

# Genetic Characterization of Highly Pathogenic Avian Influenza (H5N8) Virus from Domestic Ducks, England, November 2014

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Genetic sequences of a highly pathogenic avian influenza (H5N8) virus in England have high homology to those detected in mainland Europe and Asia during 2014. Genetic characterization suggests this virus is an avian-adapted virus without specific affinity for zoonoses. Spatio-temporal detections of H5N8 imply a role for wild birds in virus spread.

Aquatic birds are considered to be the natural reservoir of low pathogenicity avian influenza viruses of subtypes H1–H16; in these birds, including ducks, they generally do not cause clinical signs (*1*). In contrast, highly pathogenic avian influenza (HPAI) viruses of certain H5 and H7 strains cause high death rates in poultry with substantial economic losses and are thought to be derived from low pathogenicity avian influenza viruses of wild bird origin. China, Japan, and South Korea have reported outbreaks of highly related HPAI (H5N8) virus in poultry and migratory birds since early 2014 (*2,3*). Germany and the Netherlands have reported outbreaks among poultry with closely related H5N8 viruses in turkey, chicken, and duck farms since early November 2014. On November 16, 2014, an outbreak of HPAI (H5N8) virus was confirmed on a duck breeding farm in East Yorkshire, England, UK (*4*). The premises contained 6,000 breeding ducks  $\approx$ 60 weeks of age housed in 3 sheds; the ducks showed only mild clinical signs of illness.

## The Study

Thirty-seven of 120 individual swab samples and 5 of 12 tissue pools (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/6/14-1954-Techapp1.pdf>) were confirmed positive for avian influenza virus by identification of the matrix (M) gene and H5 real-time reverse transcription PCR (rRT-PCR) as described (*5*). A highly pathogenic pathotype was confirmed by RT-PCR, then Sanger sequencing of the hemagglutinin (HA) gene as described (*5*). This process showed the polybasic cleavage site PLRERRRKR/GLF from multiple bird swab and tissue samples from each shed. HPAI virus was isolated in 9- to 10-day-old specific pathogen-free embryonated hens' eggs after incubating 2 days as described (*6*). A primary isolate, A/duck/

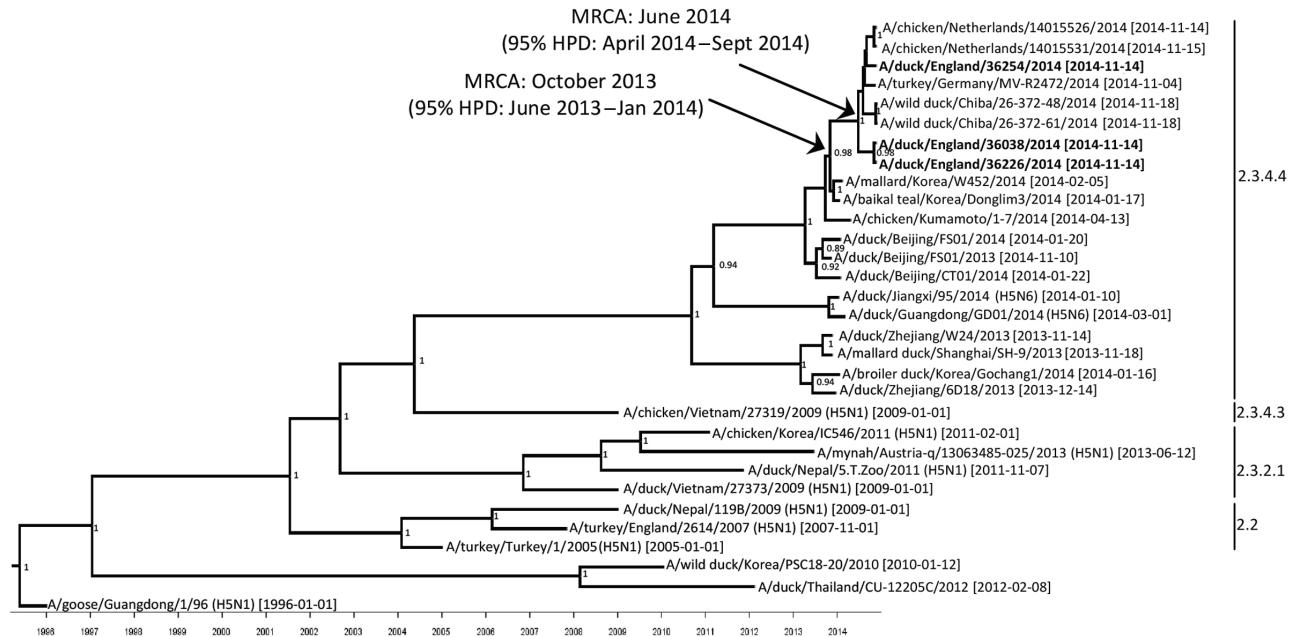
England/36254/2014 (dkEng14), was obtained after egg inoculation with pooled intestinal samples from 1 shed and was typed as H5N8 by using standard protocols (*6*). We obtained full genome sequence from dkEng14 by next-generation sequencing (online Technical Appendix). Full genome sequences of 2 more isolates, A/duck/England/36038/2014 and A/duck/England/36226/2014, derived from pooled cloacal swabs and pooled viscera from 2 different sheds, respectively, were also assessed by next generation sequencing and analyzed. We entered genome sequences on the Global Initiative on Sharing All Influenza Data EpiFlu database (*7*) under accession nos. EPI547670–EPI547677, EPI550848–EPI550849, and EPI558000–EPI558013. Initial maximum likelihood phylogenetic analysis of the HA gene was conducted as described (*5*) and the evolutionary analyses performed by using MEGA6 (*8*).

