be the low vaccination coverage in China before the 1980s and differences in vaccine coverage between urban and rural areas. Another reason might be the closed borders.

In Japan, divergence of Prn and Ptx between vaccine strains and circulating isolates (*26–28*) has been reported. Pw vaccines were introduced in Japan in 1958; the vaccine strain used was *prn1*/*ptxA2*. In 1971, reported vaccination coverage was 50% (*8*). In 1976, vaccination coverage dropped to only 9%, and pertussis returned. In 1981, a Pa vaccine containing Ptx and filamentous hemagglutinin was introduced in Japan (*27*). The strain used for production of the Pa vaccines was Tohama I (*prn1*/*ptxA2*), isolated in the 1950s. When 107 isolates collected from 1988 through 2001 were studied, the nonvaccine type *prn2*/*ptxA1* appeared in 1994 and was found in 27%–42% of isolates from 1994 through 2001 (*26*). A recent study reported similar frequency (33%) of the nonvaccine type *prn2/ptxA1* in Japan when 60 isolates collected during 1991–2007 were analyzed (*28*).

TcfA has been shown to be crucial for *B. pertussis* colonization (*29*). A total of 9 *tcfA* alleles have been reported

(*30,31*), and the most common allele is *tcfA2* (*20,24*). Our finding that 94% of isolates studied contained *tcfA2* agreed with findings from earlier studies (*20,24*). Allele *tcfA1* has been described only for reference strain 18323. In our study, 3 clinical isolates recovered during 1953–1958 were found to contain *tcfA1*. All 3 isolates were recovered from the same geographic area. Allele *tcfA1* contains a 75-bp segment not found in other *tcfA* variants. The strain with *tcfA1* was postulated to be the progenitor of the strains with *tcfA2*, *tcfA3,* or *tcfA5* (*20*).

Several studies have demonstrated that serotype Fim3 is predominant in vaccinated populations, whereas serotypes Fim2 or Fim2,3 are predominant in unvaccinated populations (*14,32,33*). In Sweden, before 1979 when Pw vaccines were first used, 70% of circulating strains were serotype Fim3 (*32*). During 1979–1995, when pertussis vaccination was stopped, Fim2 started to increase and reached 64% in the early 1990s. In 1996, when general vaccination with Pa vaccines was reintroduced, prevalence of Fim2 declined and Fim3 strains emerged rapidly. In 2002 and 2003, Fim3 was found in 96% of fully vaccinated persons. In China, before introduction of vaccination, the prevalent serotypes were Fim2,3 and Fim2. After vaccination, the frequency of serotype Fim2,3 decreased and Fim2 became predominant. The possible explanation for the predominance of Fim2 strains after vaccination is that the 2 vaccine strains used in China express Fim2,3 and Fim3. The shift from serotype Fim2 to Fim3 was observed in 1998 and coincided with the introduction of Pa vaccines in this country. Pa vaccines without fimbrial antigens may have some effect on fimbrial serotypes of circulating isolates, as was observed in Sweden (*32*); however, the exact reason remains to be shown.

In our study, most strains from China had different PFGE profiles than did those from Europe. However, many PFGE profiles detected among the strains from China fell into the same clusters as those reported in Europe (*5,22*). For example, the most common profile, BpCHR15, fell into the same cluster (cluster III) as BpSR23 and BpSR127 (*14*). Cluster III includes most strains circulating in Europe before the 1990s (*5,32*). In China, strains with BpCHR15 had been prevalent during 1965–1983. Although the strains with BpCHR15 were recovered from several different regions and over 20 years, the possibility that some strains were isolated during outbreaks cannot be excluded.

The major PFGE cluster circulating in Europe since the 1990s is cluster IV (*5,32*). Cluster IV can be divided into 3 subgroups (α, β, and γ), the frequency of which differs among countries. However, since the late 1990s in many countries in Europe, subgroup IV-β became more prevalent than the other 2 subgroups (*13*). In our study, only 2 isolates with BpSR11 belonged to group IV-β, whereas no isolates fell into subgroups IV-α or IV-γ. This PFGE result

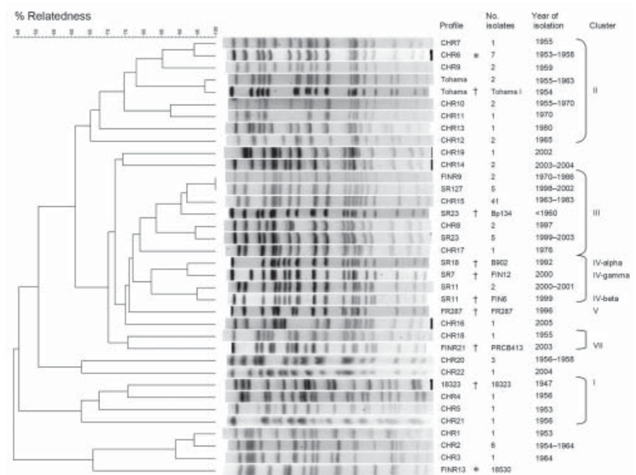

Figure 3. Dendrogram analysis of 27 pulsed-field gel electrophoresis profiles of *Bordetella pertussis* isolates circulating in China during 1953–2005. The unweighted pair group method with arithmetic mean with 1% band tolerance and 1% optimization settings was used as the clustering method. * indicates international reference strains (*17,23*); † indicates vaccine strains from China. Vaccine strains P3s10 and CS represent BpCHR6, and vaccine strain 18530 represents BpFINR13.

correlates with genotyping results.

When we examined the association of 6 common PFGE profiles with different allele combinations, we found that of the 51 isolates with BpCHR15, BpSR23, or BpSR127, 94% contained *ptxA1*/*prn1*/*tcfA2*/*fim2–1*/*fim3–1*. Of the 10 isolates with BpCHR6 or BpCHR20, 100% contained *ptxA2*/*prn1*/*tcfA2*/*fim2–1*/*fim3–1*. Of the 6 isolates with BpCHR2, all contained *ptxA3*/*prn7*/*tcfA2*/*fim2–2*/*fim3–1*.

The emergence of *B. pertussis* strains carrying a novel allele (*ptxP3*) for the Ptx promoter has been recently observed in countries with long histories of high vaccination coverage, such as the Netherlands (*34*). Furthermore, all strains from the Netherlands with BpSR11 were found to carry the *ptxP3* allele. In our study, only 2 isolates from China were found to have BpSR11, suggesting that *ptxP3* strains are not common in China.

The limitations of this study include the small number of *B. pertussis* isolates collected during the study period and recent isolates collected mainly from Beijing and its surrounding area. Therefore, our data should be interpreted with caution, and more epidemiologic studies with a larger number of isolates should be conducted in this country.

In conclusion, *B. pertussis* strains in China may differ from those in countries with long histories of high vaccination coverage. Continuous monitoring of *B. pertussis* strains is needed.

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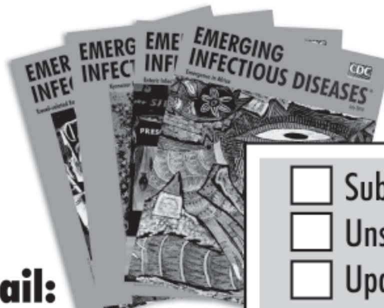

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Lymphotropism of Merkel Cell Polyomavirus Infection, Nova Scotia, Canada

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To test the hypothesis that Merkel cell polyomavirus (MCPyV) can infect cells of the lymphoid system, we analyzed 353 specimens, including 152 non-Hodgkin lymphomas, 44 Hodgkin lymphomas, 110 benign lymph nodes, 27 lymph nodes with metastasis, and 20 extranodal tissue samples. MCPyV DNA was detected by quantitative PCR in 13 (6.6%) of 196 lymphomas, including 5 (20.8%) of 24 chronic lymphocytic leukemia specimens, and in 11 (10%) of 110 benign lymph nodes, including 8 (13.1%) of 61 samples of reactive hyperplasia and 3 (10.3%) of 29 normal lymph nodes. Other samples were MCPyV negative. Sequence analysis of 9 virus-positive samples confirmed the identity of MCPyV; 3 viral strains were represented. Immunohistochemical testing showed that 1 T-cell lymphoma expressed MCPyV T-antigen. These findings suggest that the lymphoid system plays a role in MCPyV infection and may be a site for MCPyV persistence.

Merkel cell polyomavirus (MCPyV) was first described in 2008 (*1*) as a new human virus associated with Merkel cell carcinoma (MCC), an uncommon but aggressive form of skin cancer. Subsequent studies have reported the presence of MCPyV in 24%–100% of MCCs from patients from the United States, Germany, France, the Netherlands, and Australia (*1–11*). Findings of the clonal integration of MCPyV in tumor cell genomes, tumor-associated mutations in the large T-antigen (T-ag) gene, and large T-ag expression in tumors suggest that MCPyV is not only associated with MCC, but that it may be the causative agent (*1*,*12*,*13*).

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However, the natural reservoir of MCPyV in infected hosts remains to be identified. MCPyV DNA has been detected at low copy number in some non-MCC skin tumors, in normal tissues of skin and the gastrointestinal tract, and in a few nasopharyngeal aspirates and blood samples, including inflammatory monocytes (*1*,*5*,*6*,*11*,*14–16*).

Lymphocytes can disseminate viruses throughout a host and may provide sites of viral persistence. Human polyomaviruses are known to establish persistent infections in healthy persons, to undergo periodic reactivation and replication, and to cause disease in susceptible hosts. Some polyomaviruses are lymphotropic; BK virus, simian virus 40, and JC virus DNA sequences have been detected in human lymphoid tissues, blood cells, and lymphomas (*17–20*). Recently, Shuda et al. (*13*) reported the presence of MCPyV in a low percentage (2.2%) of hematolymphoid malignancies. In this study, we investigated the presence of MCPyV in benign lymph nodes and malignant lymphomas in patients from Canada.

Design and Methods

Patients and Samples

This study was approved by the Capital Health Research Ethics Board, Halifax, Nova Scotia, Canada, and by the Baylor College of Medicine Institutional Review Board, Houston. A total of 353 frozen specimens from various body sites were analyzed. Tissues from 196 malignant lymphomas, including 152 non-Hodgkin lymphoma (NHL) and 44 Hodgkin lymphoma (HL) samples, were retrieved from the Department of Anatomical Pathology, Queen Elizabeth II Health Sciences Center, Halifax, Nova Scotia, Canada.

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All cases were diagnosed during 1994 through 2001 and classified according to World Health Organization criteria by using morphologic and immunohistochemical evaluation (*21*). The NHL samples were classified as B-cell lymphoma (n = 133), NK/T-cell lymphoma (n = 18), or were unclassified (n = 1). The HL samples were classified as classical HL (n = 41) or nodular lymphocyte predominant HL (n = 3). Also included in our study were 157 non-lymphoma frozen tissue specimens, including 110 benign lymph node biopsy specimens from healthy patients or patients with inflammatory disease, 27 lymph nodes from patients with metastatic carcinoma or melanoma, 7 biopsy specimens of inflammatory tissues, and 13 other neoplastic (non-MCC) tissue samples.

Lymphoma specimens were obtained from 96 women and 98 men; patients' ages ranged from 15 to 88 years (mean 54.5 years). Of the 110 benign lymph nodes, 53 were from women and 57 from men, ranging in age from 17 to 85 years (mean 46 years). Lymph nodes with metastatic tumors were obtained from 16 women and 10 men, ranging in age from 22 to 86 years (mean 60.6 years). Of the 7 inflammatory tissue samples, 3 were from women and 4 were from men 19–84 years of age (mean 52 years). Finally, the non-MCC tumors were obtained from 8 men and 5 women, ranging in age from 22 to 72 years (mean 50.5 years). Data were not available for 3 patients, 2 with lymphoma and 1 with metastatic cancer. The cancers of all lymphoma patients were staged according to the Ann Arbor system (*22*).

Formalin-fixed, paraffin-embedded biopsy specimens of skin cancers (4 MCCs and 4 melanomas), obtained from the archives of the Department of Pathology, Baylor College of Medicine, Houston, Texas, USA, served as controls for PCR. Specimens were collected during 1998–2008.

Immunohistochemical Analysis

The expression of MCPyV T-ag was detected by immunohistochemical analysis by using a BenchMark XT IHC (Ventana Medical Systems, Inc., Tucson, AZ, USA) system. Tissue sections on microslides were deparaffinized with xylene, hydrated in serially diluted alcohol, and endogenous peroxidase activity was quenched. The sections were then treated with slightly basic Tris-based buffer for 30 min for antigen retrieval. Sections next were incubated with CM2B4, a monoclonal antibody against MCPyV large T-ag (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:250, followed by a standard polymer detection kit (diaminobenzidine) that served as the chromogen. The slides were counterstained with hematoxylin, dehydrated, and mounted for examination. A sample from a patient with MCC served as a positive control.

DNA Extraction

DNA was extracted from frozen tissues by lysis buffer/proteinase K treatment and phenol-chloroform extraction as previously described (*20*), omitting the deparaffinization step. For the paraffin-embedded specimens, sections were deparaffinized, and DNA was extracted by using our previously described protocol (*20*). During sample processing, stringent precautions were taken to avoid cross-contamination between samples. The microtome was cleaned carefully, and the blade was replaced for sectioning of each tissue. Other safety measures included working within a biosafety hood located in a dedicated room free from plasmids and viruses and using a dedicated set of pipettors and barrier filter tips. In addition, a negative extraction control that lacked tissue was processed in parallel.

Real-Time Quantitative PCR

Samples were first screened for the single-copy human RNase P gene by real-time quantitative PCR (qPCR), as described (*20*,*23*). Briefly, 5 µL of each DNA sample (undiluted and 1:10 diluted) was used in 50-µL qPCRs. This strategy detected potential PCR inhibitors in the DNA preparations, determined the human cell equivalents in each DNA sample, and normalized MCPyV viral loads to human cell numbers.

MCPyV was detected by qPCR with primers and TaqMan probe (Applied Biosystems, Foster City, CA, USA) designed to detect sequences from the unique coding region of the small tumor antigen (t-ag) gene of MCPyV. The sequences of the oligonucleotides were as follows: LT3-fwd primer 5′-AGTGTTTTTGCTATCAGTGCTTTA TTCT-3′, corresponding to nt 632–659 of MCPyV350 (GenBank accession no. EU375803); LT3-rev primer 5′-CCACCAGTCAAAACTTTCCCA-3′, corresponding to nt 702–682; and fluorogenic probe 5′-FAM-TGGTTT GGATTTCCTC-MGB-3′, corresponding to nt 661–676. The pCR.MCV350 plasmid described by Feng et al. (*1*) was used as a positive control. Amplifications were performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by using the following cycling parameters: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and of 1 min at 60°C. All PCRs were performed in duplicate. Reactions were considered positive if $\geq$10 viral genome copies/reaction were detected.

Conventional PCR

Conventional PCR was performed on a subset of DNA samples to validate the presence of MCPyV. The primer set used was the following: MC_F2 (5′-CTCATCCTCTGGATCCAGTAGC-3′) and MC_R2 (5′-CAGAAGAGATCCTCCCAGGTG-3′) specific for

a conserved region between nt positions 1142 and 1267 of the large T-ag gene of MCPyV (GenBank accession no. EU375803) and gave a 126-bp fragment. The pCR. MCV350 positive control plasmid was added to the control PCR outside the clean room after tubes containing test DNAs and negative controls (without DNA template) were closed. PCRs were performed in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems). Thermal cycling parameters were as follows: 94°C for 2 min, followed by 45 cycles at 94°C for 15 s, 60°C for 15 s, and 72°C for 15 s, with a final extension step at 72°C for 7 min. Amplified fragments were visualized by electrophoresis on a 3% agarose gel and stained with ethidium bromide. All PCR products were then purified by using the DNA Clean & Concentrator-5 system (Zymo Research, Orange, CA, USA) and sequenced by Lone Star Labs, Inc. (Houston, TX, USA).

Statistical Analysis

The z score of difference for proportions was used to test for a difference between selected groups and an outcome. Fisher exact test was used to test the distribution of clinical stage of disease and 5-year survival category between MCPyV-status groups; a p value <0.05 was considered significant.

Results

DNA Recoveries

DNA was extracted from the specimens and screened for suitability for qPCR analysis by amplification of the cellular RNase P gene. Serial dilutions were tested to determine whether PCR inhibitors were present in the DNA samples and to select noninhibitory dilutions for MCPyV analysis. Total DNA yields ranged from 41×10^4 to 403×10^6 cell equivalents (median 51×10^6) for the lymphoma samples, from 64×10^3 to 216×10^6 cell equivalents (median 29×10^6) for the benign lymph node samples, from 13×10^5 to 117×10^6 cell equivalents (median 21×10^6) for the lymph node samples with metastatic cancer, from 30×10^5 to 78×10^6 cell equivalents (median 39×10^6) for the other inflammatory tissues, and from 16×10^5 to 126×10^6 cell equivalents (median 20×10^6) for the other neoplastic tissues.

MCPyV Detection in MCC

We tested 4 MCC and 4 melanoma samples (fixed and paraffin-embedded) for the presence of MCPyV by qPCR. MCPyV sequences were detected in 2 of 4 MCC samples; none were detected in the 4 melanoma samples (Table 1). MCCs contained an average of 0.29 (range 0.02–0.56) MCPyV genome copies per cell. MCPyV in 1 sample was confirmed by conventional PCR and sequence analysis. The sequence of the amplified fragment showed 98% similarity with the MCC350 reference sequence (GenBank accession no. EU375803) and 100% homology with MCC349 (GenBank accession no. FJ173813).

Table 1. MCPyV DNA from lymphoid and nonlymphoid samples, Halifax, Nova Scotia, Canada, and from MCC tissues, Houston, Texas, USA, 1994–2008*

Type of specimen	No. samples tested	No. (%) MCPyV positive
Frozen samples from Halifax, Nova Scotia, Canada		
Malignant lymphomas	196	13 (6.6)
Benign lymph nodes	110	11 (10.0)
Lymph nodes with non-MCC metastatic cancer	27	0
Other inflammatory tissues	7	0
Other neoplastic non-MCC tissues	13	0
Totals	353	24 (6.8)
Fixed tissues from Houston, Texas, USA		
MCC	4	2 (50.0)
Melanoma	4	0

*MCPyV, Merkel cell polyomavirus; MCC, Merkel cell carcinoma.

MCPyV Detection in Malignant Lymphomas, Lymphoid Tissues, and Other Inflammatory or Neoplastic Tissues

A total of 196 frozen malignant lymphoma samples were tested for MCPyV sequences. The classification of those samples is summarized in Table 2. Viral DNA was detected in 13 (6.6%) of the lymphomas (Tables 1, 2). As determined by qPCR, the viral copy numbers were relatively low. An average of 4.6 copies/10^4 cells (range 0.16–27 copies/10^4 cells, median 0.94 copies/10^4 cells) was detected in the MCPyV-positive lymphomas. Among the lymphomas, the overall frequency of MCPyV between NHL and HL cases was similar (6.6% and 6.8%; p = 1.0) (Table 2).

MCPyV was identified most frequently in chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) (20.8%), a tumor type known to occur in MCC patients (Table 2). Patients with CLL/SLL had a high prevalence of MCPyV compared to all other B-cell lymphomas (20.8% vs. 2.8%; p = 0.01). The virus loads in CLL/SLL were similar to those found in patients with other types of lymphoma. Characteristics of MCPyV-positive and MCPyV-negative CLL/SLL patients are shown in Table 3. None of the 24 CLL/SLL patients had MCC.

A total of 110 frozen benign lymph node samples, 27 lymph nodes with metastatic tumors, and 20 other inflammatory or neoplastic tissues were tested in parallel (Table 4). Eleven of 110 (10%) benign lymph nodes were MCPyV positive (Tables 1, 4). Viral loads in MCPyV-positive lymph nodes averaged 2.3 copies/10^4 cells (range 0.44–6.0 copies/10^4 cells, median 2.1 copies/10^4 cells). Of the 11 benign lymphoid specimens positive for MCPyV DNA, 8 were among 61 (13.1%) reactive hyperplasia sam-

Table 2. Presence of MCPyV in malignant lymphomas, Nova Scotia, Canada, 1994–2001*

Pathology	No. samples tested	No. (%) MCPyV positive
Non-Hodgkin lymphoma	152	10 (6.6)
B-cell lymphoma	133	8 (6.0)
Burkitt lymphoma/Burkitt cell leukemia	2	0
Chronic lymphocytic leukemia/small lymphocytic lymphoma	24	5 (20.8)
Diffuse follicular center lymphoma	1	0
Diffuse large B-cell lymphoma	52	0
Diffuse large B-cell lymphoma/T-cell/histiocyte-rich type	1	0
Extranodal marginal zone B-cell lymphoma	2	0
Follicular lymphoma	35	2 (5.7)
Lymphoblastic leukemia/lymphoma	1	0
Lymphoplasmacytic lymphoma	4	0
Mantle cell lymphoma	7	0
Posttransplant lymphoproliferative disorder, polymorphic	1	0
Splenic marginal zone lymphoma	1	0
Unclassified	2	1 (50.0)
NK/T-cell lymphoma	18	2 (11.0)
Anaplastic large cell lymphoma	8	1 (12.5)
Angiocentric T cell lymphoma (nasal type)	1	0
Angioimmunoblastic T-cell lymphoma	2	1 (50.0)
Extranodal NK/T-cell lymphoma, nasal type	2	0
Lymphoblastic lymphoma	4	0
Peripheral T-cell lymphoma	1	0
Unclassified	1	0
Hodgkin lymphoma	44	3 (6.8)
Classical Hodgkin lymphoma	41	3 (7.3)
Mixed cellularity	10	2 (20.0)
Nodular sclerosis	29	1 (3.4)
Unclassified	2	0
Nodular lymphocyte predominant Hodgkin lymphoma	3	0
All	196	13 (6.6)

*MCPyV, Merkel cell polyomavirus.

ples and 3 were from 29 (10.3%) normal lymph nodes. All other samples tested were negative for MCPyV (Table 4).

Sequence analysis confirmed the qPCR results. Conventional PCR with MCPyV-specific primers was performed on 9 viral DNA-positive samples (4 lymphoma, 5 reactive hyperplasia). DNA amplification products of the expected size (126 bp) from the large T-ag gene were obtained, and sequence analysis confirmed the identity of MCPyV. Three of 4 MCPyV-positive lymphoma samples shared 100% sequence homology with strain MCC344 (GenBank accession no. FJ173807), whereas sequences of the remaining sample were identical to those of MCC349 (GenBank accession no. FJ173813). Among the 5 MCPyV-positive reactive hyperplasia samples, 4 had 100% homology to strain MCC344 and 1 to strain MCC339 (GenBank accession no. EU375804).

Expression of MCPyV T-antigen

A series of 17 lymphoid specimens (7 of which were positive for MCPyV DNA by PCR), consisting of 11 lymphomas and 6 reactive hyperplasia samples, were tested for expression of MCPyV T-ag. Immunohistochemical staining with the CM2B4 monoclonal antibody was carried out without knowledge of the PCR results. One sample, classified as an angioimmunoblastic T-cell lymphoma, expressed detectable T-ag in scattered lymphocytes (Figure). This sample was positive for MCPyV DNA by PCR. The remaining samples tested were negative for T-ag expression by immunohistochemical test.

Clinical Follow-Up

Five-year clinical follow-up information was available for 114 of 196 patients who had malignant lymphoma. Of those patients not included in follow-up analysis, 14 had died from other causes, 47 had received a diagnosis of lymphoma within the past 5 years, and 21 were lost to follow-up. Among the 114 patients with a 5-year follow-up, 49 had died of the disease and 65 were alive (24 with lymphoma and 41 without). Analyses showed no difference in the distributions among survival categories relative to MCPyV status (Table 5). Of the MCPyV-positive patients for whom follow-up data were available, 3 (42.9%) of 7 were alive and in remission compared with 38 (35.5%) of 107 MCPyV-negative patients. There were 3 (42.9%) deaths among the MCPyV-positive group and 46 (43.0%) deaths among the MCPyV-negative group ($p = 0.88$).

Table 3. MCPyV infection and characteristics of patients with chronic lymphocytic leukemia/small lymphocytic lymphoma, Nova Scotia, Canada, 1994–2001*

Characteristic	No. (%) cases	No. (%) MCPyV positive	No. (%) MCPyV negative
Patient sex			
M	16 (66.7)	2 (12.5)	14 (87.5)
F	8 (33.3)	3 (37.5)	5 (62.5)
Patient age, y			
≤60	6 (25.0)	0	6 (100.0)
>60	18 (75.0)	5 (27.8)	13 (72.2)
Disease stage†			
I	9 (40.9)	1 (11.1)	8 (88.9)
II	5 (22.7)	3 (60.0)	2 (40.0)
III	0	0	0
IV	8 (36.4)	1 (12.5)	7 (87.5)
Patient survival‡			
Alive in remission	2 (15.4)	1 (50.0)	1 (50.0)
Alive with disease	6 (46.1)	0	6 (100.0)
Dead from disease	5 (38.5)	1 (20.0)	4 (80.0)
Total	24	5 (20.8)	19 (79.2)

*No patients with chronic lymphocytic leukemia/small lymphocytic lymphoma had Merkel cell carcinoma. MCPyV, Merkel cell polyomavirus.
†Stage of disease for 2 MCPyV-negative patients was unknown.
‡Five-year follow-up survival information. Data were available for 13 patients, excluding 10 who had received a diagnosis within the past 5 y and one who was lost to follow-up. Disease refers to original diagnosis of either lymphoma or leukemia.

Table 4. Presence of MCPyV in benign lymph nodes, lymph nodes with metastatic cancer, and other inflammatory or neoplastic tissues, Nova Scotia, Canada, 1994–2001*

Pathologic feature	No. samples tested	No. (%) MCPyV positive
Benign lymph nodes	110	11 (10)
Acute lymphadenitis	1	0
Atypical hyperplasia	2	0
Dermatopathic lymphadenopathy	3	0
Florid follicular hyperplasia	1	0
Mycobacterium avium–intracellulare infection	2	0
Necrotizing granulomas	3	0
Normal lymph node	29	3 (10.3)
Reactive hyperplasia	61	8 (13.1)
Sarcoidosis	6	0
Systemic mast cell disease	1	0
Toxoplasmosis	1	0
Lymph nodes with metastatic tumors	27	0
Carcinoma	25	0
Melanoma	2	0
Other inflammatory tissues	7	0
Chronic sialadenitis	1	0
Follicular bronchiolitis	1	0
Hemophagocytic syndrome, spleen	1	0
Helicobacter pylori–associated gastritis	1	0
Hemochromatosis, liver	1	0
Hyperplasia and chronic perifolliculitis, skin	1	0
Interstitial pneumonitis	1	0
Other neoplastic tissues	13	0
Mixed mullerian tumor	1	0
Neurofibroma	1	0
Sarcoma	4	0
Schwannoma	2	0
Thymoma	5	0

*MCPyV, Merkel cell polyomavirus.

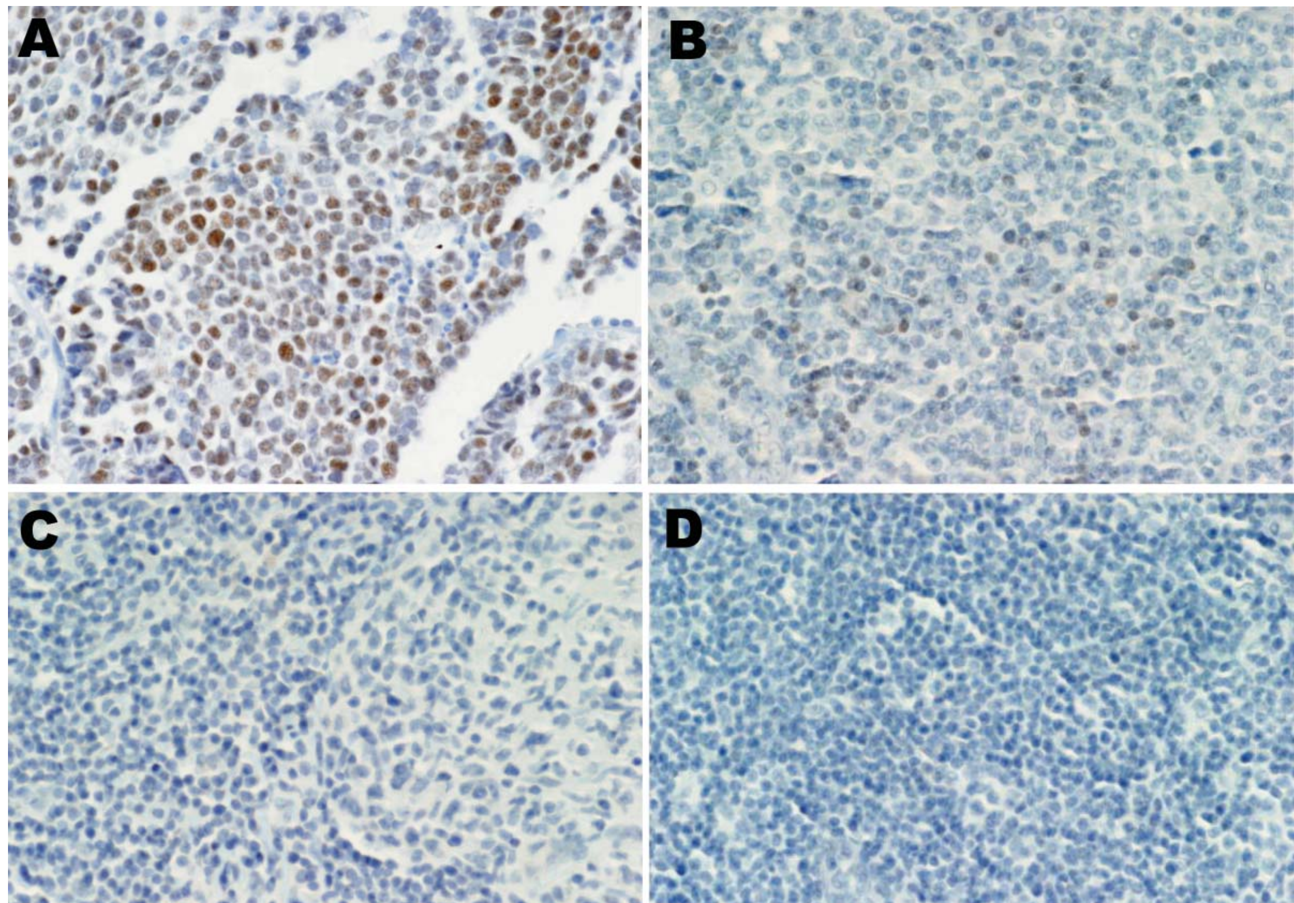

Figure. Merkel cell polyomavirus (MCPyV) large T-antigen (T-ag) expression in human tissues. A) Merkel cell carcinoma stained with CM2B4 antibody as a positive control; MCPyV T-ag was detected. B) Expression of MCPyV T-ag in small lymphocytes in an MCPyV DNA–positive angioimmunoblastic T-cell lymphoma, stained with CM2B4. C) MCPyV DNA–positive reactive lymphoid hyperplasia sample reacted with CM2B4; no T-ag was detected. D) MCPyV DNA-negative chronic lymphocytic leukemia/small lymphocytic lymphoma sample stained with CM2B4; no T-ag was detected. Original magnification ×40.

Discussion

This study describes the presence of MCPyV DNA in benign lymph nodes and malignant lymphomas in specimens from patients living in Canada. MCPyV was detected in 6.6% of lymphomas and in 10% of nonneoplastic lymph node samples. These results, together with those of Shuda et al. (*13*) and Mertz et al. (*14*), support the hypothesis that lymphocytes and monocytes may serve as a tissue reservoir for MCPyV infection. Because serologic assays have indicated that MCPyV primary infections frequently occur in children (*24–26*), we favor the interpretation that the MCPyV genomes detected in the adult lymphoid tissues reflect the presence of persistently infected cells. Only 1 specimen among MCPyV DNA–positive samples tested expressed T-ag, which suggests that most infected lymphoid cells are not producing detectable levels of viral protein. However, because MCPyV DNA copy numbers in the samples were low, a few antigen-expressing cells in the tissues may have escaped detection in the immunohistochemical assays.

The data from this study do not suggest that MCPyV caused the lymphoid tumors that were virus positive. However, more comprehensive studies are necessary to exclude the possibility that MCPyV may have lymphomagenic potential under certain conditions. An observation of interest was the presence of MCPyV in 5 (20.8%) of 24 CLL/SLL cases. CLL is a type of leukemia that is now regarded as being identical to SLL (*27*). The most recent World Health Organization classification scheme for hematopoietic malignancies considers CLL and SLL to be different manifestations of the same disease and combines these entities into 1 disease category (CLL/SLL) (*21*). Some studies have found that CLL co-exists with MCC, making the association rare but well recognized (*28–34*). CLL and MCC are age-related with an increased risk in those >60 years of age. Koljonen et al. (*35*) recently showed that MCPyV DNA

Table 5. Clinical outcomes for lymphoma patients over a 5-year period, Nova Scotia, Canada*

Characteristics	No. (%) MCPyV positive	No. (%) MCPyV negative	p value
Disease stage†			
I	2 (28.6)	35 (35.4)	
II	2 (28.6)	14 (14.1)	
III	0	11 (11.1)	
IV	3 (42.9)	39 (39.4)	0.62
Patient survival			
Alive in remission	3 (42.9)	38 (35.5)	
Alive with disease	1 (14.3)	23 (21.5)	
Died from disease	3 (42.9)	46 (43.0)	0.88

*The 5-year period refers to the time frame for each patient from time of diagnosis until follow-up 5 years later. MCPyV, Merkel cell polyomavirus.
†Clinical stage of disease at time of lymphoma diagnosis; information was not available for 8 MCPyV-negative patients.

is frequently present in MCCs that occur in CLL patients. The basis for the association between CLL and MCPyV is unclear. The link may be coincidental or may reflect some influence of the MCPyV-infection process.

The present study provides evidence of the presence of MCPyV in samples of reactive lymphoid hyperplasia. (Reactive lymphoid hyperplasia refers to a benign, reversible enlargement of the lymph node as a consequence of proliferation of some or all of its cellular components.) This is a normal response of the lymph nodes to an antigenic stimulus, such as infection or inflammation. Viruses, e.g., Epstein-Barr virus, induce reactivity of lymphoid cells in lymphoid tissues from healthy persons (*36*). In our study, 8 (13.1%) of 61 reactive hyperplasia specimens were shown to harbor MCPyV DNA at low copy number. Whether MCPyV infection prompted those cases of reactive hyperplasia is unknown.

In conclusion, our findings of the presence of MCPyV in malignant lymphomas, reactive hyperplasia, and normal lymph nodes support the hypothesis that MCPyV is lymphotropic. Our findings also suggest that the lymphoid system plays a role in MCPyV infection and may be a site for MCPyV persistence.

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Comparison of 3 Infrared Thermal Detection Systems and Self-Report for Mass Fever Screening

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Despite limited evidence regarding their utility, infrared thermal detection systems (ITDS) are increasingly being used for mass fever detection. We compared temperature measurements for 3 ITDS (FLIR ThermoVision A20M [FLIR Systems Inc., Boston, MA, USA], OptoTherm Thermoscreen [OptoTherm Thermal Imaging Systems and Infrared Cameras Inc., Sewickley, PA, USA], and Wahl Fever Alert Imager HSI2000S [Wahl Instruments Inc., Asheville, NC, USA]) with oral temperatures (≥100°F = confirmed fever) and self-reported fever. Of 2,873 patients enrolled, 476 (16.6%) reported a fever, and 64 (2.2%) had a confirmed fever. Self-reported fever had a sensitivity of 75.0%, specificity 84.7%, and positive predictive value 10.1%. At optimal cutoff values for detecting fever, temperature measurements by OptoTherm and FLIR had greater sensitivity (91.0% and 90.0%, respectively) and specificity (86.0% and 80.0%, respectively) than did self-reports. Correlations between ITDS and oral temperatures were similar for OptoTherm (ρ = 0.43) and FLIR (ρ = 0.42) but significantly lower for Wahl (ρ = 0.14; p<0.001). When compared with oral temperatures, 2 systems (OptoTherm and FLIR) were reasonably accurate for detecting fever and predicted fever better than self-reports.

Advancements in transportation coupled with the growth and movement of human populations enable efficient transport of infectious diseases almost anywhere in the world within 24 hours (*1*). This recognition has prompted the evaluation of rapid mass screening methods to delay the importation of infection into healthcare settings, communities, and countries *(1–4)*. Because fever is a common indicator of many infectious diseases, the rapid identification of fever is a major component of screening efforts. Such screening was used by many countries during the severe acute respiratory syndrome outbreak in 2003 and the influenza A pandemic (H1N1) 2009 outbreak (*2*,*3*,*5*–*8*). Despite widespread implementation of fever screening, its value for detecting highly communicable diseases has mainly been established through mathematical modeling rather than through studies in humans (*9*,*10*).

One approach to fever screening is to simply ask persons if they have a fever. In healthcare settings, this information is routinely obtained in the chief complaint or review of symptoms and in some situations by querying persons as they enter the facility (*11*). In travel settings, many countries have used a written health declaration to screen travelers arriving at international ports of entry (*2*). However, limited information exists on the accuracy of self-reported fever, which is biased by its subjective nature and reliance on travelers' awareness of fever status and willingness to report (*12*,*13*). Indeed, a clinical trial suggested that traditional thermometry is superior to self-reported fever for identifying patients with seasonal influenza (*14*). However, traditional thermometry methods are time-consuming and require close contact with potentially infectious patients.

Infrared thermal detection systems (ITDS) offer a potentially useful alternative to contact thermometry. This technology was used for fever screening at hospitals, airports, and other mass transit sites during the severe acute re-

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spiratory syndrome and influenza A pandemic (H1N1) 2009 outbreaks (*2*,*3*,*5*–*8*,*15*). ITDS appeared to enable early detection of febrile persons entering healthcare facilities, where the undetected introduction of communicable diseases can lead to outbreaks among patients and staff (*5*,*16*–*18*).

Although ITDS have the potential to serve as rapid, noninvasive screening tools for detecting febrile persons, previous studies provide conflicting information about their utility for mass fever screening (*15*,*16*,*19*–*25*). In addition, there are few published comparisons of the efficacy of different ITDS and their suitability for mass fever screening (*19*). Finally, no studies on the relative accuracy of self-reported fever and ITDS for fever screening or the value of combining these 2 methods have been published. These questions and the potential need to rapidly screen for fever during an emerging pandemic prompted us to conduct this study to validate different ITDS temperatures and self-reported fevers with oral temperatures.

Methods

Study Setting

A cross-sectional study comparing 3 ITDS was conducted in 3 urban tertiary-care hospital emergency departments in the United States: Albuquerque, New Mexico; Atlanta, Georgia; and Chicago, Illinois. Emergency departments were selected as the evaluation setting because of a potential high prevalence of fever compared with its prevalence in healthy populations and the routine measurement of each patient's oral temperature. The 3 hospitals were selected because of their estimated patient volume of >200 patients per day.

Human Subject Research Protections

The study was approved by the Institutional Review Board (IRB) of the Centers for Disease Control and Prevention (CDC) and the IRBs of the hospitals in Atlanta and Chicago. The Albuquerque hospital's IRB reviewed the protocol but deferred to CDC's IRB for approval.

Device Selection

ITDS were selected for evaluation through a competitive bidding process. Selection criteria included specifications suitable for fever screening: view field captures human heights (0.5–2.5 meters), temperature discrimination ≤0.2°C, smallest available sensor temperature range encompassing human temperatures (–40°C to 120°C), tripod/stationary mount, operational distance ≥2 meters, internal/external calibration standards, temperature capture time ≤1 second, and price ≤$25,000. Of 6 devices submitted to CDC, 3 met the above criteria and were selected for testing: the FLIR ThermoVision A20M (FLIR Systems Inc., Boston, MA, USA), the OptoTherm Thermoscreen (OptoTherm Thermal Imaging Systems and Infrared Cameras Inc., Sewickley, PA, USA), and the Wahl Fever Alert Imager HSI2000S (Wahl Instruments Inc., Asheville, NC, USA). Manufacturers provided training and consultation on the assembly and operation of the ITDS per company practices but were otherwise uninvolved in the study.

Participants and Eligibility

Adults (≥18 years of age) were recruited consecutively among patients who sought care at the emergency departments of 1 hospital in each city: Chicago (September 15–29, 2008), Atlanta (October 6–24, 2008), and Albuquerque (February 17–26, 2009). Patients were approached after they had been registered in the emergency department from 7:00 AM to 11:00 PM, 7 days per week, at all 3 sites and were enrolled in the study if they were willing to participate and gave verbal consent. Patients who were nonambulatory, mentally incompetent, arrested or incarcerated, <18 years of age, or required immediate medical attention were excluded from the study. Pregnant women were excluded in Chicago and Atlanta at the request of the hospitals' IRBs.

Sample Size

We estimated that 61 febrile patients were necessary to evaluate the sensitivity of ITDS for fever detection (assumed to be 80% from previous research) to within ±10% with 95% confidence. With an estimated fever prevalence of 2% among a population of patients at emergency departments, a total sample size of ≈3,000 patients was needed for the study.

Temperature Measurements

The 3 ITDS were positioned at the optimal distance (2–3 m) from each participant as recommended by the manufacturers. Each ITDS camera field of view was preset to capture the patient's face and neck. Participants were asked to remove eyeglasses and hats and instructed to stand facing the cameras until temperature measurements from all 3 devices had been recorded.

To account for ambient temperature, the Wahl device was manually calibrated on each morning before data collection, per manufacturer recommendation. In Albuquerque, where room temperatures varied during the day, the Wahl was additionally calibrated after noticeable changes in ambient temperature. The OptoTherm and FLIR have automated calibration systems to adjust for ambient conditions, diurnal variations in temperature, and thermal drift and therefore did not require manual calibration.

Unadjusted skin temperatures detected by ITDS were included in the analysis to enable direct comparison with oral temperature measurements. The FLIR and Wahl cameras did not display fixed temperature readings but rather readings that fluctuated by tenth of a degree increments.

For these 2 cameras, operators recorded the highest temperature displayed for each person. Measurements recorded by the FLIR during periods when the camera was not properly focused were excluded from the analysis.

Oral temperatures were measured by clinical staff using a DinaMap ProCare digital thermometer (General Electric Company, Freiburg, Germany) in Albuquerque and Atlanta and a Welch Allyn SureTemp Plus 692 Electronic Thermometer (Welch Allyn Inc, San Diego, CA, USA) in Chicago, per each hospital's established patient care standard. ITDS temperature measurements were taken either immediately after (Chicago and Atlanta) or just before (Albuquerque) each oral measurement. Confirmed fever was defined as an oral temperature ≥100°F (≥37.8°C). Room temperatures were recorded hourly by using a standard digital room thermometer.

Patient Self-Reports

Upon enrollment, patients were asked, "Do you feel like you have a fever now?" (self-reported fever) and whether they had taken medication for pain or fever (analgesic or antipyretic drugs) in the previous 8 hours. When needed, patients were given examples of trade and generic names of common antipyretic drugs. Their responses, along with each patient's age and sex, date, and time of temperature measurement were recorded.

Data Analysis

Symptom questionnaire responses, oral temperature measurements, and ITDS-recorded data were entered into an Excel (Microsoft Corp., Redmond, WA, USA) database and analyzed by using SAS Version 9.2 (SAS Institute Inc, Cary, NC, USA). Patient responses of "Don't know" to the question, "Do you feel like you have a fever now?" were analyzed as "No." ITDS and oral temperature measurements were compared by using descriptive statistics and bivariate analysis (χ^2 tests, *t* tests, and correlations). Generalized linear modeling was used to investigate the effects of covariates and potential confounders (age, sex, recent antipyretic use, study site, self-reported fever, time of day, and room temperature) on temperature measurements and to identify factors that influenced the difference between oral and ITDS temperature measurements, given site-specific effects.

Sensitivity (the proportion of those with confirmed fever who were identified as febrile by ITDS) and specificity (the proportion of those without confirmed fever who were identified as nonfebrile by ITDS) were calculated and used to plot the receiver operating characteristic (ROC) curves for all possible fever temperature thresholds on each ITDS. Optimal ITDS fever thresholds were defined as the temperature that yielded the highest combined sensitivity and specificity for fever detection for each device as determined by the ROC curves. Positive predictive value (PPV), the proportion of patients identified as febrile by ITDS who had a confirmed fever by oral temperature, was compared with self-report. The accuracies (sum of sensitivity and specificity) of the following 3 fever screening methods were compared by using oral thermometry as reference: 1) self-reported fever, 2) ITDS at optimal fever detection threshold, and 3) combination of ITDS and self-reported fever with a positive result on either method considered a fever.

Results

Of 3,345 eligible patients, we enrolled a total of 2,873 (85.9%): 1,511 (52.6%) in Chicago, 1,040 (36.2%) in Atlanta, and 322 (11.2%) in Albuquerque. The remaining 472 (14.1%) patients refused to participate. Men accounted for 1,514 (52.7%) participants; the mean age was 42 years (range 18–92 years). The mean oral temperature was 97.9°F (range 92.8°F–104.4°F); 64 (2.2%) patients had confirmed fever, including 48 (10.1%) of 476 patients reporting fever. Antipyretic or analgesic drug use within 8 hours was reported by 1,121 (39.0%) patients, including 225 (45.8%) who self-reported fever and 39 (60.9%) who had confirmed fever.

Correlations of ITDS and oral temperatures were similar for OptoTherm ($\rho = 0.43$) and FLIR ($\rho = 0.42$) but significantly lower for Wahl ($\rho = 0.14$; $p<0.001$). The areas under the ROC curves (AUC) for OptoTherm (96.0%) and FLIR (92.0%) were not significantly different but were significantly greater than the AUC of Wahl (78.2%; $p<0.001$; Figure 1). At their respective optimal threshold temperatures, sensitivities of fever detection of the 3 ITDS were not significantly different from each other, but specificities and PPVs of OptoTherm and FLIR were significantly higher than those of Wahl (Table 1; $p<0.001$). At fixed specificities, the sensitivities of each ITDS varied (Figure 2).

Compared with oral thermometry, sensitivity for self-reported fever was 75%, specificity was 84.7%, and PPV was 10.1%. Sensitivities of the 3 ITDS at their respective optimal thresholds did not differ significantly from that of self-reported fever (Table 1). However, specificities and PPVs of OptoTherm and FLIR at optimal thresholds were significantly greater than those of self-reported fever ($p<0.001$ for both comparisons), and specificity and PPV of Wahl were significantly lower than those of self-reported fever ($p<0.001$). The addition of self-report decreased the accuracy of fever detection at optimal thresholds for FLIR and OptoTherm (increase in sensitivity was less than decrease in specificity) but improved accuracy for Wahl with a greater increase in sensitivity than the decrease in specificity (Table 1). Conversely, adding OptoTherm or FLIR temperature measurements to self-reported fever increased accuracy, but adding Wahl temperature measurements decreased accuracy (Table 1).

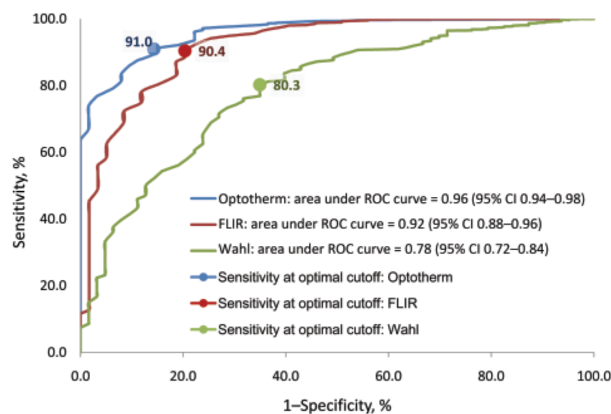

Figure 1. Receiver operating characteristic (ROC) curves of 3 infrared thermal detection systems (ITDS) for detecting fever (oral temperature ≥100°F): FLIR ThermoVision A20M (FLIR Systems Inc., Boston, MA, USA), OptoTherm Thermoscreen (OptoTherm Thermal Imaging Systems and Infrared Cameras Inc., Sewickley, PA, USA), and Wahl Fever Alert Imager HSI2000S (Wahl Instruments Inc., Asheville, NC, USA). CI, confidence interval.

Bivariate analyses revealed higher oral and ITDS temperatures among younger patients and later in the day (Table 2). Oral temperatures were higher in women, and ITDS temperature measurements were higher in men. ITDS temperature measurements increased with increasing room temperatures. Temperatures detected by oral thermometers, OptoTherm, and FLIR were higher in patients who reported recent antipyretic or analgesic drug use.

When we controlled for study site, multivariate analyses showed that 2 variables (sex and room temperature) were most strongly ($p<0.001$) associated with the size of the gap between oral and ITDS temperature measurements (Table 3). Smaller differences between ITDS and oral temperatures were found among men than among women. Differences between ITDS and oral temperatures became smaller with increasing room temperatures and as the day progressed (with the exception of FLIR). Site-specific effects indicated that, on average, differences between ITDS and oral temperatures were smaller among participants from Albuquerque and Atlanta than among those from Chicago. With the exception of Wahl measurements, the difference between ITDS and oral temperatures was greater in older patients. Differences between oral and OptoTherm temperatures tended to be smaller for those reporting antipyretic drug use.

Discussion

Our evaluation of 3 ITDS in emergency department settings found that the FLIR and OptoTherm reliably identified elevated body temperatures. The high AUCs for these 2 systems suggest that they can differentiate between febrile and afebrile persons with relatively high sensitivity and specificity at an optimal fever cutoff. The relatively high correlation with oral temperature measurement also supports the utility of these 2 ITDS, which predicted fever better than self-reports of patients and more accurately alone than in combination with self-reported fever.

Our study is one of few that simultaneously examined the effects of multiple external and internal factors (age, sex, time of day, room temperature, and antipyretic drug use) on ITDS and oral temperature measurement accuracy. We found that ITDS and oral temperature measurements were strongly influenced by site and time of day, which may be a real effect or a result of variations in oral measurement techniques. The effects of age and time of day on body temperature found in this study have been well established

Table 1. Comparisons of 3 infrared thermal detection system results and self-reported fever with oral temperature among patients in 3 emergency departments, USA, 2008–2009*

Characteristics	OptoTherm, n = 2,507 patients	FLIR, n = 2,515 patients	Wahl, n = 2,061 patients	Self-reported fever, n = 2,389 patients
Mean temperature, °F (SD)	94.3 (1.26)	95.7 (1.38)	89.4 (2.56)	–
Optimal fever threshold, °F	95.3	96.4	89.3	–
Fever (oral temperature ≥100°)				
No. (%) identified as febrile by each method	275 (11.0)	247 (9.8)	577 (28.0)	404 (16.9)
Sensitivity (95% CI)	91.0 (85.0–97.0)	90.0 (84.0–97.0)	80.0 (76.0–85.0)	75.0 (64.4–85.6)
Specificity (95% CI)	86.0 (81.0–90.0)	80.0 (76.0–84.0)	65.0 (61.0–69.0)	84.7 (83.4–86.1)
Positive predictive value (95% C)	17.9 (13.6–22.2)	18.4 (13.7–23.0)	5.7 (4.1–7.3)	10.1 (7.4–12.8)
Negative predictive value (95% CI)	99.6 (99.3–99.8)	99.5 (99.1–99.7)	99.1 (98.6–99.5)	99.3 (98.9–99.6)
Febrile by either ITDS or self-report				
No. (%) identified as febrile by each method	597 (23.8)	586 (23.3)	793 (38.5)	–
Sensitivity (95% CI)	93.8 (87.8–99.7)	89.1 (81.4–96.7)	93.8 (87.8–99.7)	–
Specificity (95% CI)	78.0 (76.4–79.5)	78.4 (76.9–80.0)	63.3 (61.6–65.1)	–
Positive predictive value (95% CI)	9.0 (6.9–11.2)	8.8 (6.8–11.3)	5.6 (4.3–7.1)	–
Negative predictive value (95% CI)	99.8 (99.5–99.9)	99.7 (99.3–99.9)	99.8 (99.4–99.9)	–

*OptoTherm Thermoscreen (OptoTherm Thermal Imaging Systems and Infrared Cameras Inc., Sewickley, PA, USA), FLIR ThermoVision A20M (FLIR Systems Inc., Boston, MA, USA), and Wahl Fever Alert Imager HSI2000S (Wahl Instruments Inc., Asheville, NC, USA). CI, confidence interval; ITDS, infrared thermal detection system.

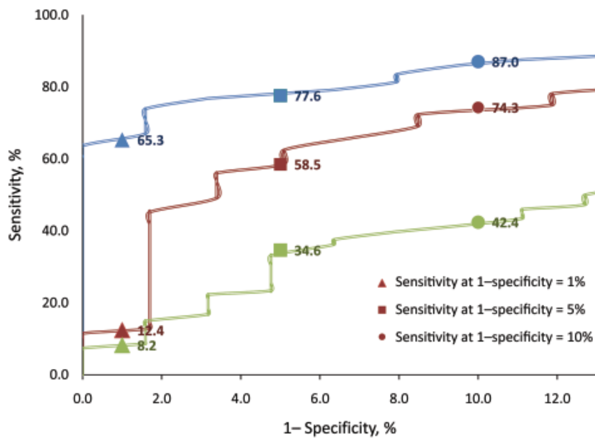

Figure 2. Enhanced view of receiver operating characteristic curves of 3 infrared thermal detection systems for detecting fever (oral temperature ≥100°F) showing sensitivities at false-positive rates (FPR) of 1%, 5%, and 10%. Red, FLIR ThermoVision A20M (FLIR Systems Inc., Boston, MA, USA); blue, OptoTherm Thermoscreen (OptoTherm Thermal Imaging Systems and Infrared Cameras Inc., Sewickley, PA, USA); and green, Wahl Fever Alert Imager HSI2000S (Wahl Instruments Inc., Asheville, NC, USA).

by previous research (*26–28*). We observed strong associations between ITDS and room temperatures. Similar observations with room temperatures and extended exposure to hot or cold environments have been reported (*22*,*25*,*29*,*30*). The unexpected association between higher temperature measurements (oral and OptoTherm) and recent antipyretic drug use may result from patients with higher fevers taking antipyretic drugs, inadequate antipyretic drug dosage, or both. The finding that men had relatively higher ITDS measurements than women has not been previously reported and may be because of differences in facial hair, use of cosmetics, or subcutaneous fat composition (*31*). Similar associations across multiple ITDS underscore the strength of these findings. By controlling for these covariates, we were able to measure the relationship between ITDS and oral temperatures with greater precision.

Although the sensitivity, specificity, and AUC of the devices we tested were similar to those found in previous studies, we observed a higher correlation between ITDS temperature measurements and confirmatory temperature measurements (*15*,*16*,*19–25*). Several factors may have contributed to these differences. The higher correlation between ITDS and body temperatures reported here may be related to the use of oral temperature measurement as reference. Although oral temperature measurements better reflect core temperatures than infrared tympanometric measurements, most previous investigations of ITDS have used the latter as reference (*19*,*23*,*24*,*32–35*). The preference for oral temperatures as reference is supported by an evaluation of methods for measuring body temperature conducted by the American College of Critical Care Medicine and the Infectious Diseases Society of America; researchers found that rectal temperatures were the most accurate of the peripheral thermometry methods, fol-

Table 2. Associations between temperature measurements by 3 infrared thermal detection systems and potential covariates, using bivariate analysis, among patients in 3 emergency departments, 2008–2009*

Characteristics	Oral thermometer, n = 2,873 patients	OptoTherm, n = 2,809 patients	FLIR, n = 2,314 patients	Wahl, n = 2,848 patients
Gender				
Male mean temperature, °F (SD)	97.85 (0.91)	94.36 (1.25)	95.77 (1.33)	89.52 (2.40)
Female mean temperature, °F (SD)	97.95 (0.87)	94.19 (1.27)	95.59 (1.40)	89.23 (2.73)
p value (*t* test)	0.002	<0.001	0.002	0.003
Age				
Correlation coefficient r	–0.12	–0.15	–0.10	–0.10
p value	<0.001	<0.001	<0.001	<0.001
Time of day				
Correlation coefficient r	0.08	0.24	0.19	0.27
p value	<0.001	<0.001	<0.001	<0.001
Antipyretic/analgesic use				
Yes (mean temperature °F) (SD)	97.96 (1.01)	94.39 (1.34)	95.76 (1.47)	89.47 (2.61)
No (mean temperature °F) (SD)	97.86 (0.81)	94.22 (1.20)	95.64 (1.32)	89.35 (2.54)
p value (*t* test)	0.003	<0.001	0.048	0.21
Room temperature				
Correlation coefficient r	0.01	0.19	0.19	0.19
p value	0.77	<0.001	<0.001	<0.001
Oral temperature				
Correlation coefficient r	–	0.43	0.42	0.14
p value	–	<0.001	<0.001	<0.001

*FLIR ThermoVision A20M (FLIR Systems Inc., Boston, MA, USA), OptoTherm Thermoscreen (OptoTherm Thermal Imaging Systems and Infrared Cameras Inc., Sewickley, PA, USA), and Wahl Fever Alert Imager HSI2000S (Wahl Instruments Inc., Asheville, NC, USA).

Table 3. Association between measured temperature, difference, and covariates, general linear regression with site-specific fixed effects in 3 emergency departments, USA, 2008–2009

Characteristics	Oral, n = 1,865 patients	OptoTherm, n = 1,851 patients	Difference (oral–OptoTherm)	FLIR, n = 1,360 patients	Difference (oral–FLIR)	Wahl, n = 1,856 patients	Difference (oral–Wahl)
Intercept (SE)	98.220 (0.936)	15.027 (3.467)	14.426 (1.337)	15.777 (4.150)	14.309 (1.559)	13.160 (6.541)	21.769 (2.489)
Variable in model*							
Oral temperature	–	0.701†	–	0.693†	–	0.645†	–
Male sex	–0.055‡	0.254†	–0.271†	0.237†	–0.260†	0.501†	–0.522†
Age	0.011‡	–0.160§	0.019§	–0.029§	0.034§	–0.001‡	0.005‡
Age squared	–0.0002§	0.0001‡	–0.0002‡	0.0003§	–0.0004§	–0.002‡	0.0001‡
Site¶							
Albuquerque	–0.498†	0.915†	–1.061†	–0.214§	0.058‡	4.256†	–4.431†
Atlanta	–0.309†	0.514†	–0.603†	0.302§	–0.399†	0.043‡	–0.149‡
Time of day	0.104§	0.156§	–0.126§	0.131§	–0.100‡	0.352†	–0.315†
Time of day squared	–0.003§	–0.004§	0.003‡	–0.003‡	0.002‡	–0.008§	0.007§
Antipyretic use	0.106§	0.137§	–0.106§	0.118*	–0.086‡	0.075‡	–0.039‡
Room temperature	–0.010‡	0.133†	–0.137†	0.160†	–0.162†	0.131†	–0.135†
Self-reported fever (No)	0.432†	0.148§	0.022‡	0.149‡	0.003‡	–0.115‡	0.264§

*FLIR ThermoVision A20M (FLIR Systems Inc., Boston, MA, USA), OptoTherm Thermoscreen (OptoTherm Thermal Imaging Systems and Infrared Cameras Inc., Sewickley, PA, USA), and Wahl Fever Alert Imager HSI2000S (Wahl Instruments Inc., Asheville, NC, USA). Value of β coefficient (β) for each variable in the model is listed in the columns.
†p<0.001.
‡Not significant (p≥0.05).
§p<0.05.
¶Referent site is Chicago.

lowed by oral, tympanic, and axillary temperature measurements, respectively (*32*).

Many types of ITDS are available, ranging from inexpensive hand-held point-and-shoot devices with laser sighting to hand-held cameras with light-emitting diode displays, wall-mounted cameras, and portable cameras on tripods such as the ones used in this study (*19*,*23*,*29*). To maximize potential efficacy, we evaluated technically advanced ITDS that were recently developed for human temperature detection. Other studies used more basic systems and did not compare different devices. Although the costs of the OptoTherm and FLIR were comparable at $22,000 and $16,000 per system, respectively, the Wahl was relatively less expensive ($8,000). Testing 3 different models at various price ranges allowed us to demonstrate substantial differences among ITDS. These differences are likely to affect their sensitivity and utility for fever screening. The systems used in this study require the person to stand in front of the camera for ≈2–3 seconds to capture a temperature. Other differences, such as moving persons, could have further affected the sensitivity of ITDS for fever detection.

Although addition of a health declaration form would allow screening to also consider recent travel history, previous fever, and other symptoms or illness exposures, health declarations have variable compliance rates and depend on a person's ability to understand questions and accurately assess symptoms as well as willingness to report (*12*,*13*,*36*,*37*). In our study, in which patients had no disincentive to report, we found that one fourth of febrile patients did not report having fever, which suggests true unawareness of fever among some persons. Only one tenth of those who reported having a fever were actually found to be febrile. Our results, therefore, probably underestimated the benefit of ITDS over self-reports of fever. In other settings, ill persons may be less likely to report symptoms for fear of adverse consequences such as travel delays, involuntary isolation of ill persons, or quarantine of exposed contacts. In settings such as travel sites (e.g., airports) and the workplace, ITDS could provide an objective means for the mass detection of fever as part of a comprehensive public health screening strategy because ITDS had greater accuracy than self-reports.

Mass health screening during a pandemic will certainly be influenced by several other factors, including perceived and actual pandemic severity, as well as the potential consequences of illness detection, either negative or positive, which can affect the sensitivity of screening that uses self-report. If being detected as febrile is perceived as harmful, travelers may hide their symptoms (*12*). Alternatively, during a pandemic with high mortality rates, incentives for reporting symptoms might be present, such as access to scarce antiviral medications and medical care. In both situations, a comprehensive screening approach may be necessary, which uses ITDS for fever screening and a health questionnaire to detect other symptoms or exposures that would increase specificity of the screening process. Finally, the usefulness of any infectious disease screening must take into account temperature fluctuations, use of antipyretic medications, transmission risks, prevalence of infections, and asymptomatic infections.

This study had several limitations. Measurement error resulting from variation in digital oral thermometer measurement and technique may have decreased the correlation between ITDS and oral temperature measurements (*38*). For FLIR and Wahl, varying readouts by different operators may have led to increased variability. This method, although necessary for direct temperature comparisons, may have decreased the accuracy of FLIR and Wahl. Use of alarm features as recommended by the manufacturers could minimize these differences but might lead to more false-positive results. In addition, unlike the other 2 devices, Wahl required calibration to ambient temperature once per day, but room temperatures varied within the day. We evaluated only systems submitted by manufacturers to the bid process, thus limiting the generalizability of our results to other devices.

To assess the sensitivity and specificity of different ITDS for fever detection and to determine their optimal thresholds, we validated each measurement by oral thermometry, which required a clinical setting. Thus, generalizability to settings such as airports and border crossings may be limited. Substantial delays to travelers and ethical concerns such as follow-up treatment made it impractical to conduct this study in an airport setting. In addition, although a few studies have examined screenings in airports, they confirmed temperature only in febrile persons, thus sensitivity and specificity of ITDS could not be established from such studies.

The sensitivity and specificity of screening by using ITDS are determined by the selected fever temperature cutoff, which tends to be 2–3 degrees lower than the standard fever threshold because of differences between skin and core temperatures. Increasing or decreasing sensitivity causes a reciprocal change in specificity. For example, lowering OptoTherm's threshold from the optimal 95.7°F to 94.5°F would achieve almost 100% sensitivity but would reduce specificity to 63.6% and increase the false-positive rate to 36.4%; to reach near 100% specificity with the OptoTherm by using cutoff of 100°F for ITDS, sensitivity decreases to 6.4%.

Maximizing accuracy by choosing the optimal cutoff with the highest sensitivity and specificity may not be practical in a real-world setting, considering the relative costs of false-positive and false-negative results. In settings where secondary evaluation is available or during a pandemic with high illness severity, ITDS temperature can be set at a lower cutoff to ensure fewer false negatives, each of which represents a potential public health threat. However, setting the cutoff to achieve very high sensitivity can result in many false positives, which could have adverse consequences to the population being screened (e.g., unnecessary travel delays, missed work) and increase the workload of staff who are conducting the screening. In settings where confirmatory testing may not be feasible or high costs may be associated with a false-positive result, a higher ITDS temperature cutoff may be preferable.

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Decrease in Shigellosis-related Deaths without *Shigella* spp.–specific Interventions, Asia

Pradip Bardhan, A.S.G. Faruque, Aliya Naheed, and David A. Sack

In 1999, a review of the literature for 1966–1997 suggested that ≈1.1 million persons die annually of shigellosis, including ≈880,000 in Asia. Our recent review of the literature for 1990–2009 indicates that ≈125 million shigellosis cases occur annually in Asia, of which ≈14,000 are fatal. This estimate for illnesses is similar to the earlier estimate, but the number of deaths is 98% lower; that is, the lower estimate of deaths is associated with markedly reduced case-fatality rates rather than fewer cases. *Shigella* spp.–related deaths decreased substantially during a period without *Shigella* spp.–specific interventions. We speculate that nonspecific interventions, e.g., measles vaccination, vitamin A supplementation, and improved nutrition, may have led to the reduced number of shigellosis-related deaths.

In 1999, Kotloff et al. reviewed the literature to estimate the global incidence of shigellosis. On the basis of studies published during 1966–1997, they estimated ≈1.1 million shigellosis-related deaths annually, resulting from ≈164.7 million cases. Of these, ≈163.2 million cases occurred in developing countries, ≈80% of which occurred in Asia (*1*). These high estimates of illness and death have increased interest in identifying interventions, including new vaccines, that might reduce these astonishing numbers (*2*–*5*).

Several changes have occurred that might have altered this incidence. Shigellosis might be increasing because of increasing populations in *Shigella* spp.–endemic areas; because of increasing resistance to antimicrobial drugs among shigellae, especially in *S. dysenteriae* type 1 (the Shiga bacillus) (*6*–*8*); or because of increasing rates of HIV infection and AIDS in many countries, which might be influencing shigellosis incidence. On the other hand, incidence might be decreasing because of improved nutrition in many countries, improved delivery of healthcare in some areas, and more widespread use of measles vaccine (*9*,*10*) and vitamin A supplementation (*11*), which might reduce the severity of intestinal infections. The availability of fluoroquinolones, often used without prescription, also might lead to changing treatment practices because families might use antimicrobial drugs earlier during diarrheal illness or for other illnesses (*12*).

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Shigellosis incidence might also have changed because of the overall reduction in diarrhea-related deaths through case management, including rehydration therapy and proper feeding (*13*). Because shigellosis is not primarily a dehydrating condition, hydration is not critical for patients with dysentery. Nonetheless, the consistent use of oral rehydration therapy for diarrhea may reduce illness from the persistent effects of repeated episodes of diarrhea, which is common in developing counties.

Thus, at the request of the World Health Organization, we reviewed the literature for 1990–2009 to estimate the current incidence of shigellosis. The earlier study by Kotloff et al. attempted to extrapolate from data from developing countries; however, most of the data were from Asia. Because the epidemiology of shigellosis may differ in Africa, we restricted our review to studies in Asian populations.

Materials and Methods

Our review comprised studies identified through Medline. The initial studies were identified by a computer search of the multilingual scientific literature published since 1990. Articles derived by using the keywords *Shigella*, dysentery, bacillary, and shigellosis were linked with

a set of other articles obtained by using the keywords incidence, prevalence, public health, death rate, mortality, surveillance, burden, distribution, and permutations of the root word epidemiol-. We conducted searches for each Asian country, except Japan and Israel. The resulting cross-linked set contained 319 articles, which we culled to 164 articles that were relevant to the goal of the search. Additional sources were located through consultations with experts in the field, proceedings of expert meetings, and the ongoing Diarrheal Diseases Surveillance Programme of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B). To enable comparison over time, we adopted the methods of the previous review on shigellosis (*1*).

We created an algorithm to estimate the number of *Shigella* infections that occurred each year in Asia. In a preliminary step, the world's population was divided into 4 age strata (0–11 months, 1–4 years, 5–14 years, and ≥15 years). Published rates of diarrhea for each of the 4 strata were used to estimate the diarrheal disease incidence. An increase in the severity of a patient's illness influences the proportion of diarrheal episodes attributable to *Shigella* spp. This correlation can be presumed to strengthen as the proportion of *Shigella* infections increases because sampling progresses from cases of diarrhea detected by household surveillance to those among outpatients to persons admitted to hospital (*14*). Thus, we subdivided the total diarrheal disease incidence into these 3 settings: estimates of mild cases in persons who stayed at home; more severe cases needing care at a clinic but not hospitalization; and cases requiring hospitalization.

The total number of diarrhea cases attributable to shigellosis was calculated for the <1-year and 1–4-year age groups by multiplying the number of diarrhea cases in the 2 settings (community and treatment facilities) by the percentage of diarrhea cases from which *Shigella* spp. were isolated (Table 1). For older children and adults, we calculated total cases by multiplying the median percentages of diarrhea cases attributable to shigellosis in persons 5–14 years and ≥15 years of age by the number of diarrhea cases in these 2 age groups according to clinical setting (Table 2).

We adopted the estimates of Kosek et al. (*15*) to calculate the number of diarrhea episodes per person per year within countries in Asia (Table 1). These estimates were based on the review of 13 longitudinal studies of stable populations in 8 countries in Asia, where active surveillance was conducted during 1984–1995.

We estimated the proportion of diarrheal episodes in each stratum that can be attributed to shigellosis by analyzing only studies in which surveillance was conducted since 1990 and that used microbiologic confirmations to report the percentage of *Shigella* spp.–related diarrhea cases for the specified age group. An overall median percentage of shigellosis was then calculated for each stratum and multiplied by the total number of diarrheal cases in the stratum to derive the number of shigellosis cases in each stratum. In addition to the median, a weighted mean with 95% confidence intervals (CIs) was calculated for these analyses by using Freeman-Tukey transformed proportions. Weights used were equal to the inverse standard errors of these transformed proportions (*18*,*19*). The numbers of shigellosis cases were added to give an overall estimate of shigellosis-related illness. Case-fatality rates (CFRs) for persons hospitalized with *Shigella* infection at the ICDDR,B hospital were used to calculate age-specific rates of *Shigella* spp.–associated death. This hospital treats >100,000 diar-

Table 1. Estimated annual number of diarrheal episodes in children 0–4 years of age, Asia, 1990–2009

Characteristic	Age group		
	0–11 mo	1–4 y	0–4 y
Total population, × 1,000	78,533	282,719	361,252
No. diarrheal episodes/child/y (*15*)	3.2	2.3	
Total diarrheal episodes, × 1,000	251,306	650,252	901,559
At home (*14,16*)	221,651	597,583	819,234
At treatment facility (*14,16*)	29,655	52,670	82,325
In outpatient department	25,884	51,370	77,254
Hospitalized	3,771	1,300	5,071
Median caused by shigellosis, %			
At home*			4.4%
In treatment facility†	5.8	9.4	
Weighted mean caused by shigellosis, % (95% confidence interval)			5.1 (4.0–5.7)
No. shigellosis cases			
At home, × 1,000			39,669
In treatment facility, × 1,000	1,720	4,951	6,671
Total, × 1,000			46,340
95% confidence interval of weighted mean, × 1,000			44,924–57,316

*Online Technical Appendix Table 1 (www.cdc.gov/EID/content/16/11/1718-Techapp.pdf).
†Online Technical Appendix Table 2.

Table 2. *Shigella* spp.–associated diarrhea in older children and adults, Asia, 1990–2009

Characteristic	Age group, y	
	5–14	≥15
Population (× 1,000)	742,911	2,833,857
Diarrhea episodes/person/y (*17*)*	0.65	0.50
Total diarrhea episodes, × 1,000	482,892	1,416,929
At home	473,234	1,388,590
Treatment facility (*1*)†	9,658	28,339
Median caused by *Shigella* spp., %		
At home*	4	4
At treatment facility†	11.6	10.7
Proportion caused by shigellosis (weighted mean), %		
At home*	4.6 (4.0–5.1)	
At treatment facility†	8.3 (7.7–9.0)	
Annual no. episodes of *Shigella* spp.–associated diarrhea, × 1,000		
At home	18,929	55,544
At treatment facility	1,120	3,032
Total no. episodes of *Shigella* spp.–associated diarrhea, × 1,000	20,049	58,576
95% confidence interval of weighted mean, × 1,000	19,673–24,898	57,726–73,057

*Online Technical Appendix Table 3 (www.cdc.gov/EID/content/16/11/1718-Techapp.pdf).
†Online Technical Appendix Table 4.

rhea patients annually and is the same hospital used for CFRs in the earlier study.

Illness was expressed as episodes of diarrhea per person-year from which shigellae were recovered. Studies were included in the death estimates if deaths caused by *Shigella* spp. could be ascertained through active surveillance. The review comprised prospective and retrospective studies but not studies based on vital statistics only. Death was considered to have been caused by diarrhea only if diarrhea was listed as the primary cause.

Results

Approximately 3,938,020,000 persons resided in Asia during 2005. This estimate included 78,533,000 infants <1 year of age and 361,252,000 children 1–4 years of age (*20*,*21*).

Shigellosis Incidence

The median frequency of *Shigella* spp. isolation from diarrheal cases in the community in children 0–4 years of age was 4.4% (range 3.1%–13.4%; weighted mean 5.1%, 95% CI 4.4%–5.7%). Because only 1 study broke this rate down into the <1-year and 1–4-year ranges, the median of the 2 values for the combined range was calculated. The median frequencies of *Shigella* spp. isolation rates from persons with diarrhea reporting to the treatment facilities were 5.8% (range 2.4%–9.3%) among children <1 year of age and 9.4% (range 2.4%–23.5%) among children 1–4 years of age. The weighted mean of the combined group was 6.6% (95% CI 6.0%–7.2%). Details of these studies are found in online Technical Appendix Tables 1, 2 (www.cdc.gov/EID/content/16/11/1718-Techapp.pdf).

Approximately 39,669,000 (weighted mean 45,980,000, 95% CI 39,669,000–51,389,000) shigellosis cases occurred in children <5 years of age in the community and 6,671,000 (weighted mean 5,433,000, 95% CI 5,256,000–5,927,000) in treatment facilities, totaling 46,717,000 (95% CI 44,924,000–57,316,000) cases among Asian children <5 years of age annually. The proportions of cases with shigellosis are detailed in online Technical Appendix Tables 1, 2.

The median percentage of diarrhea in the community was 4.0% (range 1.6%–13.5%; weighted mean 4.6%, 95% CI 4.0%–5.1%). The median percentages for patients treated at facilities were ≈11.6% (range 4.7%–17.3%) and ≈10.7% (range 4.1%–27%) respectively (weighted mean of the combined groups 8.3%, 95% CI 7.7–9.0%). (The proportions of shigellosis cases are detailed in online Technical Appendix Tables 3 and 4.) *Shigella* infections among children 5–14 years of age and persons ≥15 years of age were ≈20,049,000 (95% CI 19,673,000–24,898,000) and ≈58,576,000 (95% CI 57,726,000–73,057,000), respectively.

We combined the number of shigellosis episodes in all age groups. The total annual number of shigellosis cases in Asia was ≈125 million (95% CI 122 million–155 million).

Shigellosis-associated Deaths

Median CFRs for hospitalized shigellosis patients <1 year and 1–4 years of age and patients >5 years of age were 0.89%, 0.01%, and 0 respectively (Table 3), according to data from the ICDDR,B hospital surveillance program during 1990–2007. The weighted means for patients <1 and 1–4 years of age were 0.8% (95% CI 0.5%–1.0%) and 0.1% (95% CI 0.02%–0.25%), respectively. No deaths were reported from large studies in other countries in Asia. By using median CFRs from Bangladesh for these age groups, we determined that ≈1,960 shigellosis deaths occurred in

Table 3. Case-fatality rates for hospitalized patients with *Shigella* infections, Asia, 1990–2009

Country	Area	Year	Case-fatality rate by age group		
			0–11 mo	1–4 y	≥5 y
Bangladesh*	Urban/rural	1990–1999	2.73	1.42	0.33
Bangladesh†	Urban	2000–2008	1.26	0	0
Bangladesh‡	Rural	2000–2008	0.51	0.02	0
People's Republic of China (*22*)	Rural	2002	0	0	0
Thailand (*22*)	Rural	2000–2003	0	0	0
Indonesia (*22*)	Rural	2001–2003	0	0	0
Vietnam (*22*)	Urban/rural	2001–2003	0	0	0
Pakistan (*22*)	Rural	2002–2003	0	0	0
Bangladesh (*22*)	Periurban	2002–2004	0	0	0
Median			0.89	0.02	0
Weighted mean, % (95% confidence interval)			0.80 (0.59–1.03)	0.1 (0.02–0.25)	

*International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB,B) hospital surveillance, 1990–1999.
†ICDDR,B hospital surveillance (urban), 2000–2008.
‡ICDDR,B hospital surveillance (rural), 2000–2008.

Asia among hospitalized patients annually (Table 3). By using the 95% CIs, we estimated that the number of deaths ranged from 1,347 to 2,595.

A study from Bangladesh found that only 17.8% of shigellosis-related deaths occurred in treatment facilities; another study from the Gambia reported that only 12% of deaths associated with *Shigella* infection among children occurred in a health center (*23*,*24*). Thus, the true number of shigellosis-associated deaths may be 6–8× higher than deaths recorded in the hospital records. Hence, the estimates of the in-hospital shigellosis-associated deaths were multiplied by a factor of 7 in all age groups to correct for out-of-hospital mortality. This increased the number of deaths in all age groups to ≈13,720 shigellosis-related deaths across all the age groups per year in Asia (Table 4).

Discussion

Our review calculated that ≈125 million cases of endemic shigellosis occur annually in Asia, of which ≈14,000 (0.011%) cases result in death. Children <5 years of age are at highest risk for *Shigella* spp.–related illness and death. Although this estimate suggests that shigellosis incidence is substantial and similar to the earlier estimate, the updated death estimate is 98% lower than the estimate by Kotloff et al. (*1*) that used data primarily from the 1980s. Assuming that the population of Asia is ≈80% of the total population of the developing countries, ≈130 million *Shigella* infections and ≈880,000 deaths occurred in Asia according to the earlier estimate.

With such a large difference in estimated incidences, one estimate may be more accurate than the other. Alternatively, *Shigella* spp.–related deaths may have decreased substantially since the 1980s, even in the absence of specific interventions against shigellosis. We believe the latter explanation best explains the large difference in estimates of deaths.

The major variable that was lower in our calculations was the CFR for hospitalized patients, especially children. In the earlier estimate, a CFR of 11% was used from the ICDDR,B hospital (*24*). Recent data from the same hospital indicate the rate is now ≈0.01% overall and only 0.89% for the youngest age group. A recent estimate from Africa found a CFR of <1% during an outbreak associated with *S. dysenteriae*, suggesting that this low CFR may not be limited to Asia (*25*).

The decrease in CFRs could be explained by >1 factor. Case management might have improved, strains might be less virulent, or children might be healthier when they become infected and therefore have less severe complications. Case management in the hospital is unlikely to have changed substantially, and in fact the increasing resistance of current strains to antimicrobial drugs makes case man-

Table 4. Estimated annual number of deaths and case-fatality rates for hospitalized persons with *Shigella* infection, Asia, 1990–2009*

Characteristic	Age group	
	0–11 mo	1–4 y
No. persons with diarrhea, × 1,000†	3,771	1,300
No. persons with *Shigella* infection, × 1,000 (% total persons with diarrhea)†	219 (5.8)	122 (9.4)
No. *Shigella* spp.–related deaths (95% CI)	1,949 (1,292–2256)	24 (24–305)
Case fatality rate, %‡	0.89	0.01
Corrected no. *Shigella* spp.–related deaths, × 1,000 (95% CI)§ (*23*,*24*)	13,643 (9,044–15,792)	168 (168–2,135)
Total no. *Shigella* spp.–related deaths (95% CI)	13,811 (9,212–17,927)	

*CI, confidence interval.
†Table 1.
‡Table 3.
§Corrected for out-of-hospital deaths.

agement more difficult. Case management in the home may have changed, however, because antimicrobial drugs are widely available, and families may purchase effective antimicrobial drugs, e.g., ciprofloxacin, and begin treatment earlier in the course of the illness (*12*). Virulence of infecting strains could be lower; infections with S. *dysenteriae* type 1 are unusual. During past epidemics with this serotype, however, the CFR for *S. flexneri* was as high as it was with *S. dysenteriae* type 1 (*24*). Thus, virulence is unlikely to explain the decrease in the number of deaths.

Improved health of children who become infected appears to best explain the decreased CFR. Nutritional status of children in Bangladesh has continued to improve slowly (*26*). Perhaps more essential is the high proportion receiving measles vaccine and vitamin A (*27*). Anecdotally, in children dying of shigellosis during the 1980s, postmeasles dysentery was often diagnosed, and measles increased the severity of diarrhea, including shigellosis (*9*,*10*). Measles with dysentery is rarely seen now in Bangladesh.

Our review has some limitations. Although we reviewed all available published data on shigellosis in Asia since 1990, few sites conduct active surveillance for this infection, and only one estimates CFRs. With this large population, extrapolating accurately to the entire continent might not be possible. Nevertheless, the same methods were used in this and the earlier review. The large multicenter study on shigellosis in Asia did not record any deaths, suggesting that fatalities from shigellosis are not common (*22*).

Second, the review included only Asia, and the situation in Africa is possibly (even likely) different (*28*). The higher rates of HIV infection and AIDS and malaria, different nutritional deficiencies, different rates of measles vaccination, and different health systems and civil disturbance might suggest higher *Shigella* spp.–related deaths in Africa. Unfortunately, until recently, no long-term surveillance for diarrhea has existed in Africa on which to base estimates.

Third, the data in the review were based on microbiologic diagnosis of *Shigella* infections. Although isolation of *Shigella* spp. from fecal samples is the most specific diagnostic test for shigellosis, the culture method has limited sensitivity because of the relatively fastidious nature of the organism. Adoption of improved specimen transport methods and newer and more sensitive molecular laboratory diagnostic methods (e.g., PCR) reportedly having high sensitivities may detect more infections (*29*) but is unlikely to alter the death estimates.

The remarkable 98% decrease in deaths from shigellosis in the absence of a *Shigella* spp.–specific intervention suggests that other nonspecific interventions have helped to lower *Shigella* spp.–specific deaths. These, we believe, include measles vaccine, vitamin A supplements, and overall improvement in nutrition. Although the ready availability of antimicrobial drugs encourages the development of antibiotic drug resistance because of frequent abuse, we cannot rule out the possibility that the rapid availability of these antibiotics (especially fluoroquinolones) also have benefited children with dysentery who may be receiving treatment more quickly than they previously did.

The findings from our review may provide lessons regarding other infectious diseases. Approximately 50% of deaths among children <5 years of age have malnutrition as an underlying cause (*30*). Also, malnutrition and infection are clearly related, with one leading to the other. By reducing rates of other common infections, e.g., measles, and improving the nutritional status, including micronutrient nutrition, of children, diseases from other infections, such as *Shigella* spp., may decrease.

The 4 species and numerous serotypes of *Shigella* spp. are a challenge for vaccine developers, but shigellosis remains high, and increasing resistance to antibiotic drugs continues to make treatment difficult. An effective *Shigella* spp. vaccine may have substantial benefits, but our study suggests that *Shigella* spp.–related deaths can be, and have been, substantially reduced with currently available interventions and that such interventions do not need to be *Shigella* spp. specific.

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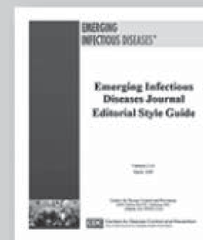

Measles Virus Strain Diversity, Nigeria and Democratic Republic of the Congo

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We investigated the genetic diversity of measles virus (MV) in Nigeria (2004–2005) and the Democratic Republic of the Congo (DRC) (2002–2006). Genotype B3 strains circulating in Kinshasa, DRC, in 2002–2003 were fully replaced by genotype B2 in 2004 at the end of the second Congo war. In Nigeria (2004–2005), two genetic clusters of genotype B3, both of which were most closely related to 1 variant from 1998, were identified. Longitudinal analysis of MV strain diversity in Nigeria suggested that only a few of the previously described 1997–1998 variants had continued to circulate, but this finding was concomitant with a rapid restoration of genetic diversity, probably caused by low vaccination coverage and high birth rates. In contrast, the relatively low genetic diversity of MV in DRC and the genotype replacement in Kinshasa reflect a notable improvement in local measles control.

Despite >90% reduction in the annual measles mortality rate in the World Health Organization (WHO) African Region during 2000–2006 (*1*), measles remains a major cause of deaths in children in sub-Saharan Africa (*2*,*3*). During this period, routine coverage of measles-containing vaccines increased from 56% to 73% in this region, and >200 million children were vaccinated through supplementary immunization activities (SIAs) by December 2004 (*4*). In the Democratic Republic of the Congo (DRC), vaccination coverage with a first dose of measles vaccine increased from 46% in 2000 to 70% in 2005, according to official country reports (*5*). The first major catch-up campaigns were conducted in several provinces in 2002 (Kasaï Oriental, Nord Kivu) and 2004 (Kasaï Occidental, Maniema, Katanga, Maniema, Sud-Kivu) (*6*). In Nigeria, no SIAs took place until 2005, and routine vaccination coverage was persistently low (<40%), at least until 2006 (*3*,*5*,*7*).

Molecular epidemiology has proven to be a major component of measles surveillance because it enables the effect of accelerated measles control activities to be assessed and the elimination of endemic virus strains to be documented. In Africa, indigenous measles virus (MV) genotypes seem to have a distinct geographic distribution (*8*,*9*). In the central and western parts of sub-Saharan Africa, mainly clade B viruses have been identified (*10*–*17*). The most common genotype is B3, with its 2 clusters B3.1 and B3.2 (*10*). The first B3 sequences in Africa were reported from Nigeria (1997–1998). The 41 MV isolates collected in southwestern Nigeria clustered in 2 distinct subgroups of genotype B3 (B3.1 and B3.2), with an unprecedented maximal sequence diversity of 4.6% in the C-terminus of the MV nucleoprotein hypervariable region (MVN-HVR) (*10*).

In the eastern and southern parts of Africa, genotypes D2 and D4 dominated and a new genotype (D10) was detected in Uganda in 2000 (*8*,*9*,*18*). Although MV sequence data from Africa have been greatly expanded since characterization of the first endemic strains was reported (*8*,*10*,*17*,*19*), essential genetic baseline information is still missing from many countries (*20*). For instance, from DRC, only 5 genotype B3 sequences have been reported from Kinshasa (2000) (*15*). We characterized MV strains collected during 2002 through 2006 from different locations throughout DRC and Nigeria. A comparison of the genetic diversity of MV strains showed notable differences

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in epidemiologic patterns in both countries that can be only partially explained by differences in vaccination practices.

Materials and Methods

Clinical Specimens and RNA Extraction

Clinical specimens from 84 patients with suspected measles were collected in different healthcare centers of the National Measles Surveillance Network in DRC during 2002–2006. Samples from Kinshasa (n = 53) were collected in 14 of the 35 local health districts. Additional specimens were obtained from 5 other provinces of DRC: Bas-Congo (n = 15), Kasaï Oriental (n = 6), Nord-Kivu (n = 2), Sud-Kivu (n = 3), and Maniema (n = 5).

In Nigeria, clinical specimens were collected from patients with suspected measles in Oyo (n = 16), Lagos (n = 12), Adamawa (n = 17), Borno (n = 1), and Sokoto (n = 4) states during 2004 and 2005. Most samples were from hospitalized patients, but those from Adamawa State were obtained during home visits.

Clinical sample collection and MV isolation on Vero-SLAM cells were performed as recommended by WHO (*21*). Specimens used for RNA extraction included throat swabs (n = 75), oral fluid (n = 19), MV culture supernatant (n = 19), serum (n = 12), dried blood (n = 5), urine (n = 2), and peripheral blood leukocytes (n = 2). Total RNA was extracted from 140 µL of body fluids, eluted swab specimens, or virus culture supernatant by using the QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany). Most measles cases were also serologically confirmed by measles-specific immunoglobulin M detection by using a commercial ELISA (Enzygnost anti-Measles IgM; Dade-Behring, Marburg, Germany).

Reverse Transcription–PCR and Sequencing

Specific cDNA of MV nucleoprotein was synthesized by reverse transcription by using SuperscriptIII Reverse Transcriptase (Invitrogen, Merelbeke, Belgium) and random hexamers (Invitrogen). MV cDNA was amplified by nested PCR by using primers MN5 (nt 1113–1134, 5′-GCCATGGGAGTAGGAGTGGAAC-3′ [*22*]) and MN6 (nt 1773–1754, 5′-CTGGCGGCTGTGTGGACCTG-3′ [*22*]) for the first round and primers Nf1a (nt 1199–1224, 5′-CGGGCAAGAGATGGTAAGGAGGTCAG-3′) and Nr7a (nt 1725–1703, 5′-AGGGTAGGCGGATGT TGTTCTGG-3′) for the second round. Both PCRs were performed in a total volume of 25 µL that contained 1.8 mmol/L $MgCl_2$, 1× PCR buffer, 0.2 mmol/L dNTPs, 0.5 U Platinum Taq (Invitrogen), and 0.8 µmol/L forward and reverse primer (Eurogentec, Seraing, Belgium). One microliter of cDNA or 5 µL of first-round product (diluted 50× in water) was added as template. Cycling conditions were initial denaturation at 94°C for 2 min; 35 (first round) or 30 (second round) cycles of amplification at 94°C for 30s, 55°C (first round) or 58°C second round) for 1 min, and 72°C for 1 min; and a final extension at 72°C for 5 min.

Nested PCR products were purified by using the Jetquick PCR product Purification Spin Kit (Genomed, Lohne, Germany). Twenty-five cycles of cycle sequencing (2-min elongation) were performed by using a BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Nieuwerkerk, the Netherlands) with Nf1a or Nr7a primers (0.5 µmol/L) and 10 ng of purified PCR product. Cycle sequencing products were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences were aligned by using ClustalW (*23*), and phylogenetic trees were constructed by using the neighbor-joining method (Kimura 2-parameter) and MEGA4 software (*24*). All new sequences were submitted to GenBank under accession nos. FN985102–FN985162.

Results

Kinshasa, DRC, 2002–2003

Eight MV strains collected in Kinshasa during September 2002–January 2003 were assigned to genotype B3.1 on the basis of their MVN-HVR sequences (Figure 1). These strains were obtained at the peak of a large epidemic that occurred in Kinshasa during January 2002–December 2003 (*25*). Their sequences differed by only 1 or 2 nt from earlier genotype B3.1 variants found in Kinshasa and Brazzaville, Congo, in 2000 (*15*), which suggests that MV continuously circulated in Kinshasa during 2000–2003, and that the overall genetic diversity of strains throughout this outbreak was relatively low (0.4% in the MVN-HVR).

Kinshasa, 2004–2006

In 2005, another large measles epidemic with >36,000 reported cases and >400 deaths occurred in Kinshasa. Increasing numbers of cases were reported from the last quarter of 2004 until the epidemic peak was reached in epidemiologic week 36 in 2005 (Figure 2). Thereafter, the incidence steadily decreased and fewer cases were reported in the beginning of 2006. Variable numbers of cases were reported from the 35 health districts throughout 2005 (Figure 2), but the case distribution over time suggested 1 large epidemic with a variable effect in the different health districts in the capital of DRC rather than a series of smaller epidemics, as was suggested for the outbreak in 2002–2003 (*25*).

MVN-HVR sequences were obtained from 45 strains collected in 14 of the 35 health districts of Kinshasa during December 2004–February 2006. All 45 viruses belonged to genotype B2, and essentially 3 sequence variants (B2KIN-A, B2KIN-B, and B2KIN-C; Figure 1) were identified. Eleven viruses collected during December

2004–May 2005 had identical sequences in the MVN-HVR (variant B2KIN-A). Five strains collected during February–May 2005 corresponded to the second variant (B2KIN-B), differing by only 1 nt from B2KIN-A in the same gene segment. The third sequence variant (B2KIN-C) was first detected in May 2005 and continued to circulate at least until January 2006. This variant differed by 1 and 2 nts from B2KIN-B and B2KIN-A, respectively, and was identified in 18 strains collected during this period. Eleven other sequence variants were obtained, each from 1 patient, and all but 1 were collected after the peak of the epidemic. Their sequences differed by 1 to 3 nts from B2KIN-C and by 2–5 nts from B2KIN-A and B2KIN-B (Figure 1). Thus, 2 different MV genotypes, B3 and B2, were associated with 2 consecutive measles epidemics in Kinshasa, suggesting that MV circulation had been temporarily interrupted during the intraepidemic phase in 2004.

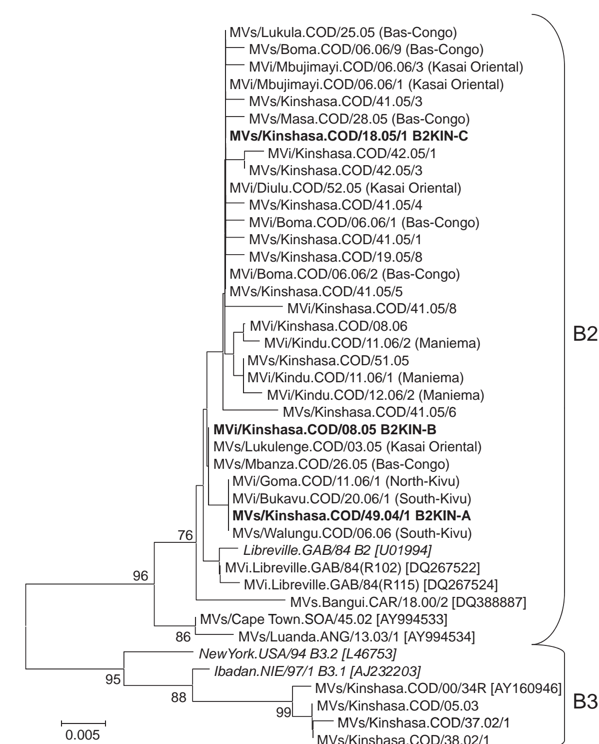

Figure 1. Phylogenetic tree including genotype B2 and genotype B3 of measles virus (MV) strains from the Democratic Republic of the Congo 2000–2006, and World Health Organization (WHO) reference strains (*italics*) of the corresponding genotypes and some other genotype B2 strains available in GenBank (accession numbers in brackets). MV strains were named according to WHO nomenclature: MVi/City of isolation.Country/epidemiologic week.year of isolation/isolate number. Sequences obtained from RNA extracted from isolates (MVi) or clinical material (MVs) were distinguished. The main genotype B2 variants from Kinshasa (B2KIN-A, B2KIN-B, and B2KIN-C) are indicated in **boldface**. Except for B2 strains from Kinshasa, the provinces of Democratic Republic of the Congo where strains were collected are indicated in brackets. The phylogenetic tree was calculated on the basis of the 450-nt region that codes for the C-terminus of the MV N protein by using MEGA4 software (*24*) and the neighbor-joining method (Kimura 2-parameter, 1,000 bootstraps). Scale bar indicates nucleotide substitutions per site.

Other DRC Provinces, 2005–2006

Genotype B2 strains detected during 2005 and 2006 in Kasaï Oriental, Bas-Congo, and Maniema provinces were most closely related to those strains identified during the same period in Kinshasa (Figure 1). MV cases from these regions thus seemed to be epidemiologically linked to the epidemic in Kinshasa. All 5 sequences obtained from North and South Kivu during February–May 2006 were identical to B2KIN-A, which was last identified ≈8 months earlier in Kinshasa. In the absence of any information on measles incidence in North and South Kivu during 2004–2006, one may speculate that B2-KIN-A strains could have been imported from the east into Kinshasa in 2004 but continued to circulate at the same time in this region bordering Rwanda. Alternatively, B2-KIN-A strains from Kinshasa might have been introduced into the region around Lake Kivu at an early stage of the epidemic because they were not detected after May 2005 in Kinshasa.

Nigeria, 2004–2005

The MVN-HVR sequence was obtained from 58 viruses collected in 5 states in Nigeria (Oyo, Lagos, Adamawa, Borno, Sokoto) during 2005 and 2006. All sequences were identified as genotype B3 and grouped in 2 separate clusters (Figure 3) with a minimal genetic distance of 5 nts (1.1%) between clusters 1 and 2. Sequence variants of cluster 1 were mainly (81.3%) found in the southwestern states (Oyo, Lagos), whereas most cluster 2 strains (70.6%) were from northern and northeastern states (Adamawa, Borno, Sokoto). Compared with MV sequences obtained in Nigeria during 1997–1998 (*10*), both clusters were most closely related to 1 particular strain (MVi/Ibadan.NIE/7.98/3).

The maximal sequence diversity identified in Lagos, Ibadan, Yola, and Mubi ranged from 1.8% to 2.9%, despite collection of specimens in each city within a period of <2 months. Seventeen sequence variants, differing by 1–11 nts (0.22 to 2.4%) from each other, were distinguished among 28 MV strains collected in January and February 2004 in Lagos (2.2% diversity; Lagos State) and Ibadan (2.2% diversity; Oyo State) (Figure 3).

Ten sequence variants differing by 1–13 nts (0.2%–2.9%) were identified among 17 strains collected in the northeastern Nigeria (Adamawa State) in June 2005. Eight of these sequence variants were detected in 1 city (Yola) during a period of only 2 weeks (2.9% diversity). Another cluster 2 strain (MVs/Maiduguri.NIE/02.05/1), which had a minimum of a 3-nt difference with strains from Adamawa State, was identified a few months earlier in neighboring Borno State. Finally, 4 additional sequence variants (2.7%

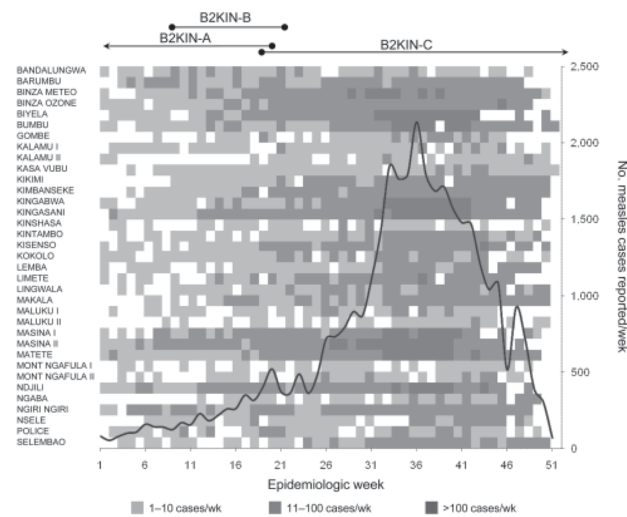

Figure 2. Epidemic curve of measles epidemic in Kinshasa, Democratic Republic of the Congo, 2005. Numbers of reported measles cases per week are shown by epidemiologic week, and measles incidence per week in the 35 health districts of Kinshasa is illustrated by gray shading. The periods during which the main genotype B2 variants (B2KIN-A, -B, and -C) were identified in Kinshasa are indicated above the epidemic curve.

diversity) were obtained during January 2005 in Sokoto State in northwestern Nigeria. Thus, multiple lineages of genotype B3 were identified in Nigeria, similar to the situation in the late 1990s, indicating that measles continued to be highly endemic during the study period.

Discussion

This study compared the genetic diversity of MV in 2 countries in Africa of similar size during a similar period. In Nigeria, all viruses belonged to the same genotype (B3.1) and were most closely related to viruses found in the same country in 1997–1998 (Figure 3) (*10*), which suggests an uninterrupted endemic transmission of MV in Nigeria during 1997–2005. MV strains from 2004–2005 formed 2 clusters within subgroup B3.1, both of which were most closely related to 1 particular variant identified in Nigeria in 1998 (MVi/Ibadan.NIE/7.98/3). No B3.2 strains or descendants of most other B3.1 variants from 1997–1998 were detected in 2004–2005, even in samples from the same cities (Ibadan, Lagos) (Figure 3). In 1997–1998, we observed a genetic diversity of 3.1% in Lagos and 4.2% in Ibadan. The phylogeny of the 2004–2005 strains from Nigeria suggests that the 2004–2005 diversity was not caused by multiple chains of transmission sustained since 1997–1998, but by only a few chains of transmission paired with a rapid restoration of a high genetic diversity (2.2% in both cities). Epidemiologic bottlenecks that reduce virus diversity include reduction of susceptible persons, low population size and density, and seasonality of the disease (*15*,*26*–*28*), but the factors that promote viral diversity are less well understood. In Nigeria, the population density is high (e.g., ≈24,000 inhabitants/km² in Lagos in 2005) and vaccination coverage was consistently low at ≈40% throughout the study period (*3*,*5*,*7*). Furthermore, measles incidence declined suddenly at the start of the rainy season (J.R. Kremer, pers. comm.), as was reported for other countries in sub-Saharan Africa (*26*).

The longitudinal analysis of MV strain diversity in Nigeria confirmed that even in populations in which measles is highly endemic, periods with low measles incidence

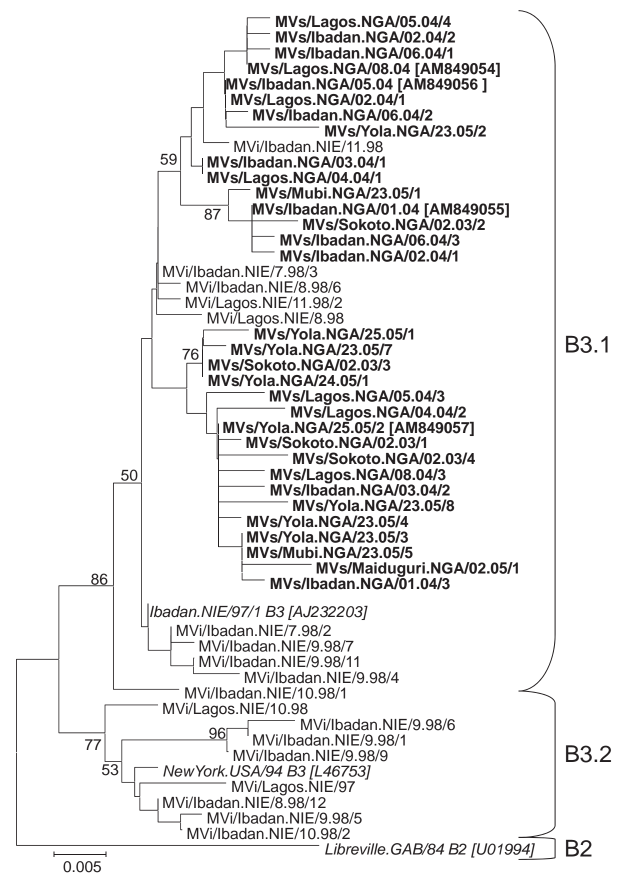

Figure 3. Phylogenetic tree including genotype B3 strains of measles virus (MV) from Nigeria collected in 1997–1998 and 2003–2005 (**boldface**) and World Health Organization (WHO) reference strains of genotypes B3.1, B3.2, and B2 (*italics*). Measles strains were named as indicated in the legend to Figure 1. For all strains from 2003–2005, which have been published, the GenBank accession number is given in brackets. For all strains from 1997–1998, NIE had been used as a 3-letter code for the country (*10*). For the more recent strains, the official WHO 3-letter code NGA was used. The phylogenetic tree was calculated on the basis of the 450-nt region that codes for the C-terminus of the MV N protein by using MEGA4 software (*24*) and the neighbor-joining method (Kimura 2-parameter, 1,000 bootstraps). Scale bar indicates nucleotide substitutions per site.

must occur, during which only a few transmission chains are sustained. The rapid restoration of a high genetic diversity was probably caused by low vaccination coverage, high birth rates and population density, and perhaps MV importation from neighboring countries. Phylogenetic comparison of genotype B3 strains from Nigeria with those from other countries in Africa suggests that transmissions of MV between different countries in Africa are frequent and that the diversity of this genotype in Nigeria reflects the overall genetic diversity of B3 in Africa (Figure 4). In contrast, a noticeably lower genotype B3 strain diversity was found in Sudan during 1997–2000 (1.3% in MVN-HVR) and in Burkina Faso in 2001 (1.5%); these findings were attributed to a higher vaccination coverage or more limited cross-border movement (in the case of Sudan) (*15*,*17*). On the other hand, MV strain diversity in Nigeria was similar to that in the People's Republic of China (1995–2003), where multiple lineages (5.3% diversity in MVN-HVR) of

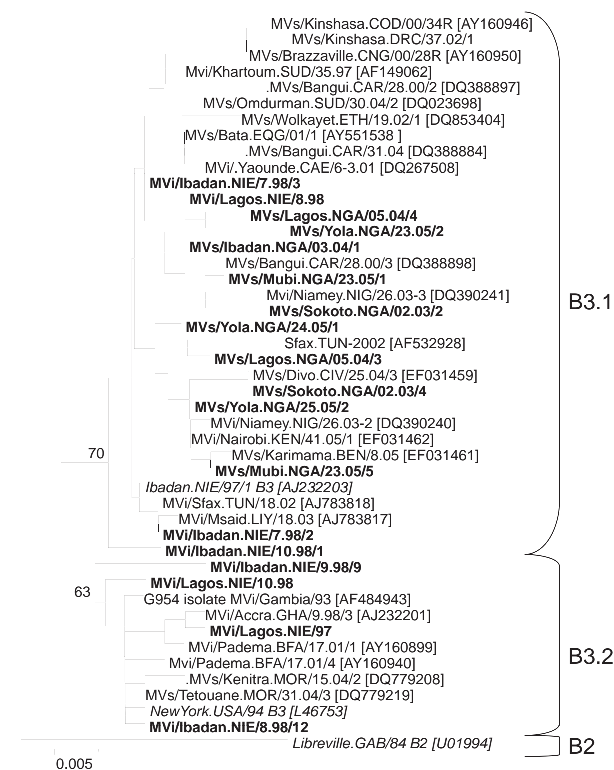

Figure 4. Phylogenetic tree showing a comparison of genotype B3 strains of measles virus (MV) from Nigeria 1997–1998 and 2003–2005 (**boldface)** and representative genotype B3 strains from other countries in Africa available in GenBank (accession numbers in brackets) and World Health Organization (WHO) reference strains of genotypes B3.1, B3.2, and B2 (*italics*). Naming of MV strains and tree calculation were performed on the basis of the 450-nt region that codes for the C-terminus of the MV N protein by using MEGA4 software (*24*) and the neighbor-joining method (Kimura 2-parameter, 1,000 bootstraps). Scale bar indicates nucleotide substitutions per site.

1 genotype (H1) co-circulated without obvious geographic restriction (*29*).

In contrast to the situation in Nigeria, where the co-circulation of different B3 viruses continued for years, in Kinshasa, MV circulation was apparently interrupted. The B3 lineage that caused the outbreak in 2002–2003 and that had been detected in 2000 (*15*) in Kinshasa had disappeared in 2004. Genotype B2 strains, which caused another large epidemic in Kinshasa ≈1 year later, seem to have fully replaced genotype B3 strains. It is commonly believed that ≥95% of a population needs to be immune to interrupt MV transmission (*30*). Because measles vaccination coverage in Kinshasa was suboptimal (<80%, Ministry of Health, DRC) additional epidemiologic constraints must have led to the observed genotype replacement (*31*). The emergence in Germany of genotype D7, which had gradually replaced the indigenous genotypes C2 and D6 during 1999–2001, raised the question of whether genotype D7 strains had a selective advantage with respect to cross-neutralizing antibodies acquired through vaccination or natural infection with other genotypes (*22*).

It has been shown in stochastic models that the likelihood of genotype replacement increases with vaccination coverage and frequency of virus importation, but that it may also occur by chance (*28*). Interestingly, the time point of genotype replacement in Kinshasa coincides with the political reunification of DRC, after the country had been divided into 4 self-governed regions during the second Congo war (1998–2003). The emergence of genotype B2 in Kinshasa may thus have been caused by the massive influx of persons from other provinces where genotype B2 was circulating. Identification of B2KIN-A strains in north-eastern DRC (North and South Kivu provinces) in 2006 and detection of B2KIN-B strains in neighboring Rwanda during late 2005 (S. Smit, pers. comm.) is compatible with an importation of genotype B2 from the eastern region of DRC into Kinshasa. On the other hand, B2 strains identified in other provinces seemed to be derived from the epidemic strains in Kinshasa. Epidemics in Bas-Congo and Kasaï Oriental did not start until ≈1 year after the steady increase in measles incidence in Kinshasa, although they were connected to the capital by much frequented roads. Thus, a vaccination campaign during the early outbreak may have prevented many measles cases and deaths not only in Kinshasa, but also in the neighboring provinces, as has been suggested (*32*–*34*).

Genotype B2 was first reported from Gabon in 1984 (*20*) with no similar virus being detected for >15 years. Therefore, genotype B2 was considered inactive, until recent variants of this genotype were found in the Central African Republic (2000), South Africa (2002), and Angola (2003) (*13*,*35*). Viruses obtained from these 3 countries had a minimum genetic distance of 6–8 nts in the MVN-

HVR compared with the closest variant (MVi.Libreville. GAB/84[R102]) obtained from Gabon almost 20 years earlier. The B2KIN-B variant differed by only 1 nt from the closest strain identified 20 years earlier in Libreville (Gabon, Figure 1). Thus, several lineages of genotype B2 have probably circulated continuously in central and southern Africa since at least the 1980s, but were never reported because of suboptimal molecular surveillance. Genotype B2 may have circulated in DRC and perhaps even in Kinshasa long before it was first detected in 2004. No MV sequences were available from DRC before this study, except for a few B3 strains from Kinshasa, 2000 (*15*). However, even if we cannot exclude the possibility that genotype B2 was already present during the 2002–2003 epidemic in Kinshasa, B3 seemed to predominate at that time.