Phylogenetic analysis showed 99.9%–100% similarity among the 3 duck isolates from the single premises in England and 99.4–100% similarity among HPAI (H5N8) sequences from China, Japan, South Korea, Germany, and the Netherlands; all belonged to Asian lineage H5 clade 2.3.4.4. Sequence comparisons based on 1,608 nt of the HA gene from each of the 3 duck isolates identified A/turkey/Germany/MV-R2472/2014 (H5N8) as the closest match, at 99.8% similarity. We implemented further phylogenetic analysis using Bayesian Markov chain Monte Carlo simulation in the BEAST package version 1.7 (*9*). The maximum clade credibility tree (Figure 1) had a similar topology to that observed for the maximum-likelihood tree.

The time elapsed between the detection of the isolates in England and the most recent common ancestor (MRCA) of the H5N8 virus cluster from Europe and Japan was  $\approx$ 5 months (95% highest posterior density [HPD] range 2.7–7.7 months; approximately June 2014). This cluster shares homology of the HA gene with viruses detected in South Korea in early 2014. The ancestor of the viruses from continental Europe, Japan, and Korea occurred  $\approx$ 13 months (11–15.5 months, 95% HPD; approximately October 2013) before the detection of the English isolates. The separation between the H5N8 HA sequences from England is attributed to 2 nonsynonymous mutations coding for amino acid substitution S181P and H273Y (HA numbering based on the mature H5 protein). Position 181 is in close proximity to the receptor binding site and antigenic site Sb (*10*); however, the effect of this condition on antigenicity has not yet been defined.

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**Figure 1.** Maximum clade credibility tree of 31 H5 sequences derived from the hemagglutinin gene of avian influenza viruses (1,608 nt). Sampling dates and locations are included on the tip labels; where specific dates were unknown, '01' was assigned. Node labels indicate significant posterior probabilities (>0.75). The dates for the most recent common ancestor (MRCA) of the currently circulating viruses circulating in Europe and Japan are indicated at the relevant nodes with 95% highest posterior density (HPD) levels. Sequences relate to H5N8 subtype unless otherwise noted. Sequences reported in this study are in bold.

The HA sequence variation observed within 1 premises in which ducks were infected in England is potentially the consequence of virus adaptation within the flock subsequent to a single introduction. Full-genome comparison of the 3 viruses in England showed amino acid differences in the HA and NA genes only (Table 1). Further analyses with sequences from the NA gene indicated a similar most recent

common ancestor ( $\approx 5$  months) for the European lineage. However, each of the 3 virus sequences detected in England grouped together with those from the Netherlands (Figure 2).