Conclusions

The difference in genetic diversity of MV in DRC and Nigeria is consistent with the level of disease control in both countries during the study period (*5*). In Nigeria, genotype B3 has circulated continuously, at least during 1997–2005. Although transmission of most lineages from 1997–1998 had apparently been interrupted, the genetic diversity observed in 2004–2005 was notable, suggesting that the genetic diversity of MV can rapidly increase in settings with low vaccination coverage and high birth rates.

In DRC, MV circulation has probably decreased because of a notable increase in routine vaccination coverage and SIAs in several provinces. However, the emergence of genotype B2 in 2004–2006 showed that measles incidence can rapidly rise in settings with high birth rates and massive migration of large populations. Of all countries in Africa, DRC and Nigeria reported the largest numbers of measles cases (12,461 and 9,960) in 2008 and the highest average annual measles incidence per 100,000 population during 2005–2008, despite the vaccination of >18 million (DRC) and 60 million children (Nigeria) during recent SIAs (2006–2008) (*7*). Sequence analysis of more recent MV strains from Nigeria and DRC is warranted to evaluate whether these SIAs had any effect on MV strain diversity in both countries.

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Dr Kremer is technical supervisor of the WHO Regional Reference Laboratory for Measles and Rubella in Luxembourg. His main research interest is the genetic and phenotypic diversity of measles virus.

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Outbreaks of Pandemic (H1N1) 2009 and Seasonal Influenza A (H3N2) on Cruise Ship

Kate A. Ward, Paul Armstrong, Jeremy M. McAnulty, Jenna M. Iwasenko, and Dominic E. Dwyer

To determine the extent and pattern of influenza transmission and effectiveness of containment measures, we investigated dual outbreaks of pandemic (H1N1) 2009 and influenza A (H3N2) that had occurred on a cruise ship in May 2009. Of 1,970 passengers and 734 crew members, 82 (3.0%) were infected with pandemic (H1N1) 2009 virus, 98 (3.6%) with influenza A (H3N2) virus, and 2 (0.1%) with both. Among 45 children who visited the ship's childcare center, infection rate for pandemic (H1N1) 2009 was higher than that for influenza A (H3N2) viruses. Disembarked passengers reported a high level of compliance with isolation and quarantine recommendations. We found 4 subsequent cases epidemiologically linked to passengers but no evidence of sustained transmission to the community or passengers on the next cruise. Among this population of generally healthy passengers, children seemed more susceptible to pandemic (H1N1) 2009 than to influenza (H3N2) viruses. Intensive disease control measures successfully contained these outbreaks.

During April 2009, pandemic (H1N1) 2009 (pandemic influenza) virus began to circulate worldwide. In Australia, public health efforts were initially focused on delaying the entry of the virus into the country. By May 24, 2009, a total of 14 cases had been identified nationally, 2 in New South Wales (NSW), and all were associated with international travel.

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On May 24, the Australian Quarantine Inspection Service reported that 6 passengers of a cruise ship had respiratory symptoms, and a point-of-care test showed positive influenza A virus results for all. The ship had departed from Sydney on a 10-day cruise in the Pacific Ocean on May 16 (cruise A) and stopped at 2 islands, neither of which had reported circulation of pandemic influenza virus. None of the sick passengers had been in countries known to be affected by this influenza strain in the week before boarding. Thus, with no reason to suspect that the pandemic strain was circulating on board, passengers were allowed to disembark in Sydney on May 25.

On May 25, the 4 available respiratory samples taken from sick passengers were quickly couriered to the South Eastern Area Laboratory Service (the major public health viral laboratory serving eastern Sydney) for influenza virus nucleic acid testing (NAT) by real-time reverse transcription–PCR (RT-PCR). Of these 4 samples, 2 were positive for pandemic influenza virus and 2 were positive for influenza A (H3N2) (seasonal influenza) virus.

In response, NSW Health requested that all passengers (1,963 from Australia and 7 from elsewhere) who were experiencing influenza-like illness (ILI) isolate themselves from healthy persons and that all asymptomatic passengers quarantine themselves for 7 days after disembarkation (or 7 days after onset of symptoms if they developed). This advice was communicated to passengers on the day of disembarkation through media alerts, the NSW Health website, and telephone information lines. Subsequently, passengers were contacted by telephone to ensure that they understood containment measures (how to prevent virus spread). Oseltamivir treatment (75 mg 2×/d for 5 days) was recommended for passengers or crew members with ILI (defined as $\geq$2 of the following: cough, fever, runny nose, or blocked nose) within 48 hours of onset and oseltamivir prophylaxis

(75 mg 1×/d for 10 days) for those in close contact with patients with laboratory-confirmed cases.

On May 25, all crew members were assessed for illness. Symptomatic members were isolated on shore, and the rest were given oseltamivir prophylaxis and continued to serve on the ship's next voyage (cruise B), which departed later the same day. Cruise B traveled along the northern coast of Australia for 7 days and made a short stop at Brisbane before returning to Sydney on June 1. To minimize the risk for infection, enhanced cleaning regimens were conducted before cruise B, and NSW Health sent a public health doctor on the cruise to conduct intense surveillance for symptomatic passengers and crew.

Outbreaks of influenza have previously been reported on cruise ships (*1–6*), but the circumstances and extent of transmission have not been well documented. The cocirculation of pandemic and seasonal influenza viruses on cruise ship A provided a unique opportunity to compare symptoms, severity, and attack rates of pandemic and seasonal strains. We describe our outbreak investigation, compare the epidemiology of the 2 influenza virus subtypes, and explore effectiveness of control measures.

Methods

Case Definition

We defined a confirmed influenza A case as illness in a cruise A passenger in whom influenza A virus was detected by NAT during the cruise or within 7 days after disembarkation (regardless of symptoms). A case of pandemic influenza was defined as illness in a person with positive RT-PCR results for that virus. Further subtyping was conducted for 44 of 100 patients with positive influenza A but negative pandemic influenza virus results by NAT; all had positive results for seasonal influenza virus. Consequently, we defined a case of seasonal influenza as illness in a person with positive influenza A virus results by NAT but negative pandemic influenza virus results and in whom influenza subtyping for seasonal influenza virus by RT-PCR either produced positive results or was not conducted. A primary case was defined as illness in the first person in a cabin to report ILI symptoms; a co-primary case, as illness in a person who reported symptom onset within 24 hours after a primary case; and a secondary case, as illness in a person whose symptoms developed ≥24 hours after symptom onset in the primary case-patient. Case-patients were considered infectious for 24 hours before and 7 days after symptom onset. For the childcare center investigation, children who remained asymptomatic throughout the cruise were considered susceptible to influenza infection at each childcare session attended. Children in whom ILI developed were considered susceptible before the infectious period began.

Case Detection

We obtained a list of the names, sex, dates of birth, nationality, contact details, and cabin numbers of all passengers and crew members on cruise A. We reviewed the cruise ship's medical records to find passengers who had sought treatment for ILI during cruises A and B. Isolated symptomatic passengers from cruise A were referred to nearby hospitals for testing. Quarantined asymptomatic passengers were asked to report if symptoms developed; if so, laboratory testing was conducted. Crew members and passengers on cruise B were asked to immediately report fever or respiratory symptoms to medical staff and were tested for influenza by at least 2 point-of-care tests taken >24 hours apart. In all 8 Australian states and territories, public health legislation requires diagnostic laboratories to report confirmed influenza cases to the jurisdictional health department (*7*). The names of influenza case-patients reported after completion of cruise A were checked against the ship's manifest.

Data Collection

Because the investigation was part of a public health control initiative, formal ethics committee review was not required. Experienced public health staff interviewed case-patients at the time of diagnosis and used a standardized questionnaire to determine symptoms, hospitalization status, and oseltamivir use. This information was entered into a statewide database. Passengers who shared a cabin with case-patients who had pandemic influenza were also interviewed about respiratory symptoms. Laboratory testing initially focused on identifying pandemic influenza cases by using the specific RT-PCR; samples determined negative for pandemic influenza virus by NAT were tested for influenza A (including seasonal influenza virus) several weeks after passengers had disembarked.

Approximately 6 weeks after disembarking, all 50 passengers who had had pandemic influenza were reinterviewed about the duration and severity of their illness. These passengers included 3 interstate residents who had been treated in NSW (and excluded 28 non–NSW case-patients as a convenience sample) and the 45 NSW case-patients who had seasonal influenza (excluding 55 non-NSW case-patients and 17 NSW case-patients for whom test results were not available at the time of interview). Ultimately, 62 cases of seasonal influenza were identified among NSW passengers; complete symptom data from 50 passengers who were interviewed at the time of diagnosis were recorded in the statewide database.

Childcare Center Investigation

On-board childcare activities were provided in 3 daily sessions (9:00 AM–10:00 PM) in 3 areas of the ship for 3 age groups: 3–6, 7–12, and ≥13 years of age. Because the

pandemic outbreak appeared to begin in and primarily affect children 3–6 years of age, the epidemiologic investigation focused on this group. Most childcare activities for this group took place in 1 room. We examined childcare attendance records for this group and, ≈6 weeks after disembarkation, interviewed the parents of all children in this group about symptoms, vaccination history, and composition of the traveling group. All specimens collected from childcare attendees were tested for pandemic and seasonal influenza subtypes.

Compliance Assessment

To assess compliance with isolation and quarantine recommendations, we interviewed all 66 households in which at least 1 person with pandemic influenza was isolated, 32 NSW households with at least 1 person with seasonal influenza, and 45 randomly selected quarantined NSW passengers. (NSW passengers were selected as a convenience sample.) Interviews were conducted by experienced public health interviewers who used a standardized questionnaire.

Laboratory Investigation

NAT detection of pandemic influenza virus was performed by using real-time RT-PCR with primers targeting the hemagglutinin gene of the pandemic influenza virus provided by the Centers for Disease Control and Prevention and following recommended protocol or by using an in-house pandemic influenza virus–specific real-time RT-PCR. Seasonal influenza virus was identified by using a 2-target RT-PCR containing primers targeting pandemic and seasonal influenza virus strains (Unité de Génétique Moléculaire des Virus Respiratoires, Institut Pasteur, Paris, France) or a commercial influenza A subtyping assay (*Easy*-Plex Influenza profile 6; AusDiagnostics, Sydney, NSW, Australia).

Statistical Analyses

We analyzed data by using Epi Info version 3.5.1 (www.cdc.gov/epiinfo). Relative risks were used to compare age (as a categorical variable split into 7 groups), sex, and place of residence. Fisher exact test results were used for cell sizes <5. A Mantel-Haenszel value of $p<0.05$ was considered significant. χ^2 tests were used to compare proportions. To compare the rates of pandemic and seasonal influenza infection in childcare attendees, the number of sessions a child attended while susceptible were summed, and cases per child-sessions at risk and exact Poisson confidence intervals were calculated. Nonoverlapping confidence intervals were considered significantly different.

Results

A total of 1,970 passengers and 734 crew members were on cruise A. Median age of passengers was 46 years (range 1–94 years), 57% were female, and most were from Australia (Table 1). Median age of crew members was 31 years (range 19–62 years), and most were born overseas (not in Australia). ILI developed in 13 (0.7%) passengers who sought medical attention during the cruise; and influenza A results from point-of-care testing were positive for 6. NAT of samples from persons who were sick during the cruise or during the 7 days after disembarkation showed positive pandemic influenza virus results for 76 (3.9%), positive seasonal influenza results for 98 (5.0%), and positive co-infection results for 2 (0.1%). ILI in the 7 days before disembarkation was reported by 15 (2.0%) crew members; NAT showed positive pandemic influenza results for 3 crew members and positive seasonal influenza results for none. These crew members were isolated on shore. The remaining 719 crew members were given oseltamivir prophylaxis and continued to work during cruise B; among these, 5 reported ILI (all within 24 hours of cruise B departing), and 3 had positive NAT results for pandemic influenza virus. Therefore, 20 (2.7%) crew members from cruise A reported ILI, and 6 (0.8%) of these had positive pandemic influenza test results; none had positive seasonal influenza test results. Given the relatively low attack rate for the crew, we focused further investigation on the passengers, among whom the attack rate for pandemic influenza was highest for children 3–6 years of age, followed by children 7–12 years of age. For seasonal influenza, the attack rate was similar among children in all age groups (Table 1).

Symptoms and Severity of Illness

In total, 2 (3%) patients with pandemic influenza and 8 (8%) patients with seasonal influenza were hospitalized ($p = 0.16$); none died. Among the 50 passengers with the pandemic strain and 50 with the seasonal strain who were interviewed, symptoms were similar, although coryza was reported significantly more often by those with pandemic influenza (Table 2). Duration of illness was similar for passengers with either strain, but a higher proportion of seasonal influenza patients reported that illness was severe enough to limit their activities.

Epidemiologic Investigation

According to date of symptom onset, the pandemic influenza outbreak began in the childcare center on May 18, which was 2 days after embarkation, and peaked on May 25, the final day of cruise A. The first reported seasonal influenza case was in an adult whose symptoms began on May 17; the second, seemingly unrelated, infection developed in a childcare attendee on May 21. The number of seasonal influenza cases also peaked on May 25 (Figure).

Childcare Center Investigation

Of the 48 passengers 3–6 years of age, 45 (94%) attended childcare. Among these 45 were 8 pairs of siblings.

Table 1. Demographics for passengers with influenza after 10-day cruise that departed Sydney, NSW, Australia, on May 16, 2009*

Demographic	No. (%) passengers, n = 1,970	Confirmed pandemic (H1N1) 2009, n = 78			Confirmed influenza A (H3N2), n = 100		
		No. (%)	RR (95% CI)	p value	No. (%)	RR (95% CI)	p value
Age group, y							
<3	13 (1)	0	0	1.00	1 (1)	1.52 (0.23–10.16)	0.67
3–6	48 (2)	20 (26)	17.43 (10.45–29.09)	<0.001	4 (4)	1.64 (0.62–4.36)	0.32
7–12	119 (6)	13 (17)	4.57 (2.40–8.69)	<0.001	6 (6)	1.00 (0.44–2.27)	0.99
13–18	114 (6)	2 (3)	0.73 (0.18–3.06)	1.00	5 (5)	0.87 (0.35–2.12)	0.75
19–35	369 (19)	18 (23)	2.04 (1.13–3.70)	0.020	19 (19)	1.02 (0.61–1.69)	0.95
36–65	1,046 (53)	25 (32)	Referent	–	53 (53)	Referent	–
>65	261 (13)	0	0.00 (undefined)	0.005	12 (12)	0.91 (0.50–1.70)	0.76
Sex							
M	842 (43)	35 (45)	Referent		47 (47)	Referent	
F	1,128 (57)	43 (55)	0.92 (0.59–1.42)	0.70	53 (53)	1.19 (0.60–1.2)	0.38
Residence							
NSW	1,135 (58)	47 (60)	Referent	–	62 (62)	Referent	–
Victoria	433 (22)	11 (14)	1.12† (0.72–1.74)	0.63	16 (16)	0.83† (0.56–1.24)	0.36
QLD	165 (8)	10 (13)			12 (12)		
SA	109 (6)	4 (5)			4 (4)		
WA	54 (3)	0			4 (4)		
ACT	39 (2)	3 (4)			1 (1)		
Tasmania	12 (1)	1 (1)			0		
NT	2 (0)	0			0		
Not Australia	7 (0)	2 (3)			1 (1)		
Unknown	14 (1)	0			0		

*Diagnosis received during 7-day period after the cruise. RR, relative risk; CI, confidence interval; –, not applicable; NSW, New South Wales; QLD, Queensland; SA, South Australia; WA, Western Australia; ACT, Australian Capital Territory; NT, Northern Territory.
†Relative risks compared NSW residents with non–NSW residents. Two co-infected case-patients have been counted in both influenza categories. The 2 case-patients with positive results from point-of-care testing on board but no further subtyping results are excluded from this table.

One child received seasonal influenza vaccine in 2008 and no child received the vaccine in 2009. The first case of pandemic influenza was in a child from Victoria, Australia, in whom symptoms developed on the third day of cruise A. The child attended childcare for 4 sessions while infectious. After the index case was identified, 19 additional cases of pandemic influenza were identified (including in 2 sets of siblings: 2 related children in whom symptoms developed at the same time and in 1 child in whom ILI developed 2 days after symptom onset in her sibling); all but 1 had attended the childcare while a known infectious case-patient was present. The first child for whom seasonal influenza was diagnosed had attended childcare the afternoon and evening before symptom onset on May 21 and for 6 sessions while symptomatic. Subsequently, an additional 3 unrelated cases of seasonal influenza were identified among childcare attendees. The 3 children who did not attend childcare remained healthy.

Among the 45 childcare attendees, NAT results for pandemic influenza were positive for 18, for seasonal influenza were positive for 2, and for both were positive for 2. ILI developed in an additional 10 children, but these children had negative influenza results by NAT; ILI developed in another 6 children who were not tested. Of these 16 children, 8 had traveling companions with positive pandemic influenza virus results and 2 had travelling companions with positive seasonal influenza virus results. The remaining 7 children remained asymptomatic. Of the 45 children who attended childcare, 44 attended concurrently with an infectious pandemic influenza case-patient and 43 attended concurrently with an infectious seasonal influenza case-patient. Considering the number of sessions attended by susceptible children, we determined that the risk for pandemic influenza infection was significantly higher (19 cases from 344 sessions = 0.055 child-sessions at risk, 95% confidence interval [CI] 0.033–0.086) than was the risk for seasonal influenza (3 cases from 279 sessions = 0.011 child-sessions at risk, 95% CI 0.002–0.031).

Secondary Attack Rates for Pandemic Influenza

A total of 66 pandemic influenza case-patients in 53 cabins were infectious while on cruise A. Excluding the co-primary case-patients, 91 passengers shared a cabin with an infectious primary case-patient. Of these 91 passengers, symptoms developed in 50 (55%). Of these 50 case-patients, 34 were tested and 12 (35%) had positive pandemic influenza results. The secondary attack rate for those ≤12 years of age (16/21) was significantly higher than for those >12 years of age (34/70) (76% vs. 49%; p = 0.03). Of the 66 case-patients, 1 received oseltamivir treatment within 48 hours of symptom onset. Information about provision of oseltamivir prophylaxis was available for 34 (83%) of 41 asymptomatic contacts. Of these, 3 (75%) of 4 children <12 years of age and 17 (57%) of 30 children >12 years

Table 2. Clinical data for 100 passengers with influenza after 10-day cruise that departed Sydney, New South Wales, Australia, on May 16, 2009

Clinical data	Pandemic (H1N1) 2009, no. (%), n = 50*	Influenza (H3N2), no. (%), n = 50†	p value
Cough	46 (92)	48 (96)	0.40
Fever (self-reported or measured)	39 (78)	34 (68)	0.26
Coryza	39 (78)	28 (56)	0.019
Fatigue	28 (56)	30 (60)	0.68
Sore throat	27 (54)	31 (62)	0.42
Headache	21 (42)	28 (56)	0.16
Myalgia	19 (38)	23 (46)	0.42
Dyspnea	12 (24)	10 (20)	0.63
Vomiting	5 (10)	4 (8)	0.73
Diarrhea	3 (6)	7 (14)	0.18
Severity of illness			
Limited activities	19 (38)	29‡ (58)	0.011
Antiinfluenza treatment	12 (24)	19§ (42)	0.06

*Patient median age 25.8 years (range 3–53 years); 20 male and 30 female; median duration of illness 5.0 days (range 0–17 days).
†Patient median age 32.4 years (range 3–82 years); 25 male and 25 female; median duration of illness 7.0 days (range 1–35 days). Duration data from 45 reinterviewed case-patients. Excludes 5 case-patients for whom symptom data was collected at the time of testing but for whom laboratory results confirming influenza subtype H3N2 were not available at the time of reinterview.
‡Data from 45 reinterviewed case-patients. Excludes 5 case-patients for whom symptom data was collected at the time of testing but for whom laboratory results confirming influenza subtype H3N2 were not available at the time of reinterview.
§Treatment given with 48 hours of symptom onset.

of age began receiving antiviral drug prophylaxis within 7 days of their first exposure to pandemic influenza virus. Of these 20, only 1 received prophylactic drug within 3 days of first exposure to the pandemic strain. Despite being asymptomatic, 11 (27%) of 41 passengers underwent laboratory testing and were negative for pandemic influenza virus by NAT.

Isolation and Quarantine

After disembarking, patients with pandemic and seasonal influenza were isolated in 149 discrete (family or household-like) groups. Of the 98 (66%) interviewed, 37% reported that they were first made aware of the need for isolation through media reports, 27% by their treating doctor, 26% by public health staff, 6% by the ship's staff, and 5% by fellow passengers. Of the 45 quarantined passengers interviewed, 52% were initially informed of the need for quarantine through media reports, 25% by work or school colleagues, 11% from the ship's staff, 7% from a friend or relative, and 5% from public health staff. All influenza case-patients reported that they had obeyed isolation requirements, and 43 of 45 quarantined passengers reported that they had remained in quarantine for 7 days after disembarkation. Of the 2 passengers who did not follow quarantine requirements, 1 reportedly attended work by private vehicle and cancelled all other outings; the other denied knowledge of the requirements.

Further Virus Transmission

Three secondary pandemic influenza infections among family contacts of case-patients from cruise A were identified; a subsequent case-patient was identified as a contact of 1 person who had secondary infection. Other than these cases, no evidence of transmission to the community or to passengers of cruise B was found.

Discussion

We identified dual outbreaks of pandemic and seasonal influenza among passengers on a cruise ship. Cruise ships provide ideal conditions for rapid spread of respiratory viral illnesses (e.g., many persons living closely together, frequently interacting in enclosed and partially enclosed environments, and often originating from both hemispheres). Although infections spread rapidly among passengers and to some crew members during the cruise, further spread to the community and the next cruise was avoided through intensive disease control measures.

After identification of the outbreak, it became apparent that undetected local transmission of pandemic influenza virus was occurring in Victoria before cruise A (*8*) and that the virus was probably introduced to the ship by the index case-patient from Victoria. The pandemic virus spread rapidly among other childcare attendees and their close contacts and to other passengers and crew. Seasonal influenza virus was the predominant influenza virus circulating in NSW before the appearance of pandemic influenza virus (NSW Health, unpub. data).

The cocirculation of both strains in the childcare center provided a unique opportunity to compare attack rates. The pandemic strain seems to have spread among children more readily than the seasonal strain. This difference in transmissibility could have resulted from innate differences in the viruses themselves or from a level of immunity from past infection with the seasonal strain. Consistent with findings

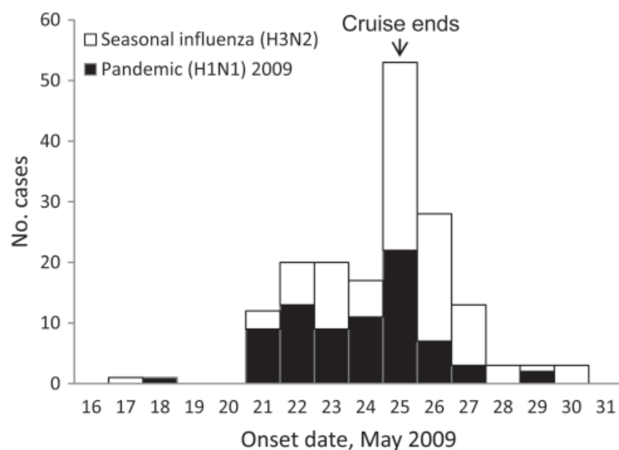

Figure. Date of onset of first symptoms for cruise ship passengers, by influenza subtype. Excludes 1 influenza A (H3N2) case-patient for whom onset date was unavailable and 1 pandemic (H1N1) 2009 case-patient and 2 influenza A (H3N2) case-patients who were asymptomatic but whose laboratory test results were positive.

in other studies, the symptoms of pandemic and seasonal influenza were similar (*9–12*). After adjusting for underlying medical conditions, we found that hospitalization rates and activity-limiting effects were higher for case-patients with seasonal than with pandemic influenza; however, this finding may be explained in part by differences in the age-specific attack rates. The secondary attack rate for pandemic influenza among cabin contacts of 55% was higher than that reported for household contacts (*13,14*), despite a small proportion of these persons having received antiviral drug prophylaxis, and may reflect the close living arrangements in a ship's cabin.

The intense passenger follow-up enabled us to assess the sensitivity of the ship's medical clinic for identifying influenza cases. Before this outbreak, ships had active containment measures in place to minimize the spread of seasonal influenza, including use of point-of-care influenza testing for patients seeking treatment for ILI and oseltamivir treatment and isolation to reduce further spread. Our active case-finding efforts identified 79 influenza cases on cruise A, yet the ship's clinic identified only 6 (8%) of these. Despite enhanced community awareness of the emerging pandemic, the ship's medical clinic staff underestimated the case count by 13-fold. The number of passengers who sought treatment at the ship's medical clinic does not accurately reflect the extent of the influenza outbreak on board, possibly because the decision to seek treatment may have been influenced by a number of factors including cost, severity of symptoms, and unwillingness to be isolated while on holiday.

Our investigation had several limitations. First, the case definition depended on NAT detection of virus in clinical samples, which may have resulted in misclassification of cases. Second, although the epidemiology is consistent with the first cases of pandemic influenza appearing in the childcare center, undetected or asymptomatic infected passengers or crew could have carried the viruses onto the ship. However, this scenario is unlikely because the symptoms developed in the index case-patient 2 days after embarkation. Third, although most ill passengers were interviewed within 2 days after onset of illness, interviews about severity, length of illness, and the experience in isolation and quarantine were conducted some weeks later, introducing possible recall bias. Fourth, although 2 cases of co-infection were detected, only the first 2 pandemic influenza–positive specimens from childcare attendees were subtyped for other influenza A subtypes; it is possible that some of the remaining pandemic influenza case-patients were also infected with seasonal influenza. Fifth, some of the remaining pandemic influenza case-patients may have been co-infected. Sixth, the secondary attack rate for cabin contacts may be an overestimate because passengers with negative NAT results were not tested for other respiratory infections, and passengers with onset of symptoms >24 hours after symptoms developed in a cabinmate were assumed to be secondary, rather than co-primary cases.

Mathematical modeling suggests that containment of influenza is possible if appropriate resources are devoted. In some countries, isolation and quarantine measures have been used in response to severe acute respiratory syndrome (*15–18*), but these measures have rarely been used for influenza control. In the influenza outbreaks reported here, direct follow-up of passengers in isolation and quarantine, supported by intense media coverage, resulted in a high degree of compliance and successful outbreak containment. Additionally, providing oseltamivir prophylaxis for crew members may have contributed to the successful containment of the infection during cruise B. Although the robust application of containment measures can stop the spread of novel influenza viruses, public health resource requirements are labor-intensive and expensive and may not be sustainable except for the most virulent of pandemic viruses.

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Enhanced Surveillance of Coccidioidomycosis, Arizona, USA, 2007–2008

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Learning Objectives

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

- Evaluate the impact of coccidioidomycosis on patients and the broader health system.
- Evaluate the performance of a laboratory-based system of diagnosis for coccidioidomycosis and incorporate that information into effective diagnostic strategies.

Editor

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Coccidioidomycosis is endemic to the southwestern United States; 60% of nationally reported cases occur in Arizona. Although the Council of State and Territorial Epidemiologists case definition for coccidioidomycosis requires laboratory and clinical criteria, Arizona uses only laboratory criteria. To validate this case definition and characterize the effects of coccidioidomycosis in Arizona, we interviewed every tenth case-patient with coccidioidomycosis reported during January 2007–February 2008. Of 493 patients interviewed, 44% visited the emergency department, and 41% were hospitalized. Symptoms lasted a median of 120 days. Persons aware of coccidioidomycosis before seeking healthcare were more likely to receive an earlier diagnosis than those unaware of the disease (p = 0.04) and to request testing for *Coccidioides* spp. (p = 0.05). These findings warrant greater public and provider education. Ninety-five percent of patients interviewed met the Council of State and Territorial Epidemiologists clinical case definition, validating the Arizona laboratory-based case definition for surveillance in a coccidiodomycosis-endemic area.

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Coccidioidomycosis, or valley fever, is a fungal disease endemic to the southwestern United States, parts of Mexico, and Central and South America. Infection probably occurs when arthroconidia from disrupted soil are inhaled,

causing lung infection (*1*,*2*). Signs and symptoms occur 1–4 weeks after exposure and can include fever, cough, fatigue, shortness of breath, headache, joint and muscle aches, and rash. *Coccidioides* spp. can spread to the central nervous system, skin, joints, or bones in <1% of those infected, resulting in extrathoracic dissemination (*3*,*4*).

Each year, an estimated 150,000 persons in the United States become infected with *Coccidioides* spp., and ≈50,000 of these become ill (*5*). Most cases are not reported to health departments, resulting in an underestimated number of coccidioidomycosis cases (*6*). In 2006 in the United States, 8,917 coccidioidomycosis cases were reported, with 5,535 cases reported in Arizona (*7*). The number of reported coccidioidomycosis cases in Arizona began to increase in 1990. From 1990 through 1995, the annual number of reported coccidioidomycosis cases increased from 255 (7/100,000 population) to 623 (15/100,000 population) (*8*). This increase led the Arizona Department of Health Services (ADHS) to change its reporting rules to make coccidioidomycosis a laboratory-reportable illness in 1997.

Since laboratory reporting became mandatory, coccidioidomycosis case reports have rapidly increased in Arizona. In 2006, the number of cases peaked at 5,535 cases (89/100,000 population) and decreased to 4,815 cases (75/100,000 population) in 2007 and to 4,768 cases (73/100,000 population) in 2008. During 2006–2008 in Arizona, the median age of patients with coccidioidomycosis was 52 years (mean 51 years). Fifty-four percent of patients with coccidioidomycosis were male (84/100,000 population), and 46% were female (72/100,000 population).

The Council of State and Territorial Epidemiologists (CSTE) and the Centers for Disease Control and Prevention require laboratory and clinical criteria to meet the case definition for coccidioidomycosis. The laboratory criteria consist of culture, histopathologic, or molecular evidence; or immunologic evidence in the form of detection of immunoglobulin M or immunoglobulin G by immunodiffusion, enzyme immunoassay, latex agglutination, tube precipitin, or complement fixation. Clinical criteria require influenza-like signs and symptoms; pneumonia or other pulmonary lesion; erythema nodosum or erythema multiform rash; involvement of bones, joints, or skin by dissemination; meningitis; or involvement of viscera and lymph nodes (*9*).

Because of Arizona's large number of cases, ADHS uses only the laboratory component of the CSTE case definition. In 2007, ADHS initiated enhanced coccidioidomycosis surveillance (which included patient interviews) to validate a laboratory-exclusive case definition for coccidioidomycosis and to characterize the effects of the disease on Arizona's population, healthcare system, and economy. The purpose of this study was to validate this case definition and characterize the effects of coccidioidomycosis in Arizona during January 2007–February 2008.

Methods

Study Design

ADHS conducted a population-based investigation of coccidioidomycosis cases reported from January 2007 through February 2008 in Arizona. Every tenth patient with newly identified coccidioidomycosis reported through the statewide surveillance system was sent a letter informing them of the investigation, and all possible methods were used to collect telephone information for each patient. If telephone information was obtained, each selected patient was contacted by telephone and interviewed with the aid of a 15-minute standardized questionnaire. If the patient could not be reached after 3 attempts or refused to be interviewed, the subsequent case-patient was sent an enrollment letter and contacted for an interview. If a patient was <18 years of age, either the parent or guardian was interviewed or the patient was interviewed with a parent or guardian present. Interviewees were asked about the signs and symptoms of coccidioidomycosis they experienced, their healthcare-seeking behavior, their medical treatment information, and the effects of the disease on their daily lives. Interviewees self-reported their race as either white, African American, Asian or Pacific Islander, Native American or Alaska Native, or Other and their ethnicity as either Hispanic or non-Hispanic. Interviewees were asked whether they took any immunosuppressive drugs and were provided the following examples: chemotherapy medications, steroids, prednisone, dexamethasone, infliximab, and interferon. If a patient who spoke only Spanish was contacted, a Spanish-speaking interviewer called back to conduct the interview in Spanish.

Symptom information obtained from the interviews was used to determine whether the case-patients met the clinical portion of the CSTE case definition. Case-patients met the CSTE clinical case definition if they had ≥1 of the following: fever, cough, sore throat, chills, dyspnea, chest pain, hemoptysis, headache, rash, stiff neck, myalgias, or arthralgia.

Questions from the standardized questionnaire were added to the Arizona Behavioral Risk Factor Surveillance System (BRFSS), 2008, an annual population-based telephone survey about health behavior and opinions. Data from BRFSS in 2008 (n = 6,165) were used to represent the general population of Arizona and were compared with data from interviews with coccidioidomycosis patients to understand how the coccidioidomycosis patients' understanding differed from the BFRSS population's knowledge of coccidioidomycosis (*10*).

Data from the Arizona Hospital Discharge Database 2007 were used to examine charges for hospitalizations of patients with a primary or secondary diagnosis of coccidioidomycosis (*11*). Hospitalizations were identified by use

of the International Classification of Diseases, 9th Revision for coccidioidomycosis (codes beginning with 114).

Data Management and Analysis

Data were entered into Microsoft Access (Microsoft Corp., Redmond, WA, USA) and analyzed by using SAS software (SAS Institute Inc, Cary, NC, USA). χ^2 tests were used to detect significant differences between groups, and *t* tests were used to analyze continuous variables. All statistical tests were 2-tailed, and a p value $\leq$0.05 was considered significant. Interquartile ranges (IQRs) at the 25th and 75th percentiles were also determined. The positive predictive value of using only the laboratory portion of the CSTE case definition in Arizona was calculated.

Results

Study Population

ADHS received reports of 5,664 coccidioidomycosis cases from January 2007 through February 2008. Of the 5,664 reported case-patients, 851 (15%) were sent letters for enrollment, and 493 (9%) were successfully enrolled. Of the 851 patients who received enrollment letters, more than half (493 or 58%) were successfully enrolled; 41 (5%) refused to be interviewed; 15 (2%) were deceased, incapacitated, or incarcerated; 228 (27%) were lost to follow up; and 74 (9%) were in the process of being contacted when the study ended. Interviewed case-patients were similar in age and sex to those case-patients who were not interviewed. However, the number of Native Americans and Hispanics was significantly lower among the interviewed patients than among those not included in the enhanced surveillance population (Table 1).

Comparing data from the interviewed patients with the Arizona population data from the US Census 2000 (*12*), we showed that 449 (91%) of those interviewed had health insurance at the time they sought care compared to 86% of the Arizona population (p<0.01). Interviewed patients differed slightly from BRFSS survey respondents: median age of interviewed patients was significantly lower (54 years vs. 58 years, respectively; p<0.01), and significantly fewer women were interviewed (odds ratio [OR] 1.9, 95% confidence interval [CI] 1.6–2.3). Also, persons with a diagnosis of coccidioidomycosis had lived in Arizona for a median of 12 years compared with BRFSS respondents, who lived in Arizona for a median of 22 years (p<0.01). Among the 493 interviewed patients, 97 (20%) were classified as immunocompromised at the time of illness; 140 (28%) reported a history of heart or lung disease; and 164 (33%) reported having no underlying disease at time of diagnosis (Table 2).

Effects on Patients

Interviewed coccidioidomycosis patients reported the following common symptoms: fatigue (84%), cough (67%), dyspnea (59%), and fever (54%). Patients sought healthcare a median of 11 days (range 0–2,669 days, IQR 2–31 days) after onset of symptoms. Median time between seeking healthcare and coccidioidomycosis diagnosis was 23 days (range 0–10,280 days, IQR 6–74 days). Thirteen (3%) patients did not know their diagnosis until $\geq$2 years after they had seen a doctor for their symptoms.

Patients reported a median of 2 visits (range 0–63 visits, IQR 1–3 visits) to a healthcare provider before coccidioidomycosis testing occurred. Symptoms lasted a median of 42 days (range 0–511 days, IQR 14–65 days) for patients

Table 1. Demographic characteristics of reported coccidioidomycosis patients compared with enhanced surveillance patients, Arizona, USA, January 2007–February 2008

Characteristic	Total reported, N = 5,664	Enhanced surveillance, n = 493	p value
Male sex, no. (%)*	3,003 (54)	259 (54)	0.7
Age, y			
Mean	51	52	0.2
Median	52	54	
Range	3 d–100 y	8 mo–100 y	
Race, no. (%)†			
White	1,685 (82)	385 (80)	0.2
African American	158 (8)	33 (7)	0.5
Asian or Pacific Islander	58 (3)	16 (3)	0.6
Native American or Alaska Native	110 (5)	11 (2)	<0.01
Other	37 (2)	37 (8)	<0.01
Unknown	0	1 (0)	
Hispanic ethnicity, no. (%)‡	319 (24)	63 (13)	<0.01

*Sex data were available for only 5,601 reported coccidioidomycosis cases.
†In Arizona, race and ethnicity are recorded in separate variables. Therefore, race data do not include a specific category for Hispanic ethnicity but include Hispanics. Race is categorized as White, African American, Asian or Pacific Islander, Native American or Alaska Native, or Other. Race data were available for 2,048 reported coccidioidomycosis patients and for 483 enhanced surveillance patients.
‡In Arizona, race and ethnicity are recorded in separate variables. Ethnicity is categorized as either Hispanic or non-Hispanic. Ethnicity data were available for 1,345 coccidioidomycosis patients and for 482 enhanced surveillance patients.

who had recovered at the time of interview (n = 167); 157 days (range 4–5,224 days, IQR 110–277 days) for those who had not recovered at the time of interview (n = 253); and 120 days (range 0–5,224 days, IQR 49–198 days) for the 2 groups combined (n = 420) (Table 3).

Of the 493 patients interviewed, 225 (46%) were employed and 178 (36%) were retired. Of those employed, 167 (74%) missed a median of 14 workdays (range 0–365 workdays, IQR 5–30 workdays) because of their illness (Table 3). Of interviewed patients, 63 (13%) were attending school when their illness began; 37 (59%) of these students missed a median of 9 days of school due to their illness. When asked about their ability to perform their usual daily activities, 369 (75%) patients said that their illness prevented their performance of usual daily activities at some point during the illness. Among these patients, daily activities were interrupted for a median of 47 days (range 0–1,825 days, IQR 15–120 days) (Table 3).

Table 2. Clinical and behavioral characteristics of enhanced surveillance coccidioidomycosis patients, Arizona, USA, January 2007–February 2008*

Characteristic	No. (%)
Coexisting condition	
Heart disease	62 (13)
Lung disease	90 (18)
Asthma requiring inhaler	47 (10)
COPD or emphysema	27 (6)
Other	29 (6)
Malignancy	70 (14)
Transplant	11 (2)
HIV	9 (2)
Diabetes mellitus	72 (15)
None	164 (33)
Immunosuppression†	97 (20)
Smoker	
Active	76 (15)
Past	203 (41)
Never	202 (41)
Unknown	12 (2)
Site of infection, n = 282	
Primary pulmonary‡	240 (85)
Disseminated§	42 (15)

*N = 493. COPD, chronic obstructive pulmonary disease.
†Immunosuppression is defined as having HIV/AIDS, a solid-organ transplant, or a bone marrow transplant or taking immunosuppressive medications. Immunosuppressive medications refer to medications that suppress the immune system and include chemotherapy medications such as steroids, prednisone, dexamethasone, infliximab, or interferon, as self-reported by patients.
‡Primary pulmonary infection is defined as the lungs being the only site of infection, as self-reported by patients.
§Disseminated infection is defined as infection spread to other parts of the body, including the central nervous system, bone, and entire body, as self-reported by patients.

Effects on Healthcare and the Economy

Almost half (46%) of the patients interviewed reported ≥1 visit to the emergency room during the course of illness; 111 (23%) first sought care in an emergency room. Approximately one fourth (26%) of patients visited a healthcare provider >10 times during the course of their illness. Two hundred patients (41%) were hospitalized overnight for their illness, and the median length of hospital stay was 6 days (range 0–306 days, IQR 4–10 days).

Data from the Arizona Hospital Discharge Database (*11*) show that 1,093 hospital visits occurred with a primary diagnosis of coccidioidomycosis in Arizona in 2007, accounting for a total of $59 million in hospital charges and a median of $33,000 per coccidioidomycosis-related hospital visit (Table 3). For the 1,735 visits with a primary or secondary diagnosis of coccidioidomycosis, total charges were $86 million, and the median charge was $30,000 per visit.

Knowledge of Coccidioidomycosis

Patients who knew about coccidioidomycosis before seeking healthcare were more likely to be diagnosed earlier than those patients who were unfamiliar with the disease (median 20 days [range 0–3,653 days, IQR 6–56 days] vs. 25 days [range 0–10,280 days, IQR 7–144 days], respectively; p = 0.04). Interviewed patients who had prior knowledge of coccidioidomycosis were also twice as likely (95% CI 1.0–3.2, p = 0.05) to request testing for coccidioidomycosis from their healthcare provider. White patients were more likely to have knowledge about coccidioidomycosis before diagnosis than were patients of other racial groups (95% CI 1.7–4.3, p<0.01).

In addition, compared with the general population, interviewed patients were more likely to learn about coccidioidomycosis from their social circles, families, friends, or co-workers (OR 2.3, 95% CI 1.8–2.9). In contrast, those persons contacted through BRFSS were more likely to hear about coccidioidomycosis from the media (TV, radio, newspaper, or Internet) than were persons with coccidioidomycosis (OR 3.3, 95% CI 2.4–4.6). Coccidioidomycosis patients were less likely than BRFSS respondents to have heard about coccidioidomycosis from their doctors (OR 2.2, 95% CI 1.3–3.6).

Treatment

Antifungal treatment for coccidioidomycosis was prescribed for 303 (61%) patients interviewed. Compared with patients who reported no common symptoms, patients who had symptoms were more likely to be treated with antifungal medication if they reported either chills (68% vs. 57%), shortness of breath (67% vs. 56%), or weight loss (75% vs. 58%; p≤0.02 for each). Those with sore throat were significantly less likely to be treated with antifungal medication than those without sore throat (52% vs. 66%, p<0.01). About 60% (289) of patients were treated with antibacterial agents. Of those, 92 (32%) received >1 course of antibacterial drugs (range 2–10 courses, IQR 2–3 courses).

Table 3. Characteristics of enhanced surveillance coccidioidomycosis patients, and statewide hospital charges identified through the 2007 Arizona Hospital Discharge Database

Description	Mean	Median	Range	Interquartile range	Total
Days from symptom onset to diagnosis date, n = 422	209	55	0–10,280	22–143	
Days from first seeking health care to diagnosis date, n = 422	156	23	0–10,280	6–74	
Days of symptom duration,* n = 420	202	120	0–5,224	49–198	
Days missed from work,† n = 159	31	14	0–365	5–30	
Days missed from school,‡ n = 35	17	9	0–120	3–15	
Days unable to perform daily activities,§ n = 352	96	47	0–1,825	15–120	
Per visit hospital charges for coccidioidomycosis primary diagnoses, n = 1,093	$54,000	$33,000	$113–$1,474,795		$59 million
Per visit hospital charges for coccidioidomycosis primary or secondary diagnoses, n = 1,735	$49,000	$30,000	$113–$1,474,795		$86 million

*Symptom duration analysis includes patients who had recovered and those who had not yet recovered from their symptoms at the time of interview.
†Number of days missed from work was available for 159/167 patients who reported that they missed work due to their disease.
‡Number of days missed from school was available for 35/37 patients who reported that they missed school due to their disease.
§Number of days patients were unable to perform daily activities was available for 352/369 patients who reported that their disease interfered with their daily activities.

Comparison to CSTE Case Definition

Of the 493 patients interviewed, 469 (95%) met the CSTE clinical case definition. Thirteen (3%) case-patients reported no symptoms. Ten (2%) had only 1 symptom consistent with coccidioidomycosis but had no symptoms that met the case definition; 1 person had only 1 symptom unrelated to coccidioidomycosis. Case-patients who met the clinical case definition were similar to those who did not meet the case definition in age, race, ethnicity, and gender.

Discussion

This investigation provides the largest population-based estimate of the effects of coccidioidomycosis in Arizona. We identified substantial personal and economic costs due to coccidioidomycosis among Arizonans with respect to duration and severity of illness, healthcare use, and healthcare costs. We also found marked delays in diagnosis as well as long duration of symptoms.

In our cohort, persons with coccidioidomycosis had prolonged symptoms for a median of 120 days, substantially longer than previous reports that indicated that coccidioidomycosis symptoms typically last <21 days (fatigue may last longer) (*2*,*13*). A study conducted among US Navy SEALS, a presumably healthy and relatively young population, reported median symptom duration of 19 days (range 2–63 days) (*14*). Our investigation identified a high number of missed workdays (median 14 days, range 0–365 days, IQR 5–30 days) and days during which persons could not perform their daily activities (median 47 days, range 0–1,825 days, IQR 15–120 days). In a study among military members, persons with coccidioidomycosis lost an average of 35 days from work (*15*). These data support the finding that coccidioidomycosis greatly affects a person's ability to function and remain productive once the disease develops.

In addition, we found a substantial delay between symptom onset and disease diagnosis. The delay in seeking medical care needs to be addressed by increasing public education about the signs and symptoms of coccidioidomycosis and the importance of seeking care early to obtain an accurate diagnosis. Our data show that the delay between seeking healthcare and ordering a diagnostic test may also be shortened by patient education. Persons who knew about coccidioidomycosis before seeking healthcare were more likely to request coccidioidomycosis testing and were more likely to receive a diagnosis earlier than those who were not familiar with the disease. Our data show that 46% of patients sought medical care without a fever, making recognition of the disease difficult for physicians and patients and possibly contributing to delays in diagnosis. Additionally, the nonspecific manifestation of respiratory illness in coccidioiodomycosis patients is indistinguishable from the manifestation of community-acquired pneumonia, which makes accurate diagnosis even more difficult (*16*).

Besides the effects on patients, this disease greatly affects the healthcare system. In our investigation, ≈25% of patients visited a healthcare provider >10 times during the course of their illness, and 41% of all interviewed patients were hospitalized. In 1993, a study conducted by Kerrick et al. showed that college students who had coccidioidomycosis visited their doctor an average of 7 times before the disease resolved (*17*). Similarly, Leake et al. found that patients >60 years of age had a median of 4 medical visits (range 1 to >30 visits) during the course of their illness (*18*). In this same study, 59% of patients were hospitalized for a median of 7 days. A study among military personnel by Crum et al. found that 22% of those with pulmonary disease and 40% with disseminated disease were hospitalized (*15*). Our study is consistent with this literature, but, being population-based, is more representative of the disease's effects on Arizona residents.

In addition to the costs generated by excess healthcare visits, we found the costs associated with hospitalizations

to be higher than costs found in previous studies. Our data show hospital charges totaling $86 million (mean $49,000 per hospitalization) among Arizona patients who had primary or secondary diagnoses in 2007. This total is much higher than that found in a previous analysis, which showed total annual hospital charges of $2 million in 1998, increasing to $19 million in 2001 (*19*). Our data clearly show the growing costs of coccidioidomycosis and its effect on healthcare costs in Arizona.

Persons with a diagnosis of coccidioidomycosis reported living in Arizona for significantly fewer years (median 12) than a sample of the general population (median 22). This finding is consistent with previous studies, which showed that relatively recent relocation to Arizona from a non-disease–endemic area is a risk factor for developing the disease (*18*,*20*,*21*). Leake et al. examined patients ≥60 years of age and identified a median duration of residence in Arizona of 6.5 years for coccidioidomycosis patients compared with 19.5 years for controls from the same geographic area (*18*).

We also found that the modified surveillance case definition used by Arizona is appropriate and has a high positive predictive value for the population in this coccidioidomycosis-endemic area. Arizona originally adopted a modified coccidioidomycosis case definition that includes only the laboratory criteria for several reasons. First, clinical information is rarely reported to public health agencies, and with >4,000 cases reported each year, obtaining this information for each case is resource intensive. Second, our experience suggests that most *Coccidioides* tests are performed on symptomatic patients (i.e., persons sick enough to seek medical attention). The data from this investigation confirm that our modified case definition is highly specific: 95% of cases reported to ADHS met both the laboratory and clinical criteria specified in the CSTE definition; the other 5% either had no symptoms or had symptoms that were inconsistent with the coccidioidomycosis case definition. These findings suggest that eliminating clinical criteria from the coccidioidomycosis case definition allows for simpler surveillance methods and requires fewer resources yet still accurately estimates prevalence and incidence of the disease in endemic regions.

Our investigation has several limitations. Coccidioidomycosis surveillance in Arizona requires a laboratory diagnosis of the disease. Because patients without a laboratory-confirmed diagnosis are missed, the number of reported coccidioidomycosis cases underestimates the actual number of cases. These reports come from outside sources, so a minor chance of error in data collection exists. Additionally, because case-patients reported to the health department are usually persons who are sick enough to seek medical attention for their symptoms and receive testing, this study is biased toward more patients with severe coccidioidomycosis cases and toward those with medical insurance. Furthermore, this enhanced surveillance data relied on self-reporting by patients. Because patients often lack medical knowledge or may refuse to answer questions during the interview, information reported may be missing or inaccurate. For instance, we relied on self-reported use of immunosuppressive medications, and these data were not verified by medical records or by physicians.

Self-reporting is subject to recall bias. However, most case-patients were contacted within a few months of their diagnosis, minimizing recall bias as much as possible. In addition, because telephone interviews were conducted, the data are limited to information from persons who were at home and who had telephones. These factors might explain the underrepresentation of Native American and Hispanics in the enhanced surveillance cohort, although every effort was made to capture information from persons who spoke only Spanish. Table 1 shows that Native Americans were underrepresented in the enhanced surveillance sample compared with the statewide coccidioidomycosis cases reported from January 2007 through February 2008 (2% vs. 5%; $p<0.01$), a difference possibly caused by lack of telephone contact information in this community. Another possible contributor to the underreporting of Native Americans is the fact that tribal entities are not required to report coccidioidomycosis cases to the state health department; however, most tribes in Arizona do voluntarily report infectious diseases to ADHS. Last, the data from the BRFSS survey may not accurately reflect the general population because respondents tended to be older and were more likely to be female than the Arizona population reported in the US Census 2000 (*22*).

This population-based cohort investigation illustrates the severe effects that coccidioidomycosis has on patients, the healthcare system, and the economy in Arizona. The data emphasize the need for effective education campaigns aimed at the general public and healthcare providers to decrease delays in diagnosis of coccidioidomycosis, which would probably reduce unnecessary use of antimicrobial drugs, relieve patient anxiety, and enable early recognition and treatment of the disease. Furthermore, the data validate a case definition that uses only laboratory criteria for coccidioidomycosis surveillance in disease-endemic areas. This information could be used to propose changes to the national CSTE coccidioidomycosis case definition in other US disease-endemic areas and thus reduce resources needed for an accurate assessment of the extent of the disease and its effects.

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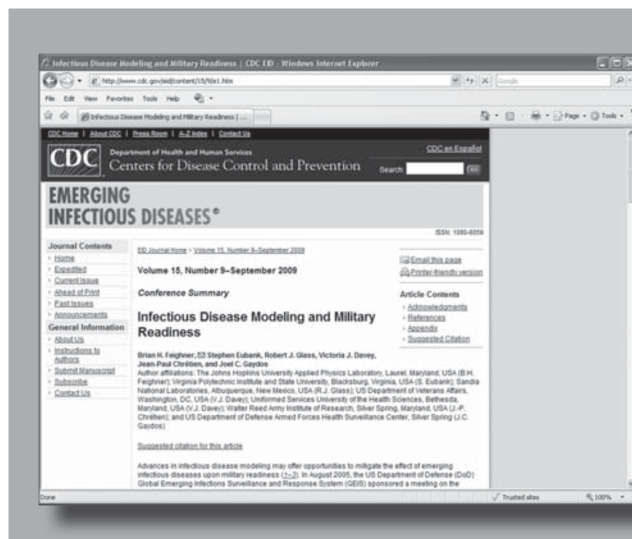

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Experimental Pandemic (H1N1) 2009 Virus Infection of Cats

Judith M.A. van den Brand, Koert J. Stittelaar, Geert van Amerongen, Marco W.G. van de Bildt, Lonneke M.E. Leijten, Thijs Kuiken, and Albert D.M.E. Osterhaus

To demonstrate that pandemic (H1N1) 2009 virus may cause respiratory disease in cats, we intratracheally infected cats. Diffuse alveolar damage developed. Seroconversion of sentinel cats indicated cat-to-cat virus transmission. Unlike in cats infected with highly pathogenic avian influenza virus (H5N1), extrarespiratory lesions did not develop in cats infected with pandemic (H1N1) 2009 virus.

Soon after pandemic (H1N1) 2009 virus emerged in North America, infections in domestic cats were reported (*1*,*2*). Infection with highly pathogenic avian influenza (HPAI) virus (H5N1) leads to severe and often fatal diffuse alveolar damage and systemic virus spread in cats (*3*–*5*). In contrast, seasonal human influenza viruses do not cause disease in cats (*6*). To elucidate the pathogenesis of pandemic (H1N1) 2009 virus infection in cats, we studied 8 laboratory cats intratracheally infected with this virus.

The Study

Pandemic (H1N1) 2009 virus (A/Netherlands/602/2009) was isolated from a 3-year-old girl from the Netherlands who had mild influenza after she visited Mexico in early 2009. Virus was cultured in embryonated chicken eggs and passaged once in MDCK cells (*7*).

We used 2 groups (4 cats/group) of 16-week-old, purpose-bred, specific pathogen–free, European shorthair cats that were seronegative for hemagglutination-inhibition (HI) antibodies against pandemic (H1N1) 2009 virus and circulating seasonal influenza A viruses. These cats were intratracheally infected with a $10^{6.0}$ tissue culture infectious dose ($TCID_{50}$) of pandemic (H1N1) 2009 virus. A third group of 3 sentinel cats were housed with these 2 infected groups (1 with group 1 and 2 with group 2) from 2 days postinfection (dpi) onward. Serum samples were obtained on 0, 4, 7, and 21 dpi and stored at –20°C until tested for HI antibodies against pandemic (H1N1) 2009 virus (*8*).

All 11 cats were monitored daily for clinical signs, and body temperature was measured at 15-min intervals. Nasal, pharyngeal, and rectal swab specimens were obtained daily from all cats. After being anesthetized with ketamine, all cats were killed by exsanguination. Cats in groups 1 and 2 were killed at 4 dpi and 7 dpi, respectively. Sentinel cats were killed at 21 dpi. Experiments were performed under BioSafety Level 3 by using protocols approved by our Institutional Animal Welfare Committee.

Necropsies were performed according to a standard protocol. Lung, nasal turbinate, nasal septum, larynx, trachea, bronchus, tracheobronchial lymph node, nictitating membrane, tonsil, heart, liver, spleen, kidney, pancreas, duodenum, jejunum, colon, adrenal gland, brain, and olfactory bulb samples were obtained, were fixed in formalin, and processed to obtain sections for staining with hematoxylin and eosin.

For detection of viral antigen, tissue sections were stained with viral nucleoprotein–specific antibody (*6*). Alveolar epithelial cells were phenotyped by using a destaining–restaining technique (*9*). After organ samples were weighed and stored at –80°C, infectious pandemic (H1N1) 2009 virus was quantified by limiting dilution virus isolation in MDCK cells (*10*).

Cats in groups 1 and 2 infected with pandemic (H1N1) 2009 virus showed mild-to-moderate clinical signs (lethargy, appetite loss, rapid and labored breathing, and protruding nictitating membrane) after 1 dpi or 2 dpi onwards. Average body temperatures increased after 1 dpi, showed a maximum increase of ≈1.5°C by 2 dpi, and returned to baseline values within 4–5 dpi (Figure). Sentinel cats showed no clinical signs. Two cats in group 1 (2 pharyngeal samples) and 2 cats in group 1 and 1 cat in group 2 (1 pharyngeal sample) had low virus titers during 1–4 dpi ($\leq 10^{1.8}$ $TCID_{50}$/g). Nasal swab specimens from all sentinel cats and pharyngeal and rectal swab specimens from 2 were virus positive by reverse transcription–PCR (cycle threshold ≥35) 2–6 days after first contact with infected cats. No virus was isolated from these swab specimens.

On 4 dpi, high virus titers were found in lungs, bronchi, and tracheas from 4 infected cats ($10^{5.5–6.3}$, $10^{2.9–4.6}$, and $10^{3.1–3.8}$ $TCID_{50}$/g, respectively). Tonsils from 2 cats, intestines from 1 cat, and the spleen from 1 cat also had high virus titers ($10^{3.0}$, $10^{4.2}$, $10^{1.6}$, and $10^{1.6}$ $TCID_{50}$/g, respectively). On 7 dpi, virus was detected in lung from 1 cat and trachea from 1 cat ($10^{3.0}$ and $10^{1.6}$ $TCID_{50}$/g, respectively). Infectious virus ($10^{1.2–2.2}$ $TCID_{50}$/g) was found in liver, intestine, brain, adrenal glands, and nictitating membranes of individual cats. The olfactory bulb of 1 cat was virus posi-

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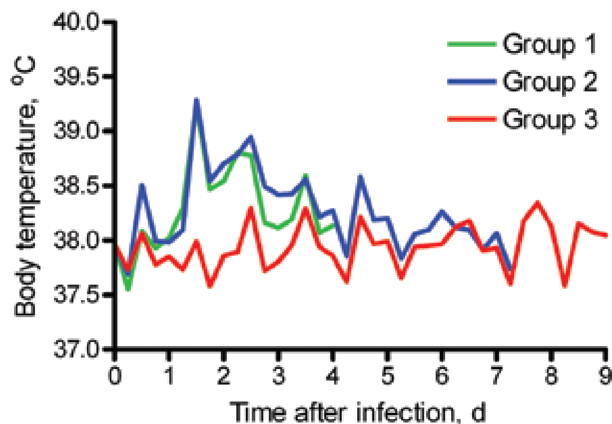

Figure. Average body temperatures of 2 groups of cats experimentally infected with pandemic (H1N1) 2009 virus (groups 1 and 2) and sentinel cats (group 3).

tive ($10^{3.0}$ $TCID_{50}$/g). No other organs from any cats were virus positive (Table).

No serum HI antibodies (titer <20) were found in group 1 cats on 4 dpi. All group 2 cats had serum HI antibodies (titers 30–120) on 7 dpi. One sentinel cat was seropositive on 15 dpi (titer 40); all cats were positive on 21 dpi (titer 80).

All infected cats showed multifocal or coalescing pulmonary consolidation, ranging from 30% to 50% on 4 dpi and from 10% to 30% on 7 dpi (online Technical Appendix, www.cdc.gov/EID/content/16/11/1746-Techapp.pdf). All tracheobronchial lymph nodes were enlarged 3–5×. Palatine tonsils were enlarged ≈2× on 7 dpi. All sentinel cats showed mild multifocal consolidation; 5%–10% of lung parenchyma were affected. Two cats had tracheobronchial lymph nodes enlarged 2–5×.

Histopathologic analysis (online Technical Appendix) identified pulmonary consolidation indicative of diffuse alveolar damage. Alveolar and bronchiolar lumina showed edema and contained variable numbers of macrophages, neutrophils, and erythrocytes mixed with fibrin and cellular debris. Alveolar walls were thickened and showed necrosis of lining epithelium and type II pneumocyte hyperplasia.

Bronchiolar walls were moderately infiltrated by neutrophils and had multifocal epithelial necrosis and multifocal peribronchiolar moderate infiltration by macrophages and lymphocytes and few neutrophils and plasmacytes. Bronchial lumina harbored few neutrophils and scant edema, fibrin, and cellular debris. There were few peribronchial infiltrates with a small number of lymphocytes, plasmacytes, and macrophages. Lung lesions seen on 4 dpi and 7 dpi were comparable except for more extensive type II pneumocyte hyperplasia on 7 dpi. Tracheobronchial lymph nodes and palatine tonsils had severe sinus histiocytosis and lymphocytolysis and moderate infiltration by neutrophils. Histologic changes in lung parenchyma of all sentinel cats were consistent with chronic lesions resulting from those seen in the other cats. No lesions were seen in other organs of all cats.

Virus antigen expression was more prominent on 4 dpi than on 7 dpi and was closely associated with histologic lesions (online Technical Appendix). Virus antigen expression was seen in many type II pneumocytes, few type I pneumocytes, alveolar macrophages, bronchiolar ciliated and nonciliated epithelial cells, and rare bronchial ciliated epithelial cells. Type I and II pneumocytes were identified by double-staining with cytokeratin. No virus antigen was observed in sentinel cats.

Conclusions

Intratracheal infection of domestic cats with pandemic (H1N1) 2009 virus resulted in mild-to-moderate clinical signs and virus replication throughout the respiratory tract, which caused diffuse alveolar damage. The pathogenesis in the respiratory tract in cats was similar to that occurring in humans, macaques, and ferrets (*7*,*11*–*13*). Seroconversion of sentinel cats indicated cat-to-cat transmission.

Unlike infection with seasonal human influenza viruses, infection with pandemic (H1N1) 2009 virus causes respiratory disease in cats. To compare infections with these viruses, we used our unpublished data for cats intratracheally infected with $10^{5.0}$ $TCID_{50}$ of HPAI virus (H5N1) (A/Indonesia/5/2005) at 4 dpi and 7 dpi (*4*) and for sham-infected cats. Histopathologic and immunohistochemical findings in lungs of cats infected with these viruses coincided, which indicated a similar pathogenetic process and increased severity in cats infected with HPAI virus (H5N1).

Table. Test results for 8 cats intratracheally infected with pandemic (H1N1) 2009 virus*

	No. positive			
	IHC analysis		Virus isolation†	
Tissue source	4 dpi	7 dpi	4 dpi	7 dpi
Respiratory				
Lung	4	3	4	1
Bronchus	2	0	4	0
Trachea	0	0	4	1
Nasal turbinates	0	0	2	0
Extrarespiratory				
Liver	0	0	0	1
Intestine	0	0	1	1
Olfactory bulb	0	0	0	1
Brain	0	0	0	1
Spleen	0	0	1	0
Tonsil	0	0	2	0
Adrenal gland	0	0	0	2
Nictitating membrane	0	0	0	2

*Four cats were examined on each day. IHC, immunohistochemical; dpi, days postinfection.
†No virus was isolated from tracheobronchial lymph node, pancreas, heart, or kidney of any cats.

However, in contrast to HPAI virus (H5N1), pandemic (H1N1) 2009 virus does not cause extrapulmonary lesions in infected cats. Our data show that pandemic (H1N1) 2009 virus may cause respiratory disease in cats and that human-to-cat transmission is the most likely route of infection.

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Reassortment of Ancient Neuraminidase and Recent Hemagglutinin in Pandemic (H1N1) 2009 Virus

Priyasma Bhoumik and Austin L. Hughes

Sequence analyses show that the outbreak of pandemic (H1N1) 2009 resulted from the spread of a recently derived hemagglutinin through a population of ancient and more diverse neuraminidase segments. This pattern implies reassortment and suggests that the novel form of hemagglutinin conferred a selective advantage.

Influenza virus A is a single-strand, negative-sense RNA virus whose genome consists of 8 RNA segments that encode 10 proteins (*1*). Influenza A is endemic in wild waterfowl, from which new strains periodically emerge to infect mammals, including humans and domestic pigs (*2*). Strains of influenza A viruses are categorized according to serotypes for hemagglutinin (HA) and neuraminidase (NA) proteins. These proteins cover the surface of the virus, are the main targets of the host's cellular immune response, and play major roles in the infection process (*1*,*3*,*4*).

In 2009, a novel strain of influenza A virus, pandemic (H1N1) 2009 virus, appeared in the human population, infecting thousands and causing many deaths (*2*,*5*–*8*). Phylogenetic analyses support a close relationship between the new strain and the strains that infect swine (*6*–*9*). Because different segments of the pandemic (H1N1) 2009 virus genome show different patterns of relationship to previously identified clades of influenza A virus sequences, these analyses support a role for intersegment reassortment in the origin of the new strain (*6*–*9*). For example, HA of pandemic (H1N1) 2009 virus shows a close relationship to that of classical swine influenza A virus, and NA shows a close relationship to that of Eurasian swine influenza A virus (*6*–*9*).

The Study

To examine the effects of intersegment reassortment on sequence diversity, we analyzed the pattern of nucleotide substitutions in pandemic (H1N1) 2009 virus and compared it with that of other influenza A virus genotypes (see www.biol.sc.edu/~austin). In pandemic (H1N1) 2009 virus, synonymous (π_S) and nonsynonymous (π_N) nucleotide diversity (online Technical Appendix, www.cdc.gov/EID/content/16/11/1748-Techapp.pdf) was significantly greater in NA than in HA (Table 1). In pandemic (H1N1) 2009 virus, π_S in NA was >100× that in HA, and π_N in NA was >50× times that in HA (Table 1). By contrast, in pre-2009 influenza virus subtype H1N1, π_S and π_N were similar in HA and NA (Table 1). Likewise, in influenza virus subtypes H3N2 and H5N1, π_S and π_N were similar in HA and NA (Table 1). Thus, pandemic (H1N1) 2009 virus was unique among serotypes in showing a marked difference in sequence diversity between HA and NA.

To test whether the difference between HA and NA in pandemic (H1N1) 2009 virus resulted from sampling error, we applied the same analysis to 92 epidemiologically matched pairs of HA and NA sequences from pandemic (H1N1) 2009 virus (see www.biol.sc.edu/~austin) collected in the same month (the same date, when possible) and from the same state (or the same country if not of US origin). π_S was significantly greater in NA (mean ± SE 0.2537 ± 0.0183) than in HA (0.0030 ± 0.0011; $p<0.001$ by z-test). Likewise, in epidemiologically matched pairs, π_N was significantly greater in NA (0.0215 ± 0.0022) than in HA (0.0012 ± 0.0003; $p<0.001$ by z-test).

In HA and NA genes of serotypes of influenza subtypes H1N1 (pre-2009), H3N2, and H5N1, π_S was significantly greater than π_N (Table 1). For pandemic (H1N1) 2009, π_S was significantly greater than π_N in NA (Table 1); π_S was also greater than π_N in HA, but the difference was not significant because diversity was low at synonymous and nonsynonymous sites (Table 1). π_S was significantly greater than π_N for each of the other 6 genes (online Technical Appendix Table). A pattern of π_S greater than π_N indicates past purifying selection that has eliminated deleterious nonsynonymous mutations (*10*).

To obtain evidence regarding slightly deleterious variants subject to ongoing purifying selection (*11*–*13*), we examined gene diversity at synonymous and nonsynonymous polymorphic single-nucleotide polymorphism (SNP) sites in HA and NA genes (Table 2). In the NA genes of pandemic (H1N1) 2009 virus, subtypes H1N1 (pre-2009), H3N2, and H5N1, the gene diversity at nonsynonymous SNP sites was significantly lower than that at synonymous SNP sites (Table 2). The same pattern was seen in SNP sites in the HA gene of all serotypes except pandemic (H1N1) 2009 virus. Thus, the HA gene of pandemic (H1N1) 2009 virus showed a unique pattern in the absence of evidence of ongoing purifying selection decreasing the frequency of slightly deleterious variants.

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Table 1. Synonymous and nonsynonymous nucleotide diversity in hemagglutinin and neuraminidase genes of influenza A virus genotypes*

	HA			NA		
Genotype	No. sequences	π_S ± SE	π_N ± SE	No. sequences	π_S ± SE	π_N ± SE
Pandemic H1N1 (2009)	397	0.0041 ± 0.0015	0.0012 ± 0.0003	171	0.4626 ± 0.0493†	0.0616 ± 0.0065†
H1N1 (pre-2009)	105	0.0926 ± 0.0063	0.0171 ± 0.0017	105	0.0842 ± 0.0088	0.0126 ± 0.0016
H3N2	562	0.1178 ± 0.0094	0.0229 ± 0.0028	357	0.0871 ± 0.0077	0.0213 ± 0.0020
H5N1	109	0.0918 ± 0.0080	0.0189 ± 0.0026	116	0.1034 ± 0.0082	0.0194 ± 0.0027

*HA, hemagglutinin; NA, neuraminidase; π_S, synonymous nucleotide diversity; π_N, nonsynonymous nucleotide diversity. There was a significant difference (p<0.001) between π_S and π_N in all cases except HA of pandemic (H1N1) 2009.
†Significant difference between π_S or π_N in NA and corresponding value in HA (z-test; p<0.001).

Table 2. Mean ± SE gene diversity at synonymous and nonsynonymous polymorphic nucleotide sites in hemagglutinin and neuraminidase genes of influenza A virus serotypes*

	HA		NA	
Genotype	Synonymous	Nonsynonymous	Synonymous	Nonsynonymous
Pandemic H1N1 (2009)	0.0120 ± 0.0007 [173]	0.0112 ± 0.0006 [839]	0.2535 ± 0.0173 [179]	0.0863 ± 0.0060 [706]†
H1N1 (pre-2009)	0.0798 ± 0.0063 [198]	0.0506 ± 0.0019 [814]†	0.0765 ± 0.0083 [152]	0.0453 ± 0.0017 [712]†
H3N2	0.0710 ± 0.0086 [203]	0.0331 ± 0.0030 [793]†	0.0760 ± 0.0087 [177]	0.0332 ± 0.0029 [688]†
H5N1	0.1195 ± 0.0106 [184]	0.0552 ± 0.0027 [834]†	0.1120 ± 0.0098 [157]	0.0482 ± 0.0024 [645]†

*HA, hemagglutinin; NA, neuraminidase. Numbers of polymorphic nucleotide sites are indicated in brackets.
†Gene diversity at nonsynonymous sites significantly different from that at synonymous sites (p<0.001; randomization test).

At 9 aa positions in HA, a residue not seen in our sample of pre-2009 influenza (H1N1) virus was fixed (100% frequency) in our sample of pandemic (H1N1) 2009 virus (Figure). The following amino acid replacements were involved; residue(s) in pre-2009 influenza (H1N1) are listed first: F/I/L88S, N101S, T256K, N/S275E, A/D/G277N, Q382L, G/R391E, F454Y, and S510A. Of these positions, 4 (88, 101, 275, and 391) were among those listed as having unique amino acid residues in pandemic (H1N1) 2009 virus on the basis of a smaller sequence sample by Ding et al. (*9*).

Conclusions

Analysis of nucleotide sequences of HA and NA from 4 serotypes of influenza A virus showed a unique pattern of polymorphism in pandemic (H1N1) 2009 virus. In other serotypes, diversity of synonymous and nonsynonymous nucleotides was similar in HA and NA; in pandemic (H1N1) 2009 virus, HA showed much lower nucleotide diversity at synonymous and nonsynonymous sites than did NA. Of all serotypes analyzed, NA showed evidence of past and ongoing purifying selection against deleterious nonsynonymous mutations, and HA showed evidence of past and ongoing purifying selection of all serotypes except pandemic (H1N1) 2009 virus. These unique features of HA of pandemic (H1N1) 2009 virus imply that it has a more recent common ancestor than NA of the same serotype and that it has spread rapidly by frequent reassortment into a background of a much more ancient NA genotype.

The recent spread of HA of pandemic (H1N1) 2009 virus implies multiple events of reassortment, creating a population of viruses with an ancient and diverse NA gene and a much less diverse HA gene. The polymerase basic protein 1 gene also showed low diversity (online Technical Appendix Table), suggesting similar reassortment. Other genes of pandemic (H1N1) 2009 virus showed a level of diversity intermediate between that of HA and NA, suggesting that their association with this ancient and diverse NA may have resulted from earlier reassortment events. The bottleneck in the history of HA of pandemic (H1N1) 2009 virus explains the low genetic diversity and the absence of evidence of ongoing purifying selection because purifying selection is most effective when the population is large (*11–13*). Absence of ongoing purifying selection is thus consistent with a recent population expansion, of which pandemic (H1N1) 2009 virus shows evidence (*14*).

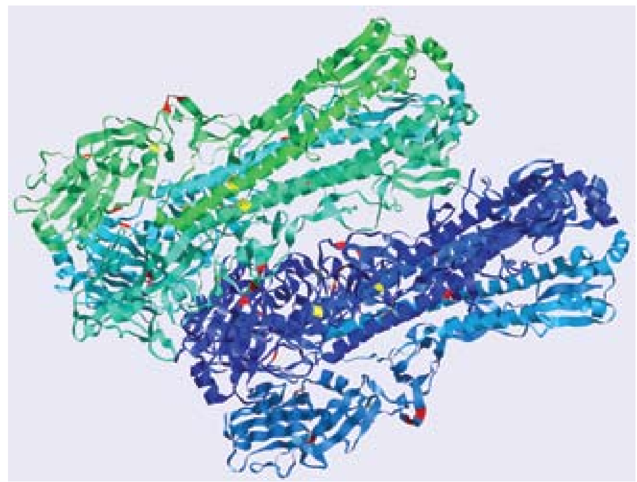

Figure. Structure of the pandemic (H1N1) 2009 virus hemagglutinin homotrimer, indicating (in red) the 9 aa positions in hemagglutinin at which a residue not found in pre-2009 influenza (H1N1) was fixed (100% frequency) in pandemic (H1N1) 2009 virus.

One factor that might have favored the spread of a recently evolved HA segment in the pandemic (H1N1) 2009 virus population would be the occurrence of ≥1 selectively favored aa replacements, causing a selective sweep (*15*) and reducing diversity at the HA locus. Such replacements in the ancestor of pandemic (H1N1) 2009 virus would likely be conserved in the pandemic (H1N1) 2009 virus population. The 9 aa residues in HA not found in our sample of pre-2009 influenza (H1N1), but fixed in our sample of pandemic (H1N1) 2009 virus, are candidates for selectively favored amino acid replacements in pandemic (H1N1) 2009 virus. Low diversity in ≥1 genes may be a recurring feature of newly emerged influenza A pandemics, supporting the need for vaccine development early in a pandemic to minimize mutation accumulation in viral genes of low initial variability.

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Prevalence and Genetic Structures of *Streptococcus pneumoniae* Serotype 6D, South Korea

Eun Hwa Choi, Hoan Jong Lee, Eun Young Cho, Chi Eun Oh, Byung Wook Eun, Jina Lee, and Min Ja Kim

To determine prevalence and genetic structures of new serotype 6D strains of pneumococci, we examined isolates from diverse clinical specimens in South Korea during 1991–2008. Fourteen serotype 6D strains accounted for 10.4% of serogroup 6 pneumococci from blood, sputum, nasopharynx, and throat samples. Serotype 6D strains consisted of 3 sequence types.

Streptococcus pneumoniae is a common cause of invasive infection in infants, children, and adults. The polysaccharide capsule of *S. pneumoniae* is the major virulence factor that protects the organism from host phagocytosis (*1*). Recently, 2 new serotypes of serogroup 6 pneumococci, 6C and 6D, were genetically and biochemically characterized (*2,3*). Serotype 6C was identified in 2007 on the basis of its distinct binding patterns with 2 monoclonal antibodies; serotype 6C had previously been typed as 6A according to the standard quellung reaction. Serotype 6C produces glucose in the place of galactose in the 6A capsular polysaccharide and has the $wciN_\beta$ gene, which is ≈200 bp shorter than the corresponding *wciN* gene in 6A (*2,4*). After the discovery and characterization of 6C through genetic and biochemical studies, a new experimental serotype, 6X1 (later named 6D), was created by mutating the critical nucleotide in the *wciP* gene of the 6C capsule gene locus or by inserting the $wciN_\beta$ gene into the 6B capsule gene locus (*3*). However, this putative serotype, 6D was thought to not exist in nature until recently, when 2 studies found 6D strains in nasopharyngeal aspirates from children in Fiji during 2004–2007 (*5*) and in 2 nasopharyngeal aspirates from children in South Korea in 2008 (*6*). Although serotype 6C has only recently been described, several studies indicate that serotype 6C pneumococci have been circulating in many countries, including the United States, the Netherlands, Australia, Israel, and South Africa (*7–10*). However, reports of naturally occurring serotype 6D pneumococci are limited.

We investigated the prevalence of serotypes 6C and 6D in 2 collections of pneumococci isolated from clinical specimens in South Korea. We compared the genetic diversity and antimicrobial drug susceptibility patterns of the 4 serotypes, 6A, 6B, 6C, and 6D.

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The Study

Of the 2 collections of pneumococcal isolates, the first consisted of 587 clinical specimens obtained from infants and children at Seoul National University Children's Hospital, Seoul, South Korea, from May 1991 through May 2008. The second collection consisted of 225 clinical specimens obtained from adults at 2 participating hospitals in Seoul from March 2004 through August 2007. When >1 isolate was recovered from the same person, only the initial isolate was included in the study. From these 2 sample collections (n = 812), we redetermined serotypes for 134 isolates previously assigned to serogroup 6.

Serotyping was performed by using the quellung reaction with antiserum for serogroup 6, factor 6b, and factor 6c (Statens Serum Institute, Copenhagen, Denmark). To assign serotypes 6C and 6D, we screened all strains for $wciN_\beta$ and $wciP_{6B}$ by using 2 simplex PCRs and subsequent sequencing analysis. The *wciN* gene was amplified with the forward primer (5106) 5′-TAC CAT GCA GGG TGG AAT GT-3′ and the reverse primer (3101) 5′-CCA TCC TTC GAG TAT TGC-3′, resulting in product sizes of 1.8 kb for serotypes 6C and 6D for the $wciN_\beta$ gene (*2*). The *wciP* gene was amplified by using the forward primer 5′-AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG -3′ and the reverse primer 5′-TTA GCG GAG ATA ATT TAA AAT GAT GAC TA-3′ (*11*). Presence of $wciN_\beta$ and $wciP_{6B}$ was confirmed by sequencing analysis. A characteristic of 6B *wciP* is the presence of an A at nucleotide position 584 (according to the sequence of *wciP* [*12*]), which creates a codon for asparagine at residue 195 of the 6B *wciP* protein. Antimicrobial drug susceptibility testing, multilocus sequence typing (MLST), and eBURST analyses were performed as described (*13*).

Capsular swelling reactions indicated 63 serotype 6A and 61 serotype 6B strains. However, 10 strains were not distinguished by the standard method (quellung reaction) because they reacted with both factors, 6b and 6c. Sequencing analysis showed that 6 serotype 6A strains were sero-

type 6C according to the presence of $wciN_\beta$ but the absence of $wciP_{6B}$. Subsequently, 4 serotype 6B strains and 10 undistinguished strains were identified as serotype 6D on the basis of the presence of $wciN_\beta$ and $wciP_{6B}$.

Serotypes tested by using the molecular method were 6A (n = 53, 39.6%), 6B (n = 61, 45.5%), 6C (n = 6, 4.5%), and 6D (n = 14, 10.4%). The earliest recovery of a serotype 6D isolate was in 1996, and the earliest recovery of a serotype 6C isolate was in 1993. Two serotype 6D strains were obtained from adults, and the remaining 12 strains were obtained from infants or children. Sources of serotype 6D isolates were blood (n = 5), sputum (n = 6), nasopharynx (n = 2), and throat (n = 1) specimens (Table). All serogroup 6 isolates except a 6C strain showed multidrug resistance to at least 3 classes of antimicrobial drugs. According to MLST, 3 sequence types (STs) were found in serotype 6D pneumococci (ST189 [n = 7], ST3171 [n = 4], and ST282 [n = 3]), which fell into 2 clonal complexes according to eBURST analysis (Figure). ST189 and ST282 were closely related to clonal complex 81, which clustered with serotype 6A strains. All 4 ST3171 strains were isolated from blood. Each ST exhibited distinct antimicrobial drug susceptibility patterns and genes for macrolide resistance (Table).

Conclusions

We identified 14 naturally occurring serotype 6D strains among 134 serogroup 6 pneumococci collected from diverse clinical specimens in South Korea during 1991–2008. The prevalence rate of serotype 6D among serogroup 6 isolates was 10.4%, slightly higher than that of serotype 6C (4.5%). Although serotype 6D was only recently discovered, we demonstrated that serotype 6D strains have been circulating since at least 1996. Serotype 6D was identified from various clinical sources, including blood, sputum, throat swab, and nasopharynx specimens, contrasting with findings of 2 previous studies (*5,6*).

The genetic structures of serotype 6D pneumococci in the MLST database (www.mlst.net) were single isolates of ST4241 (Australia); ST982, ST4190, ST5085, and ST5086 (China); and 2 isolates of ST282 (South Korea). Of those, 3 strains from China (ST982, ST5085, and ST5086) were closely related to the ST3171 strain from South Korea. This cluster of serotype 6D strains was associated with serotype 6A and 6B isolates from 3 countries in Asia. A single isolate of ST4241 was related to STs associated mostly with serotype 6B, but the ST4170 strain did not seem to be linked to other STs. This study demonstrated that 7 serotype 6D strains of ST189 and 3 serotype 6D strains of ST282 were related to clonal complex 81, which had previously been associated with only serotype 6A isolated from South Korea. However, this clonal complex also included several STs associated with many other global serotypes, such as 23F, 19F, and 19A. Although the mechanism is not completely clear, available data indicate that capsular switching from serotypes 6A, 23F, 19F, or 19A to serotype 6D is possible; this switching could occur in addition to replacement of the $wciN_\beta$ gene into the 6B capsule gene locus. A previous study indicated capsular switching as the possible event for formation of serotype 6C isolates (*14*).

In a recent study, factor 6d antiserum was validated for accurate serotyping of 6C (*10*) and is now commercially available, but antiserum for detection of 6D has not yet been developed. Further studies will be required to investigate the prevalence and genetic relatedness of serotype

Table. Genetic structures and phenotypes of 14 strains of *Streptococcus pneumoniae* serotype 6D, Seoul, South Korea*

CC or ST and year of isolation	Patient age	Sample source	MIC, µg/mL		Macrolide resistance gene	
			Penicillin	Cefotaxime	*mef*A	*erm*B
CC81						
ST189						
2000	5 y	Throat swab	1.50	0.75	Present	Absent
2004	81 y	Sputum	2.00	1.00	Present	Absent
2006	1 y	Sputum	1.50	1.00	Present	Absent
2006	2 y	Sputum	1.00	0.75	Present	Absent
2007	5 y	Sputum	1.50	0.75	Present	Absent
2007	6 y	Nasopharynx	1.50	0.75	Present	Absent
2007	73 y	Blood	2.00	1.00	Present	Absent
ST282						
2004	4 mo	Sputum	1.50	0.75	Present	Absent
2005	8 mo	Nasopharynx	1.50	0.75	Present	Absent
2005	9 mo	Sputum	1.50	0.75	Present	Absent
ST3171						
1996	1 y	Blood	0.06	0.05	Absent	Present
1997	3 y	Blood	0.06	0.50	Absent	Present
1997	14 y	Blood	0.06	0.50	Absent	Present
1997	15 y	Blood	0.06	0.50	Absent	Present

*CC, clonal complex; ST, sequence type. Antimicrobial drug susceptibility testing, detection of *mef*A/*erm*B, multilocus sequence typing, and eBURST analyses were performed as described (*13*). All strains were resistant to at least 3 antimicrobial drug classes.

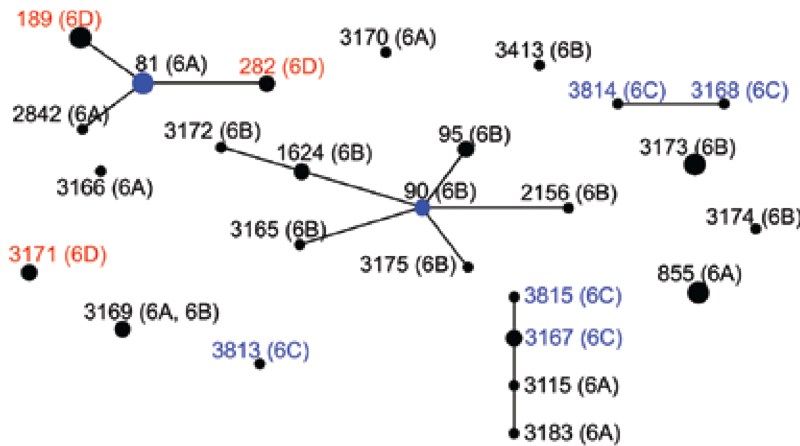

Figure. eBURST analysis of 134 strains of *Streptococcus pneumoniae* serogroup 6. Serotypes are indicated in parentheses. Circle size correlates with number of strains of each sequence type. Blue circles indicate predicted founders (original sequence types within the cluster). Serotype 6D is shown in red, serotype 6C in blue.

6D pneumococci in different countries and to evaluate the effect of pneumococcal conjugate vaccine on serotype distribution.

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Dr Choi is a specialist in pediatric infectious diseases and an associate professor at Seoul National University College of Medicine. Her research interests are primarily related to pediatric respiratory infections and pneumococcal diseases.

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Multidrug-Resistant *Salmonella enterica* Serovar Infantis, Israel

Ohad Gal-Mor, Lea Valinsky, Miriam Weinberger, Sara Guy, Joseph Jaffe, Yosef Ilan Schorr, Abraham Raisfeld, Vered Agmon, and Israel Nissan

To determine whether rapid emergence of *Salmonella enterica* serovar Infantis in Israel resulted from an increase in different biotypes or spread of 1 clone, we characterized 87 serovar Infantis isolates on the genotypic and phenotypic levels. The emerging strain comprised 1 genetic clone with a distinct pulsed-field gel electrophoresis profile and a common antimicrobial drug resistance pattern.

Nontyphoid *Salmonella enterica* (NTS) is a common cause of foodborne illnesses worldwide. In industrialized countries, *S. enterica* serovars Enteritidis and Typhimurium are responsible for most NTS infections (*1*). In Israel, the distribution of NTS infections differs from the global epidemiology for NTS by having a larger representation of serogroups C1 and C2 (serovars Virchow, Hadar, and Infantis) in addition to serovars Enteritidis and Typhimurium (*2*,*3*).

Analysis of annual trends of NTS infections in Israel during 1995–2009 shows a steady decrease in the incidence of these infections, from 86.9 cases/100,000 persons in 1995 to 31.4/100,000 in 2005. During this period, the predominant serovars were Enteritidis, Typhimurium, Virchow, and Hadar, followed by Infantis. Since 2006, annual incidence of NTS has started to increase, rising to 44.0 cases/100,000 persons in 2009. This trend coincided with a sharp increase in incidence of serovar. Infantis from 1.2 cases/100,000 persons in 2001 to 14.7/100,000 in 2009, a 12-fold rise (Figure 1, panel A). The proportion of serovar Infantis increased from <10% of NTS in 1995–2005 to 34% in 2009 (Figure 1, panel B). Furthermore, this steep increase in serovar Infantis from clinical (human) sources correlated with an elevated frequency of serovar Infantis

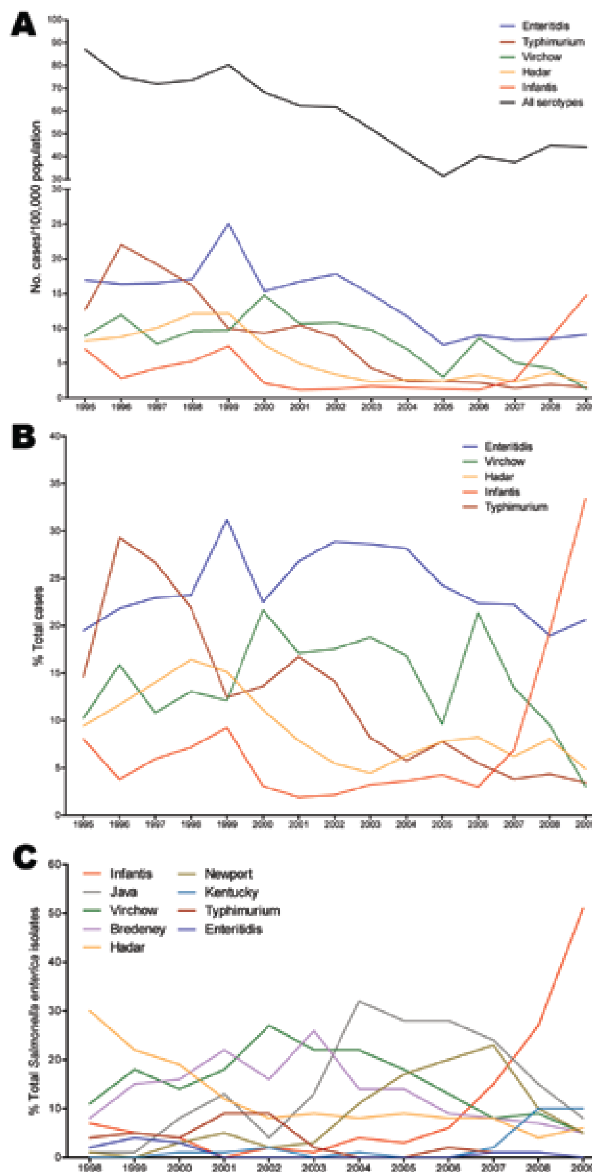

Figure 1. Salmonellosis epidemiology in Israel, 1995–2009. A) Annual incidence of salmonellosis in Israel. Laboratory-confirmed cases of *Salmonella* infections per 100,000 population caused by all *Salmonella* serotypes (black) and by the 5 leading serotypes in Israel. B) The relative contribution (in percentages) of each serotype to the total annual number of *Salmonella* serotypes. *Salmonella* infection incidences were constructed according to the number of human *Salmonella* isolates submitted to the Government Central Laboratories during January 1, 1995–December 31, 2009 (after excluding repeated isolates from the same patient). Data on the Israeli population were derived from the publications of the Israeli Bureau of Statistics. C) Prevalence of *S. enterica* serovar Infantis and other leading serotypes in poultry. The proportion of different *Salmonella* serotypes as percentage from the total *Salmonella* isolates in poultry was analyzed according to routine surveillance in poultry processing plants conducted by veterinary services in 1998–2009. *Salmonella* isolates have been received, identified, and documented in the National *Salmonella* Reference Center of Israel.

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from poultry that became apparent after 2006. Serovar Infantis became the predominant serotype in poultry during 2007–2009, while the prevalences of serovars Enteritidis, Typhimurium, Virchow, Bredeney, Newport, and Paratyphi B var. Java decreased (Figure 1, panel C).

The Study

Molecular analysis was used to study whether the rapid emergence of *S. enterica* ser. Infantis resulted from a general increase in different biotypes or a successful spread of 1 clone. Seventy-one randomly selected isolates of *S. enterica* ser. Infantis identified in Israel during 2007–2009 (21 human sources, 28 poultry sources, and 22 food sources) and 16 historical strains isolated during 1970–2005 (12 human sources, 2 poultry sources, and 2 food sources) were subjected to pulsed-field gel electrophoresis (PFGE). Macrorestriction with the *Xba*I enzyme discriminated the isolates into 23 distinct profiles (pulsotypes), designated I1–I23. Although the historical isolates showed high diversity in their PFGE patterns, most (58/71, 82%) recent (2007–2009) isolates were homogeneous and showed an indistinguishable PFGE profile (pulsotype I1), which was not found among the historical isolates (Figure 2; online Appendix Table, www.cdc.gov/EID/content/16/11/1754-appT.htm). These results indicate that most of the emerging isolates belong to 1 genetic clone that probably started to spread in Israel sometime during 2005–2007. Furthermore, comparison of the I1 pulsotype with other PFGE profiles through PulseNet (www.cdc.gov/pulsenet/) and PulseNet Europe (www.pulsenetinternational.org/networks/europe.asp) indicated a pattern not reported elsewhere, suggesting the emerging clone is endemic to Israel.

To further characterize the isolates, we performed susceptibility tests to 16 antimicrobial compounds. Overall, resistance to 11 antimicrobial agents was detected (Table; online Appendix Table). Two clear differences were found between the strains isolated before and after 2007. First, although 6/16 (38%) of the historical strains were sensitive to all tested antimicrobial agents and 5/16 (31%) were resistant to only 1 (nitrofurantoin), none of the 2007–2009 isolates were sensitive to all of the tested antimicrobial agents. Most (68/71, 96%) of the recent isolates were resistant to ≥3 antimicrobial agents, which suggests a process of resistance acquisition over time. Second, whereas isolates from 1970–2005 did not share any obvious resistance pattern, most (66/71, 93%) of the 2007–2009 strains showed a combined resistance pattern to nalidixic acid, nitrofurantoin, and tetracycline with or without additional resistance to trimethoprim/sulfamethoxazole (Table). The convergence of the recent serovar Infantis clones to a dominant resistance pattern is consistent with their common PFGE profile and shows that they share high similarity on phenotypic and genotypic levels.

Next, we characterized the molecular mechanisms responsible for the common antimicrobial drug–resistance phenotype. In bacteria, an efficient means of acquisition and dissemination of resistance genes is through mobile genetic elements such as plasmids, transposons, or integrons (*5*). Plasmid analysis for 15 emerging (2007–2009) and 7 historical (1970–2005) randomly selected isolates demonstrated that all possessed 1 large plasmid of ≈100 kb. To

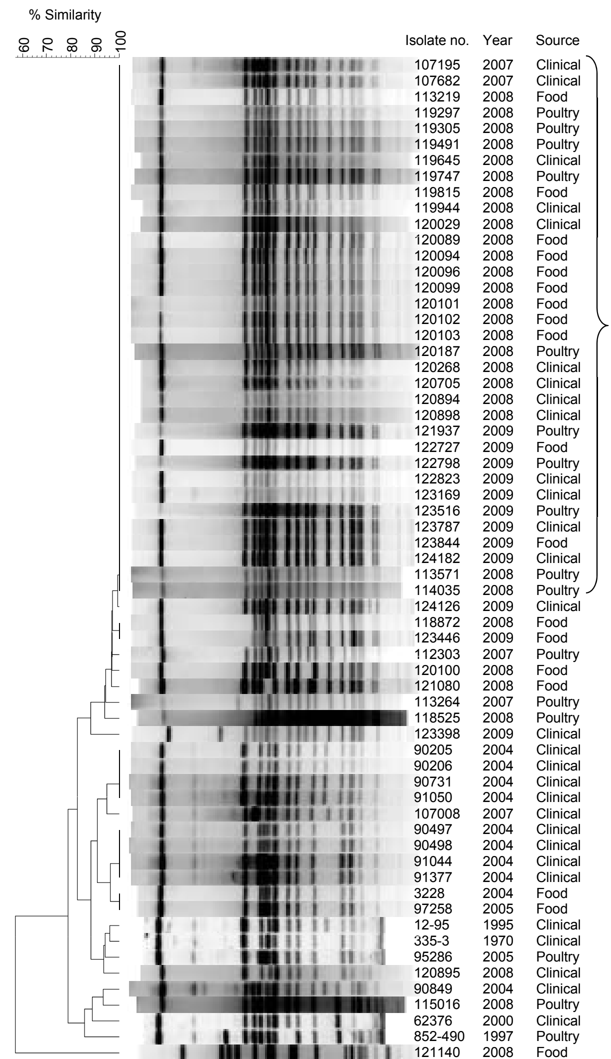

Figure 2. Pulsed-field gel electrophoresis (PFGE) patterns of *Salmonella enterica* serovar Infantis isolates from clinical, food, and poultry sources isolated in Israel, 1970–2009, showing a high degree of clonality. Isolate number, year of isolation, and source are indicated. Bracket indicates I1 pulsotype pattern. Macrodigestion performed using *Xba*I restriction enzyme and genetic similarity (in %) was based on dice coefficients. PFGE was conducted according to the standardized *Salmonella* protocol Centers for Disease Prevention and Control PulseNet as described (4) by using *S. enterica* ser. Braenderup H9812 strain as a molecular size standard. Because of space limitations, only 34/58 pulsotype I1 clones are shown. A complete list is provided in the online Appendix Table (www.cdc.gov/EID/content/16/11/1754-appT.htm)

Table. Antimicrobial drug resistance patterns of *Salmonella enterica* serovar Infantis isolates, sorted by isolation year, Israel*

	PFGE pattern								
	1970–2005		2007		2008		2009		Total
Antimicrobial drug resistance profile	I1	D	I1	D	I1	D	I1	D	
Ampicillin, cefuroxime, ceftriaxone, cephalothin, nitrofurantoin, trimethoprim/sulfamethoxazole		1							1
Ampicillin, cefepime, ceftriaxone, cephalothin, cefuroxime, tobramycin		1							1
Nitrofurantoin		5							5
Nitrofurantoin, tetracycline				1					1
Levofloxacin, nalidixic acid						1			1
Nalidixic acid, nitrofurantoin, tetracycline			1	1	37	3	8	3	53
Nalidixic acid, nitrofurantoin, tetracycline, cephalothin						1			1
Nalidixic acid, nitrofurantoin, tetracycline, trimethoprim/sulfamethoxazole		2	1	1	10		1		15
Nalidixic acid, tetracycline, trimethoprim/sulfamethoxazole						1			1
Nalidixic acid, tetracycline						1			1
Nalidixic acid, trimethoprim/sulfamethoxazole		1							1
Sensitive to all tested antimicrobial drugs		6							6
Total		16	2	3	47	7	9	3	87

*PFGE, pulsed-field gel electrophoresis; I1, emerging PFGE pattern; D, different from the emerging pattern.

identify antimicrobial drug resistance genes that are possibly encoded on this plasmid, mating experiments were conducted with a plasmid-free, rifampin-resistant *Escherichia coli* J5–3 strain and recent *S. enterica* ser. Infantis isolates harboring tetracycline, nalidixic acid, and nitrofurantoin resistance genes. Conjugation experiments showed the obtained *E. coli* transconjugants received the large (≈100-kb) plasmid and acquired the tetracycline resistance phenotype but remained susceptible to nalidixic acid and nitrofurantoin. We concluded the tetracycline resistance gene(s) is encoded on the conjugative plasmid. Molecular analysis by PCR showed the *tetA* gene encoded within the Tn*1721* transposon in 6 of 6 randomly selected emerging isolates but in only 1 of 5 older historical strains.

We examined class 1 integrons using PCR primers designed to amplify the variable region of class 1 integrons. All 6 recent isolates bore 1 integron with a variable region of ≈1 kb. Sequencing of the resulting amplicon showed the *dfrA1* gene cassette conferring resistance to trimethoprim–sulfamethoxazole followed by the *orfC* gene of unknown function. In contrast, 3/5 historical isolates did not possess any integron, and 2/5 contained a disparate integron with a variable region of ≈1.3 kb. Sequencing analysis indicated a different cassette encoded by the aminoglycoside adenyltransferase *aadA1* gene conferring resistance to spectinomycin and streptomycin.

Resistance to quinolones is often associated with point mutations in the quinolone-resistance determining region of the *gyrA* gene (*6*). To examine this possibility, we determined the *gyrA* sequence from 6 recent naladixic acid–resistant and 4 naladixic acid–sensitive isolates. All resistant clones showed the same nucleotide substitution from guanine to thymine at position 259 (G259T) in the *gyrA* gene, resulting in the exchange of asparagine in position 87 to tyrosine (Asp87Tyr) in the quinolone resistance–determining region domain. No mutations were found in the *gyrA* sequence of the naladixic acid–sensitive isolates, suggesting that the Asp87Tyr point mutation is responsible for the observed naladixic acid–resistance phenotype.

Conclusions

It is likely that environmental selective pressure caused by use of antimicrobial drugs has led to the distribution of appropriate resistant genes. Nitrofurans and sulfonamides, for example, have been widely used to treat infections and promote growth of livestock (*7*). Because the emerging clone was dominant in all levels of the food chain, including broiler chickens, it is possible that the emerging clone was originally introduced from a poultry source. Recent studies from other countries identified healthy poultry as a potential reservoir of *S. enterica* ser. Infantis (*8–10*).

Molecular and phenotypic characterization of recent *S. enterica* ser. Infantis isolates from different sources and regions in Israel showed high homogeneity of emerging isolates that differ genetically and phenotypically from previously isolated strains. We showed that the emerging clone is multidrug resistant and is characterized by a large conjugative plasmid harboring the Tn*1721* transposone and *tetA* gene, which provides reduced susceptibility to tetracyclines. Additional characteristics include a class 1 integron containing the *dfrA1* cassette, a *gyrA* mutation that mediates nalidixic acid resistance and furthers resistance to nitrofurantoin. Our results suggest the recent emergence of serovar Infantis is an outcome of a clonal expansion and establishment of a specific biotype that took place during a

relatively short period. Virulence mechanisms contributing to this phenomenon are the subject of an ongoing study.

Acknowledgments

We thank Noemi Nógrády for providing the *Escherichia coli* J5-3 Rif[R] strain.

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Extended-Spectrum β-Lactamase–producing *Escherichia coli* in Neonatal Care Unit

Sarah Tschudin-Sutter, Reno Frei, Manuel Battegay, Irene Hoesli, and Andreas F. Widmer

An outbreak of extended-spectrum β-lactamase–producing *Escherichia coli* in a neonatal care unit began with transmission from a mother to her newborn twins during vaginal delivery. Subsequently, infection spread by healthcare worker contact with other neonates; a healthcare worker also was infected. Knowledge about transmission may improve infection control measures.

Gram-negative *Enterobacteriaceae* expressing extended-spectrum β-lactamase (ESBL) are among the most multidrug-resistant pathogens in hospitals and are spreading worldwide (*1–3*). Infections caused by ESBL–producing organisms have resulted in poor outcomes, reduced rates of clinical and microbiological responses, longer hospital stays, and greater hospital expenses (*4,5*). Multiple outbreaks of ESBL-producing *Enterobacteriaceae* in intensive care units (ICUs) and increased rates of illness and death, especially in neonatal ICUs, have been reported (*6–10*). Physical contact is the most likely mode of transmission. The gastrointestinal tract of colonized or infected patients is the most frequent reservoir. Several studies indicate that transient carriage of bacteria on the hands of healthcare workers (HCWs) may lead to transmission to patients (*7,11*).

We report an outbreak of ESBL-producing *Escherichia coli* (ESBL *E. coli*) in a neonatal intermediate care unit. Initial transmission was from a mother to her newborn twins and subsequently by physical contact of HCWs with other patients; an HCW also was infected.

The Study

The Department of Obstetrics and Gynecology of the University Hospital, Basel, Switzerland, has 94 beds; ≈2,000 babies are delivered there each year. The neonatal unit includes 12 beds for healthy newborns and 9 beds for infants requiring intermediate care.

A 29-year-old woman with dichorionic twin pregnancy was admitted to the antenatal care unit at 32 weeks' gestation because of spontaneous preterm rupture of membranes of the first twin. Her medical history was unremarkable. Screening results for gestational diabetes, as well as urinary controls and vaginal swabs for group B *Streptococcus*, were negative. After confirmation of preterm rupture of membranes by ultrasound and vaginal examination, therapy was initiated with amoxicillin/clavulanic acid (3 × 2.2 g/d) for 10 days, tocolysis with betamimetics (hexoprenaline) until 34 weeks' gestation, and 1 course of steroids for lung maturation (betamethasone 2 × 12 mg with an interval of 24 h).

Five weeks later, the woman spontaneously delivered 2 healthy boys (1,920 g, Apgar scores 9/10/10; and 2,045 g, Apgar scores 8/9/9) under epidural analgesia with placement of a urinary catheter. Two days after delivery, an asymptomatic urinary tract infection with ESBL *E. coli* was detected in the mother; it was treated with trimethoprim/sulfamethoxazole for 7 days. Follow-up urinalysis was negative for ESBL *E. coli*; however, rectal swab performed to document colonization was positive for ESBL *E. coli*. This pathogen persisted for >7 months after delivery, after which the patient was lost to follow-up.

Both twins were initially admitted to the neonatal intermediate care unit because of their prematurity. Six days after birth, screening rectal swabs confirmed colonization with ESBL *E. coli* in both neonates. The twins did not show clinical signs of infection and were discharged on their 20th day.

Screening of the 6 other neonates in the neonatal intermediate care unit during the twins' stay showed that 3 were colonized. In addition, rectal screening of 31 HCWs indicated that 2 (7%) were positive for ESBL *E. coli*. Invasive infection did not develop in any of the 3 neonates colonized with ESBL *E. coli*.

Monthly follow-up screening was performed for the 2 HCWs who were positive for ESBL *E. coli*. They continued working after reeducation about general hygiene precautions. One HCW left her job at the hospital and was lost to follow-up; the other was negative for ESBL *E. coli* at 2-month follow-up.

Rectal swab specimens for surveillance of intestinal carriage were obtained from all patients in the neonatal intermediate care unit during the outbreak and at 2 weeks, 5 months, and 7 months after the outbreak. Screening for ESBL *E. coli* carriage among HCWs was performed by obtaining rectal swabs.

Cultures were performed by using CHROMagar orientation medium (Becton Dickinson BBL Diagnostics, Sparks, MD, USA). ESBL production was identified ac-

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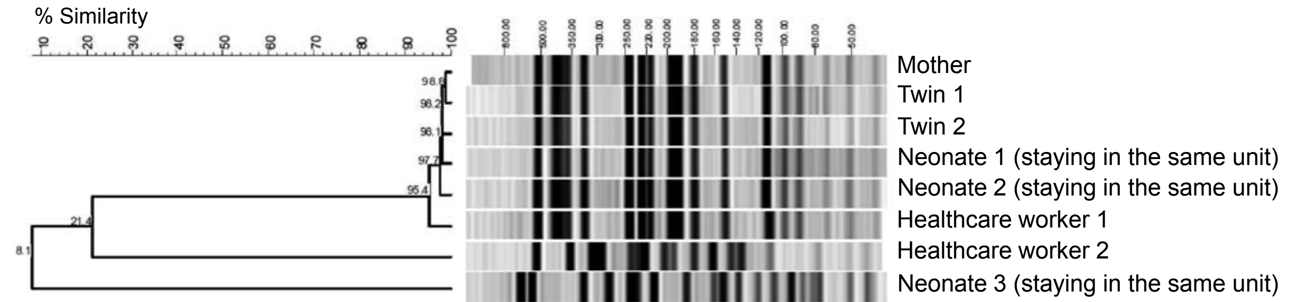

Figure 1. Molecular typing of extended-spectrum β-lactamase–producing *Escherichia coli* isolates by pulsed-field gel electrophoresis. Dendrogram shows a cluster of 6 isolates with identical banding pattern and 2 isolates with 2 distinct patterns.

cording to the guidelines of the Clinical Laboratory Standards Institute (*12*). Routine susceptibility testing was performed by microbroth dilution (Micronaut-S; Merlin, Bornheim-Hersel, Germany). Four cephalosporins (cefpodoxime, ceftriaxone, ceftazidime, and aztreonam) were used for screening. If ≥1 of the cephalosporins showed increased MICs, ESBL *E. coli* was confirmed with Etest strips (AB Biodisk, Solna, Sweden) containing cefotaxime or ceftazidime, each with and without clavulanic acid.

Molecular typing was performed by pulsed-field gel electrophoresis (PFGE). ESBL was molecularly confirmed by PCR amplifying genes for TEM, SHV, and CTX-M β-lactamases. Amplicons were sequenced by using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Genotyping by PFGE showed 1 dominant ESBL *E. coli* strain; 2 different genotypes were found in 1 HCW and in 1 of the screened neonates staying in the same unit as the twins (Figure 1). The outbreak strain was found in the index patient, her twins, 2 neonates staying in the neonatal intermediate care unit at the same time, and 1 HCW (Figure 2). Sequencing of the ESBL gene showed TEM-29 type. Surveillance cultures performed on all patients in the neonatal intermediate care unit indicated no further ESBL *E. coli* was present at 2 weeks, 5 months, and 7 months after the outbreak.

Before the outbreak, a quaternary ammonium–based disinfectant was used daily to clean the neonatal unit. HCWs routinely cared for healthy babies without using gloves but did use an alcohol-based hand sanitizer. Products for patient care were shared among neonates; in particular, no protective covering was used for clinical thermometers.

After screening showed ESBL *E. coli*, reinforced infection control strategies were established. A schedule of training sessions emphasizing proper hand hygiene, routine use of protective covering for clinical thermometers, environmental cleaning using an aldehyde-based disinfectant, and routine use of gloves and gowns for any patient contact (particularly changing diapers) was instituted. Furthermore, separate care products were used for each neonate.

Conclusions

We report an outbreak caused by transmission of ESBL *E. coli* from a mother to her newborn twins and subsequent spread to other neonates and 1 HCW. The mother was most likely colonized before hospitalization, and a urinary tract infection developed peripartum. Transmission by contact during vaginal delivery of the twins and transmission by physical contact to 1 of the HCWs and the other neonates was the most likely mode of transmission. We interpret the detection of ESBL *E. coli* infection in 1 of the neonates and the other HCW as a coincidence because both had a different genotype (TEM-12) and PFGE pattern type of ESBL *E. coli*.

Because we screened only for ESBL *E. coli*, we might have underestimated the true extent of the outbreak. However, the ESBL-encoding gene, which is on a plasmid, could have been transferred to other *Enterobacteraceae* and would have been missed. Risk factors for colonization in newborns include low birthweight, duration of hospitalization, total parenteral nutrition, previous use of antimicrobial drugs, and mechanical ventilation in a neonatal ICU (*13*). In the intermediate care setting, breastfeeding was associated with a lower risk for ESBL-producing

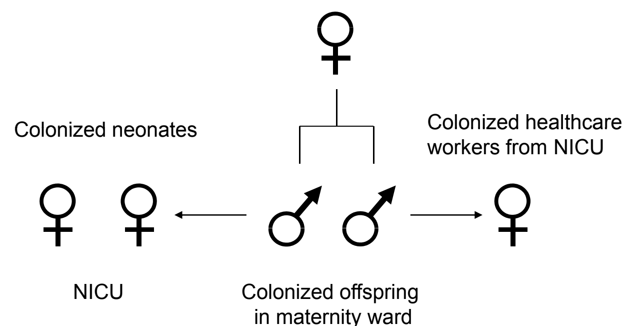

Figure 2. Spread of extended-spectrum β-lactamase–producing *Escherichia coli* outbreak. NICU, neonatal intensive care unit.

Enterobacteriaceae (*14*) because breastfed neonates have more contact with their mothers and therefore are possibly less frequently handled by HCWs. Our patients had only 1 identified risk factor: the twins from the colonized mother had low birthweight; the other neonates had no risk factors. Improved infection control strategies may be necessary to limit spread of ESBL *E. coli* in maternity wards because transmission to neonates during delivery is possible. A feasible approach could be to screen mothers whose neonates need to be transferred to ICUs; an outbreak in this setting would be particularly harmful.

Dr Tschudin-Sutter is a board-certified internal medicine physician currently completing a fellowship in hospital epidemiology and infectious diseases at the University Hospital, Basel, Switzerland. Her research emphasis is multidrug-resistant pathogens.

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Hepatitis E Virus Infection in Sheltered Homeless Persons, France

Mamadou Kaba, Philippe Brouqui, Hervé Richet, Sekené Badiaga, Pierre Gallian, Didier Raoult, and Philippe Colson

To determine the prevalence of hepatitis E virus (HEV) infection among sheltered homeless persons in Marseille, France, we retrospectively tested 490 such persons. A total of 11.6% had immunoglobulin (Ig) G and 2.5% had IgM against HEV; 1 person had HEV genotype 3f. Injection drug use was associated with IgG against HEV.

Initially considered a leading cause of acute hepatitis in tropical and subtropical countries, hepatitis E virus (HEV) is endemic to industrialized countries (*1*). Although substantial data indicate that HEV infection is a porcine zoonosis, more information is needed about the epidemiology and transmission of this virus in industrialized countries (*1–3*). Homeless persons are at higher risk than other persons for viral hepatitis (A, B, and C) because their lifestyle might include injection drug use (IDU) and poor hygiene (*4*), but data on HEV prevalence among them are scarce (*5*,*6*). In Marseille in southeastern France, ≈1,500 persons are homeless (*4*). Since 2000, shelter-based surveys have been conducted yearly to monitor infectious diseases in homeless persons (*4*). This work determined the prevalence of HEV infection in this population.

The Study

The surveys were reviewed and approved by the Institutional Review Board (CCPPCRB99/76) (Comité de Protection des Personnes Sud-Méditerranée II; www.cpp-sudmed2.fr/) and the Ethics Committee of the Medical School, University of the Mediterranean, Marseille). Participating homeless persons were examined by a physician and interviewed by using a standardized questionnaire, and serum samples were collected from each participant for laboratory testing. Epidemiologic, clinical, and biologic data that were collected varied from 1 year to another.

Serum samples collected from 490 homeless persons in 2003, 2005, and 2006 in 2 shelters in Marseille (online Appendix Table, www.cdc.gov/EID/content/16/11/1761-appT.htm) were tested retrospectively for immunoglobulin (Ig) G and IgM (EIAgen HEV kits; Adaltis Italia SpA, Rome, Italy) against HEV and for HEV RNA by using an in-house real-time reverse transcription–PCR specific for open reading frame 2 (*7*). HEV RNA sequencing was performed when HEV RNA was detected, and genotype was assigned through phylogenetic analysis of open reading frame 2 partial sequences (*7*). Serologic testing for hepatitis A, B, and C and for HIV were performed by using Axsym Abbott assays (Abbott Diagnostics Division, Wiesbaden, Germany). Statistical analysis was performed by using STATA version 10.1 software (StataCorp, College Station, TX, USA). Pearson χ^2 test, Fisher exact test, Kruskal-Wallis test, or logistic regression model were used when appropriate.

Mean ± SD age of homeless persons was 43 ± 14 years, and their mean ± SD duration of homelessness was 49 ± 84 months. Most (96.3%) were men and were born in North Africa (40.2%) or in France (33.3%) (online Appendix Table). Previous or ongoing IDU was reported for 4/176 (2.3%). Overall prevalence of anti-HEV IgG and IgM was 11.6% (95% confidence interval [CI] 8.9%–14.8%) (57/490) and 2.5% (95% CI 1.3%–4.2%) (12/490), respectively. Mean optical density ratio (optical density/cutoff value) was 3.0 (range 1.1–6.9) and 2.0 (range 1.1–4.6) for IgG and IgM, respectively. Three (0.6%; 95% CI 0.1%–1.8%) homeless persons were concurrently positive for HEV IgM and IgG, whereas 9 (1.8%; 95% CI 0.8%–3.5%) were positive only for IgM and 54 (11%; 95% CI 8.4%–14.1%) were positive only for IgG.

HEV RNA was detected in 1 homeless person, a 50-year-old man from Romania concurrently seronegative for HEV IgM and IgG and for hepatitis B and C viruses. He reported excessive alcohol intake but no IDU. HEV genotype was 3f (Figure), and sequence analysis showed 98% nt identity with sequences previously recovered from persons in Spain and France. Alanine aminotransferase (ALT) level had been assessed in only 2/12 HEV IgM–positive homeless persons and was elevated in 1 person (177 IU/L), in association with an increased γ-glutamyl transferase level (788 IU/L). Among the 19 homeless persons sampled in 2 different years, 1 seroconverted; he was seronegative for HEV IgM and IgG in 2005 then positive in 2006 (optical density ratio 1.14 and 4.3, respectively). Results of HEV RNA testing were negative in both serum samples, and ALT level had not been tested.

The prevalence of HEV IgG or IgM in homeless persons did not differ by year of survey; sex; place of birth; or serologic status for hepatitis A, B, or C viruses (online Ap-

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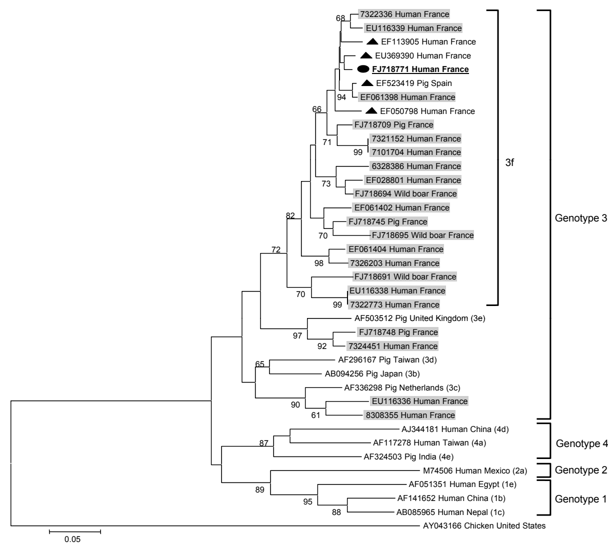

Figure. Phylogenetic tree based on partial nucleotide sequences (275 bp) corresponding to the 5′-end open reading frame 2 region of the hepatitis E virus (HEV) genome. Phylogenetic analysis included HEV sequence recovered in the present study (black circle, **boldface** and underlined; GenBank accession no. FJ71877) and sequences corresponding to the HEV sequences hits with the highest BLASTn score (http://blast.ncbi.nlm.nih.gov) to this sequence (black triangles), previously recovered in our laboratory (**boldface**), and of previously determined genotypes and subtypes (*2*) (in parentheses). Shading indicates sequences previously isolated in our laboratory Bootstrap values >60% of 1,000 resamplings of the data are indicated. Avian HEV sequence AY043166 was used as an outgroup. The names of HEV sequences are labeled as follows: GenBank accession no., host, and country of origin where recovered. Scale bar indicates number of nucleotide substitutions per site.

pendix Table). In addition, mean age, duration of homelessness, and ALT, aspartate aminotransferase, and γ-glutamyl transferase levels did not differ among homeless persons who were positive or negative for HEV antibodies (Table). In the univariate analysis, previous or ongoing IDU (3/4 vs. 19/172; p = 0.006), HIV seropositivity (2/3 vs. 16/183; p = 0.03), and having scabies (6/20 vs. 48/462; p = 0.02) were significantly associated with HEV IgG. In multivariate analysis that used variables that were statistically significant in the univariate analysis as covariates, only IDU was independently associated with anti-HEV IgG (adjusted odds ratio 26.3, 95% CI 2.5–267.1; p = 0.006).

Conclusions

We found that 11.6% (95% CI 8.9%–14.8%) of homeless persons in Marseille were positive for HEV IgG, whereas 2.6% (95% CI 1.3%–4.2%) had HEV IgM or HEV RNA, indicating recent or ongoing HEV infection. The HEV IgG prevalence is similar to that previously found (*6*) in homeless persons in Los Angeles, California, USA (13.5%), and much lower than that found in 98 homeless children in Cochabamba, Bolivia (66.3%) (*5*). This prevalence falls between seroprevalences recently assessed among blood donors in northern (3.2%) and southwestern (16.6%) France (*8*,*9*).

These comparisons should take into account the use of different serologic assays, in addition to differences in epidemiologic settings. Indeed, substantial differences have been observed regarding the performances of some HEV IgG tests (*10*). Moreover, the sensitivity and specificity of the HEV IgG assay used in our study have not been previously evaluated, which warrants a cautious interpretation of the results. A preliminary study conducted in 2008 of 194 blood donors in Marseille with the same assay as that used in the present study found that the prevalence of HEV IgG was 9% (P. Gallian, unpub. data), which is similar to the seroprevalence we found in homeless persons. The HEV IgM assay we used showed good performance in patients with HEV genotype 3 infections in reference to PCR testing (*11*). Thus, sensitivity, specificity, and negative predictive value were 90%, 100%, and 98.8%, respectively. In addition, sensitivities and specificities of this test and of the HEV IgM ELISA 3.0 (MP Diagnostics, Singapore) did not differ significantly.

Although based on a small subset of homeless persons, our finding of the association of IDU with serologic results indicating past HEV infection is intriguing. This result is

Table. Age, duration of homelessness, and liver biochemical test results for 490 homeless persons, Marseilles, France, 2003, 2005, and 2006*

	HEV IgM			HEV IgG		
Variable	Positive	Negative	p value	Positive	Negative	p value
Age, y, mean ± SD	37 ± 12	43 ± 14	0.13	45 ± 15	43 ± 14	0.24
Duration of homelessness, mo, mean ± SD	39 ± 38	49 ± 85	0.36	48 ± 78	49 ± 85	0.28
Liver biochemical tests,† mean ± SD, IU/L						
Alanine aminotransferase levels‡	93 ± 119	32 ± 31	0.99	36 ± 32	32 ± 33	0.49
Aspartate aminotransferase levels§	49 ± 54	34 ± 43	0.99	34 ± 32	34 ± 44	0.79
γ-glutamyl transferase¶	401 ± 547	65 ± 137	0.92	76 ± 136	68 ± 147	0.44

*HEV, hepatitis E virus; Ig, immunoglobulin.
†Assessed only in 2005 for 209 homeless persons.
‡Data missing for 11 persons.
§Data missing for 10 persons.
¶Data missing for 9 persons.

surprising because the proportion of IDUs in our homeless population was low (2.3%) and much lower than proportions previously reported (9%–83% (*12–15*). HEV IgG prevalence of 2.2%–62% has been described in IDUs, but a significant difference with the control group was found in only 2 of these studies (*12–15*). HEV IgG prevalence in Sweden was 62% (21/34) in patients who acquired acute hepatitis B through IDU compared with 25% (9/36) in patients with sexually acquired hepatitis B ($p<0.005$) (*13*). Moreover, in Italy, the prevalence was 5.4% (16/179) in IDUs compared with 2.6% (49/1,889) in the general population ($p<0.00001$) (*12*). In summary, our data indicate that HEV infection occurs in homeless persons, and further studies are needed to determine whether IDU is responsible for HEV transmission.

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Enterovirus 71 Infection with Central Nervous System Involvement, South Korea

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We assessed neurologic sequelae associated with an enterovirus 71 (EV71) outbreak in South Korea during 2009. Four of 94 patients had high signal intensities at brainstem or cerebellum on magnetic resonance imaging. Two patients died of cardiopulmonary collapse; 2 had severe neurologic sequelae. Severity and case-fatality rates may differ by EV71 genotype or subgenotype.

Several major outbreaks of enterovirus 71 (EV71) have been reported since 1974 (*1*,*2*). Countries of the Asian Pacific Rim particularly have been recently affected by large outbreaks of EV71-associated hand-foot-and-mouth disease (HFMD). Most patients with HFMD experience a mild disease course, but recent reports on the outbreak of EV71 infection in various countries, including Taiwan, People's Republic of China, and Malaysia, indicate that some EV71-infected persons have severe neurologic complications or systemic disease (*3*,*4*).

The varying prevalences of neurologic complications of EV71 infection among outbreaks are assumed to have been driven by differences of genotypes and co-infection with other viruses, such as a newly characterized adenovirus; however, the exact reasons remain unclear (*3*,*5*,*6*). We report an outbreak of EV71 infection with neurologic involvement on the basis of information from a prospective, clinical, and virologic study that was collected through South Korea's nationwide surveillance system.

The Study

The EV surveillance system in South Korea consists of 62 clinics (8 primary clinics, 14 secondary hospitals, and 40 tertiary hospitals located nationwide) managed by pediatric physicians (Figure). During 2009, a total of 2,427 cases of viral disease were reported to the Korea Centers for Disease Control and Prevention through a web-based system. In addition, an experienced neurologist (W.-S.R.) collected detailed clinical information about, and results of imaging studies of, patients reported to have central nervous system (CNS) involvement. We monitored the patients until they were discharged or for 3 weeks if duration of hospitalization was >3 weeks. Patient outcome was classified into 1 of 4 groups; no sequelae (neurologic dysfunction without dependency), mild sequelae, severe sequelae (neurologic dysfunction requiring assistance), or death.

EV genome detection was attempted by real-time reverse transcription–PCR (RT-PCR) by using TaqMan technology (Applied Biosystems, Foster City, CA, USA). Briefly, viral RNAs were extracted by using the magnetic bead–based viral nucleic acid purification protocol described by Boom et al. (*7*). Subsequently, 1-step real-time RT-PCR was performed by using a dual-labeled fluorogenic EV-specific probe and primers designed on the basis of previous data (*8*). For genotyping, seminested RT-PCR was used to amplify part of the viral protein (VP) 1 gene of EV, based on the Korea Centers for Disease Control and Prevention protocol for detection of pan-EV, and sequencing analysis for VP1 amplicon was performed by using automatic sequencer and DNAstar software package (*9*).

In 2009, a total of 2,427 patients were recruited. Of these patients, 519 had symptoms of HFMD or herpangina. EV was detected in 461 (19%) of all patients and in 321 (66%) of patients with suspected HFMD and herpangina. Samples from 331 (72%) of the 461 EV-seropositive patients were available for genotyping (Table 1). In addition,

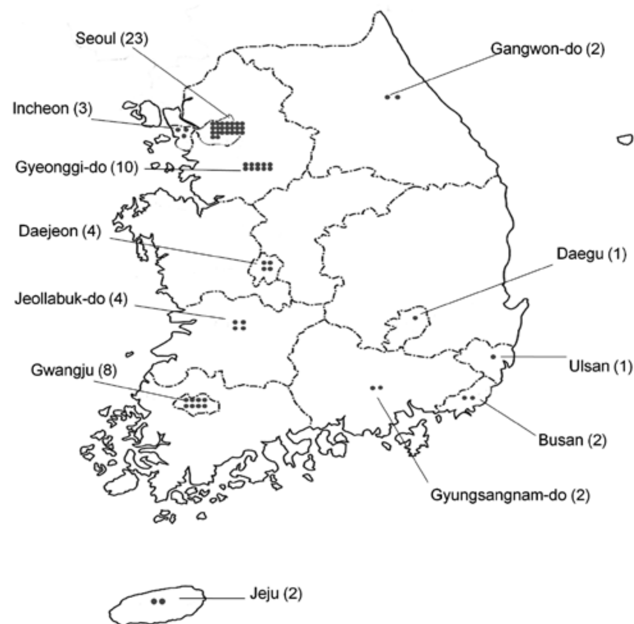

Figure. Geographic distribution of clinics participating in enterovirus surveillance, South Korea, 2009.

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Table 1. Genotype distribution of enteroviruses, South Korea

Genotype	No. cases (%)
CA2	24 (7.3)
CA5	35 (10.6)
CA6	32 (9.7)
CA12	3 (0.9)
CA16	29 (8.8)
CB1	8 (2.4)
E3	8 (2.4)
E6	3 (0.9)
E9	2 (0.6)
E11	8 (2.4)
E30	2 (0.6)
E33	3 (0.9)
E71	174 (52.6)
Total	331

we found 112 cases of HFMD with CNS complications (meningitis or encephalitis); EV was detected in 95 (85%) and EV71 in 92 (82%) case-patients. Furthermore, EV71 was detected in 2 of the 187 case-patients in which meningitis without HFMD or herpangina was diagnosed. Thus, 94 patients were enrolled in this study. The incidence of EV71 infection peaked in July and decreased drastically in August.

Fifty-nine (63%) patients were boys. Mean (SD) patient age was 46 (29) months (range 1 month–12 years); 12 (13%) patients were <1 year of age. Initial diagnoses were viral meningitis (60 patients), encephalitis (20), acute cerebellar ataxia (12), acute transverse myelitis (1), and Guillain-Barré syndrome (1). Rash and fever were the most common initial symptoms (85% and 81%, respectively). Approximately 50% of patients had headache, vomiting, and neck stiffness. Cerebrospinal fluid (CSF) profile was available for 77 patients. Median leukocyte count was 111 cells/mm^3 (interquartile range 48–318 cells/mm^3), mean (SD) protein level was 43 (18) mg/dL, and mean (SD) glucose level was 69 (13) mg/dL. Of the 32 (34%) patients who underwent magnetic resonance imaging of the brain, 24 (75%) had normal results, 4 (13%) had meningeal enhancement on T1-enhanced imaging, and 4 (13%) had high signal intensity at the brainstem or cerebellum on T2-weighted or fluid-attenuated inversion recovery imaging. All brain parenchymal lesions were located in the brainstem or cerebellum, and the 4 patients with brain lesions subsequently experienced ataxia.

EV71 was found in 72 (92%) of 78 lower gastrointestinal tract samples, 37 (60%) of 62 upper respiratory tract samples, and 2 (5%) of 37 CSF samples. On the basis of sequence analysis, C4a, with high similarity to strains from China in 2008, was a dominant serotype of EV71 (76%); and C1 was found in 2 patients and C5 in 1 patient.

The results of this and previous studies are summarized in Table 2. The partial VP1 sequences of Korean EV71 strains were registered in GenBank (HM443164–644), and viral genetic identity belonged to C4a genotype, which was not a prevailing genotype in the previous reports of other Asian Pacific countries.

Conclusions

The severity of, and case-fatality rate for, EV71 infection in our population were relatively low compared with those of previous reports in which the case-fatality rate ranged from 10% to 26% (*12*). This discrepancy has some plausible explanations. In previous studies, the prevailing genotype of EV71 was B (*1*,*14*). In contrast, the predominant genotype in our patients was C4, particularly C4a, which has been prevalent in China since 2008 (*11*). The case-fatality rate also was low in China, with 3 patients dying of the 1,149 reported with EV71 infection (*11*). Therefore, the virulence of the C4a genotype may be milder than that of other genotypes.

We detected EV71 rarely in the CSF of our patients. Possible explanations include the transient presence of the virus in CSF, a lower viral load in CSF, and use of an EV PCR assay that had not been optimized to detect EV71. In our population, the CSF profile of EV71 infection appeared to be broadly similar to that of other cases of vi-

Table 2. Enterovirus 71 outbreaks, Asia*

Reference	Outbreak location, year	HFMD		Complicated HFMD		Genotypes detected	GenBank accession nos.
		No. patients	No. patients with EV71	No. patients	No. patients with EV71		
Zhang et al. (*10*)	Fuyang, PRC, 2008	151	59	112	42	C4a	EU703812–14, GQ121417–41
Zhang et al. (*11*)	Shandong, PRC, 2007	105	55	11	6	C4a	EU753365–417
AbuBakar et al. (*1*)	Brunei, 2006	≥100	34	NA	NA	B4, B5	FM201328–61
Ooi et al. (*12*)	Sarawak, Malaysia, 2000–2004	773	277	102	56	B4, C1, B5	AY905549–50, AY794036, AF376069
Lin et al. (*13*)	Taiwan, 1998	NA	NA	405	78	C2	AY055194–97, FJ357343
This study	South Korea, 2009	519	168	112	92	C4a, C1, C5	HM443164–644

*HFMD, hand-foot-and-mouth disease; EV71, enterovirus 71; PRC, People's Republic of China; NA, not available.

ral encephalitis or meningitis. Thus, analyzing the CSF of patients with suspected EV71 infection may provide minimal information.

Several studies have shown that EV71 infection rate was most common during the warmer season (*15*). In our study, the seasonality of EV71 infection initially was similar to that of previous reports. However, the prevalence of EV71 infection decreased drastically in August, the warmest month in South Korea. A possible reason for this difference could be that in 2009, influenza pandemic (H1N1) 2009 affected South Korea; with the first death caused by it in South Korea reported in August. As a consequence, personal hygiene practices, such as handwashing and covering one's cough or sneeze, were emphasized to prevent virus spread. Considering the transmission route of EV71 infection, the emphasis on personal hygiene may thus have hindered the spread of EV71, as well as of the influenza virus.

We report 94 cases of PCR-confirmed EV71 infection with CNS involvement, including 2 deaths, and provide additional clinical and virologic information about EV71. We confirmed that EV71 commonly involved the brainstem and cerebellum, and therefore ataxia is not uncommon in EV71 infection with CNS involvement. In addition, our study supports the hypothesis that the severity of and case-fatality rates for EV71 infection may differ by genotype or subgenotype of EV71.

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Genome Sequence Conservation of Hendra Virus Isolates during Spillover to Horses, Australia

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Bat-to-horse transmission of Hendra virus has occurred at least 14 times. Although clinical signs in horses have differed, genome sequencing has demonstrated little variation among the isolates. Our sequencing of 5 isolates from recent Hendra virus outbreaks in horses found no correlation between sequences and time or geographic location of outbreaks.

Hendra virus (HeV) (family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Henipavirus*) was first isolated in 1994 during an outbreak of respiratory disease in horses at a stable in Hendra, a suburb of Brisbane, Queensland, Australia. During the outbreak, all horses at the affected property were tested. Thirteen horses died; 7 recovered with seroconversion, some without clinical signs; and 9 remained uninfected. A horse trainer and a stable worker also became infected, and the trainer subsequently died. In October 1995, HeV infection was diagnosed for a third person, who lived in Mackay, ≈1,000 km north of Brisbane. Retrospective analysis demonstrated that an outbreak had occurred in Mackay in August 1994, where the infected person had assisted with necropsies on 2 horses. At the time, he recovered from a mild undiagnosed infection. Fourteen months later, fatal encephalitis developed, suggesting either virus persistence or late onset of disease symptoms (*1*). Since 1994, a total of 12 outbreaks have occurred (Table 1).

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Table 1. Hendra virus outbreaks affecting horses and humans, Australia, August 1994–May 2010*

Date	Location	Horses, no. cases	Humans, no. cases/no. deaths
1994 Aug	Mackay, QLD	2	1/1
1994 Sep	Hendra, QLD	20	2/1
1999 Jan	Trinity Beach, QLD	1	0/0
2004 Oct	Gordonvale, QLD	1	1/0
2004 Dec	Townsville, QLD	1	0/0
2006 Jun	Peachester, QLD	1	0/0
2006 Oct	Murwillumbah, NSW	1	0/0
2007 Jun	Peachester, QLD	1	0/0
2007 Jul	Clifton Beach, QLD	1	0/0
2008 Jul	Redlands, QLD	5	2/1
2008 Jul	Proserpine, QLD	3	0/0
2009 Jul	Rockhampton, QLD	3	1/1
2009 Sep	Bowen, QLD	3	0/0
2010 May	Tewantin, QLD	1	0/0
Total		44	7/4

*QLD, Queensland; NSW, New South Wales.

Serologic evidence identified flying foxes (genus *Pteropus*) as the likely reservoir host, and HeV was subsequently isolated from 2 species of pteropid bats (*2*). Serologic evidence of HeV in bats has been demonstrated along the east coast of Australia to Melbourne and west across northern Australia to Darwin. Seroprevalence can be as high as 25% (*3*). In recent years, the regularity of spillover events has increased. Increased monitoring of bat population sizes, virus in bat populations, and virus characterization is necessary to better learn about trigger(s) for spillover events.

Nipah virus (NiV) is the only other known species within the genus *Henipavirus*. NiV was first identified during a major outbreak of diseases in pigs and humans in peninsular Malaysia during 1998–99. NiV reemerged in Bangladesh in 2001, with recurrence resulting in human infection almost annually in Bangladesh and India (*4*). Serologic evidence of NiV or NiV-related viruses has been demonstrated in bats in Thailand (*5*), Indonesia (*6*), People's Republic of China (*7*), Madagascar (*8*), and west Africa (*9*); virus has been isolated from flying foxes in Malaysia (*10*) and Cambodia (*11*).

A major characteristic of henipavirus infections is their systemic spread, with evidence of infection in multiple organ systems. HeV infection in horses typically produces an acute, febrile respiratory disease (*12*) with a high case-fatality rate. The 2008 Redlands outbreak was the largest in horses since the first identified outbreak in 1994. During this outbreak, infected horses showed atypical signs of HeV infections, with clinical features of a more neurologic nature (*13*). Before the outbreak was attributed to HeV, 2 persons became infected, resulting in 1 death and the potential exposure of >50 persons. The reason for the altered clinical picture during this spillover event is unknown.

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The Study

Little is known about the genetic variation of henipaviruses because few sequences are available; most sequences came from the NiV outbreak in Malaysia and Singapore. From the NiV outbreak in Malaysia, sequences were obtained for isolates from 4 humans, 4 pigs, and 1 bat. One sequence is available for the original HeV isolate and 1 isolate each of NiV from Bangladesh and India. A limited sequence for the *N* and *G* genes for 2 isolates from Cambodia has been deposited in GenBank.

Five additional isolates from horses were obtained from outbreaks in Murwillumbah (2006), Peachester (2007), Clifton Beach (2007), Redlands (2008), and Proserpine (2008). These isolates provided us with a unique opportunity to examine HeV sequence variation over time and geographic separations.

Analysis of the sequences demonstrated the extreme conservation at the genome and protein levels. All isolates had the identical genome length of 18,234 nt, with the sequence variation across the full genome being ≤1%. All open reading frames (ORFs) were the same length as those of the original HeV isolate (Hendra virus/Australia/horse/1994/Hendra). Minor nucleotide changes were seen in the ORFs and the noncoding regions. The *N* ORF had the highest percentage of changes in both nucleotide and amino acid variation (Table 2). The number of nucleotide changes was lower than those in other RNA viruses; for example, the *N* gene of measles virus showed up to 7% variation in nucleotide sequence (*14*). The predicted size of each of the *P* gene products (V, W, and C) was conserved in all isolates. The small basic (SB) protein ORF identified in the original HeV isolate of HeV, but not seen in any of the NiV strains, was present in all isolates with an identical length of 65 aa. The variation in this ORF was higher than that for other ORFs, with 0–6% aa variation. Although SB is in an alternate frame within the *P* gene, the variation was much higher than that of the *C* ORF, which is also in an alternate ORF.

Phylogenetic analysis was performed by using both DNA and amino acid sequences for individual genes and for the complete genome. The multiple branches of the phylogenetic tree, particularly for the *N* gene (Figure), suggest that all these isolates branched from an ancestor earlier than 1994 when the first identified outbreak occurred. For example, the 2006 isolate from Murwillumbah shows greatest similarity to the 1994 isolate, whereas the other isolates are in a separate branch on the tree.

The data reported here are consistent with each of these individual spillover events that occurred after exposure to viral variants coming from a large pool of quasispecies in the bat population in Australia. This genetic conservation in HeV isolates may suggest that HeV is genetically stable in the reservoir bat population, although because of the lack of HeV isolates from bats, the possibility cannot be excluded that this genetic similarity resulted from selection both for variants that can infect horses and selection within the infected horse. Further analysis of this sequence data and future sequence information will help with understanding of differences in the clinical picture and may provide evidence to explain the apparent increase in regularity of recent spillover events.

Conclusions

Our results demonstrated that HeV isolates from horses are genetically similar, with variation at both the nucleotide and amino acid levels, uniformly small, and <1% across the 18.2-kb genome. Furthermore, data show that different outbreaks resulted from independent spillover events from a pool of HeV quasispecies in the fruit bat populations in Australia. The genetic relatedness of HeV isolates from horses is not correlated with the time the corresponding HeV outbreaks occurred. The presence of SB ORF in all HeV isolates, but not in NiV isolates, warrants further functional analysis of this intriguing putative protein.

Acknowledgments

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Table 2. Nucleotide and amino acid residue changes in recent Hendra virus isolates compared with the original isolate, Australia

Isolate	Full genome variation (%)	Open reading frame (length in nt): *N* (1,599) nt	*N* aa	*P* (2,124) nt	*P* aa	*M* (1,059) nt	*M* aa	*F* (1,641) nt	*F* aa	*G* (1,815) nt	*G* aa	*L* (6,735) nt	*L* aa
Hendra virus/Australia/horse/2006/Murwillumbah (GenBank accession no. HM044318)	171 (0.94 %)	16	6	5	4	6	2	2	1	4	2	39	10
Hendra virus/Australia/horse/2007/Peachester (GenBank accession no. HM044319)	87 (0.47 %)	2	1	13	4	5	1	10	1	12	2	57	11
Hendra virus/Australia/horse/2007/Clifton Beach (GenBank accession no. HM044321)	139 (0.76 %)	12	4	6	3	5	1	11	1	5	0	48	11
Hendra virus/Australia/horse/2008/Redlands (GenBank accession no. HM044317)	186 (1.02 %)	16	4	14	2	4	1	13	1	11	0	59	12
Hendra virus/Australia/horse/2008/Proserpine (GenBank accession no. HM044320)	183 (1.00 %)	17	4	15	4	7	2	9	1	11	1	64	11

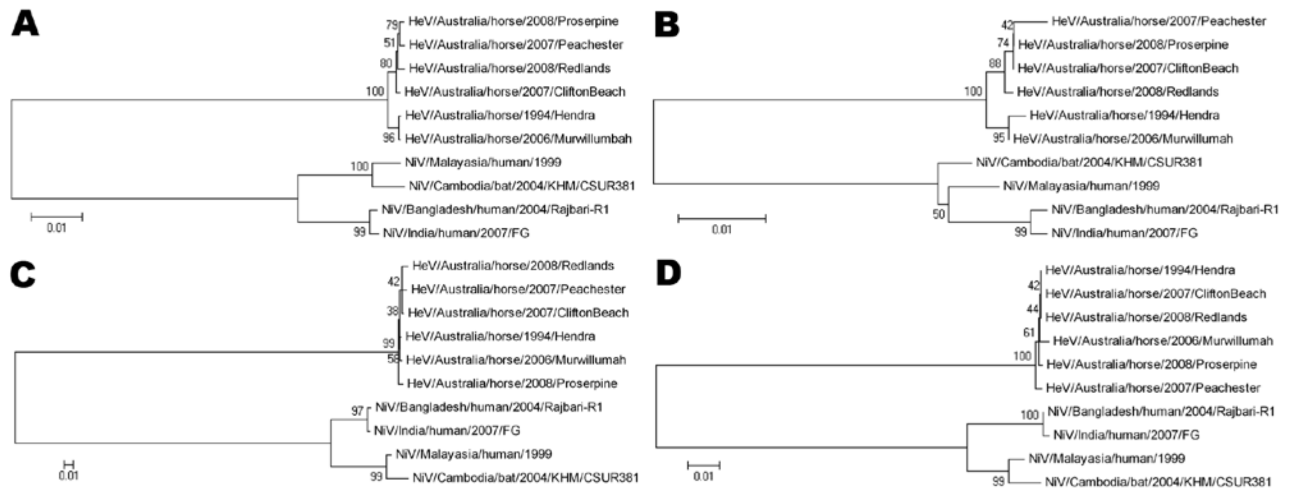

Figure. Phylogenetic trees based on the *N* open reading frame (ORF) (A, B) and the *G* ORF (C, D), with DNA sequences used for A and C and amino acid sequences for B and D. All sequences were compared with the reference sequences for each of the known henipavirus strains; Hendra virus/Australia/horse/1994/Hendra (GenBank accession no. AF017149), Nipah virus/Malaysia/human/1999 (GenBank accession no. AF212302), Nipah virus/Bangladesh/human/2004/Rajbari, R1 (GenBank accession no. AY988601), Nipah virus/Cambodia/bat/2004/KHM/CSUR381 (GenBank accession no. AY858110 [*N* ORF] and AY858111 [*G* ORF]) and Nipah virus/India/human/2007/GF (GenBank accession no. FJ513078). Phylogenetic trees were constructed by using the neighbor-joining algorithm in the MEGA4 software package (*15*). Scale bars represent substitutions per site. HeV, Hendra virus; NiV, Nipah virus.

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Importation of Dengue Virus Type 3 to Japan from Tanzania and Côte d'Ivoire

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Travelers can introduce viruses from disease-endemic to non–disease-endemic areas. Serologic and virologic tests confirmed dengue virus infections in 3 travelers returning to Japan: 2 from Tanzania and 1 from Côte d'Ivoire. Phylogenetic analysis of the envelope gene showed that 2 genetically related virus isolates belonged to dengue virus type 3 genotype III.

Dengue virus (DENV) is an arthropod-borne virus that infects ≈100 million persons each year in Southeast Asia, Central and South America, and Africa. Infection with any 1 of the 4 DENV serotypes causes a wide range of disease, from dengue fever to the more severe dengue hemorrhagic fever. Epidemics of dengue-like illness have occurred in Africa, but information about etiology is limited (*1*). Transmission of another arthropod-borne virus, chikungunya virus, which causes disease similar to dengue, has been documented in Tanzania. Although Tanzania is on the list of countries at risk for DENV transmission (*2*), to our knowledge, no DENV isolates have yet been identified and studied there.

In Japan as of June 16, 2010, a total of 50 cases of imported dengue had been reported. Among these cases, 2 were dengue fever that had developed in 2 travelers after they returned from Tanzania. We report the molecular characterization of 2 DENV type 3 (DENV-3) isolates from 1 of the travelers who had visited Tanzania in 2010 and from a traveler who had visited Côte d'Ivoire in 2008.

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The Study

In 2010, a 55-year-old man (patient 1) and a 23-year-old woman (patient 2) returned to Japan from Tanzania; high fever and thrombocytopenia developed in each on days 1 and 3 days after return, respectively. In 2008, a 65-year-old man (patient 3) returned to Japan from Côte d'Ivoire and subsequently experienced high fever. Serum samples from each of the 3 patients were sent to the National Institute of Infectious Diseases, Japan, for laboratory examination. DENV serotypes were determined by serotype-specific reverse transcription–PCR (RT-PCR) (*3*). DENV-specific immunoglobulin (Ig) M was detected by Dengue Fever Virus IgM Capture ELISA (Focus Diagnostics, Inc., Cypress, CA, USA) used according to the manufacturer's instructions. Dengue IgG Indirect ELISA (Panbio Ltd, Sinnamon Park, Queensland, Australia) was used to detect anti-DENV IgG according to the manufacturer's instructions. Serum from patient 1 was negative for anti-DENV IgM and anti-DENV IgG; serum from patients 2 and 3 was positive for anti-DENV IgM and IgG. All 3 serum samples had positive DENV nonstructural protein (NS) 1 antigen results according to NS1 capture ELISA (Platelia Dengue NS1 Antigen assay; Bio-Rad Laboratories, Marnes-la-Coquette, France) and negative chikungunya viral RNA results by RT-PCR.

DENV-3 (D3/Hu/Tanzania/NIID08/2010) was isolated from patient 1, and DENV-3 (D3/Hu/Côte d'Ivoire/NIID48/2008) was isolated from patient 3 by using the *Aedes albopictus* mosquito cell line C6/36 and FcγR-expressing baby hamster kidney cells (*4*). DENV-3 RNA was detected in serum from patient 2 by RT-PCR, but the virus was not isolated. The viral RNA was extracted by using a High Pure Viral RNA Extraction kit (Roche Diagnostics, Mannheim, Germany), transcribed to cDNA, amplified by PCR, and sequenced as described (*3*).

Nucleotide sequences of the isolates were compared with selected sequences of DENV-3 (Table). Sequence alignment and phylogenetic analysis was performed by the Genetyx analysis program (Genetyx Corp., Tokyo, Japan). The phylogenetic tree was constructed by using the neighbor-joining method. The selected DENV-3 strains were grouped into 5 genotypes (*5*). Confidence values for virus groupings were assessed by bootstrap assembling analysis of 1,000 replicates. The 2 DENV-3 isolates belonged to DENV-3 genotype III (Figure). The envelope (E)-protein sequence showed that the DENV-3 (D3/Hu/Tanzania/NIID08/2010 strain) isolated from patient 1 had a sequence homology of 98% to the DENV-3 D3/Hu/Côte d'Ivoire/NIID48/2008 strain and 99% to a DENV-3 6805 strain isolated in Saudi Arabia in 2004 (GenBank accession no. AM746229) (*6*).

Conclusions

DENV transmission has occurred in western Africa and some parts of eastern Africa (*7–10*). We isolated

DENV-3 from 2 patients in Japan in whom dengue fever developed after they returned from Côte d'Ivoire (western Africa) and Tanzania (eastern Africa). Detection of DENV-3 in patients 1 and 2 suggests local DENV-3 transmission in Tanzania. As of April 2010, at least 17 suspected cases of dengue have been reported among residents of Dar es Salaam, Tanzania, but the molecular epidemiology of DENV in Tanzania has not been analyzed (*11*). In the absence of such analyses, data on molecular epidemiology of DENV isolated from returning travelers offers timely information to countries where dengue surveillance is not routinely performed. Data and reports of the presence of a competent DENV vector, *Ae. aegypti* mosquitoes, suggest the need for further studies on local DENV transmission in Tanzania (*12*).

It is assumed that travelers increase the risk for introduction of DENV serotypes or strains from disease-endemic to non–disease-endemic areas where competent vectors such as *Ae. aegypti* or *Ae. albopictus* mosquitoes are present. The sequence homology among the DENV-3 strain isolated from the traveler to Tanzania (patient 1), the DENV-3 strain isolated from the traveler from Côte d'Ivoire in 2008 (patient 3), and a DENV-3 strain isolated in Saudi Arabia in 2004 ranged from 98% to 99% (GenBank accession nos. AB549332, AB447989, AM746229, respectively). Because the E-protein gene of the isolate from Tanzania was highly similar to those in viruses circulating regionally and the Middle East, the disease could have been introduced or reintroduced into the country from neighboring areas. These data suggest the need for further studies of the route of disease dissemination and surveillance of dengue in Africa. Genetic differences among subtypes may result in differences in virus virulence and epidemic potential (*13*). DENV-3 genotype III, previously isolated from several parts of Africa, Latin America, and the Indian subcontinent, has been associated with higher incidence of major

Table. Comparison of dengue virus type 3 sequences from travelers returning from Tanzania and Côte d'Ivoire with selected dengue virus type 3 sequences*

Year isolated	Name	Strain	Isolate origin	GenBank accession no.
2010	Tanzania2010	D3/Hu/Tanzania/NIID08/2010	Tanzania	AB549332
2008	Côte d'Ivoire2008–1	D3/Hu/Côte d'Ivoire/NIID48/2008	Côte d'Ivoire	AB447989
2008	Côte d'Ivoire2008–2	2008/00510	Côte d'Ivoire	FM213456
2007	Bhutan2007–1	SV0786_07	Bhutan	FJ606712
2007	Bhutan2007–2	SV0837_07	Bhutan	FJ606708
2004	Saudi Arabia2004–1	6805	Saudi Arabia	AM746229
2004	Saudi Arabia2004–2	6475	Saudi Arabia	AM746232
2004	Colombia2004	22379_MEDELLIN/04	Colombia	FJ389910
2003	India2003	GWL-25	India	AY770511
2001	Venezuela2001	LARD6667	Venezuela	AY146773
2000	Brazil2000	68784	Brazil	AY038605
2000	Venezuela2000	LARD6315	Venezuela	AY146767
2000	Cambodia–India2000†	00–28–1HuNIID	Cambodia/India	AB111081
1997	Thailand1997	D97–0106	Thailand	AY145728
1995	Honduras1995	HN179	Honduras	FJ189469
1992–1994	Malaysia1992–1994–1	LN1746	Malaysia	AF147458
1992–1994	Malaysia1992–1994–2	LN2632	Malaysia	AF147459
1992	Fiji1992	29472	Fiji	L11422
1991	Sri Lanka1991	2783	Sri Lanka	L11438
1990	Sri Lanka1990	SK698	Sri Lanka	FJ189449
1989	Tahiti1989	2167	Tahiti	L11619
1989	Sri Lanka1989	260698	Sri Lanka	L11437
1986	Thailand1986	D86–007	Thailand	L11441
1985	Mozambique1985	1559	Mozambique	L11430
1984	India1984	1416	India	L11424
1983	Philippines1983	168.AP-2	Philippines	L11432
1981	Malaysia1981	29586	Malaysia	L11427
1963	Puerto Rico1963–1	PR6	Puerto Rico	L11433
1963	Puerto Rico1963–2	BS-PRico63	Puerto Rico	AY146762
1956	Philippines1956	H87	Philippines	L11423
2001	DENV-1	01–44–1HuNIID	Tahiti	AB111070
2005	DENV-2	D2/Hu/OPD030NIID/2005	East Timor	AB219135
2001	DENV-4	MY01–22713	Malaysia	AJ428556

*DENV, dengue virus.
†Strain 00–28–1HuNIID was isolated from a traveler who returned to Japan after visiting Cambodia and India.

epidemics of dengue and dengue hemorrhagic fever (*14*). The DENV isolates from Tanzania and Côte d' Ivoire were closely related to a DENV-3 genotype III strain isolated from a major DENV outbreak in northern India in 2003–2004; the E-protein gene homology was 98% (*15*).

Emergence of DENV-3 genotype III in geographically diverse areas may thus result from higher epidemic potential of the virus, although further studies are needed to understand the clinical and epidemiologic implications of emergence or reemergence of the virus in Tanzania and Côte d' Ivoire. Whereas other vector-borne diseases such as malaria and yellow fever have been well studied in Africa, dengue needs more attention with regard to identification of epidemics, clinical implications, and disease management.

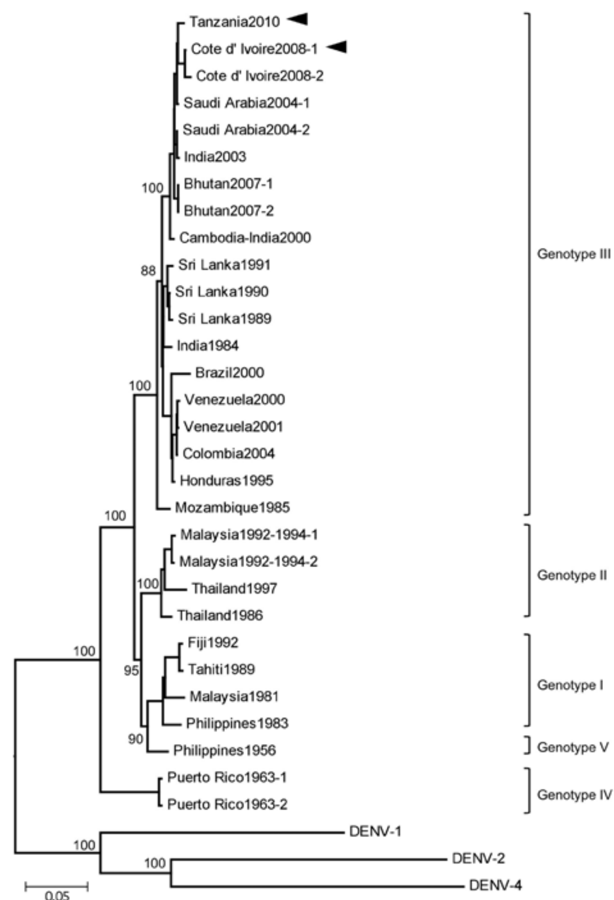

Figure. Phylogenetic tree based on the envelope genome sequence of selected dengue virus type 3 (DENV-3) strains. The tree was rooted to DENV-1, DENV-2 and DENV-4. Multiple sequence alignments were performed, and the tree was constructed by using the neighbor-joining method. The percentage of successful bootstrap replication is indicated at the nodes. DENV-3 genotypes are indicated on the right. The isolated DENV-3 strains, D3/Hu/Tanzania/NIID08/2010 strain (Tanzania2010) and D3/Hu/Côte d'Ivoire/NIID48/2008 strain (Côte d'Ivoire2008), are indicated with arrowheads. Scale bar indicates nucleotide substitutions per site.

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Comparison of Survey Methods in Norovirus Outbreak Investigation, Oregon, USA, 2009

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We compared data from an Internet-based survey and a telephone-based survey during a 2009 norovirus outbreak in Oregon. Survey initiation, timeliness of response, and attack rates were comparable, but participants were less likely to complete Internet questions. Internet-based surveys permit efficient data collection but should be designed to maximize complete responses.

Internet-based questionnaires are increasingly used during investigations of outbreaks; however, compared with telephone interviews, a differential response rate on the basis of exposures or outcomes might bias results (*1–6*). On September 24, 2009, the Oregon Public Health Division was notified of an outbreak of gastroenteritis that occurred among participants of a 475-mile bicycle ride during September 13–19, 2009. Five of 6 riders who independently reported illness to the event organizer and provided stool specimens were positive for norovirus (GII) infection. In responding to the outbreak, we administered a questionnaire using Internet- and telephone-based methods to directly compare data with regard to response rates, attack rates, and risk factors for illness.

The Study

The event organizer provided telephone numbers, email addresses, and age and sex information for all 2,273 registered riders, of whom 1,288 were Oregon residents. Separate samples of Oregon cyclists were randomized to participate in identically worded surveys, either over the Internet (n = 204) or by telephone (n = 93). The survey contained 95 questions, including 46 about food items eaten. Survey completion was defined as provision of an answer to the last question in the survey (did the participant become ill?), unless the respondent answered "yes." An affirmative answer led to additional questions about symptoms of illness. Each survey took ≈10–15 minutes to complete.

The Internet survey was formatted with Inquisite Survey (Inquisite, Inc., Austin, TX, USA). We sent an email message that included a link to the survey to the riders. Among 204 riders selected for the Internet survey, 201 had valid email addresses. A reminder was emailed to nonresponders after 5 days. Of the 93 riders selected for the telephone survey, 91 had valid telephone numbers. Oregon Public Health Division interviewers attempted at least 5 times to telephone each participant, including during the evening.

We defined a case as vomiting or ≥3 loose stools within 24 hours in an event rider with onset during September 11–22, 2009 (i.e., a period that included the 2 days before and the 3 days after the ride). Analyses were conducted in SAS 9.1 (SAS Institute, Inc., Cary, NC, USA). Statistical tests were performed by using χ^2 tests with significance determined as $p<0.05$.

Although similar proportions of participants initiated each survey type (153/201 [76%] Internet vs. 76/91 [84%] telephone) (Table 1), participants in the Internet survey were less likely to complete the survey (129/201 [64%] vs. 72/91 [79%]; p = 0.01 for difference in overall completion rate). Within each subgroup, participants were less likely to complete the Internet survey than the telephone survey (Table 2), although the differences were not significant in each subgroup. Within the Internet survey cohort, riders ≥50 years of age were more likely to complete the survey (80/114 [70%]) than were riders <50 years (48/86 [56%]; $p<0.05$).

Both cohorts completed the survey within 2 days (92/129 [71%] Internet vs. 47/72 [65%] telephone; p = 0.44) (Table 1, Figure 1). Only 74 (57%) of 129 riders who completed the Internet survey answered ≥90% of the food item questions, compared with 68 (94%) of 72 riders in the telephone survey ($p<0.0001$).

Three Internet survey respondents reported illness that did not meet the case definition; they were excluded from analysis. Among the remaining 126 Internet respondents, illness of 23 (18%) met the case definition, as did illness of 13 (18%) of 72 telephone interviewees. The attack rate for the Internet survey cohort who responded within 2 days after survey release (21/91 [23%]) was higher than for those who responded later (2/35 [6%]; p = 0.02); among telephone interviewees, percentage of cases among early interviewees (8/47 [17%] did not differ significantly from those among later interviewees (5/25 [20%]).

The epidemic curve appeared consistent with propagated transmission that peaked near the end of the event (Figure 2). Illness was not significantly associated with

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Table 1. Comparison of Internet- and telephone-based survey responses among participants of September 2009 bicycle ride, Oregon, USA*

Survey response	No. respondents/total no. participants (%)†		Ratio (95% CI)
	Internet-based survey	Telephone survey	
Initiation of survey	153/201 (76)	76/91 (84)	0.9 (0.8–1.02)
Confirmed ride participation	137/153 (90)	72/76 (95)	0.9 (0.9–1.02)
Completed survey	129/137 (94)	72/72 (100)	0.9 (0.9–0.98)
Overall completion rate	129/201 (64)	72/91 (79)	0.8 (0.9–0.98)
Completed surveys within 2 days after survey release	92/129 (71)	47/72 (65)	1.1 (0.9–1.3)
Answered 90% of questions about food items	74/129 (57)	68/72 (94)	0.6 (0.5–0.7)
Attack rates‡	23/126 (18)	13/72 (18)	1.0 (0.5–1.9)

*CI, confidence interval.
†Number of riders who answered the question compared with number of riders who were asked.
‡Three respondents to the Internet-based survey who reported illness that did not meet the case definition were excluded from analysis for attack rates.

age, sex, hand-hygiene practices, reported availability of soap and water, or any of the food items in either survey cohort.

Camping in the organizer's tents during the event was not significantly associated with illness in the telephone survey (4/18 [22%] in the organizer's tents vs. 9/54 [17%] in other accommodations; risk ratio [RR] 1.3, 95% confidence interval [CI] 0.5–3.8). However, it was significantly associated with illness in the Internet survey (12/34 [35%] vs. 11/92 [12%]; RR 3.0, 95% CI 1.4–6.0) and in the combined dataset (Mantel-Haenszel summary RR 2.3, 95% CI 1.3–4.0).

Conclusions

The Internet and telephone survey methods yielded similar findings with noteworthy differences. Our Internet survey response rate was comparable with that in some reports (*1*) and higher than in others (*2*,*7*). Overall, we found a lower response rate for the Internet survey cohort, with significantly fewer complete surveys. Riders ≥50 years of age were somewhat more likely to complete the Internet survey than were their younger peers in this relatively affluent cohort.

Illness was associated with use of the event organizer's tents in the Internet survey only. Similar proportions of respondents reported illness and reported sleeping in the tents in both survey cohorts, making response bias an unlikely explanation for the different findings. Tents were reallocated at each stop; thus, riders did not use the same tent every night. Smaller sample size, leading to insufficient power in the telephone survey, could have contributed to the differing results, which might have led to different conclusions on the association of the event organizer's tents with illness. Nonetheless, an environmental source of exposure from contaminated tents is biologically plausible, given the low infectious dose of norovirus and its ability to persist on surfaces (*8*).

Our experience is relevant to other public health agencies considering Internet surveys for outbreak investigations. First, early respondents to the Internet survey were more likely to report illness than were later respondents, suggesting that a response bias was present soon after survey release that disappeared with time and the reminder email. Survey invitations and reminders must explicitly encourage all invitees, not just those in whom illness developed, to complete the survey. Second, 1 reminder after 5 days boosted response to the Internet survey; more frequent reminders initiated earlier would have required minimal time and might have boosted overall response further. Third, a disadvantage of Internet questionnaires is the absence of a prompter to encourage survey completion and address questions. Implementing mandatory data-entry

Table 2. Overall survey completion rate among participants in September 2009 bicycle ride, Oregon, USA, 2009

Stratification variable	No. respondents/total no. participants (%)*		p value
	Internet-based survey	Telephone survey	
Sex			
M	95/153 (62)	44/56 (79)	0.03
F	34/48 (71)	28/35 (80)	0.34
Age, y†			
<50	48/86 (56)	31/37 (84)	0.001
≥50	80/114 (70)	41/54 (76)	0.44
Living accommodations			
Event organizer's tents	36/56 (64)	18/21 (86)	0.07
Not in event organizer's tents	93/145 (64)	54/70 (77)	0.03

*Number of riders who answered the question compared with number of riders who were asked.
†Age was missing for 1 participant in the Internet-based survey.

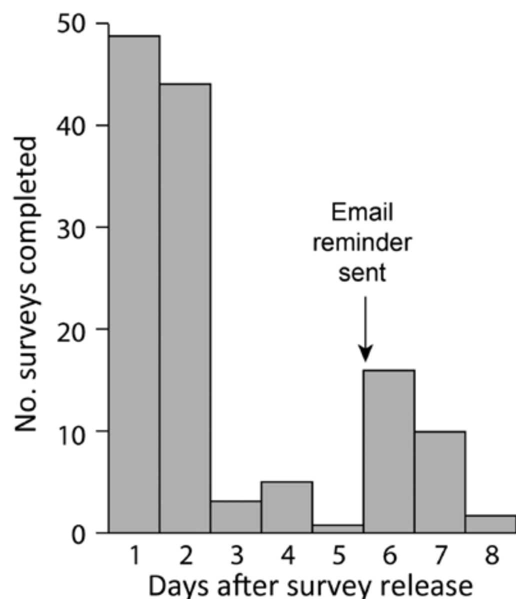

Figure 1. Timeliness of completed Internet-based surveys among participants in September 2009 bicycle ride, Oregon, USA.

checks to advance through the survey might lead to more complete survey data. Internet survey methods might be more practically suited for relatively shorter, straightforward questionnaires that do not risk respondent fatigue and early termination and do not attempt to assess complex arrays of potential exposures that might require interviewer clarification and assistance.

This study has certain limitations. Our findings may not be generalizable to groups with different patterns of Internet access or use (*9*). Also, delays in administering our survey (the first notification came 5 days after the event) might have influenced response rates and exposure recall. Finally, we did not formally quantify and compare the costs of designing and conducting these 2 surveys.

Internet surveys will likely be increasingly used to investigate outbreaks. Our experience suggests that developing quality Internet surveys requires more initial time and effort (greater fixed cost), but once the survey instrument is deployed, it requires less time and expense per respondent for public health agencies (less variable cost). Accordingly, Internet surveys probably become more economical as the group to be surveyed becomes larger. Continued evaluations of Internet surveys are warranted to validate their findings, particularly among populations with lower Internet access and use.

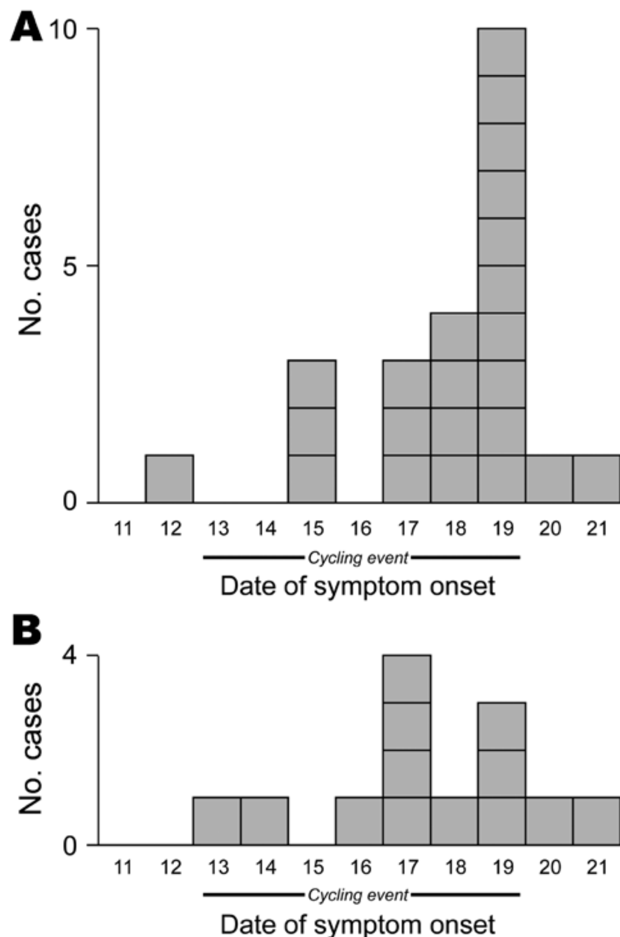

Figure 2. Comparison of epidemic curves in Internet-based survey (n = 23) (A) and telephone-based survey (n = 13) (B) for ill participants in September 2009 bicycle ride, Oregon, USA.

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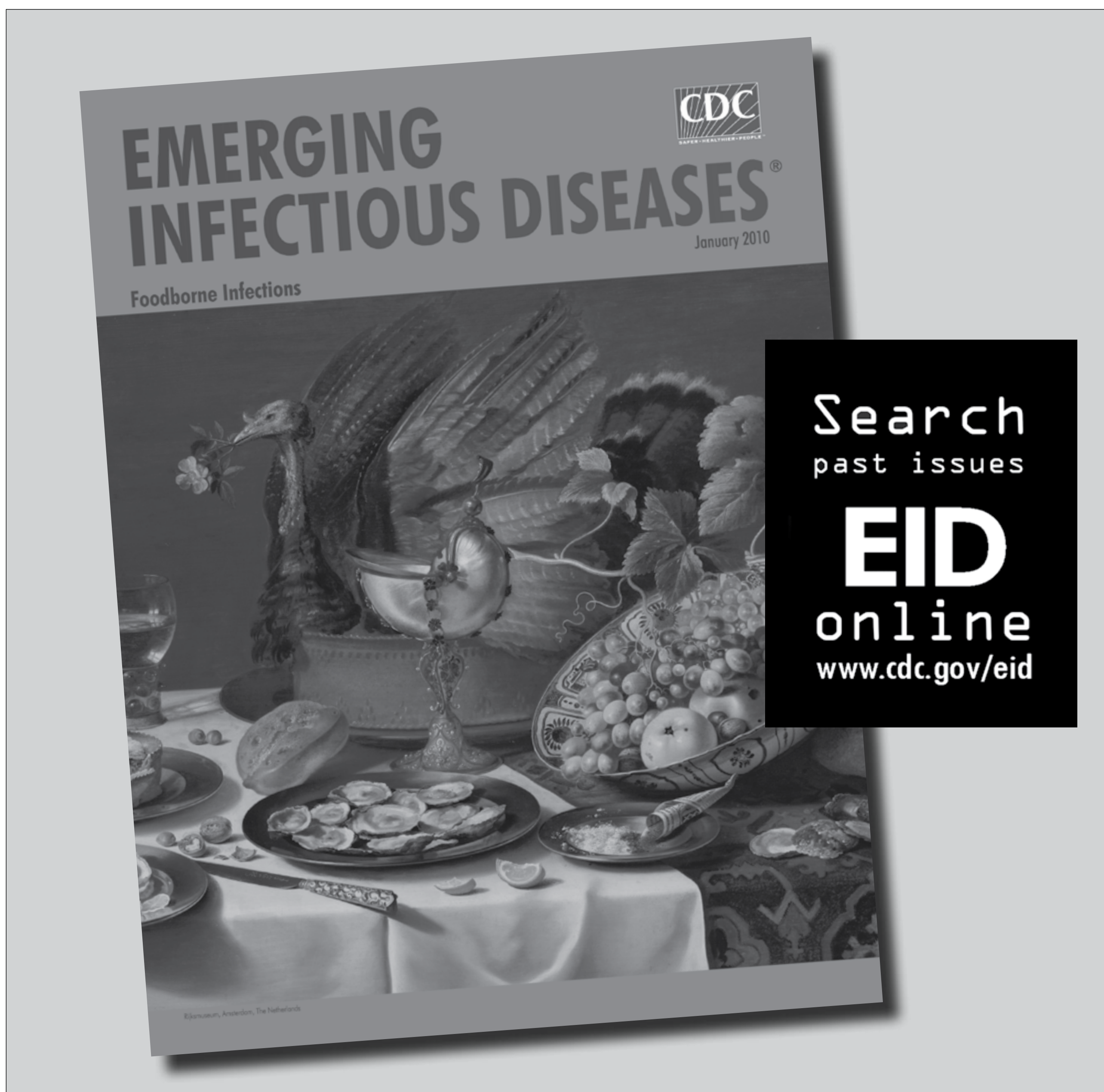

Typing of Lymphogranuloma Venereum *Chlamydia trachomatis* Strains

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We analyzed by multilocus sequence typing 77 lymphogranuloma venereum *Chlamydia trachomatis* strains from men who have sex with men in Europe and the United States. Specimens from an outbreak in 2003 in Europe were monoclonal. In contrast, several strains were in the United States in the 1980s, including a variant from Europe.

Lymphogranuloma venereum (LGV) is a sexually transmitted disease caused by *Chlamydia trachomatis* serovars L1, L2, and L3. This disease is endemic to parts of Africa, Latin America, and Asia but only occurred sporadically in Europe before 2003, at which time an outbreak of LGV occurred in the Netherlands among men who have sex with men (MSM) (*1*). Since then, a large number of LGV cases have been reported among MSM in Europe, North America, and Australia. Although the inguinal form (formation of buboes) is more common in heterosexual LGV patients, in the current epidemic among MSM, anal infections have been diagnosed in most LGV cases.

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Sequencing the highly variable outer membrane protein A (*ompA*) gene identified a new genetic variant designated L2b, which subsequently has been identified in nearly all recent LGV cases in MSM that have been investigated (*2*). This variant was also found in isolates obtained from MSM in San Francisco, California, USA, in the early 1980s (*3*). However, differences in other regions of the *C. trachomatis* genome are possible and these regions should be investigated.

Multilocus sequence typing (MLST) is a genotyping method based on amplification and sequencing of several genetic regions. Recently, 3 MLST systems for genotyping of *Chlamydiacae* bacteria have been reported. Two are based on housekeeping genes, and their resolution is comparable to that of *ompA* (*4*,*5*), which gives these 2 systems limited usefulness in *C. trachomatis* strain discrimination and outbreak investigations. A third MLST system was reported by Klint et al. (*6*) for short-term epidemiologic analysis of *C. trachomatis* strains. This system showed a 3-fold higher resolution than conventional *ompA* genotyping when applied to serovars A–K (*6*,*7*), which cause ocular trachoma and genital chlamydia infections. Recent evaluation of typing schemes confirms the considerable discriminatory potential of our system and recommends it for typing of closely related clinical strains (*8*). This system includes 5 highly variable gene regions; 2 of them (penicillin-binding protein and histone H1–like protein [*hctB*]), are subjected to selection pressure, which facilitates analysis of epidemiologic changes over limited periods.

Our objective was to deduce the nature and origin of the LGV outbreak among MSM in Europe. We used the MLST system of Klint et al. (*6*) to investigate genetic variation in LGV strains. Strains were obtained from MSM from contemporary Europe and the United States and from MSM in San Francisco during the 1980s.

The Study

All LGV specimens were obtained from MSM attending outpatient clinics. Twenty-two specimens were obtained in San Francisco during 1979–1985, and 5 specimens were obtained in the Baltimore, MD–Washington, DC, USA, area during 2007–2009. Fifty specimens were obtained from patients in Europe (Denmark [n = 7], France [n = 15], Germany [n = 1], the Netherlands [n = 9], Norway [n = 2], Spain [n = 7], and Sweden [n = 9]) during 2004–2008. DNA purification, PCR amplification of the *ompA* gene and the 5 specific highly variable gene regions (by using high-fidelity polymerase), DNA sequencing, and analysis of data (mean 5,972 nt/specimen, primers excluded) were performed as described (*6*,*9*). All novel mutations were reamplified and resequenced to confirm their authenticity.

All except 1 of the 50 specimens from Europe had an *ompA* genotype identical to the L2b reference strain L2b/

UCH-1/proctitis (AM884177.1). The nonidentical specimen came from Spain and had a previously unpublished single C→T point mutation in variable segment 2 at position 517 when compared with L2b/UCH-1/proctitis. This sequence of this specimen has been deposited in GenBank (accession no. GQ413955). All 50 specimens from Europe shared an identical MLST genotype (Table). The 5 specimens from the contemporary United States had the same *ompA* and MLST genotype as specimens from Europe (Table).

The 22 specimens from San Francisco were separated into 3 *omp*A genotypes and 5 MLST genotypes (Table). Six specimens had an *ompA* genotype identical to the L2 reference strain L2/434/Bu, and 7 had a novel genotype that differed from the L1 reference strain L1/440 by 9 point mutations. The remaining 9 specimens had an *ompA* genotype identical those of specimens from Europe and L2b reference strain L2b/UCH-1/proctitis. Three of these 9 specimens had MLST genotypes of strains from Europe, and the other 6 had a novel 108-nt deletion in the *hctB* gene region. This *hctB* variant has not been found in other specimens of *C. trachomatis* serovars A–L3 and is unique in the MLST database (http://mlstdb.bmc.uu.se/), which contains genetic profiles for 496 specimens (February 2010).

Conclusions

We aimed to deduce the nature and origin of the LGV outbreak among MSM in Europe. Because of the conserved nature of *Chlamydia* spp. genomes, high-resolution genotyping, rather than following evolutionary changes over long periods as with conventional MLST systems, was essential for investigating this outbreak. We found that the high-resolution MLST system described by Klint et al. (*6*) was most suitable for the current study.

LGV specimens from MSM in Europe in this study were monoclonal for *ompA* and the 5 highly variable regions. This finding suggests a single source of origin for the LGV outbreak among MSM in Europe. One specimen in this study contained a novel, single, point mutation in *ompA*. In 373 contemporary LGV cases in MSM in the United Kingdom, the L2b *ompA* variant comprised >90% of the specimens, and the remaining 35 specimens belonged to 4 novel sequence variants, each differing from the L2b variant by a single point mutation (*10*). These single point mutations might have occurred recently, and their presence is less likely to indicate a separate source of origin.

LGV specimens from MSM in the 1980s in San Francisco showed genetic variation in *ompA* and the 5 highly variable gene regions. MLST analysis showed that 6 of the 9 specimens from San Francisco that had the L2b *ompA* variant were genetically different from the strain in Europe; the remaining 3 specimens had an MLST profile identical to that of the variant from Europe. Genetic variation among LGV specimens from San Francisco supports the idea that LGV is endemic in MSM in the United States, a finding that has been reported (*11*).

In contrast, the epidemiologic pattern and genetic characterization of LGV in several countries in Europe indicate that the L2b type has disseminated across Europe in recent years. In Sweden, 3 LGV cases that produced clinical signs were detected in 2004 and 2005 (*12*). In a survey of 81% of patients with *C. trachomatis* infection detected among high risk MSM in Stockholm, no additional LGV cases were detected (*12*), and a total of 15 LGV cases were detected in 2007. If one considers the highly internationalized network of sexual contacts among MSM (*13*), the L2b variant may have been imported to Europe from the United States. A

Table. Genetic profiles of lymphogranuloma venereum specimens, Europe and United States*

Location	Sample years	No. specimens	MLST profile					
			hctB	CT058	CT144	CT172	*pbpB*	*ompA*
Europe†	2004–2009	49	27	13	17	13	29	28‡
Europe§	2004–2009	1	27	13	17	13	29	39¶
USA#	2007–2009	5	27	13	17	13	29	28‡
USA**	1979–1985	3	27	13	17	13	29	28‡
USA**	1979–1985	6	44††	13	17	13	29	28‡
USA**	1979–1985	7	18	13	23	13	29	40‡‡
USA**	1979–1985	5	18	13	19	6	28	22§§
USA**	1979–1985	1	18	37	19	6	28	22§§

*MLST, multilocus sequence typing; *hctB*, histone H1–like protein; *pbpB*, penicillin-binding protein; *ompA*, outer membrane protein A. Values are arbitrary designations referring to allele variants in our *Chlamydia trachomatis* MLST database (http://mlstdb.bmc.uu.se/). All MLST variants differ within regions with <5 point mutations unless otherwise indicated.
†Denmark (n = 7), France (n = 15), Germany (n = 1), the Netherlands (n = 9), Norway (n = 2), Spain (n = 6), and Sweden (n = 9).
‡*ompA* variant 28 is identical to the reference strain L2b/UCH-1/proctitis (AM884177.1).
§Spain.
¶*ompA* variant 39 contains a single point mutation compared with L2b/UCH-1/proctitis and has been deposited in GenBank under accession no. GQ413955.
#Baltimore, Maryland–Washington, DC.
**San Francisco, California.
††*hctB* variant 44 contains a novel 108-nt deletion and is unique in the MLST database.
‡‡*ompA* variant 40 contains 9 point mutations compared with reference strain L1/440 (DQ064294.1) and has been deposited in GenBank under accession no. GQ413956.
§§*ompA* variant 22 is identical to reference strain L2/434/Bu (AM884176.1).

limitation of our study is that it does not include old LGV strains from Europe. However, after widespread investigations, we believe that no such strains are available.

Our study shows that the MLST system of Klint et al. (*6*) is suitable for epidemiologic analysis of *C. trachomatis* transmission, as indicated by our ability to differentiate the L2b *ompA* variant found in San Francisco into 2 strains. Investigation of additional LGV specimens from the United States and regions to which LGV is endemic with the MLST system could help determine a more detailed epidemiologic picture of the LGV outbreak in 2003 among MSM.

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Enterovirus 75 Encephalitis in Children, Southern India

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Recent outbreaks of enterovirus in Southeast Asia emphasize difficulties in diagnosis of this infection. To address this issue, we report 5 (4.7%) children infected with enterovirus 75 among 106 children with acute encephalitis syndrome during 2005–2007 in southern India. Throat swab specimens may be useful for diagnosis of enterovirus 75 infection.

Sequences of enterovirus 75 (EV75) were first identified in cerebrospinal fluid (CSF), stool samples, and throat samples obtained during 1974–2000 in Ethiopia, Oman, Bangladesh, and the United States; the new enterovirus serotype was proposed in 2004 (*1*). Manifestations include upper respiratory tract infections and acute flaccid paralysis. In 2005–2006, EV75 was associated with aseptic meningitis in Spain (*2*,*3*). Enteroviruses have long been associated with encephalitis, but recent outbreaks of EV71 in Southeast Asia have highlighted the diagnostic difficulties that may be encountered (*4*–*6*). We report EV75 associated with encephalitis in India.

The Study

We prospectively studied children ≤16 years of age who came to the Pediatric Department at the Vijayanagar Institute of Medical Sciences, Bellary, Karnataka, southern India, with acute encephalitis syndrome during October 2005–December 2007. Children with acute fever and symptoms with an onset <14 days before coming to the hospital, a neurologic illness, and ≥1 of the following signs (change in mental status including confusion, disorientation, coma, or inability to talk; new onset of seizures excluding simple febrile seizures; photophobia, headache, or meningitis) were recruited into the study. Children with laboratory-confirmed *Plasmodium falciparum* parasitemia or a history of neurologic conditions and those whose parents removed them from the hospital were excluded. The study protocol was reviewed and approved by the ethics committees of Vijayanagar Institute of Medical Sciences and the University of Liverpool, UK. Informed consent was obtained from parents or guardians.

For each child, a completed medical history was obtained and a detailed physical examination was performed. Samples were obtained for routine diagnostics. Rectal and throat samples were also collected after December 2005. Swabs were placed in sterile vials containing viral culture media (Dulbecco's modified Eagle's medium). All samples were frozen at –20°C, stored at –70°C, and transferred to diagnostic laboratories.

To detect enteroviruses, including EV75, in throat swab extracts, RNA was extracted by using the Chemagic Viral DNA/RNA kit (Chemagen AG, Baesweiler, Germany) and a Kingfisher mL Magnetic Extractor (Thermo Fisher Scientific Inc., Waltham, MA, USA). Pan-enterovirus reverse transcription–PCR (RT-PCR) was then performed by using primers specific for the 5′ untranslated region (5′-ATT GTC ACC ATA AGC AGC CA-3′ and 5′-CCT CCG GCC CCT GAA TGC GGC TAA T-3′); these primers produced a 154-bp product (*7*). Enteroviruses identified by pan-enterovirus RT-PCR were typed by nucleotide sequencing of the viral protein 1 (VP1) region (*8*,*9*).

To further type enteroviruses, phylogenetic analysis was performed on all nucleotide sequences (from this study and others obtained from GenBank) by using MEGA4 software (*10*) (www.megasoftware.net). The remaining swab transport medium was filtered, and 100 µL of filtrate was aliquoted onto each of 3 tissue culture plates containing rhabdomyosarcoma, Vero, and 293T cell lines for virus isolation. Cell lines were chosen to facilitate culturing of a range of viruses that may have been responsible for the clinical spectrum of illness seen.

Because CSF volume for virus isolation was limited to a 100-µL sample, this sample was placed on rhabdomyosarcoma cells for isolation and identification of enteroviruses. All CSF and serum samples were tested by using an immunoglobulin M capture ELISA to detect antibodies to Japanese encephalitis virus (JEV) and dengue virus, which circulate in the study region (*11*). If a sufficient amount of CSF remained, RNA was extracted by using the Viral RNA Mini Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions for pan-enterovirus RT-PCR, as described above and for a JEV RT-PCR (*7*,*12*). Plasma samples were also tested for chikungunya virus by using RT-PCR (*13*).

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Of 243 children recruited into the study, 3 died before diagnostic samples could be obtained and 8 left the hospital against medical advice before samples were obtained. Among 232 children who satisfied the inclusion criteria, 166 CSF samples were obtained from 152 children. We also obtained 108 throat swabs specimens from 106 children and 19 rectal swab specimens from 18 children from the 171 children recruited after December 2005 when swab sampling began.

Virus isolates from throat swabs of 5 (4.7%) of 106 patients with a clinical signs of acute encephalitis syndrome were positive by pan-enterovirus RT-PCR for EV75. Sequencing indicated that VP1 regions of virus isolates obtained were similar to those of EV75. The 5 EV75-positive children were admitted to hospital on days 1–6 (median 4 days) of illness (Table). These children had a median age of 8 years (range 1.5–10 years), and 3 were girls. All 5 children had severe clinical disease (drowsiness, irritability, and reduced consciousness) at the time of admission, and 3 had seizures before admission.

Lumbar puncture was performed for 4 of the 5 children. Laboratory values were within reference ranges for 2 children, leukocyte counts were increased for 3 children, and results were not available for 1 child. The child for whom no lumbar puncture was performed had neurologic deterioration, meningism, and bilateral papilloedema; this child was treated empirically.

Sequencing showed that VP1 region homology for virus isolated in this study was similar to that observed for previously identified EV75 isolates (Figure). Sufficient CSF sample for pan-enterovirus RT-PCR was available for 162 samples from 162 patients, including 4 of the 5 EV75-positive patients; all samples were negative for JEV. These 162 samples were also negative for enteroviruses by pan-enterovirus RT-PCR (*7*,*12*). Serum samples from the 5 EV75-positive patients were negative for JEV and dengue virus by immunoglobulin M capture ELISA, for JEV by RT-PCR, and for chikungunya virus by RT-PCR. Eight patients showed positive results for chikungunya virus by RT-PCR, 48 showed positive results for JEV by ELISA, and 12 showed positive results for dengue virus by ELISA (*14*).

Conclusions

Enteroviruses are a diverse group, and preferred samples for their diagnosis differ. Species B enteroviruses such as coxsackie virus A9 and Echo viruses B1–6 are usually readily isolated from CSF, unlike species A enteroviruses such as EV71. Although EV75 is a species B enterovirus, we were unable to isolate enterovirus from CSF from any of the patients, although throat swabs specimens were positive. This finding is similar to that for EV71, which has been isolated more frequently from throat swab specimens than from CSF or vesicles (*4*). In a study from Spain, EV75 was isolated from the CSF of 5 patients and nasopharyngeal swab specimens of 3 patients (*3*). However, throat swab specimens are rarely obtained in rural hospital settings with limited diagnostic facilities.

Although EV75 infection may have been a coincidental finding for the patients, we believe that this possibility is unlikely because results for other common causes of encephalitis were negative. Results of CSF testing were negative for other circulating viruses, including JEV, dengue virus, and herpes simplex virus, and patients also showed negative results for JEV and dengue virus by ELISA.

Our results illustrate the need to confirm diagnoses of EV75 in a range of specimens and by a range of laboratory investigations. This confirmation is required in India where encephalitis is often diagnosed clinically and recent outbreaks have been attributed to JEV. Laboratory diagnosis is hampered by single samples and high cost and low reliability of diagnostic tests currently available. Our study also shows that EV75 can cause encephalitis, in addition to aseptic meningitis and acute flaccid paralysis. Thus, as for other enteroviruses, throat swab specimens may be especially useful for diagnosis of infection with EV75.

Table. Characteristics and samples tested for 5 patients with confirmed enterovirus 75 infection, southern India*

Patient no.	Age, y/sex	Illness duration/length of hospital stay, d	Clinical signs†	Sample tested for EV75
1	6/F	6/7	5 d of fever and rigors, 3 d of headache, 2 d of neck pain and cough, and 1 d of photophobia. Drowsy and irritable with brisk reflexes on admission.	Throat swab and CSF
2	1.5/M	8/8	8 d of fever, rigors, coryzal symptoms, drowsiness, and mouth twitching; and 3 episodes of GTC seizures of 5–10 min duration. Still vacant and communication reduced at discharge.	Throat swab and CSF
3	10/F	3/5	3 d of fever, headache, and vomiting; 2 d of drowsiness at home; and 1 episode of GTC convulsion at home.	Throat swab
4	8/M	8/7	8 d of fever, headache, neck pain, cough, vomiting, and reduced speech and irritability. Irritable at discharge.	Throat swab and CSF
5	8/F	2/1	2 d of fever and 1 episode of GTC convulsion at home lasting 30 min.	Throat swab and CSF

*EV75, enterovirus 75; CSF, cerebrospinal fluid; GTC, generalized tonic clonic.
†At admission to study.

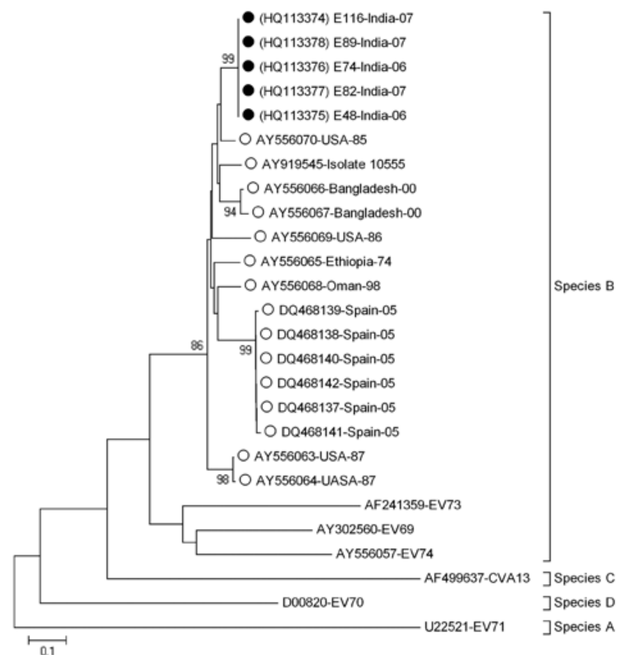

Figure. Phylogenetic analysis of viral protein 1 enterovirus 75 (EV75) nucleotide sequences. The tree was constructed by using the neighbor-joining method and the maximum-composite likelihood-substitution model. Significance of phylogenies was investigated by bootstrap analysis with 1,000 pseudoreplicate datasets. Bootstrap values >70% are indicated on the tree. Closed circles indicate isolates from India (this study) and open circles previously reported EV75 sequences. All EV75 sequences are named by using the conventional GenBank accession numbers (published sequences) or sample name (sequences from this study), country of origin, and year of isolation (where available). All prototype EV reference sequences are indicated by their GenBank accession numbers preceding serotype identification. Scale bar indicates nucleotide substitutions per site.

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Isolation of Ancestral Sylvatic Dengue Virus Type 1, Malaysia

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Ancestral sylvatic dengue virus type 1, which was isolated from a monkey in 1972, was isolated from a patient with dengue fever in Malaysia. The virus is neutralized by serum of patients with endemic DENV-1 infection. Rare isolation of this virus suggests a limited spillover infection from an otherwise restricted sylvatic cycle.

Dengue virus (DENV) is a mosquito-borne pathogen maintained in sylvatic (nonhuman primate/sylvatic mosquitoes) and endemic (human/urban/peridomestic mosquitoes) cycles. The endemic form of DENV poses a serious health threat to >100 million persons living in dengue-endemic regions (*1*). The endemic form of DENV may have originated from adaptation of sylvatic DENV to either peridomestic/urban mosquitoes or nonhuman primate hosts 100–1,500 years ago (*2*).

All 4 DENV genotypes are thought to have independently evolved from a sylvatic ancestral lineage, perhaps in Malaysia (*2*). However, only sylvatic DENV-1, DENV-2, and DENV-4 have been isolated, and monkey seroconversion against DENV-1, DENV-2, and DENV-3 has been demonstrated (*3*). Incidences of spillover infection involving sylvatic DENV-2 have been reported, but mainly in West Africa.

Sylvatic dengue may still be endemic to West Africa, especially in areas with dense human habitation near forest areas (*4*,*5*). Sporadic reports of sylvatic dengue may be the result of low incidence of severe forms of this disease in these regions. In contrast, infection with sylvatic dengue is rare in other parts of the world, especially in Southeast Asia where dengue is hyperendemic. Sylvatic DENVs (DENV-1, DENV-2, and DENV-4) were last isolated from monkeys in Malaysia in the 1970s (*3*).

During 2004–2007, a dramatic increase occurred in the number of suspected dengue cases in Malaysia; 155,424 cases and 358 deaths were reported (*6*). DENV-1 was the predominant virus isolated and accounted for 68% of all DENVs isolated. This outbreak represented a third cycle that involved DENV-1 in Malaysia since the 1960s (*7*). We report isolation of DENV-1 that shared >97% genome sequence similarity to an ancestral DENV-1 isolated from a sentinel monkey in Malaysia in 1972 (*3*).

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The Study

At least 442 DENV-1 isolates from the 2004–2007 dengue outbreak were obtained from the Diagnostic Virology Repository at the University of Malaya Medical Centre. Viral RNA was extracted from infected cell culture supernatants, and a 1-step reverse transcription–PCR amplification of the DENV-1 envelope gene was performed by using amplification primers (*8*). Amplified fragments were purified and sequenced by Macrogen Inc. (Seoul, South Korea).

DENV-1 genome sequences from study isolates and those obtained from GenBank (Table 1) were used to construct phylogenetic trees. Maximum clade credibility was inferred by using the Bayesian Markov chain Monte Carlo method implemented in BEAST version 1.5.2 (*9*). For simplicity, only 10 new DENV-1 sequences from the study and 47 from GenBank were analyzed.

Phylogenetic trees showed 6 distinct DENV-1 subgenotypes: 3 ancestral subgenotypes (Hawaii/Japan, 1940s; Thailand, 1960s; and Malaysia, 1972) and 3 major endemic subgenotypes (SI, SII, and SIII), which is consistent with reported findings (*8*). An isolate identified as D1.Malaysia.36046/05 grouped with isolate P72_1244, a sylvatic DENV-1 reportedly isolated from a sentinel monkey in Malaysia in 1972. Virus envelope gene sequence shared >97% nt sequence similarities and >99% aa sequence similarities. There was only 1 aa difference at position 55, from valine in P72_1244 to isoleucine in D1.Malaysia.36046/05.

Focus-reduction neutralization tests (FRNTs) were performed by using the D1.Malaysia.36046/05 isolate. Serum samples from patients with primary dengue caused by DENV-1 SI and SII (Figure) were pooled and used in FRNTs as described (*10*). Neutralizing antibody titer was

Table 1. Sylvatic and endemic dengue virus isolates used in the study, Malaysia

Isolate*	Year isolated	GenBank accession no.
D1.Malaysia.36046/05	2005	FN825674
D1.Malaysia.32581/04	2004	FR666923
D1.Malaysia.32858/04	2004	FR666921
D1. Malaysia.33087/04	2004	FR666922
D1. Malaysia.33370/04	2004	FR666923
D1.Malaysia.36000/05	2005	FR666924
D1.Malaysia.36139/05	2005	FR666925
D1.Malaysia.32694/04	2004	FR666926
D1.Malaysia.35765/05	2005	FR666927
D1.Malaysia.35845/05	2005	FR666928

*Isolate D1.Malaysia.36046/05 is a sylvatic type. All other isolates are endemic types.

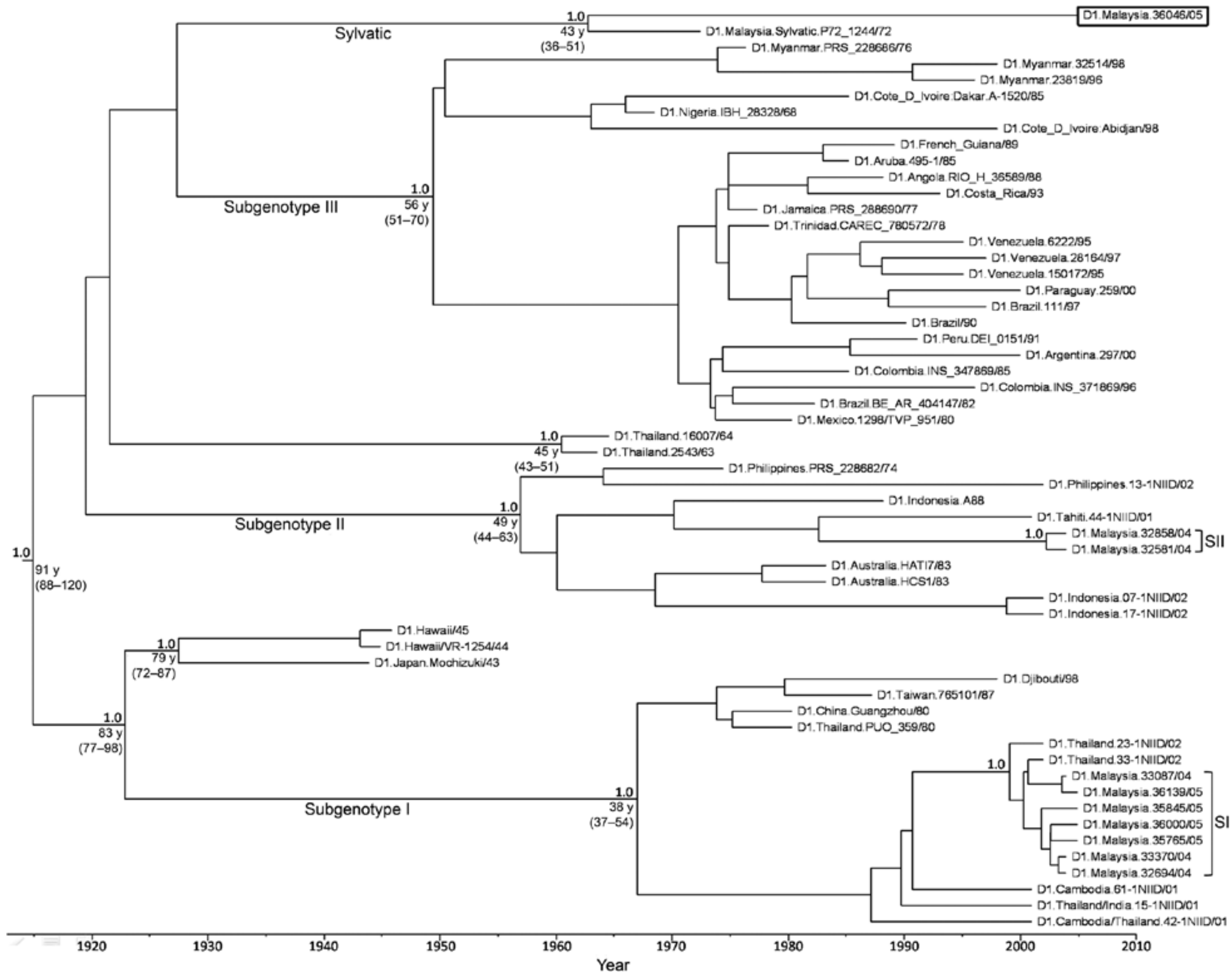

Figure. Maximum clade credibility tree of complete envelope genes of dengue virus type 1 (DENV-1) isolates. Horizontal branches are drawn to a scale of estimated year of divergence. Coalescent times with 95% highest posterior density values (ranges in parentheses) and posterior probability values (all 1.0) of key nodes are shown. Patient convalescent-phase serum samples used for neutralization assays from which virus was isolated are indicated at the end of branches according to their virus groups. Box indicates sylvatic DENV-1 isolated in the study. New sequences were used to create the phylogenetic tree are as in Table 1. SII, subgenotype II, SI, subgenotype I.

defined as the reciprocal of the highest serum dilution that reduced viral foci by 50% ($FRNT_{50}$). FRNT results after adjustment of the titer to that of respective isolates showed that the D1.Malaysia.36046/05 virus is neutralized by serum from patients with DENV-1 SI infections ($FRNT_{50}$ = 320) and samples from patients with DENV-1 SII infections ($FRNT_{50}$ = 80) (Table 2).

Laboratory and clinical records showed that D1.Malaysia.36046/05 virus was isolated from a patient who had headache, body ache, chills, rigors, and abdominal pain for 3 days and sought treatment at the University of Malaya Medical Centre. The patient was treated as an outpatient and suspected of having dengue fever. Serologic results for dengue immunoglobulin M were negative. D1.Malaysia.36046/05 was isolated and identified initially as DENV-1 by using immunofluorescent antibody staining. The patient did not return for subsequent follow-up, and efforts to locate the patient were unsuccessful. The most recent address of the patient was within a high population–density area of Kuala Lumpur. Additional sequencing of other DENV-1 isolates from the 2004–2007 outbreak did not identify any additional D1.Malaysia.36046/05–like virus.

Conclusions

Isolation of the ancestral DENV-1 after >30 years suggests that a mosquito–host transmission cycle has maintained this virus. This rare isolation of the virus suggests a restricted transmission cycle. The natural host of the virus cannot be determined conclusively because the only known

Table 2. Serum neutralization of ancestral sylvatic dengue virus isolate D1/Malaysia/36046/05, Malaysia*

Serum group*	Neutralizing antibody titer†
Mock	0
Virus	0
Medium	0
SI	320
SII	80

*Mock, controls treated with serum from healthy (no dengue infection) donors; virus, virus plus diluent; medium, serum and diluent without virus; SI, subgenotype I; SII, subgenotype II.
†Reciprocal of the highest serum dilution that reduced viral foci by 50% (50% focus-reduction neutralization test). Serum from patients infected with sylvatic virus was not available. Virus was treated with serum from patients infected with primary dengue virus type 1 SI or SII.

fact is that the virus was isolated from a patient with dengue fever. The original ancestral DENV-1 isolate P72_1244 was designated as sylvatic because it was isolated from a sentinel monkey in a rural forest (*3*). Its sylvatic origin has recently become uncertain because the virus genome is phylogenetically closer to other endemic DENV-1 lineages (*11*). However, because no virus with high sequence similarities to that of DENV-1 isolate P72_1244 has been isolated over the past 33 years, the virus may have been maintained in a sylvatic cycle through a nonhuman primate/mosquito enzootic cycle.

The estimated sequence evolution rate for D1.Malaysia.36046/05 is 5.20×10^{-4} substitutions/site/year. This rate is relatively slower than those for other endemic DENV-1 isolates used in this study (5.67×10^{-4} to 8.05×10^{-4} substitutions/site/year). The much smaller monkey:human population ratio (700,000:28,000,000) (*12*) (http://en.wikipedia.org/wiki/Malaysia) and the more restricted mobility of monkeys could have limited the virus genome sequence divergence, leading to conservation of the sylvatic virus genome sequence.

The absence of the virus from the endemic urban cycle over the past 33 years could have been caused by its inability to overcome population herd immunity after exposure to endemic DENV-1. Efficient neutralization of virus by serum from patients infected with DENV-1 SI and SII supports this possibility (*13*). Conversely, the virus may not be highly transmissible by peridomestic mosquitoes (*14*) and may be confined to the enzootic forest cycle. Therefore, isolation of the ancestral virus from a person living in Kuala Lumpur is most likely the result of a stochastic spillover event after contact with infected forest-dwelling mosquitoes.

We report isolation of an ancestral sylvatic DENV-1 from an infected person. Available evidence does not support endemic presence of the virus in an urban dengue cycle. However, a sylvatic cycle needs to be considered in any future dengue vaccination initiatives.

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Mr Teoh is a PhD candidate at the University of Malaya. His primary research interest is the mechanisms of dengue virus evolution in Malaysia.

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Estimates of the True Number of Cases of Pandemic (H1N1) 2009, Beijing, China

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During 2009, a total of 10,844 laboratory-confirmed cases of pandemic (H1N1) 2009 were reported in Beijing, People's Republic of China. However, because most cases were not confirmed through laboratory testing, the true number is unknown. Using a multiplier model, we estimated that ≈1.46–2.30 million pandemic (H1N1) 2009 infections occurred.

Infection with a novel swine-origin influenza A (H1N1) virus, currently named pandemic (H1N1) 2009 virus, first occurred in the United States and Mexico in early April 2009 (*1*,*2*) and then rapidly spread to other regions of the world. As the outbreak expanded, laboratory testing of persons with suspected cases became increasingly impractical, extremely resource intensive, and was discontinued. We assume, therefore, that the number of laboratory-confirmed cases represents only a small fraction of the actual number of infections (*3–5*). In this study, we used a multiplier model to estimate the true number of cases of pandemic (H1N1) 2009 in Beijing, People's Republic of China.

The Study

To estimate the prevalence of pandemic (H1N1) 2009 in the United States, the US Centers for Disease Control and Prevention (CDC) developed a software program (Impact2009, version 1.0) (*6*) based on the Monte Carlo approach and the multiplier model. Although this simple and useful program can be used to estimate the true number of cases in the United States, it may not be so readily applied to other countries because of uncertainties in the model parameters. To account for these uncertainties, in this study we decided to alter the way in which the baseline data assumptions were calculated. For example in the original CDC model, the prevalence was calculated on the basis of the laboratory-confirmed case data. In contrast, we calculated the baseline case number by multiplying the reported number of influenza-like illness (ILI) cases in secondary and tertiary hospitals by the positive rate of pandemic (H1N1) 2009 among ILI cases. We obtained this information from the Beijing influenza surveillance system, which encompasses data on ILI cases from all secondary and tertiary hospitals (levels 2, 3) and virologic surveillance data (*7*).

From the virologic surveillance data, we determined that positive cases of pandemic (H1N1) 2009 were identified through August 3, 2009. From this finding, we used 2 phases for the model: phase 1 (May 16, 2009, through August 2, 2009) and phase 2 (August 3, 2009 through December 31, 2009). In addition, the consultation rate for ILI cases had changed over the course of the pandemic, because of changes in strategies used to control the disease in Beijing before and after National Day (October 1). To adjust for the introduction of these strategies, we further divided phase 2 into 2 periods: period 2a (from August 3, 2009, through September 30, 2009) and period 2b (from October 1, 2009, through December 31, 2009).

During phase 1, the number of laboratory-confirmed cases was considered to reflect the true number of pandemic (H1N1) 2009 infections. However, during phase 2, we calculated the true number of infections by multiplying the baseline by the estimation coefficient, using the multiplier model. In this multiplier model, the baseline case number was equal to the sum of the product of the weekly ILI case number in level 2 and 3 hospitals and the corresponding weekly pandemic (H1N1) 2009 positive rate among case-patients with ILIs. The estimation coefficient was found by multiplying the reciprocal of the parameters in the model. The following parameters were required in our estimation: the proportion of symptomatic infection among patients with cases of pandemic (H1N1) 2009, the proportion of ILI among patients with symptomatic cases of pandemic (H1N1) 2009, the consultation rate among ILI case-patients, the sampling success rate, and the sensitivity of the test (Figure; Table 1). These terms were obtained from a review of the literature (*8–12*) and from recommendations by health professionals. We assumed that the consultation rate of ILIs in each of the 2 periods was consistent and that the syndromic profile of pandemic (H1N1) 2009 did not change greatly.

In phase 1, a total of 325 positive cases were reported (considered as the true infection number). In period 2a and period 2b of phase 2, the baseline case numbers were 6,520 and 171,899, respectively. During phase 2, a total of

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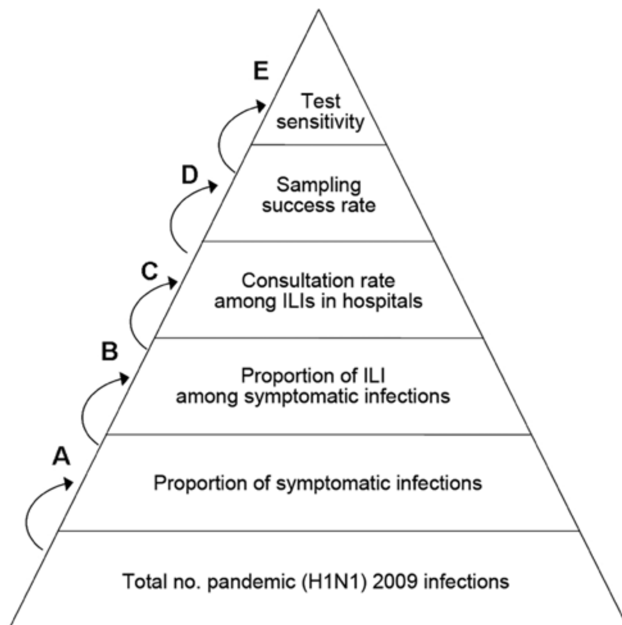

Figure. Model parameters for estimating the true number of persons infected with pandemic (H1N1) 2009 in Beijing. A, hospitals refer to level 2 and 3 hospitals in Beijing; B, sampling success rate was included in the model because not all actual positive specimens gave positive results because of the timing of collection or the quality of the specimen; C, test sensitivity was included in the model because not all actual positive specimens gave positive results due to the insensitivity of PCR reagent and unpredictable errors in experimental operations and instruments; D, proportion of true pandemic (H1N1) 2009 cases for which specimens were successfully collected; E, proportion of true positive specimens that were correctly identified by PCR reagent. ILI, influenza-like illness.

1,800,074 pandemic (H1N1) 2009 infections were estimated. Thus, by the end of 2009, the cumulative number of persons infected with pandemic (H1N1) 2009 in Beijing was estimated to be 1,800,399 (90% range 1.46–2.30 million) (Table 2). However, only 10,844 laboratory-confirmed cases were reported during the same period. One laboratory-confirmed case equaled 166 (90% range 135–212) infections in reality. According to the population size of Beijing, the overall infection rate was 10.6%. The highest infection rate was recorded in those 5–14 years of age (31.8%), followed by those 0–4 years of age (30.8%) (Table 2). In comparison, the rate in persons >60 years was only 0.9%.

Conclusion

Despite the small number of laboratory-confirmed cases (10,844), we estimated that the actual number of persons infected with pandemic (H1N1) 2009 was 1.8 million in Beijing by the end of 2009. Previous studies have claimed that the number of laboratory-confirmed cases of pandemic (H1N1) 2009 was substantially underestimated, reflecting only a very small fraction of the actual infections (*3*–*5*). This study also demonstrated that school age children were more likely to be infected with pandemic (H1N1) 2009. However, those >60 years of age were at low risk for infection.

From November 27 through December 7, 2009, a serologic survey to establish the prevalence of pandemic (H1N1) 2009 antibody was conducted in the general population of Beijing. The results showed that ≈14%–15% (*13*) of the general population had antibodies to pandemic (H1N1) 2009 virus. Based on the population size of 17 million in Beijing in 2009 (*14*) and the assumption that antibodies against pandemic (H1N1) 2009 virus are usually produced after 2 weeks of infection or vaccination (*15*), we estimated that 2.37 to 2.54 million persons were infected with pandemic (H1N1) 2009 virus as of November 13, 2009. According to data from the Beijing Center for Disease Prevention and Control, by November 13, 2009, 1.36 million persons had received the pandemic (H1N1) 2009 vaccine. After the vaccinated population were removed from the equation, the total number of pandemic (H1N1)

Table 1. Parameter values and sources of data included in the multiplier model for estimating the true number of persons infected with pandemic (H1N1) 2009, Beijing*†

Code	Parameter	Value, %	Source
A	Proportion of symptomatic infection among case-patients with pandemic (H1N1) 2009	70–75	Pandemic (H1N1) 2009, ECDC Risk Assessment.,2009; version 6, 6 Nov.
B	Proportion of ILI among symptomatic case-patients with pandemic (H1N1) 2009	26–42	Literature and unpublished clinical data
C1 (period 2a)	Consultation rate among ILI case-patients in secondary and tertiary hospitals	38	Telephone interview conducted by Beijing CDC
C2 (period 2b)	Consultation rate among ILI case-patients in secondary and tertiary hospitals	48	Telephone interview conducted by Beijing CDC
D	Sampling success rate	80–90	Previous surveillance data
E	Sensitivity of test	95–100	Professional recommendations

*ECDC, European Centre for Disease Prevention and Control; ILI, influenza-like illness; Beijing CDC, Beijing Center for Disease Prevention and Control.
†The multiplier model was only used for phase 2 in this study, and phase 2 was divided into 2 periods, period 2a and period 2b. During phase 2, the true number of infections was calculated by multiplying the baseline by the estimation coefficient, using the multiplier model. The baseline case number was equal to the sum of product of weekly ILIs number in level 2/3 hospitals and the corresponding weekly pandemic (H1N1) 2009 positive rate among case-patients with ILIs. The estimation coefficient was obtained by multiplying the reciprocal of the parameters mentioned in this table.
The baseline case numbers in periods 2a and 2b were 6,520 and 171,899, respectively.

Table 2. Estimated numbers of persons infected with pandemic (H1N1) 2009 and infection rate, by age group, Beijing*

Age group, y	Proportion of total no. persons infected, %	Estimated no. cases, median (90% CI)	Estimated rate, %, median (90% CI)
0–4	13.4	241,253 (195,910–307,571)	30.8 (25.0–39.2)
5–14	35.1	632,300 (513,459–806,111)	31.8 (25.8–40.6)
15–24	29.4	528,597 (429,247–673,902)	22.2 (18.0–28.3)
25–59	20.9	375,383 (304,829–478,571)	4.1 (3.3–5.2)
≥60	1.3	22,865 (18,568–29,150)	0.9 (0.7–1.1)
Total	100.0	1,800,399 (1,462,012–2,295,305)	10.6 (8.6–13.5)

*CI, confidence interval.

2009 cases was estimated to be ≈1.01 to1.18 million. At the same time, the number of infections was estimated at 0.87–1.28 million as of November 13, 2009, by the multiplier model (data not shown in the section of the study). The estimates of the infection matched with the actual number estimated from the serologic survey in principle.

In phase 1, the number of laboratory-confirmed cases was considered to reflect the true infection number. This assumption, however, may lead to an underestimation for 2 reasons. First, we ignored the parameters used in phase 2, and second, difficulties occurred in testing all of the samples taken from patients who sought consultation for ILIs. Nevertheless, because the pandemic did not spread in the community in phase 1, we believe that this underestimation would have been quite low.

Although, in theory, serologic surveys should provide an accurate record of the infection rate of pandemic (H1N1) 2009, they failed to provide a quicker and more representative result than the multiplier model. Given the similarities between the estimates obtained from the model and the estimates obtained from the serologic survey, we conclude that the multiplier model based on the Monte Carlo approach should be considered a useful and simple method for estimating the true number of infections during a pandemic.

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Plasmid-mediated Quinolone Resistance among Non-Typhi *Salmonella enterica* Isolates, USA

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We determined the prevalence of plasmid-mediated quinolone resistance mechanisms among non-Typhi *Salmonella* spp. isolated from humans, food animals, and retail meat in the United States in 2007. Six isolates collected from humans harbored *aac(6')Ib-cr* or a *qnr* gene. Most prevalent was *qnrS1*. No animal or retail meat isolates harbored a plasmid-mediated mechanism.

Severe *Salmonella enterica* infections are commonly treated with fluoroquinolones (e.g., ciprofloxacin) (*1*). In the United States, the antimicrobial drug susceptibility of *Salmonella* spp. isolated from humans, food animals, and retail meats is systematically monitored by the National Antimicrobial Resistance Monitoring System (NARMS). This program is a collaborative effort of the Centers for Disease Control and Prevention (CDC), the Food and Drug Administration Center for Veterinary Medicine (FDA-CVM) and the US Department of Agriculture (USDA). Antimicrobial susceptibility to fluoroquinolones among *Salmonella* spp. has been monitored since the program's inception in 1996.

Although fluoroquinolone resistance in *Enterobacteriaceae* is predominantly due to topoisomerase mutations, 3 plasmid-mediated mechanisms have been described that confer decreased susceptibility to ciprofloxacin: quinolone resistance proteins (Qnr), Aac(6′)-Ib-cr, and QepA efflux (*2*). The Qnr proteins protect the DNA-gyrase from quinolones, Aac(6′)-Ib-cr modifies quinolones with a piperazinyl group, and QepA is involved in active efflux (*2*). Because patients have experienced treatment failure when infected with *Salmonella* isolates that displayed decreased susceptibility to fluoroquinolones, plasmid-mediated mechanisms are clinically relevant (*3*).

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A survey of 12,253 NARMS non-Typhi *Salmonella* (NTS) isolates collected from humans from 1996 through 2003 identified 10 (0.08%) *qnr*-positive isolates (*4*). A second survey of NARMS NTS collected from humans during 2004–2006 showed an increase in the proportion of isolates harboring plasmid-mediated quinolone resistance mechanisms. Among 6,057 isolates, 17 *qnr*-positive isolates and 1 *aac(6')-Ib-cr*-positive isolate were detected, representing 0.3% of the NTS collected during that time (*5*).

The increase in plasmid-mediated quinolone resistance among NTS isolated from humans in the United States prompted further studies to determine continued presence among NTS of human origin and possible reservoirs of these mechanisms. In this study, we investigated plasmid-mediated quinolone resistance mechanisms among NARMS NTS isolated from humans, food animals, and retail meat in the United States in 2007.

The Study

In 2007, 54 NARMS-participating public health laboratories from all 50 states forwarded every 20th human isolate of NTS to CDC. Similarly, NTS isolated from retail meat (chicken breasts, ground turkey, ground beef, and pork chops) were submitted by 10 states that participated in CDC's Foodborne Diseases Active Surveillance Network (FoodNet) for analysis at FDA-CVM. NTS from food animals were obtained from carcass rinsates (chicken), carcass swab specimens (turkey, cattle, and swine), and ground products (chicken, turkey, and beef). Animal samples were collected by the Food Safety Inspection Service of the USDA from federally inspected slaughter and processing plants throughout the United States and sent to USDA facilities in Athens, Georgia, for further analysis.

At each agency, MICs were determined by broth microdilution (Sensititer; Trek Diagnostics, Westlake, OH, USA). Human, animal, and retail meat isolates of NTS that displayed decreased susceptibility to ciprofloxacin (MIC $\geq$0.25 mg/L) were included in our study. For each isolate, genomic DNA was prepared by lysing the bacteria at 95°C and collecting the supernatant after centrifugation. PCRs with previously described primers were used to screen isolates for *qepA*, *aac(6')-Ib-cr,* and *qnr* genes (*qnrA, B, C, D, S*) (*6–10*). Positive controls were included for *qepA* (*Escherichia coli* TOP10 pAT851), *qnrA* (*S. enterica* serotype Montevideo AM28704), *qnrB* (*S. enterica* serotype Berta AM04589), *qnrS* (*S. enterica* serotype

Table. Characteristics of non-Typhi *Salmonella enterica* isolates harboring *qnr* or the *aac(6')-Ib-cr* gene, collected through NARMS, 2007*

Isolate no.	*S. enterica* serovar	Submitting site	Resistance phenotype	Ciprofloxacin MIC, mg/L	*aac6'Ib/qnr* variant
AM31035	Thompson	NY	AMP, SUL	0.5	*aac(6')-Ib*-cr
AM30827	Typhimurium	CA	STR, SUL, TET	0.5	*qnrS1*
AM31228	Corvallis	VA	ND	0.5	*qnrS1*
AM33914	Typhimurium	NC	ND	0.5	*qnrS1*
AM31434	Enteritidis	LX	SUL, SXT, TET	0.25	*qnrB2*
AM33097	Beaudesert	CA	ND	0.25	*qnrB19*

*NARMS, National Antimicrobial Resistance Monitoring System; NY, New York; AMP, ampicillin; SUL, sulfamethoxazole or sulfisoxazole; CA, California; STR, streptomycin; TET, tetracycline; VA, Virginia; ND, none detected; NC, North Carolina; LX, Los Angeles; SXT, trimethoprim/sulfamethoxazole.

Bovismorbificans AM12888) and *aac(6')-Ib-cr* (*E. coli* 36564). For isolates with positive results in the screening, amplicons were confirmed by direct sequencing by using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Among 2,165 isolates of NTS collected from humans in 2007, 51 (2.4%) displayed decreased susceptibility to ciprofloxacin. Among 320 NTS obtained from retail meat, 5 (1.6%) showed decreased susceptibility to ciprofloxacin, and among the 1,915 isolates obtained from animal sources, 5 (0.3%) showed such susceptibility. Six (11.8%) of the 51 human isolates carried a plasmid-mediated mechanism that affected quinolones; 5 isolates harbored a *qnr* gene, and 1 isolate contained the *aac(6')-Ib-cr* gene (Table). None of the isolates harbored the *qepA* gene. Sequencing of the 5 *qnr*-positive isolates showed 3 *qnrS* and 2 *qnrB* variants among 4 serotypes (Beaudesert, Corvallis, Enteritidis, and Typhimurium) (Table). The *aac(6')-Ib-cr* gene was found in an isolate of serotype Thompson, and sequencing confirmed the 2 point mutations (Trp102Arg and Asp179Tyr) characteristic of the ciprofloxacin-modifying variant. The MIC of ciprofloxacin among the *qnr*-positive isolates ranged from 0.25 mg/L to 0.5 mg/L, whereas the *aac(6')-Ib-cr*–positive isolate displayed an MIC of 0.5 mg/L. All isolates from humans were susceptible to nalidixic acid (MIC range 8–16 mg/L). None of the isolates obtained from retail meat or those isolated from animal sources harbored plasmid-mediated mechanisms affecting quinolones. However, all retail meat and animal isolates with decreased susceptibility to ciprofloxacin were resistant to nalidixic acid (MIC $\geq$32 mg/L), which suggests the presence of topoisomerase mutations.

The 6 patients (3 male and 3 female) who were infected with a Qnr-producing or Aac(6′)-Ib-cr–producing *Salmonella* isolate had a median age of 18 (range 3–84 years). Three patients were available for interview. They reported gastrointestinal symptoms and had sought medical care for their condition. Two of the patients had received antimicrobial drug treatment (ciprofloxacin and cefdinir, respectively); none of the patients developed an invasive infection. Two patients reported a history of international travel to Mexico and Thailand, respectively.

Conclusions

Six (0.3%) NARMS NTS collected from humans in 2007 harbored a plasmid-mediated quinolone resistance mechanism, the same prevalence as in 2004–2006 (*5*). None of the isolates collected from animal and retail meat by the USDA and FDA in 2007 harbored these mechanisms. Among the human isolates, *qnr* genes predominated and *qnrS1* was most prevalent. This gene has previously been described among NARMS human NTS and was first detected in an isolate of serotype Bovismorbificans collected in 2000 (*4*). The gene was later reported in 11 isolates (serotypes Corvallis, Enteritidis, Montevideo, Saintpaul, and Typhimurium) collected by NARMS during 2004–2006 (*5*).

That *qnr* genes could only be detected among *Salmonella* isolates obtained from humans warrants further exploration. One factor that could influence the number of Qnr-producing *Salmonella* isolates among humans in the United States is the extent of travel-associated infections. Two patients in this study had a history of international travel before illness onset. Another factor that could lead to the development of Qnr-producing *Salmonella* isolates is the in vivo transfer of resistance from other *qnr*-bearing *Enterobacteriaceae.*

Our study does not suggest that food animals and meat in the United States are major sources of *Salmonella* isolates that harbor plasmid-mediated quinolone resistance mechanisms. However, animals and food have been described as reservoirs for these mechanisms elsewhere. A high prevalence of *Enterobacteriaceae* with *qnr* and *aac(6')-Ib-cr* have been reported among companion and food animals in the People's Republic of China and *qnr*-positive *Salmonella* isolates have been found in poultry in Europe (*11*,*12*). Thus, other food and meat sources, not investigated in the current study, may serve as reservoirs for these mechanisms.

Fluoroquinolone resistance among isolates of NTS has important public health implications because ciprofloxacin is commonly used to treat invasive infections of *Salmonella* spp. in adults. Although plasmid-mediated quinolone resistance mechanisms do not, by themselves, confer clinical resistance to ciprofloxacin, they may promote the selection of mutations that do (*13*). In addition, studies have shown

that patients infected with isolates that display low-level fluoroquinolone resistance may respond poorly to treatment, prompting a reconsideration of MIC breakpoints in clinical medicine (*3*,*14*). To avoid further dissemination of plasmid-mediated quinolone resistance among *Salmonella* and other *Enterobacteriaceae* isolates in the United States, prudent use of antimicrobial agents in both human and veterinary medicine will be crucial. Continued surveillance for resistant bacteria among human, animal, and food sources remains critical.

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Hemolytic–Uremic Syndrome in a Grandmother

Lane C. Crawford, Mark L. Crawford, and Sean R. Moore

In the spring of 2007, just weeks before my college graduation, my parents called to tell me that my grandmother was sick. I was worried to hear that she was in the hospital, but like the rest of my family, I had little doubt that she'd be better in no time. At a spry 82 years, my grandmother was the picture of health. She was a nonsmoker, a nondrinker, and had had no medical problems except for mild asthma. A world traveler, avid gardener, and savvy businesswoman, she has been known to do her grocery shopping, attend a bank board meeting, talk her way out of a speeding ticket, and rearrange the living room furniture all in time to prepare dinner for 12 and cut fresh roses for the table. As a loving wife, mother of 5, and grandmother of 10, she is the family matriarch and holds us together in a way I never fully appreciated until that familiar structure was suddenly threatened.

My grandmother's illness began with acute onset of abdominal cramps and watery, nonbloody diarrhea followed by nausea and vomiting. She had no fever. The diarrhea continued intermittently through the first day and night of her illness, and by the second day, when the diarrhea became grossly bloody, her internist had her admitted to a community hospital.

At the time of admission, she was normotensive and still afebrile. Physical examination findings were unremarkable except for mild bilateral lower abdominal tenderness. The leukocyte count was mildly elevated at 13,500 cells/μL, hemoglobin was within normal limits at 15.0 g/dL, and the platelet count was 263,000 cells/μL. Electrolytes were within normal limits, blood urea nitrogen was mildly elevated at 19 mg/dL, and creatinine was within normal limits at 0.7 mg/dL. Urinalysis findings were also within normal limits. A radiograph of her abdomen showed no signs of a perforated organ. Gastroenterologists were consulted. Their assessment suggested infectious diarrhea as the most likely diagnosis, followed by ischemic colitis and diverticular disease. To check for enteric infections, they ordered stool culture, stool leukocyte count, and a stool *Clostridium difficile* toxin test. Supportive treatment of intravenous hydration and antiemetics was initiated.

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On my grandmother's third day of illness, the bloody diarrhea worsened and the leukocyte count increased to 18,100 cells/μL. A stool sample contained no *C. difficile* toxin but did contain leukocytes. A contrast computed tomography (CT) scan of the abdomen showed pancolitis with 2-cm wall edema and narrowing of the colon lumen. The CT findings of colitis distributed throughout multiple vessels, sparing of the terminal ileum, and good contrast flow in the major mesenteric vessels made a diagnosis of ischemic colitis much less likely; therefore, bacterial pancolitis became the leading diagnosis. Because of increasingly severe bloody diarrhea and leukocytosis, intravenous ciprofloxacin and metronidazole were started empirically while stool culture results were pending.

Over the next 2 days, the bloody diarrhea abated, but abdominal distention and a mild tremor developed. An abdominal radiograph showed air distending the stomach and small bowel. The leukocyte count rose to 30,500 cells/μL, and the platelet count dropped to 105,000 cells/μL. Low urine output, blood urea nitrogen level of 57 mg/dL, and creatinine level of 1.9 mg/dL suggested acute kidney injury, for which increased intravenous fluids and a diuretic were ordered.

None of these developments were overly alarming; nevertheless, my grandmother looked sicker, and my family's concern deepened. My father (M.L.C.), an orthopedic surgeon, was growing increasingly uneasy, but because hemorrhagic colitis was not his area of expertise, he was content to defer to his colleagues' judgment. However, as a physician and son, he felt compelled to fully understand his mother's illness. He began by searching the literature on infectious hemorrhagic colitis and learned that the major bacterial causes are *Shigella, Salmonella, Yersinia, Vibrio, Escherichia coli, Campylobacter,* and *Clostridium* spp. (*1*). He then visited the hospital's bacte-

riology laboratory, where a technician explained the lab's stool culture protocol.

My father learned that stool culture on commonly used culture media is quite effective for identification of *Salmonella* spp. and other bacteria not normally found in human bowel. However, because *E. coli* is part of normal bowel flora, a separate strategy is needed to distinguish diarrheagenic *E. coli* serotypes from their nonpathogenic brethren. To do this, most laboratories use a selective medium called sorbitol-MacConkey agar (SMAC) to identify the pathogenic serotype *E. coli* O157:H7. Whereas most other *E. coli* serotypes ferment sorbitol and grow as pink colonies, this serotype does not ferment sorbitol and grows as colorless colonies. When my father asked how often stool culture results from patients with actual bacterial colitis were negative, the technician assured him, "almost never." Thus, when my grandmother's stool culture came back negative, my father did not question it.

On my grandmother's fifth day of illness, colonoscopy showed severe nodular and granular inflammation from rectum to cecum. The gastroenterologist excluded ischemic colitis but, given the negative culture and the colonoscopic appearance, had to consider the possibility of inflammatory bowel disease. Although new onset of this disease was unlikely in an 82-year-old patient, this possibility was covered by prescribing a full dose of steroids; ciprofloxacin was continued. In the meantime, my father continued his literature search, which reinforced his feeling that inflammatory bowel disease was unlikely.

When my father refocused his search on infectious causes of hemorrhagic colitis, *E. coli* emerged as the most frequently described culprit and seemed consistent with my grandmother's case. He further learned that the serotypes of *E. coli* that cause diarrheal illness in humans do so by producing Shiga toxin and are thus referred to as Shiga toxin–producing *E. coli* (STEC); that the O157:H7 serotype is the most frequently identified type of STEC in the United States; and that it is the most likely serotype to cause hemolytic–uremic syndrome (HUS), a life-threatening condition characterized by hemolytic anemia, low platelet count, and renal failure. He also came across several articles that gave him pause. One recent article stated that 20%–50% of all STEC infections in the United States are caused by non-O157 STEC serotypes, some of which can cause HUS (*2*). A second article explained that non-O157 STEC is not detected by SMAC because, like nonpathogenic *E. coli*, it ferments sorbitol. Instead, both O157 and non-O157 STEC could be detected by enzyme immunoassay for Shiga toxin (*3*). My grandmother's negative stool culture now seemed less conclusive.

The next morning, the sixth day of illness, my grandmother was transferred to an intensive care unit because of worsening renal failure. Her mental status had declined and her tremor had worsened. My father suspected HUS and conveyed his concern to her internist, who agreed that HUS was a possibility. My father then returned to the laboratory and inquired about the Shiga toxin assay he'd read about. The technician told him that they had recently opted not to buy the toxin assay because of its expense and their satisfaction with SMAC; however, the toxin assay was available at their reference laboratory. Unfortunately, the original stool sample had been discarded. A new specimen was collected, sent, and had negative results for Shiga toxin, but it also grew no gram-negative bacteria on culture. Three days of antimicrobial drugs had effectively sterilized the colon, rendering a false-negative result more likely. That afternoon, the hemoglobin level decreased to 12.0 g/dL, platelet count fell to 71,000 cells/μL, and lactate dehydrogenase level was markedly elevated, all of which could be consistent with developing HUS. Although no schistocytes were evident on peripheral blood smear, the internist was concerned and consulted a nephrologist at a nearby regional hospital. Upon hearing of suspected HUS, he recommended immediately transferring my grandmother for plasmapheresis and possible dialysis.

The next 12 hours were a blur of confusion and frustration for my family. When care of a patient is transferred from one medical team to another, treatment plans often change abruptly, sometimes because of differences in clinical judgment and other times because of imperfect communication between the teams. Whatever the reason, my grandmother left her local hospital with a plan for plasmapheresis and supportive care for HUS, and 1 hour later, after evaluation at the regional hospital, received a diagnosis of acute abdomen and sepsis. A general surgeon was consulted and recommended emergency total colectomy. Without it, he said, she would be dead within the hour. My family was shocked and distressed by this drastic change of plans. My father was particularly hesitant. In his reading about the recommended therapy for STEC and HUS, he had not come across any mention of the need for surgical intervention. Nevertheless, the general surgeon was adamant in his recommendation, and considering the surgical adage "You can never go wrong by looking," my father advised my grandfather to consent to the operation.

Four hours later, the surgeon returned and explained to the family that the operation had gone well. He had removed all but the distal 12 inches of the colon, which was not as severely affected. He expected that my grandmother would need mechanical ventilation, at least overnight and possibly for 1–2 days. The colon had been reddened and inflamed, but he had found no dead bowel and no perforations. He had noted 1-cm wall edema. Recalling the 2-cm wall edema on the CT image from 3 days ago, my father concluded that the colon had been recovering and regretted his decision to allow the operation.

In the week after my grandmother's operation, my family learned everything they could about STEC. After learning that it is most frequently transmitted through undercooked beef or contaminated produce, they analyzed their food history. This prompted the discovery of "The Meatloaf," now infamous in our family lore. Three days before becoming ill, my grandmother had cooked a meatloaf for dinner, which a family member recalled being a bit pink in the middle. Over the next 4 days, all 3 other family members who had eaten the meatloaf experienced mild nonbloody diarrhea but recovered fully. My 4-year-old cousin, who had refused to eat any meatloaf, showed no signs of illness. For once we were grateful for his picky eating. The ground beef in the meatloaf had come from 2 calves from the family farm; the calves had been processed at a local slaughterhouse. Another piece of the puzzle had fallen into place.

Meanwhile, my grandmother continued to need mechanical ventilation and became comatose and anuric. She was evaluated by physicians from the departments of general surgery, infectious disease, nephrology, hematology, pulmonology, and pathology. Despite my father's suggestions regarding STEC and HUS, their various assessments of her condition culminated in a diagnosis of ischemic colitis with sepsis resulting in acute renal failure, disseminated intravascular coagulation (DIC), and hypotensive brain injury, from which she was not likely to recover.

Each of these diagnoses was far more common than STEC/HUS, but several things simply didn't fit. First, the operative findings were inconsistent with ischemic colitis. Second, my grandmother lacked the fever and hypotension characteristic of sepsis. Her blood pressure had never dropped to an extent that would be expected to cause severe end-organ damage. Third, the leukocyte count remained markedly elevated despite removal of the suspected source of infection, a battery of potent antimicrobial drugs, and negative blood and urine cultures. The elevated leukocyte count did not fit with sepsis but might have represented acute inflammation associated with HUS (*4*). Finally, although hemoglobin level and platelet count remained low, other coagulation study results were all within normal limits, more consistent with HUS than DIC.

My family remained convinced of the STEC/HUS diagnosis. On postoperative day 6, my father requested that antimicrobial drugs be stopped. If she really had sepsis, she was dying from it in spite of them. If not, they were only obscuring the true diagnosis. The next day my father called the foodborne diseases division of the Centers for Disease Control and Prevention (CDC). He described my grandmother's case to the physician on call, who agreed, without reservation, with the diagnosis of STEC infection. He explained that sepsis and DIC rarely occurred with STEC infection and that surgery was almost never required. Antimicrobial drugs were not recommended and might even hasten progression to HUS. Although not of proven effectiveness, plasmapheresis was commonly used for treatment of HUS. With this further support of the diagnosis, my father again presented his case to my grandmother's physicians, and her nephrologist agreed to start a trial of daily plasmapheresis.

Over the next 4 days my grandmother remained comatose, but her urine output slowly began to increase. A nephrologist at a university medical center was consulted and recommended continuing plasmapheresis. That afternoon, my grandmother opened her eyes. By the end of the day—the 16th day of illness—she was recognizing family members, following simple commands, and mouthing words. She could turn her head to look at my grandfather, who was promising her everything in the world. As a combat pilot in World War II, he'd seen his share of battles but none like this. After weeks of feeling lost, helpless, and unable to save his war-time bride, he now regained hope. My family's relief was indescribable.

Histopathology slides of my grandmother's colon were sent to a university medical center for review, where pathologists diagnosed acute hemorrhagic necrotizing colitis consistent with STEC infection. Three days after waking from the coma, my grandmother was transferred to that medical center for the remainder of her recovery—incidentally, the same university I was attending as an undergraduate. On the morning of commencement, I was able to visit her wearing my cap and gown. After 11 days in the university hospital and 18 days in a rehabilitation facility, she was finally discharged to go home. Exceeding all expectations, her kidneys and neurologic system recovered fully, and 15 months later her colostomy bag was removed. She is now back to doing all her usual activities—serving as bank director, babysitting her 2 youngest grandchildren, flying to Hawaii for vacation, and cooking for a crowd. Just not meatloaf.

As illustrated by this case report, STEC infection may be difficult to recognize clinically, so appropriate laboratory testing is crucial for accurate and timely diagnosis. Up to 20%–50% of STEC infections in the United States, or ≈37,000 cases per year, are caused by non-O157 *E. coli* serotypes (*2*,*5*), some of which have been associated with severe disease and HUS (*6*,*7*). Failure to test for these serotypes leads to underdiagnosis and underreporting of STEC infection, to the detriment of patient care and public health surveillance, respectively. A 2006 report strongly urges clinical diagnostic laboratories to assay all stool specimens for Shiga toxin, to simultaneously culture the specimens on SMAC for organism isolation, and to forward positive specimens to a public health laboratory (*8*). Because data with regard to implementation of these recommendations are lacking, we surveyed diagnostic laboratories in Tennes-

see about their protocols for STEC detection and identified factors influencing their choice of protocol.

From July through October 2008, we contacted all clinical laboratories licensed to perform microbiologic or bacteriologic testing in Tennessee and conducted telephone and email interviews with supervisors. This survey was approved by our university's institutional review board.

From 130 laboratories, we received 117 responses, a high response rate of 90%. Of these 117 respondents, 57 (49%) performed stool cultures in house. Of these 57 laboratories, 46 (81%) included STEC in their routine testing for enteric pathogens, 8 (14%) tested for STEC only in bloody specimens or only with a physician's order, and 3 (5%) did not test for STEC at all. We further asked the 54 laboratories that tested any or all specimens for STEC about their STEC detection protocol. We found that 38 (70%) of 54 used SMAC alone; 4 (7%) of 54 used Shiga toxin assay alone; and 12 (22%) of 54 used both. Only 8 (15%) of the 54 laboratories used both tests concurrently, and 6 (11%) did so for all specimens as recommended by CDC in 2006.

The second part of our survey aimed to ascertain which factors influenced adherence to the 2006 CDC recommendations. In the 38 laboratories that used SMAC alone, 13 (35%) supervisors stated that they were not familiar with the Shiga toxin assay, and 24 (63%) claimed some familiarity. These 24 were asked to identify reasons for choosing SMAC over Shiga toxin assay. Of the 24, a total of 20 (83%) thought that SMAC alone provides adequate STEC detection, 18 (75%) said SMAC was a better fit for their laboratory's workflow and staffing situation, 17 (71%) said that they used SMAC because of its lower cost, and 12 (50%) said that they used SMAC because laboratory personnel were unfamiliar with the Shiga toxin assay.

Our survey suggests that Tennessee laboratories fall short of best practice recommendations for detection of non-O157 STEC; only 11% had fully implemented the CDC-recommended protocols. The key factors associated with nonadherence were lack of familiarity with Shiga toxin assays and limited knowledge of current recommendations.

Although laboratories operate under the constraints of third-party reimbursement, local economics, and institutional policy, knowledge of best practice recommendations is essential for making an informed choice of protocol. As evidenced in my grandmother's case, appropriate laboratory practices must be complemented by physicians' high index of suspicion and familiarity with diagnostic techniques. Ultimately, it is the treating physician's responsibility to ensure that all necessary diagnostic tests are ordered, whether in house or at a reference laboratory. The 2006 recommendations were recently emphasized in a 2009 MMWR Recommendations and Reports article (*9*). It is our hope that this recent publication will further contribute to the improved diagnosis of STEC infections, to the benefit of public health, patients, and grandmothers.

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Typhoid Fever among Children, Ghana

To the Editor: Typhoid fever (TF) remains a problem of concern in many low-income countries. *Salmonella enterica* serovar Typhi causes ≈22,000,000 symptomatic infections and 220,000 fatalities worldwide annually (*1*). However, the effect and incidence of TF in many parts of sub-Saharan Africa are largely unknown because diagnostic laboratories are lacking and fatal TF is frequently attributed to malaria (*2*,*3*). In Ghana, TF ranks among the leading 20 causes of outpatient illness, accounting for 0.92% of hospital admissions (*4*).

We conducted our study at the rural Agogo Presbyterian Hospital in the Ashanti Region of Ghana. The percentage of residents of 99 villages and household clusters of buildings (population size 18–13,559 persons, median 277 persons) with access to the study hospital was assessed in a healthcare utilization survey. A proportional-to-size number of children were randomly selected in each village, and a standardized interview was conducted. TF incidences were calculated for September 2007–November 2008 (Table). A bacteriology laboratory with BACTEC 9050 automated blood culture system (Becton Dickinson, Sparks, MD, USA) was established in the study hospital and run to assess the number of admissions with TF, the incidence of TF in the adjoining community and *S. enterica* ser. Typhi resistance to a panel of antimicrobial drugs.

The study included 1,456 children <15 years of age who were admitted to the pediatric ward of Agogo Presbyterian Hospital over the 23-month study period. Overall, 52.1% were male; mean age of children was 32.2 months (SD ± 36.0 months; median 19 months, range 0–174 months). Blood was cultured by using a BACTEC 9050 blood culture system (Becton Dickinson), and positive samples were examined by standard methods. Antimicrobial drug susceptibility testing was performed on all serovar Typhi isolates by using the Kirby-Bauer disk-diffusion method for ampicillin, chloramphenicol, tetracycline, trimethoprim/sulfamethoxazole, amoxicillin/clavulanicacid, gentamicin, ciprofloxacin, and ceftriaxone.

Children <2 years of age had the highest proportion of positive blood cultures (164/1,456, 21.3%; online Appendix Figure, www.cdc.gov/EID/content/16/11/1796-appF.htm). Of 298 blood cultures yielding positive growth for bacterial pathogens or for *Candida* spp., 37 (12.4%) isolates (2.5% of the 1,456 hospitalized children) were positive for *S. enterica* ser. Typhi. The frequency of TF was low among children <2 years of age (7/1,018, 0.7%), increased among those 2 to <11 years of age (29/417, 7.0%), and decreased among children ≥11 years of age (1/22, 4.6%) (online Appendix Figure). One (2.7%) child with TF died. Malaria parasites were detected in 2 children with *S. enterica* ser. Typhi. Pathogens other than *S. enterica* ser. Typhi were identified among 21.3% and 11.8% of children 0 to <2 years and 5 to <8 years of age, respectively. These pathogens included nontyphoidal salmonellae, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. *S. enterica* ser. Typhi isolates were resistant to chloramphenicol (73%), trimethoprim/sulfamethoxazole (71%), ampicillin/amoxicillin (70%), tetracycline (64%), gentamicin (46%), and amoxicillin/clavulanic acid (24%) but susceptible to ciprofloxacin and ceftriaxone.

TF incidence in children <5 years of age was ≈190 cases/100,000 population and highest in children 2–5 years of age (290/100,000 per year) and 5–8 years of age (200/100,000 per year) (Table). In children older than 8, incidence decreased continuously, and the number of cases was too low to enable precise age-stratified incidence calculations. The incidences in the study area point to a higher impact of TF than expected (*4*) and may reflect an underestimation of TF in other West African regions as well. Our high incidence figure may still underestimate the incidence because of a low sensitivity of standard microbiologic methods (up to 50%), which are prone to underdiagnose moderate bacteremia in *Salmonella* infections (*5*,*6*).

Compared with Asia, only limited data are available from Africa on *S. enterica* ser. Typhi drug resistance. A study from Nigeria showed that, among serovar Typhi strains isolated from hospitalized patients in Lagos during 1997–2004, resistance rates reached 87% for ampicillin and were 0.7% for ciprofloxacin, compared with 70% and 0%, respectively, in the present study. Resistance to trimethoprim/sulfamethoxazole was 59% in Nigeria, compared with 71% in Ghana. In Togo, proportions of serovar Typhi strains resistant to chloramphenicol and trimethoprim/sulfamethoxazole

Table. Estimates of *Salmonella enterica* serovar Typhi incidence in children, Ghana, September 2007–November 2008

Age group, y	No. *S. enterica* ser. Typhi isolates*	Coverage population†	Incidence‡ (95% confidence interval)
0–15	16	14,933	120 (70 to 170)
<2	1	2,133	50 (–30 to 140)
2–<5	8	3,200	290 (120 to 450)
5–<8	5	2,880	200 (50 to 340)
8–<11	1	2,880	40 (–30 to 110)
11–<15	1	3,840	30 (–20 to 80)

*Observation period 15 mo (period of complete and uninterrupted assessment of blood culture data).
†No. residents of each community with access to Agogo Presbyterian Hospital.
‡Per 100,000 persons per year.

were 33% and 46%, respectively, before 2002 and 73% and 79% in 2003–2004 (*7*) and thus similar to those in our study.

In addition, resistances to ciprofloxacin and ceftriaxone were <10%. Multidrug resistance (resistance to ampicillin, trimethoprim/sulfamethoxazole, and chloramphenicol) was observed in 63% of children in our study, compared with 7% in India, 22% in Vietnam, and 65% in Pakistan (*8–10*).

More effort is needed in Africa to enable reliable and standardized laboratory diagnoses of *Salmonella* infections and to sustain TF surveillance and drug sensitivity surveys. Moreover, introduction of a vaccination program should be discussed after more data are obtained from other areas in Ghana and West Africa. Such data currently are collected in an extensive standardized surveillance program across the continent performed by our group and others. In parallel, trials should be conducted to assess the effectiveness and cost-effectiveness of currently available and newly developed TF vaccines.

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Shigella spp. Antimicrobial Drug Resistance, Papua New Guinea, 2000–2009

To the Editor: Approximately half the *Shigella* spp. infections in developing countries are caused by endemic shigellae (*1*), which in these countries are responsible for ≈10% of all episodes of diarrhea among children <5 years of age and up to 75% of deaths from diarrhea (*2*). Deaths from epidemic *Shigella* spp. in the community are estimated to outnumber deaths within the healthcare setting. In Papua New Guinea, diarrhea is a major cause of hospital admission and death (*3*); *Shigella* spp. are among the most common causes of enteric bacterial infection (*4,5*), and *S. flexneri* is the most common serotype (*3,6*). Outbreaks of bloody diarrhea are frequently reported; however, diagnosis in remote settings is challenging, partly because the storage requirements for the organism are difficult to meet.

Multidrug resistance of shigellae is not new (*1*); many countries have

reported resistance to amoxicillin, co-trimoxazole, and chloramphenicol. For this reason, the World Health Organization recommends that all patients with bloody diarrhea be treated with either ciprofloxacin or 1 of the 3 second-line drugs: pivmecillinam, azithromycin, and ceftriaxone (*7*). The antimicrobial drug currently recommended for patients with bloody diarrhea in primary healthcare settings in Papua New Guinea is co-trimoxazole (*8*); ciprofloxacin is available only in hospitals.

In August 2009, an epidemic of multidrug-resistant *S. flexneri* infection associated with widespread illness and death across 4 provinces of Papua New Guinea was reported to health authorities. To understand the trends and to inform antimicrobial drug policy makers, we reviewed retrospective microbiological data for 2000–2009. With the exception of 3 isolates collected during an outbreak in the border regions of the 4 provinces during 2009 (excluded from analysis), all isolates in our study were obtained as part of routine surveillance. Fecal samples were collected by clinicians from any patient seeking care for severe diarrhea at Port Moresby General Hospital.

Before serologic testing was conducted, samples were spread directly on desoxycholate citrate agar and MacConkey agar plates for culture. Antimicrobial drug resistance testing was performed by using the Kirby-Bauer method.

From a total of 3,419 fecal samples cultured, 136 (4.0%) were positive for *Shigella* spp. The most commonly isolated species was *S. flexneri* (90.4%); less frequently isolated were *S. boydii* (3.7 %), *S. dysenteriae* (2.9%), and *S. sonnei* (1.5%). Of the 123 *S. flexneri* isolates, 20 (16%) were further characterized; the most frequent serovars were serovar 2 (40%) and serovar 3 (30%). Many (48%) *Shigella* spp.–positive isolates were from children <5 years of age. The highest rates of antimicrobial drug resistance of all *Shigella* spp. were to amoxicillin (96%), co-trimoxazole (86%), and chloramphenicol (60%); no resistance to ciprofloxacin and cephalexin was found (Table).

Current evidence supports the use of ciprofloxacin, ceftriaxone, and pivmecillinam for treatment of bloody diarrhea (*9*). It also suggests that dysentery rarely relapses if an infected child has received a full course of treatment with 1 of these drugs and the causative pathogen is sensitive to the drug. Reducing the risk for relapse of bacterial infections among children is beneficial because it reduces the likelihood of subsequent episodes of dysentery occurring in that child and of transmission to others (*9*). In our study, most isolates were resistant to co-trimoxazole and the other available antimicrobial drugs, indicating that their use would not have reduced illness and subsequent transmission in this setting. The lack of resistance to ciprofloxacin and cephalexin indicates that these drugs may be more effective; however, they are neither available at the primary healthcare level nor recommended in Papua New Guinea, which is cause for concern.

Surveillance for antimicrobial drug resistance is essential for the containment of antimicrobial drug resistance globally. However, international surveillance depends on strong national surveillance systems. Despite the existence of a network of subnational laboratories where fecal sample cultures had been performed, these laboratories no longer perform these cultures. In 1964, the laboratory in 1 provincial hospital analyzed and subtyped 1,000 stool samples over a 15-month period (*6*). In our study, conducted at the national referral hospital (which limits the representativeness), we analyzed 3,419 fecal samples over a 10-year period.

Outbreaks of bloody diarrhea are common in remote settings in Papua New Guinea, yet with the exception of the 3 isolates from 2009 that were excluded from analysis, no *Shigella* spp.–

Table. Antimicrobial drug resistance of *Shigella* spp., Papua New Guinea, 2000–2009*

Drug	Total no. isolates tested	Sensitivity	*Shigella* sp., no. (%) isolates					
			S. boydi	*S. dysenteriae*	*S. flexneri*	*S. sonneii*	Unknown sp.	Total
Amoxicillin	98	S	0	1 (33)	2 (2)	0	1 (100)	4 (4)
		R	3 (100)	2 (67)	87 (98)	2 (100)	0	94 (96)
Cephalexin	46	S	2 (67)	2 (100)	38 (100)	2 (100)	1 (100)	45 (98)
		I	1 (33)	0	0	0	0	1 (2)
		R	0	0	0	0	0	0
Ciprofloxacin	41	S	2 (67)	NA	35 (100)	1 (100)	2 (100)	40 (98)
		I	1 (33)	NA	0	0	0	1 (2)
		R	0	0	0	0	0	0
Chloramphenicol	114	S	0	2 (50)	9 (9)	2 (100)	1 (50)	14 (12)
		I	0	2 (50)	28 (28)	0	1 (50)	31 (27)
		R	4 (100)	0	64 (63)	0	0	68 (60)
Naladixic acid	13	S	1 (100)	0	8 (100)	1 (100)	1 (50)	11 (85)
		R	0	1 (100)	0	0	1 (50)	2 (15)
Co-trimoxazole	76	S	1 (25)	1 (33)	9 (14)	0	0	11 (14)
		R	3 (75)	2 (67)	57 (86)	2 (100)	1 (100)	65 (86)

*S, sensitive; R, resistant, I, intermediate; NA, not applicable.

positive samples have been identified during outbreaks. Molecular methods may serve as an adjunct to traditional laboratory methods by improving sensitivity and also enabling diagnosis of *Shigella* spp. outbreaks among remote populations where specimen storage and transport requirements may be challenging (*10*).

We describe extremely high rates of resistance of *Shigella* spp. to cotrimoxazole, the recommended treatment for bloody diarrhea in Papua New Guinea. Strengthening national surveillance for antimicrobial drug resistance would provide the evidence to better inform policy decision makers. A review of the national antimicrobial drug policy for management of bloody diarrhea is urgently needed.

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Fatal Avian Influenza (H5N1) Infection in Human, China

To the Editor: Since the first avian influenza virus (H5N1) was isolated from a goose in the southern region of the People's Republic of China a decade ago (*1*), no poultry outbreak has been reported in Shandong Province in eastern China, although adjacent provinces have experienced an avian influenza epidemic (*2*). In fall 2008, several rounds of investigation of poultry farms and markets were conducted in Jinan, Shandong Province, and no influenza virus (H5N1) was isolated by reverse transcription–PCR (RT-PCR) from 19,340 poultry oropharyngeal, cloacal, and cage specimens.

However, a fatal influenza (H5N1) infection in a human was identified on January 17, 2009 (*3*). The patient was a 27-year-old woman from Jinan. Influenza-like illness (ILI) developed on January 5, and the patient received intravenous ribavirin and cephalosporins on January 9. On January 11, she was hospitalized for fever (41°C) and respiratory symptoms. On January 15, extensive infiltration in both lungs developed; the diagnosis was pneumonia of unknown etiology. Early on January 17, she underwent endotracheal intubation. She died of acute respiratory distress syndrome and multiple organ failure later that day.

Two endotracheal aspirates collected on January 17 were positive for influenza virus (H5N1) and for genes encoding matrix protein by real-time PCR and RT-PCR. However, throat swabs collected on January 15 and 16 had been negative even after repeated testing (Table). The influenza virus (H5N1) was isolated on January 22 after 48-hour culture and named A/Shandong/1/2009(H5N1). Whole-genome sequencing showed that all segments were of avian origin. The

hemagglutinin gene and amino acid sequences of this virus were highly homologous with 24 strains of influenza virus (H5N1) isolated during 2005–2008 in China. There was no change in the hemagglutinin cleavage or the receptor binding sites or in the neuraminidase gene conferring oseltamivir resistance (*4*). Nevertheless, mutations in the matrix 2 gene indicated amantadine resistance (*5*).

The patient was a stay-at-home mother with a daughter 20 months of age. Her husband operated a barbecue food stand. The raw poultry ingredients (duck blood and chicken hearts) for the barbecue were washed and processed at home, and the patient may have had unprotected contact with raw poultry products. However, the patient did not raise poultry, did not have contact with sick or dead poultry, had not visited a poultry market recently, and had not consumed sick or dead poultry or raw poultry food. No influenza virus (H5N1) was detected by RT-PCR from the raw duck blood and chicken hearts saved in the patient's home refrigerator or at the poultry seller where the duck blood and chicken hearts originated. No influenza virus (H5N1) was detected by RT-PCR from 448 poultry oropharayngeal and cloacal specimens collected from the live poultry markets immediately after the virus was identified in the patient.

The patient's close contacts (157 persons), including family members and healthcare professionals, were isolated and monitored medically for 7 days according to the Chinese Center for Disease Control and Prevention guidelines, but without chemoprophylaxis because oseltamivir was unavailable. ILI did not develop in any of these persons, and all had negative results for immunoglobulin M against influenza (H5N1) virus. During January 17–23, all 9,865 clinic or hospital visitors with respiratory symptoms in the patient's residential region were interviewed; 829 had fever and 586 had ILI. All recovered quickly without treatment. Persons (537) with frequent exposure to poultry were monitored; ILI did not develop in any person.

This case was comparable to some other influenza (H5N1) infections in humans without identified sources of exposure (*6*,*7*). The virus for this case might have come from infected poultry products in the barbecue raw ingredients. Viremic blood from infected poultry can contaminate raw poultry products. It is also possible that blood was contaminated from poultry struggling during blood collection. The shared kitchen for cooking and raw poultry product processing might be the place where viral transmission occurred. Although the patient had no direct contact with raw poultry products, the shared utensils might have acted as vectors for transmission. Consequently, Jinan enhanced public education to increase awareness of personal protection for persons with direct contact with poultry or poultry raw products and their family members.

The failure to use oseltamivir resulted from lack of alertness and preparedness by healthcare professionals for influenza (H5N1) infection because no human influenza (H5N1) infection had been reported in Shandong Province. The lack of a local oseltamivir reserve also precluded timely oseltamivir use. In response to this public health incident, Jinan enhanced education on self-protection, case management, empirical oseltamivir use, and emergency response to influenza by healthcare and public health professionals, in addition to building a local oseltamivir reserve. These efforts led to preparedness and timely treatment with oseltamivir during the second case of influenza pandemic (H1N1) 2009 infection in China on May 11, 2009 (*8*).

Because influenza viruses (H5N1) can replicate efficiently only in cells of the lower respiratory tract where the avian virus receptor is prevalent (*9*), it is better to collect lower respiratory tract specimens early for laboratory testing. Given that early oseltamivir administration is critical and most effective (*10*), oseltamivir should be administered quickly to patients with pneumonia of unknown etiology without waiting for laboratory confirmation of influenza infection.

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Table. Clinical specimens collected from patient with fatal avian influenza virus (H5N1) infection and test results, Shandong Province, China*

Specimen type	Collection date, Jan 2009	Place tested	Test method	Result
Nasopharyngeal swab	15	Shandong CDC	RT-PCR	A/H5HA/AN1 negative
Nasopharyngeal swab	15	Shandong CDC	Real-time PCR	A/H5HA/H5NA negative
Nasopharyngeal swab	16	Shandong CDC	RT-PCR	A/H5HA/AN1 negative
Nasopharyngeal swab	16	Shandong CDC	Real-time PCR	A/H5HA/H5NA negative
Endotracheal aspirate	17	Shandong CDC	RT-PCR	A/H5HA/AN1 positive
Endotracheal aspirate	17	Shandong CDC	Real-time PCR	A/H5HA/H5NA positive
Endotracheal aspirate	17	China CDC	RT-PCR	A/H5HA/AN1 positive
Endotracheal aspirate	17	China CDC	Real-time PCR	A/H5HA/H5NA positive

*CDC, Center for Disease Control and Prevention; RT-PCR, reverse transcription–PCR.

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Mycobacterium heckeshornense Infection in HIV-infected Patient

To the Editor: *Mycobacterium heckeshornense* is a slow-growing scotochromogen phenotypically and phylogenetically related to *M. xenopi*. It was first identified as a cause of lung infection on the basis of unique 16s rRNA and 16S–23S spacer sequencing (*1*). Published data are limited to the original description and 5 case reports in English-language literature (*2–5*). We report disseminated *M. heckeshornense* infection in an HIV-infected patient and document its role as an emerging pathogen.

A man 40 years of age with diffuse large B-cell lymphoma had advanced HIV infection and a CD4 count <10 cells/mm^3. Antiretroviral therapy (ART) comprising abacavir/lamivudine and lopinavir/ritonavir was initiated. Ongoing night sweats and weight loss after chemotherapy prompted submission of blood cultures, which were positive for *M. heckeshornense* after 41 days' incubation.

Progressive wasting after 2 months' ART prompted treatment for *M. heckeshornense* with isoniazid 300 mg 1x/d, clarithromycin 500 mg 2×/d, moxifloxacin 400 mg 1×/d, vitamin B6 25 mg 1×/d, and rifabutin 150 mg 3×/wk. Pretreatment blood and urine cultures grew *M. heckeshornense* after 41 and 30 days' incubation, respectively. After 18 months of ART and antimycobacterial therapy, the patient's condition improved, and his mycobacterial blood culture remained negative.

Blood for cultures was collected in Vacutainers (Becton Dickinson, Sparks, MD, USA), injected into Myco-F lytic media (Becton Dickinson), and incubated at 35–37°C in a fully automated BACTEC 9000 MB (Becton Dickinson) blood culture instrument for 42 days. Ziehl-Neelsen stain confirmed acid-fast bacilli (AFB) after 41 days. Gen-Probe AccuProbe DNA probes (Gen-Probe Incorporated, San Diego, CA, USA) for *M. avium* and *M. tuberculosis* complexes were negative. The initial blood culture was subcultured to Middlebrook 7H11 media and incubated at 35°–37°C, demonstrating a growth range of 37°–45°C (optimal growth at 42°–45°C). The isolate was forwarded to the National Reference Centre for Mycobacteriology (Winnipeg, Manitoba, Canada) for partial 16S ribosomal RNA gene sequence identification. It corresponded with 100% sequence identity to the type strain of *M. heckeshornense*. The 16S rRNA gene sequence analyzed for this isolate was 1,314 bp long and presented a divergence of 2.6% from its closest species, *M. xenopi*.

Optimal growth temperature and extended time to isolation of the initial isolate prompted a second incubation period of 42 days (total of 84 days) for all subsequent cultures. A subsequent blood culture was positive at 53 days of incubation. If negative after this reincubation period, a terminal Ziehl-Neelsen smear was performed to confirm absence of AFB.

Susceptibility testing of the initial blood and urine isolates was performed by using the radiometric

broth MIC method (Becton Dickinson BACTEC 460 radiometric system; Becton Dickinson Microbiology Systems) and by microbroth dilution method (TREK Diagnostics, Cleveland, OH, USA) for isoniazid and streptomycin. Susceptibility results were as follows: amikacin MIC ≤2.0 μg/mL, ciprofloxacin MIC ≤1.0 μg/mL, clarithromycin MIC ≤16.0μg/mL, rifabutin MIC ≤0.12 μg/mL, ethambutol MIC ≤8.0 μg/mL, isoniazid MIC 1.0 μg/mL, and streptomycin MIC 8.0 μg/mL (*6*).

Disseminated disease caused by nontuberculous mycobacteria (NTM) has been described for HIV-infected patients with CD4 counts <50 cells/mm^3. More than 90% of NTM cases are caused by *M. avium–intracellulare* complex (MAC) (*7*). Reports of *M. heckeshornense* infection are limited to immunocompetent persons with lung disease (*1–3*), tenosynovitis (*4*), and axillary lymphadenitis (*5*), attesting to the organism's virulence.

No criteria exist for diagnosing disseminated NTM infection other than MAC, which is based on clinical signs and isolation from cultures of blood, lymph node, bone marrow, or other sterile sites. The most common diagnostic method is blood culture, positive for >90% of cases (*7*). *M. heckeshornense* was considered the etiologic agent for the patient reported here on the basis of repeated blood culture isolation, clinical signs, and improvement with treatment. Concomitant antiretroviral therapy and immune restoration also likely contributed to the patient's improvement.

The incidence of *M. heckeshornense* infection may be underestimated because of incubation time allowed by the BACTEC 9000 series instruments at 42 days. The positive cultures for this patient were obtained close to or after the traditional 42-day period and without reincubation may have been missed. Piersimoni et al. (*8*) found that most blood cultures for *M. xenopi* were detected with terminal AFB and visual growth inspection performed after the isolates had been determined as negative by conventional means at 42 days, which suggests a need for prolonged incubation.

The optimal treatment for *M. heckeshornense* infection has not been established. Its phenotypic and genotypic resemblance to *M. xenopi* suggests that similar treatment may be reasonable. Current recommendations for treatment for *M. xenopi* infection include isoniazid, rifampin or rifabutin, and ethambutol, with or without an initial phase of streptomycin (*9*). For HIV-infected patients, consideration should be given to prolonged treatment similar to that for disseminated MAC infection, generally ≥12 months and accompanied by a sustained (≥6 months) increase in CD4 counts to >100 cells/mm^3 (*9*).

We report disseminated *M. heckeshornense* in an HIV-infected patient, documenting the pathogen's increasing clinical spectrum. Its isolation requires prolonged incubation and may be missed by standard mycobacterial isolation instruments.

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Geographic Expansion of *Baylisascaris procyonis* Roundworms, Florida, USA

To the Editor: *Baylisascaris procyonis* roundworms are common parasites of raccoons (*Procyon lotor*) in several regions of North America, Europe, and Asia. These parasites are increasingly recognized as a cause of larva migrans in humans, an infection that often results in severe neurologic sequelae or death. In addition, larva migrans has been documented in ≈90 species of wild and domestic birds and mammals. In the United States, *B. procyonis* roundworms are most prevalent in the midwestern, northeastern, and Pacific western states. Numerous surveillance studies have been conducted in the southeastern United States, and *B. procyonis* roundworms are most common in the mountainous regions of Virginia, Kentucky, and West Virginia (*1–4*). Geographic expansion of *B. procyonis* roundworms has been recently documented in Georgia. In 2002, 22% of raccoons sampled in DeKalb County, Georgia, a highly urbanized area near Atlanta, were positive for the parasite (*5*), and recently, 10% of raccoons sampled in Clarke County, Georgia, were positive (*6*). Whether this expansion is due to natural spread of the parasite among raccoons or to translocations of infected raccoons into naive areas is unclear. We document expansion of *B. procyonis* roundworms into northwestern and southeastern Florida.

In 2006 and 2007, nine ascarids (>3 inches) were collected from the feces of an unrecorded number of raccoons admitted to a rehabilitation center in northern Florida. In September 2008, December 2009, and June 2010, one ascarid each was found in the feces of a 4- and a 6-month-old raccoon from Leon County, Florida, and a 6-month-old raccoon from Wakulla County, Florida, after routine treatment with pyrantel pamoate (20 mg/kg). In July 2010, a juvenile (6-month-old) raccoon from Broward County, Florida, which had been admitted to a rehabilitation center, passed several ascarids (2 collected for testing) in its feces after ivermectin treatment (0.2 mg/mL) for mange. The 14 ascarids were preserved in 70% ethanol, and adult males were identified as *Baylisascaris* spp. on the basis of their morphologic characteristics (perianal rough patches). The ascarids were subsequently confirmed as *B. procyonis* by sequence analysis of the 5.8S rRNA gene or the internal transcribed spacer (ITS)-1 and ITS-2 regions (*7*,*8*). The complete sequences of the 5.8S rRNA gene and ITS-2 region from 2 ascarids from northern Florida and 1 from southern Florida were identical to *B. procyonis* sequences (GenBank accession nos. AJ001501 and AB051231, respectively). ITS-1 sequences from the 2 ascarids from northern and southern Florida were 99.1% (424/428; AB053230) to 100% identical (AJ00745 and ascarids from Georgia, Kentucky, and Texas [*6*]), respectively, to *B. procyonis* sequences.

Several previous studies did not detect *B. procyonis* roundworms in raccoons or latrine sites in central Florida (n = 51 from Glades, Highlands, Hillsborough, and Orange counties), southern Florida (n = 90 from around Miami and n = 64 fecal samples on Key Largo), and numerous counties throughout Florida (n = 177) (*1*,*3*,*9*). Historically, *B. procyonis* roundworms have been absent throughout most of the Southeast, but the parasite was recently detected in north-central Georgia (*5*,*6*). How the species became established in Florida remains unclear. Establishment could have resulted from natural dispersal of infected raccoons from *B. procyonis*–endemic areas; however, recent examination of several raccoon populations in southern Georgia failed to detect such infections (*6*). Alternatively, the parasites could have been introduced from the movement of infected raccoons, exotic pets (e.g., kinkajou [*Potos flavus*]), or natural wildlife intermediate hosts (*1*).

Additionally, because domestic dogs can serve as definitive hosts, an infected dog from a *B. procyonis*–endemic area may have passed eggs into the environment (*1*). Veterinarians in Florida should be aware of this possible zoonosis and carefully examine ascarid eggs detected in fecal specimens because *B. procyonis*–infected dogs often have mixed infections with *Toxocara canis, Toxascaris leonina*, or both, which have morphologically similar eggs (*1*). Physicians, veterinarians, and wildlife biologists in Florida should be aware of this serious pathogen and the likelihood its range will increase, as highlighted by the recent detection of *B. procyonis* roundworms in a kinkajou from southern Florida (K.P. Kazacos et al., unpub. data).

This study also highlights the importance of wildlife rehabilitation centers as resources for the study of wildlife/zoonotic diseases. Animals admitted to rehabilitation centers are often ill or injured, which may increase pathogen shedding or transmission. Additionally, young raccoons are likely to be infected with *B. procyonis* roundworms, and kits as young as 3 months of age can be patent. Numerous fatal *B. procyonis* larva migrans infections have occurred among animals in rehabilitation centers and zoological parks. These infections were

likely acquired when animals were housed in enclosures previously occupied by infected raccoons or when bedding or food became contaminated with *B. procyonis*–infected raccoon feces. In *B. procyonis*–endemic areas, cages used to house raccoons should be thoroughly decontaminated by flaming, or cages should be dedicated for use by raccoons. Because *B. procyonis* roundworms can spread to other animals, persons in contact with raccoons should be alert to potential transmission routes and apply appropriate biosecurity procedures.

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Vibrio cholerae O1 Variant with Reduced Susceptibility to Ciprofloxacin, Western Africa

To the Editor: Many variants of choleragenic vibrios have emerged since the beginning of the seventh pandemic, indicating continuous evolution of this pathogenic agent. Variations occur mainly in genetic determinants of virulence and antimicrobial drug susceptibility. In September–October 2009, concurrent outbreaks of acute watery diarrhea in northeastern Nigeria (4,559 cases) and northern Cameroon (696 cases) were investigated by state ministries of health. We report reduced sensitivity to ciprofloxacin in *Vibrio cholerae* O1 strains and the atypical cholera toxin B (*ctxB*) genotype of these strains.

In September–October 2009, stool specimens from patients in Nigeria were collected on filter paper, moistened with sterile physiologic saline, and sent at room temperature to the National Reference Center for Vibrios and Cholera at the Institut Pasteur (Paris, France). Ten *V. cholerae* O1 biotype El Tor serotype Ogawa strains were isolated and identified by using standard procedures. Concurrently in Cameroon, 9 *V. cholerae* O1 Ogawa strains isolated from patient stool samples by the bacteriology laboratory of the Pasteur Center (Garoua, Cameroon) were sent to the National Reference Center for Vibrios and Cholera.

All strains were tested for antimicrobial susceptibility by MIC determination to tetracycline, trimethoprim/sulfamethoxazole, sulfonamides, ampicillin, chloramphenicol, nalidixic acid, and ciprofloxacin by using Etest (AB bioMérieux, Solna, Sweden) according to Clinical and Laboratory Standards Institute procedures and interpretative standards for *V. cholerae* (*1*). PCR amplification of the genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) and subsequent sequencing of PCR products were performed (*2*).

PCR was used to test for the presence of *ctxA* and *ctxB* genes, which encode the cholera toxin (CT), and the *tcpA* gene, which encodes the toxin-coregulated pilus. Genotyping of *ctxB* was performed by sequencing PCR products.

All isolates showed susceptibility to tetracycline (MIC 1.5 mg/L), intermediate susceptibility to ampicillin (MICs 12–16 mg/L) and chloramphenicol (MICs 8–12 mg/L), and resistance to trimethoprim/sulfamethoxazole

(MIC >32 mg/L), sulfonamides (MIC >1,024 mg/L), and nalidixic acid (MIC >256 mg/L). MICs of ciprofloxacin ranged from 0.25 to 0.5 mg/L.

Sequencing of *gyrA*, *gyrB*, *parC*, and *parE* genes among all strains detected 1 mutation in *gyrA* (substitution of serine by isoleucine at position 83) and 1 mutation in *parC* (substitution of serine by leucine at position 85). Both point mutations have been associated with quinolone resistance in clinical isolates of *V. cholerae* (*2*). None of the strains had any mutations in *gyrB* or *parE*.

The presence of *ctxA* and *ctxB* genes confirmed the toxigenicity of all isolates, and *tcpA* PCR product size and sequence identified El Tor biotype strains. The DNA sequence of *ctxB* was similar to that of the recently reported Orissa variant identified in India in 2007 (*3*). This sequence had 2 mutations resulting in histidine at position 39 and threonine at position 68 (this amino acid sequence is similar to the CT-B subunit of the reference classical strain) and a third mutation resulting in substitution of histidine by asparagine at position 20.

We report atypical El Tor strains of *V. cholerae* O1 and their reduced susceptibility to ciprofloxacin in Nigeria and Cameroon. Since the 1990s, atypical El Tor strains that produce classical CT have been increasingly reported from countries in Asia, where they have gradually replaced the prototype El Tor strains, but they have only been reported in 2 countries in Africa (Mozambique and Zambia) (*4,5*). On the basis of the CT-B subunit sequence, these variants differ from variants isolated in southern Africa and from most variants isolated in Asia by having the same modified classical CT as a strain recently isolated in Orissa in eastern India (*3*), which has not been reported elsewhere. These findings indicate evolution of *V. cholerae* O1 El Tor hybrid strains. Their presence may indicate spread of strains from eastern India to Africa (*6*).

The presence of CT-B variants in central or western Africa is of great concern because these strains may be more toxigenic (*3*). There is also concern for the strains isolated in this study because of their reduced susceptibility to ciprofloxacin. Although reduced susceptibility to fluoroquinolone is common in southern Asia (*7,8*), it was reported in Africa (Zimbabwe) only recently (*9*). Our findings, in addition to the report of Islam et al. (*9*), indicate that *V. cholera* with reduced sensitivity to a fluoroquinolone is present in southern and western Africa. These results highlight the need for continued monitoring of antimicrobial drug susceptibility and strain tracking to maintain an efficient cholera surveillance system.

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Yersinia pestis DNA Sequences in Late Medieval Skeletal Finds, Bavaria

To the Editor: *Yersinia pestis*, the causative agent of plague, is held responsible for 3 human pandemics: the Justinian plague (5th–7th century), the Black Death (13th–15th century), and modern plague (1870s to present). In 1894, Alexandre Yersin identified *Y. pestis* during an epidemic of plague in Hong Kong (*1*). However, whether *Y. pestis* was indeed responsible for the medieval epidemic is still controversial. *Y. pestis* specific DNA has been detected in medieval skeletal finds (*2*,*3*), although some investigators have failed to do so (*4*), leading to the suggestion that a viral hemorrhagic fever was the agent of these medieval pandemics (*5*).

Against this background, we investigated a mass burial site that was discovered under the sacristy of the St. Leonhard Catholic church in Manching-Pichl, near Ingolstadt in Bavaria, Germany. In 1984, during the renovation of this church, 75 human skeletons and several scattered skeletal elements were discovered under the sacristy. The skeletons lay densely packed in 4 layers. Apparently, no grave pit had been dug at all, but the bodies were obviously deposited side by side and covered with dirt layer by layer. An approximation of the time of burial was possible only by means of accompanying building structures, which were dated to the Gothic period (1250–1500 CE).

In the course of research preceding this study, our research group found *Y. pestis* DNA in 10 of 33 examined individual skeletal remains from the mass grave beneath the sacristy (*6*) by using the primer pair YP12D/YP11R (*3*). In the current study, the remains of 6 persons from the mass burial site that had positive *Y. pestis* DNA results before were further investigated. DNA from additional tooth samples of these persons was extracted, and more markers mapped on the *Y. pestis* high copy number plasmid pPCP1 were included. In addition to the primer systems YP12D/YP11R and YP11D/YP10R (*3*), the following primer systems were used. The primer pair YP14F (5′-TCCGGGTCAGGTAATATGGA-3′)/YP13R (5′-ACCAGCCTTTCACATTGAGG-3′) amplifies another sequence section (positions 6953–7082, reference: *Y. pestis* strain CO92 plasmid pPCP1 sequence AL109969.1) on the *Y. pestis pla* gene (encoding plasminogen activator). The plasminogen activator belongs to the virulence factors of *Y. pestis*. The primer pair pst-F (5′-GGTAAATCGCTGAACCGAAG-3′)/pst-R (5′-AACAGCACCTCTGACGCTTT-3′) amplifies a 129-bp sequence section (positions 5026–5154, GenBank accession no. AL109969.1) on the *pst* gene (encoding pesticin activity protein). Pesticin is a bacteriocin that is active against only a few closely related microorganisms. The primer pair PCP-F (5′-CATCCACATGCTCAACCCTA-3′)/PCP-R (5′-CTGAACGCATTTCAGTGGTG-3′) can amplify a 128–130-bp sequence section (positions 8428–8555, GenBank accession no. AF053945.1; positions 8428–8557, GenBank accession no. AL109969.1) on plasmid pPCP1.

These 3 new primer sets, designed with the aid of the software component Primer3 (*7*), were applied by using a suicide PCR method (*3*,*8*); i.e., new primer pairs targeting sequences not previously amplified in the laboratory were used. At no time during all examinations was any modern *Y. pestis* DNA included.

The sample preparation, DNA extraction, PCR setup, electrophoretic separation, and sequencing of amplicons are described elsewhere (*6*,*9*); however, we used 0.20 µmol/L of each primer, an annealing temperature

Table. Amplification results obtained from the skeletal remains of 6 persons excavated at the St. Leonhard Catholic church, Manching-Pichl, Bavaria, Germany*

Identification no.	Sex, age at death, y/sex	Tooth sample	DNA extract†	YP12D/YP11R‡		YP11D/YP10R	YP14F/YP13R	pst-F/pst-R	PCP-F/PCP-R
17-I	≈11/M	1st	I	–	+	NT	NT	–	–
		1st	II	–	–	NT	–	NT	NT
		2nd	I	–	–	–	–	NT	NT
22	20–22/F	2nd	I	–	–	–	NT	NT	NT
34-I	Young adult/M	1st	I	+	+	NT	NT	+	+
73-I	20–25/M	1st	I	(+)	(+)	NT	NT	NT	NT
		1st	II	–	–	–	–	NT	NT
		2nd	I	–	–	–	–	NT	NT
S1-I	≈8/M	1st	I	+	+	NT	NT	+	+
S4-XX	≈15/F	1st	I	+	+	NT	NT	+	–
		1st	II	+	+	+	+	+	+
		2nd	I	+	+	+	+	+	+

*Extraction controls and PCR controls had no amplicons. +, amplicon; (+), weak amplicon; –, no amplicon; NT, not tested.
†I and II represent separate extracts from the same tooth.
‡Independent PCRs with the primer pair.

of 54°C, and 45 amplification cycles. PCR blanks containing all reagents except for DNA and extraction blanks were included in every PCR set.

Results of the amplification reactions are listed in the Table. All accompanied extraction and PCR controls remained free of amplification products. All amplicons resulting from suicide PCRs were sequenced. Amplicons resulting from the use of primer pairs YP14F/YP13R and pst-F/pst-R matched the reference sequence to 100% (GenBank accession no. AL109969.1). Amplicons resulting from the use of primer pair PCP-F/PCP-R matched this reference sequence to only 97.78%. This deviation is because of a 2-bp insertion (2 Ts, positions 8531 and 8532, GenBank accession no. AL109969.1) at *Y. pestis* strain CO92 plasmid pPCP1. The sequences obtained from 3 persons' remains showed in the pPCP1 sequence section between nucleotide positions 8528–8532 only 3 Ts instead of 5 Ts described for *Y. pestis* strain CO92 plasmid pPCP1 (GenBank accession no. AL109969.1). The sequences found in this study were deposited in GenBank under accession nos. HQ290521–HQ290523.

To conclude, the successful recovery of several *Y. pestis* plasmid pPCP1 DNA sequences in skeletal finds from the mass burial site excavated in Manching-Pichl suggests that these persons died of plague. Moreover, our findings constitute a molecularly supported confirmation for the presence of *Y. pestis*, the etiologic agent of plague, in late medieval (1250–1500 CE) southern Germany. In future studies, we will attempt to recover chromosomal *Y. pestis* DNA from the mass grave skeletal remains to obtain clues as to the specific *Y. pestis* strain and the microbiology of past plague in Europe.

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Two Clusters of HIV-1 Infection, Rural Idaho, USA, 2008

To the Editor: Prevalence of HIV-1 infection in rural areas of the United States has been increasing (*1*). During 2003–2007, an average of 30 (range 24–42) cases of new HIV-1 infection diagnoses per year among Idaho residents were reported. Of the 152 reported cases during this period, 54 (36%) were related to a person living in a rural area of ≤75,000 residents and a 60-minute drive from an urban area (*2*). Of these 54 cases, 19 (35%) were in men who have sex with men (MSM), 5 (9%) were in injection drug users (IDU), and 2 (4%) were in those in both categories.

In March 2008, a cluster of newly identified HIV-1 infections that included 5 cases (cluster A) in a rural southeastern Idaho city (city A) was reported to the Idaho Department of Health and Welfare. Two patients were men and the median age was 26 years (range 18–32 years). One patient was an IDU (Table). Through epidemiologic investigation, 3 additional patients were suspected to be IDUs, but confirmation was not practicable. All reported methamphetamine use. One man and 2 women reported both male and female sex partners.

During September–December of that year, another increase in newly identified HIV-1 infections in southeastern Idaho (cluster B) was reported

to Idaho Department of Health and Welfare. Cluster B included 10 cases, all among men who reported living within a 50-mile radius of city A, with most in a rural city (city B) located <30 miles from city A. The median age of the men in cluster B was 24 years (range 18–37 years). Each case was epidemiologically linked to at least 1 other case in the cluster; each patient reported having had unprotected sex with male partners. Although we suspected transmission of HIV-1 between persons in clusters A and B, whether the clusters were linked epidemiologically remained unclear after an initial investigation.

Although the primary use of HIV-1 sequence data is to assist clinicians in selecting antiretroviral (ARV) therapy, public health practitioners can use HIV-1 sequences from cases and compare those with HIV-1 sequences from others living in the region to explore phylogenetic associations and possible HIV transmission clusters (*3*). To evaluate links between clusters A and B, HIV-1 *pol* consensus sequence data for 4 of the 5 cases from cluster A and 6 of the 10 cases from cluster B were obtained from 5 commercial laboratories. No case-patients had received ARV. Additionally, we used sequence data from a patient residing in city B who had received an HIV-1 diagnosis in December 2008 but was not epidemiologically linked to either cluster. HIV-1 control sequences from 2 Idaho HIV clinics, including 34 HIV-1–infected persons within a 275-mile radius of city B identified who had not received ARV and who had resistance testing performed during 2005–2008, were used to represent the regional epidemic. Control sequences were aligned with cluster A and B sequences and analyzed as described (*4*,*5*).

Ten of the HIV case-patients for whom nucleotide sequence data were obtained were infected with HIV-1, subtype B, and were placed into 2 distinct phylogenetic-related groupings. Group 1 contained 4 patients from cluster A and 1 patient with no known epidemiologic link to either cluster. Group 2 contained 5 patients from cluster B. The average *pol* genetic distance among virus from members of group 1 was 0.2% (median 0.1%, SD 0.2%) and from members of group 2 was 0.1% (median 0.1%, SD 0.1%). The average distance among the control sequences was 5.1% (median 5.2%, SD 1.2%). The average distance between groups 1 and 2 was 4.8% (median 4.8%, SD 0.2%), which does not demonstrate a linkage between the 2 groups. The 1 case in group 1 that was not initially identified with either cluster had a genetically related HIV-1 sequence to members of cluster A, indicating a potential previously unidentified epidemiologic link. The sequence from 1 case associated with cluster B was not genetically similar to members of either cluster and was more similar to controls. These data do not indicate from whom each patient acquired the infection.

The epidemiologic investigation combined with the molecular analysis shows transmission of HIV-1 originating from 2 sources occurred within a group of rural MSM in southeastern Idaho and indicates a separate case previously believed to be unrelated to 2 local clusters had genetic similarity to cluster A. Limitations of this investigation include the inability to obtain HIV sequences from all persons identified in clusters A and B and an inability to confirm high-risk behaviors for all identified case-patients.

Previous HIV clusters have demonstrated that infectious persons can spread HIV quickly within a social network and highlighted the importance of timely prevention activities to limit HIV transmission in a community (*6*,*7*). Use of phylogenetic analysis of HIV-1 sequences obtained from commercial laboratories showed that clusters A and B were not epidemiologically related and helped target appropriate and specific HIV prevention activities.

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Table. Sex and risk factors among patients epidemiologically linked to 2 clusters of HIV-1, southeastern Idaho, USA, 2008*

Case ID	Sex	Risk factors	Cluster†	Phylogenetic group
1	F	WSMW/SIDU	A	1
2	F	WSMW/SIDU	A	1
3	M	MSMW/IDU	A	1
4‡	M	MSW	A	ND
5	M	MSM	B	2
6‡	M	MSM	B	ND
7‡	M	MSM	B	ND
8‡	M	MSM	B	ND
9	M	MSM	B	2
10§	M	MSM	B	ND
11	M	MSM	B	2
12‡	M	MSM	B	ND
13	M	MSM	B	2
14	F	WSM	A	1
15	M	MSM	B	2
16¶	M	MSM	ND	1

*ID, identification; WSMW, women who have sex with men and women; SIDU, sex with injection drug users; MSMW, men who have sex with men and women; IDU, injection drug user; MSW, men who have sex with women; ND, not determined; MSM, men who have sex with men; WSM, women who have sex with men.
†Cluster A associated with injection drug use; cluster B associated with MSM.
‡HIV-1 sequence was not available for molecular analysis.
§HIV-1 sequence was similar to controls but not to sequences from either cluster A or B.
¶Case with no epidemiologic link to cluster A or cluster B.

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Pandemic (H1N1) 2009 and Oseltamivir Resistance in Hematology/ Oncology Patients

To the Editor: Tramontana et al. (*1*) recently described characteristics and oseltamivir resistance in hematology and oncology patients infected with pandemic (H1N1) 2009 virus. Such cases merit further study because concurrent medical problems in immunosuppressed patients may obscure and delay diagnosis and management of pandemic (H1N1) 2009 infections. Moreover, severe complications of such infection may be more likely to develop in immunosuppressed patients (*2*). During the winter of 2009, oseltamivir-resistant pandemic (H1N1) 2009 virus infection was diagnosed for 4 patients at Duke University Medical Center. We describe the clinical features of the infections, the challenges associated with diagnosis of pandemic (H1N1) 2009 virus infection, and the clinical outcome for the infected patients.

Four immunocompromised patients who received chemotherapy and immunotherapy for solid-organ and hematologic malignancies were hospitalized at our tertiary care medical center during October–November 2009, a period of peak activity of pandemic (H1N1) 2009 in surrounding communities in North Carolina (*3*). These 4 case-patients experienced symptoms attributable to pandemic (H1N1) 2009 from 0 to 14 days after hospital admission, and the diagnosis of pandemic (H1N1) 2009 was made 0–28 days after symptom onset. Illness, diagnosis, and treatment of the patients are summarized in the Table. One patient reported contact with a family member who had influenza-like illness. Three other patients likely acquired pandemic (H1N1) 2009 in the hospital. An investigation could not conclusively establish whether transmission of pandemic (H1N1) 2009 occurred between case-patients and healthcare workers or visitors (*4*). All 4 case-patients ultimately died; 2 patients recovered from pandemic (H1N1) 2009 after antiviral drug therapy but died of underlying disease and subsequent bacterial infections. One case-patient did not receive antiviral drugs because the diagnosis was made posthumously.

We learned valuable lessons regarding diagnosis and management of pandemic (H1N1) 2009 in immunocompromised patients. First, pandemic (H1N1) 2009 infection can be difficult to diagnose in immunocompromised hospitalized patients. Such patients do not exhibit consistent symptoms or signs for pandemic (H1N1) 2009. Consistent with Tramontana et al. (*1*), fever was the most common feature, followed by progressive dyspnea and intermittent cough. None of our patients reported sore throat. Moreover, such nonspecific symptoms may be inadvertently attributed to concurrent medical problems common in immu-

nocompromised patients such as bone marrow suppression, adverse effects of drugs or chemotherapy, recent surgical procedures, opportunistic infections, or line-related bloodstream infections.

Second, respiratory viruses may be imported and subsequently transmitted to hospitalized patients despite standard infection prevention measures (*5*). Clinicians should remain vigilant for hospital-onset respiratory viral infections and have a low threshold for diagnostic testing, particularly during periods of increased influenza or respiratory virus activity in the community.

Early suspicion and prompt testing may have reduced the delay in the diagnosis and management of these patients with pandemic (H1N1) 2009. However, the initial nasal wash specimen from patient 1 was negative for pandemic (H1N1) 2009 virus antigen, whereas the initial bronchos-

Table. Clinical, diagnostic, and therapeutic patient information for 4 patients hospitalized for hematologic and oncologic conditions, North Carolina, USA, 2009*

	Patient no./age, y/sex			
Patient information	1/43/F	2/58/F	3/67/F	4/61/M
Underlying disease	Relapsed acute myelogenous leukemia	Refractory mycosis fungoides	Recurrent metastatic thymoma	B-cell acute lyphoblastic lymphoma
Reason for admission	Scheduled consolidative chemotherapy	Staphylococcal sepsis; recent interferon-α; malnutrition	Progressive fevers and hypoxia; diffuse; infiltrates shown on chest radiograph	Fevers and respiratory compromise at home, after recent chemotherapy
Signs/symptoms on admission	Intermittent fever during early admission; d 14† cough, persistent fevers; d 24 progressive hypoxia	Intermittent fevers; d 27, cough; persistent fevers, progressive hypoxia	Fever for 5 d; hypoxia, widespread pulmonary infiltrates	Daily fevers for 5 d, cough, hypoxia, fatigue, generalized weakness; diffuse infiltrates on radiograph
Use of oseltamivir	Yes, for 10 d; 75 mg daily prophylaxis after known exposure 2 d prior	No	No	No
Diagnostic information	d 14, nasal wash PCR negative‡; d 25, BAL positive for pandemic (H1N1) 2009 virus; d 37, BAL remained positive, H275Y mutation	d 27 nasal wash positive for pandemic (H1N1) 2009 virus; d 44, H275Y mutation confirmed on d 27 specimen; medication modified	d 1, nasal wash positive for pandemic (H1N1) 2009 virus; d 4, d 9, bronchoscopy results positive; d 12, bronchoscopy results negative for influenza on culture and PCR; H275Y mutation detected on all positive specimens§	d 23 bronchoscopy and d 27 nasal wash results negative; d 28 bronchoscopic viral culture positive for pandemic (H1N1) 2009 virus, 1 d after patient's death; H275Y mutation detected by CDC
Factors confounding pandemic (H1N1) 2009 diagnosis	Consolidation chemotherapy; *Escherichia coli* bacteremia, presumptive fungal pneumonia, persistent leukopenia neutropenia postchemotherapy	*Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae* bacteremia; recent interferon-α therapy; salvage chemotherapy; persistent leukopenia and neutropenia at discharge	Recent thoracic radiotherapy; catheter-associated *Staphylococcus aureus* bloodstream infection.	Consolidative chemotherapy with prolonged neutropenia; significant emphysema; catheter-associated pseudomonal bloodstream infection
Treatment	Oseltamivir,75 mg 2×/d for 5 d; then, 150 mg 2×/d until death; mechanical ventilation for 15 d	Oseltamivir, 75 mg 2×/d for 9 d; then 150 mg 2×/d for 8 d; modified to renally adjusted IV zanamivir for 10 d, when mutation detected	Oseltamivir, 75 mg 2×/d for 12 d; mechanical ventilation for 16 d	Broad-spectrum antibacterial agents; antiviral agents (not influenza agents), and antifungal agents; mechanical ventilation for 48 h
Clinical outcome	Died 38 d postadmission; refractory respiratory failure and progressive ARDS	Improvement in respiratory status following zanamivir treatment; ultimately, failure of bone marrow recovery; died 4 d after discharge to hospice	Refractory respiratory failure; septic shock; decision to withdraw care	Refractory respiratory failure and elective withdrawal of care

*BAL, bronchoalveolar lavage; ARDS, acute respiratory disease syndrome; IV, intravenous; CDC, Centers for Disease Control and Prevention.
†Days postadmission.
‡All specimens, unless otherwise stated, were tested with proFlu Plus PCR (Prodesse, Waukesha, WI, USA) for influenza viruses A and B and respiratory syncytial virus. No quantitative tests were available.
§Mutation genotype confirmed at CDC. Results were available posthumously for patients 1, 3, and 4. No mutation that conferred zanamivir resistance was detected.

copy and nasal wash specimens from patient 4 also were negative for such antigens on laboratory testing. This underscores the limitations of current testing and that the sensitivity of pandemic (H1N1) 2009 diagnostic testing remains poor and needs further improvement. Thus, in patients suspected of having pandemic (H1N1) 2009 or in those who are critically ill, lower tract respiratory specimens should tested to improve diagnostic sensitivity, and clinicians should consider using immunoassay and culture methods.

All 4 patients described in this case series had viral isolates containing H275Y mutation in the neuraminidase gene of pandemic (H1N1) 2009 virus, which is specifically associated with high-level resistance to oseltamivir. Increasing data show that immunocompromised patients are at increased risk for development of drug-resistant influenza infections after oseltamivir prophylaxis or while receiving oseltamivir treatment (*6*). Fortunately, this resistance trait remains rare.

Existing evidence suggests oseltamivir-resistant pandemic (H1N1) 2009 virus is stable and retains similar transmissibility and virulence as the wild-type virus (*7*). Therefore, in immunosuppressed patients, in which the influenza mortality rate is high, clinicians should also suspect drug-resistant influenza infection if the patient does not improve. Before she died of underlying hematologic illness, patient 2 clinically improved after treatment with intravenous zanamivir (obtained through an emergency application for an investigational–new drug). As reported in other studies, pandemic (H1N1) 2009 virus was found in her nasal washes, 1 week after she received zanamivir for 10 days (*8*).

Some data suggest that pandemic (H1N1) 2009 virus has a predilection to affect the lower respiratory tract and is associated with more illness and death than is seasonal influenza (*9*). All 4 case-patients with in this series developed dyspnea, and 3 of the 4 ultimately died of refractory respiratory failure. Our observations suggest that oseltamivir-resistant pandemic (H1N1) 2009 virus is also associated with poor prognosis and may retain the same tropism for lower respiratory tract involvement as wild type.

These case-patients illustrated the complexity of the diagnosis and management of such infections in hospitalized immunocompromised patients. Vigilance and heightened clinical suspicion are needed to facilitate early diagnosis, treatment and prevention measures to limit transmission of pandemic (H1N1) 2009 virus or similar viral pathogens.

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Acute Encephalopathy and Pandemic (H1N1) 2009

To the Editor: Since the World Health Organization declared a global pandemic of influenza A pandemic (H1N1) 2009 in June 2009, the number of cases of this strain of influenza has steadily risen. Although most cases have been mild, with complete and uneventful recovery, multiple cases of severe infection with complications, including death, have been reported. Yet the neurologic complications of this virus have been rarely described. We read with interest the article by Kitcharoen et al. (*1*) concerning a patient with encephalopathy associated with pandemic (H1N1) 2009, which

progressed to produce quadriplegia with diffuse sensory loss. In that study, however, pandemic (H1N1) 2009 virus was not isolated from the patient's cerebrospinal fluid (CSF) or brain tissue or detected by reverse transcription–PCR (RT-PCR). We report a case in an adolescent patient with encephalopathy-associated pandemic (H1N1) 2009 that was confirmed by real-time RT-PCR of CSF.

On November 2, 2009, a previously healthy 16-year-old girl was admitted to Asan Medical Center, Seoul, South Korea. Five days earlier, she had sought care for cough, fever (maximum 38.5°C), and mild headache. Enzyme immunoassay (SD Bioline rapid influenza test; Standard Diagnostics Inc., Yongin, South Korea) of a nasopharyngeal swab was positive for influenza virus. Because a large outbreak of pandemic (H1N1) 2009 was concurrent, she was given a presumptive diagnosis and treated with oseltamivir, 75 mg 2×/d, for 5 days. However, her headache worsened, and she was referred to the hospital.

At admission, her temperature was 36.8°C. Examination showed no disturbance of consciousness or focal neurologic deficits except for a severe headache. Results of routine laboratory tests, including serologic tests for HIV, were negative. Real-time RT-PCR of a nasopharyngeal swab at admission was negative for pandemic (H1N1) 2009 virus; a serologic test for this virus was not performed. A magnetic resonance imaging (MRI) scan of the patient's brain at admission is shown in the Figure, panel A. Examination of CSF showed 0 cells/mm^3, protein 35.4 mg/dL, glucose 48 mg/dL; blood glucose level was 49%. No bacteria or fungi were isolated from CSF, but pandemic (H1N1) 2009 virus was detected by real-time RT-PCR (Roche Diagnostics, Mannheim, Germany). On the basis of the MRI and RT-PCR results, we diagnosed encephalopathy-associated pandemic (H1N1) 2009 infection. By hospital day 3, her headache and respiratory symptoms had improved, and she was discharged on day 10 without headache or other neurologic signs. A follow-up brain MRI, obtained 1 month later, is shown in the Figure, panel B.

Several hypotheses have been proposed regarding the pathogenesis of influenza-associated acute encephalopathy (IAAE) (*2*): the most straightforward is that it is caused by viral infection of the central nervous system. The isolation of influenza virus from the CSF of living patients (*3*) (or its detection by RT-PCR) and from brain tissue of patients who have died (*4*) supports this hypothesis. More frequently, however, influenza virus has not been detected in the CSF or brains of affected patients despite thorough attempts. Thus, other possible methods for the assessing the pathogenesis of IAAE have been proposed: elevated concentrations of several cytokines such as interleukin (IL)–6, tumor necrosis factor (TNF)–α, and soluble TNF receptor-1; or determination of renal and hepatic dysfunction (*2*). Although IAAE in adults and children was reported during the pandemic (H1N1) 2009 pandemic (*1*,*5*–*8*), this virus was not detectedby virus isolation or RT-PCR in CSF and brain tissue of these patients. The virus was detected in CSF of an infant 3 months of age with IAAE (*8*); however, the virus may have been found in CSF because of the presence of blood from a traumatic lumbar puncture.

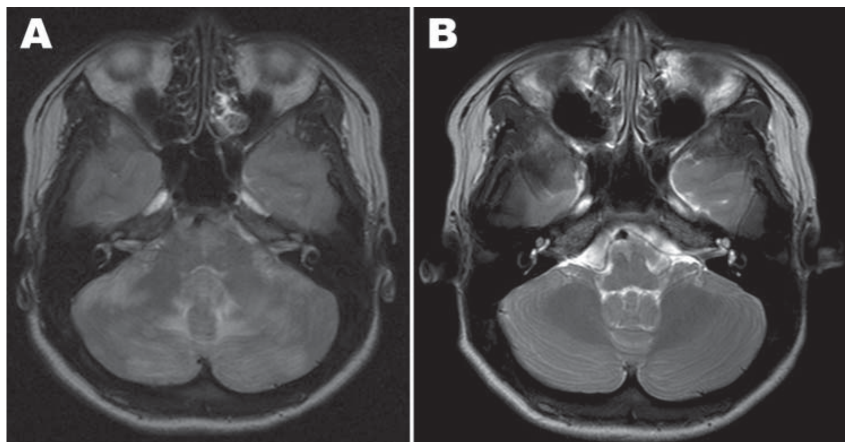

Figure. Magnetic resonance imaging (MRI) scans of case-patient's brain. A) MRI at hospital admission shows ill-defined T2 changes in both cerebellar hemispheres, periventricular white matter, and the pons. B) MRI of the brain 1 month later, showing nearly complete disappearance of the changes observed at admission.

The absence of pleocytosis and the normal protein and glucose levels in CSF from the patient described here were noteworthy. Previous reports showed that leukocyte counts within normal limits (70%–90%) were found in CSF of patients with IAAE and seasonal influenza infection (*9,10*). Recent publications on IAAE and concurrent pandemic (H1N1) 2009 virus infection also reported no increase in CSF leukocyte count and protein level (*1*,*5*,*7*). Therefore, absence of CSF pleocytosis and protein levels within normal limits are common with IAAE.

The diagnosis of IAAE in the patient reported here is probable, based on positive real-time RT-PCR results from CSF examination and brain MRI findings. However, some limitations should be mentioned. First, a positive RT-PCR result could have resulted from contamination associated with clinical procedures and laboratory assays. Nonetheless, we believe that the lumbar puncture was done aseptically and that the real-time RT-PCR performed in the hospital's clinical microbiologic laboratory was reliable. Second, the brain MRI findings were also nonspecific andcould be associ-

ated with hypoxia, edema, or other unknown processes. However, the patient had no history of hypotensive episodes, hypoxemia, abnormal metabolic and toxic processes, and other infectious disease. In conclusion, IAAE with pandemic (H1N1) 2009 may be caused by direct viral infection of the CNS and, although its pathogenesis is not clear, physicians should remain alert to this possibility.

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Oseltamivir-Resistant Pandemic (H1N1) 2009 Treated with Nebulized Zanamivir

To the Editor: In late November 2009, a 3-year-old immunocompromised boy experienced an upper respiratory tract infection caused by influenza A pandemic (H1N1) 2009 virus, as demonstrated by a positive result for real-time PCR on a nasal swab specimen. His medical history was notable for a congenital intracardiac tumor; an ABO–incompatible heart transplant at 2 months of age; and an Epstein-Barr virus–related humoral rejection 20 months later that was treated with anti-CD20 and plasmapheresis and continuous immunosuppressive therapy with tacrolimus and everolimus. Thus, a 5-day regimen of oseltamivir treatment was undertaken, and the patient's clinical signs improved.

However, 3 days after drug treatment was suspended, the child had a relapse and exhibited fever, cough, and mild respiratory distress. The patient had fine crackles in the left posterior basal lung, normal oxygen saturation, and an infiltrate in the left basal lung, observed on chest radiograph. Infection with pandemic (H1N1) 2009 virus was confirmed. He was then transferred to an isolated ward of the pediatric department, and oseltamivir treatment was again initiated and dosages of immunosuppressive drugs were reduced. However, no clinical or virologic responses were observed during the 3 weeks of drug administration.

Over the next month, the oral dosage of oseltamivir was increased twice, without substantial effects on clinical course and viral clearance of the infection (Figure). Because of persistence of infection, the viral neuraminidase gene was sequenced, which showed the H275Y mutation (*1*). We immediately requested zanamivir aqueous solution from GlaxoSmithKline (Brentford, UK), and, after the approval of the hospital's ethics committee and parents' consent were obtained, nebulized treatment was carried out for 10 days. Fever and respiratory symptoms and signs resolved after 6 days of treatment and progressive real-time PCR gave negative results. Moreover, at the end of the treatment period, chest radiograph did not show abnormal findings, and results of a hemagglutination-inhibition assay were positive for influenza. No zanamivir-related adverse events were observed, except for a mild bronchospasm that responded to albuterol.

Another notable point is that the clinical course of the disease was not severe, although the child was immunocompromised and the infection persisted for almost 2 months. However,

influenza virus persistence, possibly caused by inability of the child's immune system to clear the infection, and prolonged treatment with oseltamivir, led to the appearance of the H275Y mutation. H275Y has been described as the most common mutation that confers oseltamivir resistance in pandemic (H1N1) 2009 infection and has been found in all the resistant isolates reported worldwide (*1*). Consistent with previous reports (*2*), in the patient described here, antiviral drug resistance arose early in the treatment course. Retrospective analysis demonstrated the appearance of a mixed population after ≈2 weeks of drug use with a slow progression toward a pure H275Y variant. This latter finding may be explained by other virologic characteristics of this viral isolate, which is currently undergoing deep sequencing of the full genome.

Zanamivir represents the therapeutic option for patients infected with the H275Y mutation of pandemic (H1N1) 2009 virus. Its licensed formulation as a dry powder is suitable only for patients who can actively use inhaled drugs and thus cannot be used in children <7 years of age (*3*). Intravenous zanamivir solution has been reported to be safe and effective in experimental influenza A virus infection and as compassionate therapy in 2 immunocompromised adult patients who underwent mechanical ventilation for severe pneumonia (*4*,*5*). Moreover, successful use of intravenous zanamivir in a critically ill child, who was immunosuppressed after allogenic stem cell transplantation and infected with oseltamivir-resistant pandemic (H1N1) 2009 virus, has been reported (*6*). In this latter case, the regimen was well tolerated and was associated with a decrease in viral load.

Despite these results showing the efficacy of zanamivir intravenous administration, the inhalatory route for influenza virus–specific drugs should be the first choice, whenever possible, because it delivers therapeutic molecules directly to the site of viral replication, resulting in low systemic exposure. For this reason and because of the mild severity of the patient's disease, we decided to use zanamivir solution nebulized by aerosol. The compliance to this treatment was easily achieved, and the therapy showed good efficacy and was well tolerated by the child. The minor side effect observed has already been reported in the literature (7).

In conclusion, our experience supports the view that in immunocompromised patients with persistent infection, emergence of resistant viral strains should be strictly monitored. In this context, recently developed real-time PCRs for rapid screening of H275Y could be useful (*8*). Moreover, although a direct cause and effect has not been confirmed, this case suggests that aerosolized zanamivir solution can be considered as a therapeutic option in young children with mild respiratory symptoms who are infected with oseltamivir-resistant influenza viruses. Additional studies should be conducted in young patients with more severe disease.

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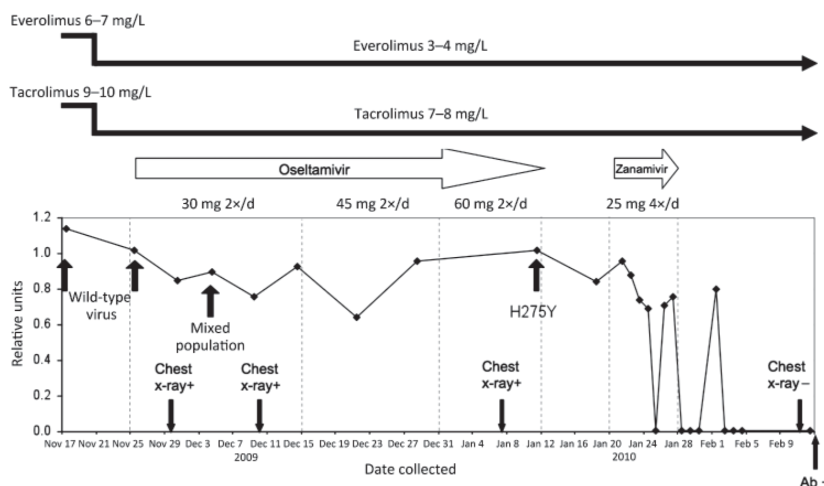

Figure. Schematic showing events surrounding oseltamivir-resistant pandemic (H1N1) 2009 virus infection in 3-year-old immunocrompromised child, Italy, in relation to viremia levels, expressed as relative units of influenza A RNA, normalized with respect to the housekeeping gene RNaseP.

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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.

Enteric Viruses in Ready-to-Eat Packaged Leafy Greens

To the Editor: Fresh produce increasingly has been implicated in viral disease outbreaks (*1*). In some instances, lettuce was contaminated before wholesale distribution (*1*). Enteric viruses can be introduced in the field if produce is exposed to human waste. Processed and packaged produce can be contaminated if equipment or wash water is not effectively sanitized. Fewer than 10 infectious viral particles are sufficient to cause disease (*2*), and these organisms are resistant to disinfectants at concentrations that reduce bacterial levels (*3*). Contamination of fresh produce could pose a health risk to humans because fresh produce is eaten raw. High levels of viral contamination can result in large outbreaks, but intermittent contamination of fresh produce accounts for some sporadic cases of norovirus and rotavirus gastroenteritis.

During April 27–November 23, 2009, we performed viral testing on 328 samples of packaged leafy greens (representing 12–14 different lots from 3–6 companies per week; no samples were taken on weeks with a statutory holiday) for norovirus or rotavirus RNA. Packaged leafy greens were purchased from retail stores in southern Ontario, Canada. Shipments maintained an average temperature of 3.8°C during transit to the testing laboratory. Each 25-g sample was spiked with 10^6 PFU of feline calicivirus (FCV) as a sample process control (*4*). Virus was concentrated by using an adsorption-elution-ultrafiltration filtration protocol (*4*).

Recovery of FCV was quantified from an RNA standard curve. FCV process control recovery was <0.01% for 55 (17%) samples. Recovery of ≥0.01% of the FCV was observed for the remaining 273 (83%) samples. Two samples from which FCV was not recovered were positive for norovirus (CE-V-09–0138) and rotavirus (CE-V-09–0129); they were considered true positive results.

Of these 275 samples, 148 (54%) were positive for norovirus by real-time reverse transcription–PCR (RT-PCR) (*5*), and 1 (0.4%) was positive for rotavirus group A by RT-PCR (*6*). To confirm detection of norovirus RNA, we amplified a second norovirus target by RT-PCR of region C (*5*). Only 40 samples (15% of total) produced a band of the expected size for this second norovirus amplicon. Of these 40 amplicons, only 16 (6% of total) could be sequenced to confirm norovirus RNA. The rotavirus-positive sample was confirmed by sequencing.

For some sample dates, multiple lots were positive; for others, no positive samples were identified (Figure). Multiple detections on the same date were not caused by cross-contamination; partial capsid sequencing showed different genetic types on dates when multiple samples were positive (Figure). Results were positive from 5 different brands, and no organic samples were confirmed positive for enteric virus contamination. Of the 16 norovirus strains confirmed, 13 belonged to genogroup I (GI) and 3 to genogroup II (GII) (Figure). All were strain types known to be human pathogens. The group A rotavirus was not subtyped; group A rotaviruses can be human or animal pathogens.

Most noroviruses detected belonged to GI. Previous reports indicate that GI norovirus are more frequently identified in foodborne or waterborne outbreaks; GII.4 noroviruses are more common in large outbreaks spread person to person (*7*). Identification of GI norovirus is consistent with occasional contamination of produce or wash water. Disinfectants and sanitation agents are used in wash water at low concentrations, at which they have limited efficacy against norovirus (*3*).

Washing and disinfecting produce before eating it can reduce the risk for infection by reducing the viral load by 10- to 1,000-fold (*8*). The median level of confirmed contamination in this study was ≈500 RNA copies for norovirus (range 1.4 copies to 9×10^6 copies).

A limitation of our findings is the inability to determine the association between molecular detection results and infectious virus. No outbreaks were related to the sequences detected here. There is no routine cell culture system for the laboratory growth of human norovirus. Genomic RNA can persist after the virus has been inactivated (*9*). The new ViroNet Canada network, which went online in April 2010, will monitor strains detected in leafy greens and other food products together with strains from community outbreaks to identify outbreaks linked to contaminated foods.

Our comprehensive surveillance study identified norovirus and rotavirus contamination of packaged leafy greens. We detected noroviruses on 6% and rotavirus on 0.4% of lots tested from retail markets in southern Ontario. Packages with confirmed positive samples were both imported into Canada and had been conventionally grown. Noroviruses have a low infectious dose (*2*), and detection of viral RNA is associated with human health risk in oysters, another commodity that is eaten raw (*10*). Our results suggest a possible risk for foodborne transmission of norovirus and rotavirus from packaged leafy greens.

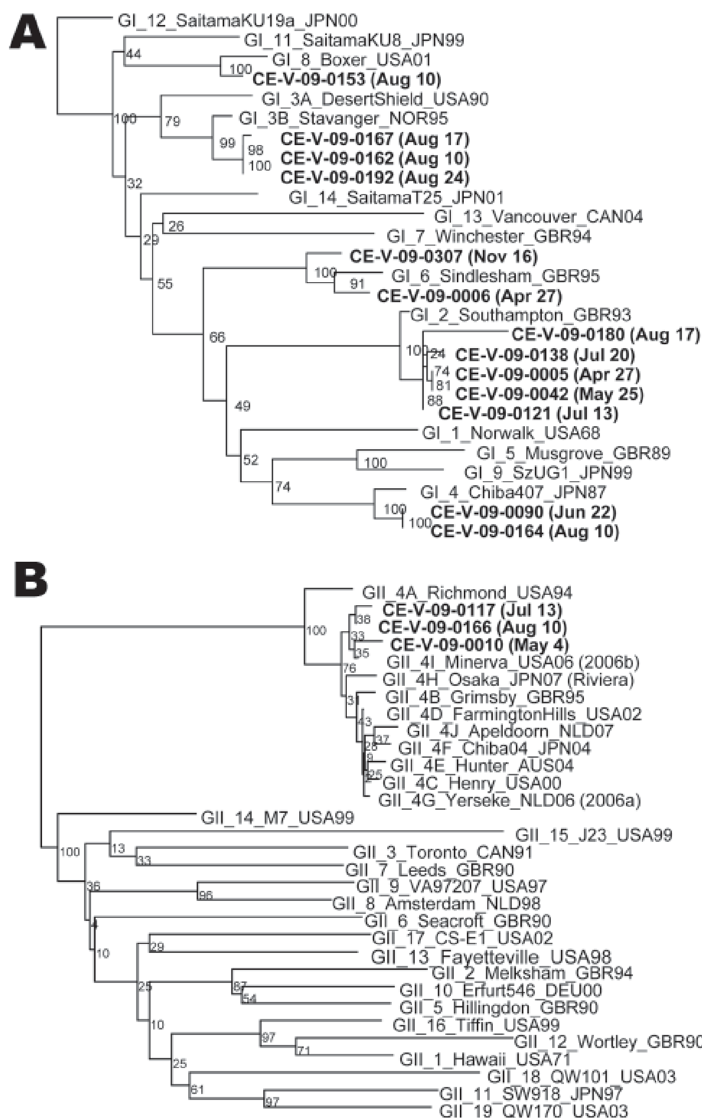

Figure. Phylogenetic analysis of the partial capsid sequence from genogroup I (A) and genogroup II (B) norovirus strains detected on leafy greens samples, Ontario, Canada, 2009, compared with the ViroNet Canada reference set for this region. Dates in parentheses are the date when testing was performed. Bootstrap scores were assigned as a percentage of 2,000 replicates.

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DOI: 10.3201/eid1611.100877

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In Response: The report by Mattison et al. about detection of noroviruses in 6% of ready-to-eat packaged leafy greens sampled in Ontario, Canada, suggests that these products could be vehicles for widespread dissemination of norovirus (*1*). As they suggest, this finding should lead to studies evaluating the potential risk from such contamination. In particular, prospective attempts to identify whether these strains may be associated with community outbreaks are necessary. However, the primary norovirus genotype identified in the leafy greens samples (GI) is not the norovirus that has primarily caused human illness in recent years (GII).

Ready-to-eat packaged leafy greens are widely eaten. One third of respondents to the 2002 FoodNet Population Survey reported eating prepackaged salad in the week before interview (*2*). In the absence of evidence linking this contamination to norovirus outbreaks, it is premature for consumers to change how they handle or eat ready-to-eat packaged leafy greens.

The authors provide data on the apparent viral loads they observed and cite data to suggest that washing and disinfecting produce before eating it could reduce viral loads below the level of an infectious dose. However, the Food and Drug Administration does not recommend rewashing prewashed produce and does not recommend washing fresh produce with soap, detergent, or commercial produce washes (*3*). Because the products sampled in the study by Mattison et al. were prewashed, whether washing them would further reduce viral loads is not clear. In addition, rewashing ready-to-eat produce creates a potential risk for cross-contamination of the produce in consumers' kitchens. Soap, detergents, or sanitizers could leave potentially harmful residues if rewashed produce is not thoroughly rinsed. These potential risks need to be weighed against the uncertain potential benefits of rewashing ready-to-eat packaged leafy greens. Given the ubiquity of these products, any change in recommended handling practices could have far-reaching consequences.

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DOI: 10.3201/eid1611.101304

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The Persistence of Influenza Infection

To the Editor: The report by Pinsky et al. (*1*) is interesting, but it raises some major questions. The finding of influenza virus in stool is not new (*2*). Of more interest is their statement regarding the prolonged shedding of influenza virus in the stool (for >2 months) and respiratory secretions (for >1.5 years). How frequently were respiratory samples collected and tested to confirm that the same virus was shed for these periods in these samples? Influenza virus, like most other acute respiratory viruses, typically does not cause long-term latent or persistent infections in humans. The authors need to exclude the possibility of frequent reinfection with contemporary circulating seasonal hemagglutinin 1 (H1) influenza viruses. However, they do not provide any data to this effect.

Currently, with the wider availability and more stringent expectations of modern molecular techniques, such

data might be obtained by collecting and sequencing several genes (ideally full genomes) from contemporary circulating seasonal H1 viruses and comparing them, phylogenetically, with the virus shed, contemporaneously, from the child, at monthly intervals, for example (if the child tolerates this testing). Even with this testing frequency, several influenza infection episodes may go undetected. Although the child's virus, if it truly persists, may undergo some minor host-induced mutations, new infections with seasonal H1 viruses will likely demonstrate a greater, sudden sequence variability, which enables them to be relatively easily distinguished from the more minor, gradually accumulated mutations that can be seen in a persisting infection (*3*).

Second, ribavirin is not recommended for treating influenza infection (*4,5*). Can the authors explain why this child was taking ribavirin for influenza infection, and how often was his condition treated with this drug during the 1.5 years when influenza H1 was shed? Was his treatment regimen eventually changed? Currently, the recommended treatment for influenza is with the neuraminidase inhibitors (oseltamivir, zanamivir), which have a much safer adverse effect profile, and their effectiveness has been shown to be cost-effective (*5*).

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DOI: 10.3201/eid1611.100974

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In Response: We appreciate Dr Tang's insightful letter (*1*) regarding our article (*2*) and are encouraged that this case report may have evoked increased interest in the phylogeny of the influenza sequences obtained during this patient's longstanding illness. We were acutely aware that we had not provided evidence that it was indeed the same influenza A virus found in these samples. Even though we proposed sequencing studies in the article's discussion section, in hindsight, we should have further expanded the discussion to include the points Dr Tang raises. At that time, however, we made a conscious decision not to include sequencing data in the manuscript for the following reasons.

First, we believed that finding that influenza A virus could be isolated from stool needed to be rapidly disseminated during the pandemic to reinforce awareness of the potential risk of acquiring influenza A infection through this source. During the summer of 2009, when we wrote this article, only influenza A (H5N1) had been reported to be culturable from human stool (*3*). Viral nucleic acids of seasonal influenza A had been demonstrated in stool in several studies (*4–6*), but of course, identification of viral nucleic acids remains an imperfect correlate to the presence of infectious virus.

Second, we considered the time, personnel, and funding required for a viral sequencing project of the type suggested and determined that we should attempt this as a follow-up study. Samples were obtained from the patient nearly every other week for >2 years, providing valuable data to further investigate this important question.

Dr Tang makes an addditional point regarding the use of ribavirin to treat the patient's condition. As laboratorians, we had similar questions and never found satisfactory answers. The patient did eventually receive oseltamivir, but this occurred >4 months after his last positive influenza A test result. The antiviral drug course was given empirically after the patient was admitted to the pediatric intensive care unit with fever and mental status changes, which were ultimately determined to be due to coagulase-negative staphylococcal septicemia.

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DOI: 10.3201/eid1611.101431

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etymologia

Baylisascaris

[ba"lis-as'kə-ris]

From the Greek term for intestinal worms, *askaris*. This genus of nematodes was named after H.A. Baylis, a parasitologist at the British Museum of Natural History, London, who studied these organisms in the 1920s and 1930s. The most common cause of baylisascariasis in humans and animals is infection with the roundworm *Baylisascaris procyonis*, which takes its name from *Procyon*, a genus of raccoons. The species was first isolated from raccoons in the New York Zoological Park in 1931.

Source: Gavin PJ, Kazacos KR, Shulman ST. Baylisascariasis. Clin Microbiol Rev. 2005;18:703–18. PMID: 16223954

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Smallpox Zero: An Illustrated History of Smallpox and Its Eradication

Jonathan Roy

African Comic Production House, Johannesburg, South Africa, 2010

ISBN: 978-0-620-43765-3
Pages: 72; Price: US $19.95

Smallpox Zero is an excellent commemoration of the 30th anniversary of smallpox eradication. This picture book tells the story of the disease from its origin through eradication and beyond (proposed fate of virus stock). The story is presented in short bubbles of text on a background of colorful, hand-drawn pictures. The children's book packaging (8½″ × 11″ × ¼″ with glossy, illustrated cover) belies the large amount of well-organized information inside. The 10 chapters draw you in with enticing titles such as No One Escaped. Once you're inside, the artwork captivates you. Practically every drawing would be suitable for framing and display. The maps, landscapes, and faces bring humanity's greatest scourge back to life for readers.

But who are the intended readers? The design and layout seem aimed at youth. A busy background with multiple drawings is right on target for teens, and the drawings are in the style of youth-oriented action comics. Even the need to reach youth is explained in the Note to Readers, "Since the battle against it [smallpox] was waged in remote areas far from the gaze of television cameras, the younger generations may have difficulty in appreciating the nature and magnitude of the task of global eradication."

The youth-attracting design, however, is mismatched with complex content. Readability scales rank average reading level at grade 12; several segments rank higher than grade 14. (For comparison, the reading level of this review is grade 11.) Multisyllabic words such as disease "transmission" rather than "spread" are the culprits. Adding to the complexity is mention of places and populations that young readers probably never heard of, like Sarawak and Hittites.

Readers can at least find many terms defined in a glossary in the back of the book. However, use of boldface or color to indicate which terms are defined would have been helpful. Today's impatient readers, accustomed to the instant gratification of online pop-up bubbles, may be unwilling to spend time flipping pages to check. Also helpful would have been a table of contents and index.

Audience and navigation tools aside, this is still a powerful book that should preserve the memory of smallpox and its heroes for future generations. Jonathan Roy, illustrator and narrator, effectively offers something for everyone, regardless of their location, education level, and native language. The global, rather than country-centric, perspective should appeal to readers worldwide; the small bites of information make each message palatable for even low-literacy readers; and the drawings eliminate language barriers. All these factors work together to increase access to the message that we should not take our freedom from smallpox for granted.

Although I cannot see Smallpox Zero being studied in a classroom, I can easily see it being browsed in a reception room, a reading room, a waiting room, or even a living room. A quick glance is enough to create an overall appreciation for the millions worldwide who suffered from smallpox and for the dedicated and selfless men and women we have to thank for its eradication.

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DOI: 10.3201/eid1611.101145

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Nicolaes Gillis (recorded 1601, active 1622–1632 in Haarlem) *Still Life on the Table* (Banquet Table) (1614) Oil on wood (110 cm × 74 cm) National Gallery in Prague, Czech Republic

A Moveable Feast[1]

Polyxeni Potter

A basket of grapes painted by Zeuxis more than 2,000 years ago was so realistic, the grapes so enticing, that birds flew down from the sky to peck at the picture, wrote Pliny the Elder in The Natural History. Food imagery, around since antiquity, expanded and flourished during the 17th century, as part of renewed interest in still-life painting in the Netherlands but also among German, French, Italian, and Spanish artists of the same period.

The rise of still-life painting from the bottom rank of artistic subject matter, especially in the cities of Antwerp, Haarlem, Leiden, and Utrecht, reflected the urbanization of society and a shift in emphasis from religious subjects to domestic scenes featuring personal possessions, earthly pleasures, and the economic prosperity following an explosion of global commerce. Paintings of food became very common, particularly in Haarlem, and formed the basis for later efforts in the genre. *Ontbijtjes*, or breakfast pieces showing foods for a snack or light meal at any time of the day, were wildly popular and witness the eating habits, class distinctions, fads, literary trends, religious beliefs, and health conventions of the period.

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The work of Nicolaes Gillis was part of this tradition. Though little is known about his life, Gillis was recorded in Haarlem and painted table or banquet scenes, one of the first in his generation to do so, along with Floris van Dijck and Floris van Schooten. These artists probably knew each other's work very well. Their mostly two-dimensional compositions showed tables arranged strictly parallel to the horizontal edges of the canvas and were additive in their presentation and arrangement of objects. Gillis may have been the oldest of the group—a 1601 date appears on one of few works by him that have survived.

Still Life on the Table, on this month's cover, is a breakfast piece showing fruits, nuts, bread, and cheeses served as dessert. Like similar paintings in this vein, it offers a glimpse of graceful living. A delicate table runner with crocheted border has been unfolded over the tablecloth for the occasion, the creases carefully running toward the back of the painting, creating the illusion of perspective. A slightly

[1]Set of Memoirs by Ernest Hemingway.

downward view allows good overall display of all the objects on the table, which are arranged side by side, touching only here and there, rarely overlapping, placed all the way to the edges of the canvas, reaching out to the viewer.

Fruit, though not an important part of the local diet, is abundantly displayed because of its visual appeal. This appeal, expertly demonstrated in Caravaggio's simple basket of fruit back in 1596, never lost its popularity. "Fine and rare fruits of various kinds are usually chosen for the dessert, for the pleasure they afford by their contrasting beauty." Lavish goblets and serving pieces provide richness and glow around a pyramid of hard cheeses. Floris van Dijck also often stacked these in two or more layers. The bottom comprises half a wheel with deep yellow color, still young. The top is smaller and darker, showing an aged, more mature product. Cheese, a common food eaten in large quantities by the masses, was also enjoyed by the affluent as part of dessert. Later, in his Physiology of Taste (1825), Jean Anthelme Brillat-Savarin would uphold this convention: "A dinner that ends without cheese is like a beautiful woman with one eye."

While in early banquet paintings the food seemed untouched, Gillis' work contains signs of consumption, a feeling of spontaneity, and bits of realism. The lemon has been cut, the seeds clearly visible; some of the fruit is peeled, the skin still attached; nut shells are scattered on the table; and along with the flower in the forefront rests a moth. The cheeses have irregular cuts made by the knife now on the platter. While food is beautiful, eating is a human need not usually included in food paintings, except perhaps in extreme situations, as in Hieronymus Bosch's *Garden of Worldly Delights*, which actually shows a figure, a half-human bird demon, eating in hell. Seventeenth-century Dutch and Flemish paintings also sometimes depicted peasants as stupid and ugly creatures eating and sometimes vomiting.

Banquet tables laden with costly exotic food and gleaming accessories have received many and sundry interpretations, apart from the obvious one of luxury. Some have dwelled on the moral undertones, suggesting the transitory nature of worldly pleasures—an argument against indulging in them—although the beauty and seductiveness of the displays argue in favor of the food. Others have commented on religious symbolism, the Lenten nature of meatless compositions, although fasting and feasting have long been argued among the various religious sects. Yet others remind that in the Golden Age, as in our times, food was often linked to health. Balance in meals was held in high regard, and foods were believed to complement each other. For this reason, game and lobsters were often paired in paintings.

Fascination with the beauty of food and interest in its connection with health remain intense and, in our times, go far beyond pairing food items, although debate on nutrition is increasing along with the growing epidemic of obesity in the United States and abroad. While not as photogenic, the utilitarian act of eating, including food consumed, and on the other end of the alimentary canal, food depleted, is fraught with danger and remains extremely central. Consumers are plagued by illnesses, among them enteric infections. The emphasis over the years has been in the prevention of foodborne disease—from the ubiquitous outbreaks of salmonellosis to more exotic fare due to new and reemerging foodborne pathogens.

But enteric infections, among them cholera and typhoid fever, until the 19th century, generally were not associated with food but rather with a variety of unsavory and unsanitary conditions. These and many other enteric infections, also not always foodborne, are with us today—some, and their complications, in this issue: *Salmonella enterica* clusters in Minnesota, extended spectrum β-lactamase–producing *Escherichia coli* in neonatal care, decreased ciprofloxacin susceptibility in *S. enterica* infections and association with foreign travel, multidrug-resistant *S. enterica* serovar Infantis in Israel, *Shigella* spp. antimicrobial resistance in Papua New Guinea, geographic expansion of *Baylisascaris procyonis* roundworms in Florida, and intentional infection with probiotics that can become invasive. Some enteric infections due to noroviruses, the most common causes of gastroenteritis in the world, also not always foodborne, are at times spread through contaminated food.

"If you are lucky enough to have lived in Paris as a young man," wrote Ernest Hemingway in his Memoirs, "then wherever you go for the rest of your life, it stays with you, for Paris is a moveable feast." The same can be said of new and reemerging infections. Prevention and control have curbed and tamed some, but the threat remains. Like a moveable feast, they are present but on their own time. And in different forms and unexpected ways, they always stay with us.

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EMERGING INFECTIOUS DISEASES

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Pandemic (H1N1) 2009 Infection in Dogs at Peak of Pandemic Activity, Italy

Complete list of articles in the December issue at http://www.cdc.gov/eid/upcoming.htm

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November 4–6, 2010
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EFIS-EJI course on Innovative Strategies for Vaccines: Malaria, TB, HIV
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November 11–13, 2010
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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.

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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

Sulfadoxine/Pyrimethamine Intermittent Preventive Treatment for Malaria during Pregnancy

CME Questions

1. Which one of the following pregnant women is likely to be most vulnerable and least protected from malaria?

A. Gravida 1 para 0 (G1P0)
B. G2P1
C. G3P2
D. G4P3

2. A 21-year-old woman residing in a stable malaria transmission area of Africa comes to the attention of the local clinic. She reports that she is feeling well and denies recent illness with or without fever.

Which of the following represents current recommendations for malaria prophylaxis in this scenario?

A. Continuous chloroquine
B. Intermittent sulfadoxine/pyrimethamine
C. Continuous sulfadoxine/pyrimethamine
D. Intermittent chloroquine

3. The clinic's staff discusses malaria prophylaxis with the young woman who questions whether it is necessary. The clinic's most appropriate response would be:

A. Prophylaxis is not indicated at this time because she is not currently infected
B. Prophylaxis will reduce her chances of having a low-birth-weight baby
C. Prophylaxis will reduce her chances of infection during pregnancy, but is unlikely to affect the outcome of the baby
D. Prophylaxis is not useful because she resides in an area with documented high resistance rates in children

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

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Article Title

Enhanced Surveillance of Coccidioidomycosis, Arizona, USA, 2007–2008

CME Questions

1. You are seeing a 52-year-old man in Arizona who has a history of cough and intermittent fever for 2 months. He was previously seen in an urgent care center and an emergency department, and he completed one course of azithromycin with little effect.

You suspect that this man might have coccidioidomycosis. Which of the following statements regarding the definition of coccidioidomycosis in the current case series is most accurate?

A. Only clinical criteria were important in the definition of coccidioidomycosis
B. The vast majority of laboratory-diagnosed cases met the clinical criteria for coccidioidomycosis
C. Both clinical and laboratory criteria were required to define coccidioidomycosis
D. Fungal culture was the sole means of laboratory diagnosis of coccidioidomycosis

2. Which additional symptom in this gentleman would support a clinical suspicion of coccidioidomycosis according to the current study?

A. Fatigue
B. Cough
C. Dyspnea
D. Fever

3. The patient from question 1 is diagnosed with coccidioidomycosis, and he is worried what this diagnosis might cost him financially and in terms of his daily function. Based on the results of the current study, what can you tell him?

A. Most patients with coccidioidomycosis do not miss work because of illness
B. Three quarters of patients report an impact on usual daily activities
C. Less than 10% of patients visit the emergency department
D. Hospitalization for coccidioidomycosis is exceedingly rare

4. Which of the following factors from the current study had the biggest impact on being diagnosed and treated for coccidioidomycosis earlier?

A. Education about coccidioidomycosis from television announcements
B. Education about coccidioidomycosis from newspaper campaigns
C. Education about coccidioidomycosis from social circles or families
D. The presence of sore throat

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

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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, and figure legends. Appendix materials and figures should be in separate files.

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. Submit figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

Videos. High-quality video files are accepted in the following formats: AVI, MOV, MPG, MPEG, and WMV. The files should be no longer than 5 minutes in length.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may 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.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 biographical sketch. Dispatches are updates on infectious disease trends and research that 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 abstract, figures, or tables. Include biographical sketch.

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. Include biographical sketch.

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. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

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.

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Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

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. Email to eideditor@cdc.gov.