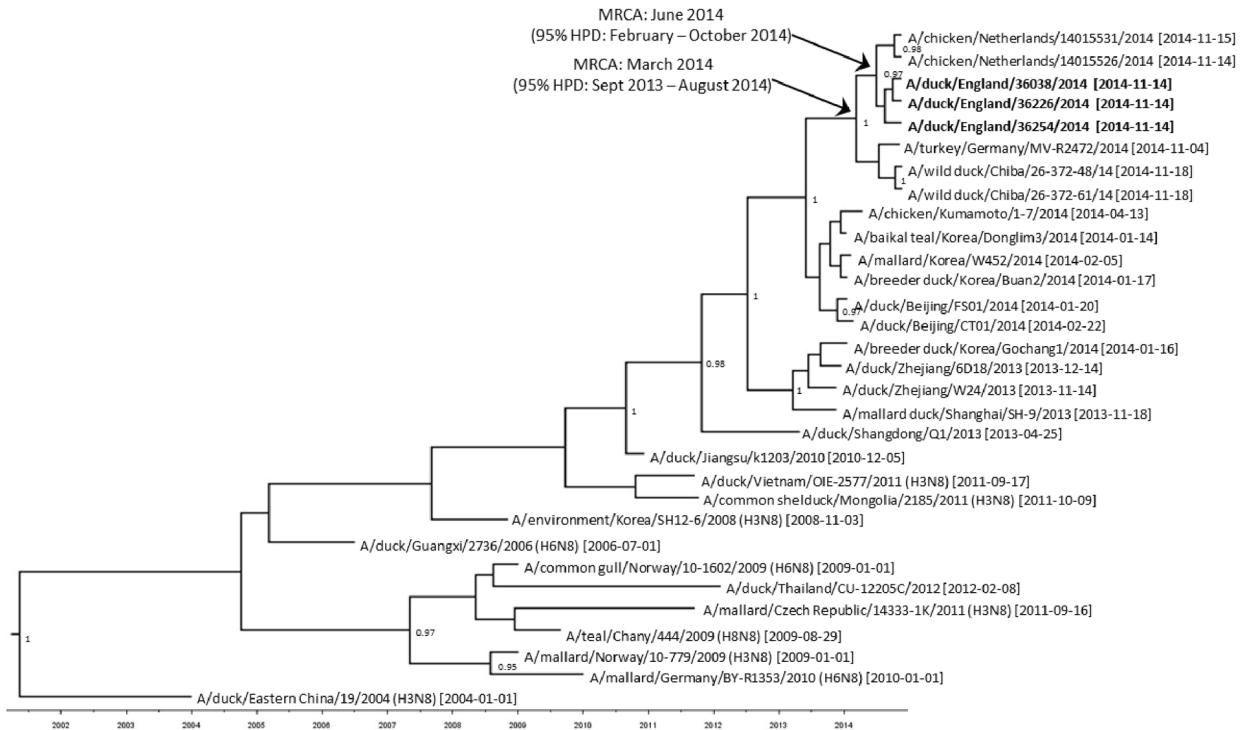
To investigate the potential zoonotic affinity of dkEng14, sequence data was compared with the H5N1 genetic changes inventory at the US Centers for Disease Control and Prevention (11) to identify single or collective mutations that might influence viral phenotypic characteristics of importance and may indicate adaptation to mammalian species or alter susceptibility to antiviral drugs. A total of 8 point mutations among 114 of interest were identified (Table 2). In addition to the polybasic (PB) cleavage site in the HA protein, positions 133 and 156 each contained an alanine residue (H5 numbering relative to A/Vietnam/1203/2004), which is reportedly related to increased virus binding to  $\alpha 2,6$  sialic acid human receptors.

An asparagine that has been associated with reduced susceptibility to amantadine and rimantadine antiviral drugs was present at position 31 in the M2 protein, but we did not detect any signature motifs associated with resistance to antivirals targeting neuraminidase. Aspartic acid at position 30 and alanine at position 215 in the M1 protein together with serine at position 42 and methionine at position 101 in the nonstructural 1 protein were observed; all have been individually linked to increased virulence in mice. A serine residue at position 66 in the PB1-F2 protein

**Table 1.** Genome sequence comparisons of 3 highly pathogenic avian influenza (H5N8) viruses from domestic ducks, England, November 2014\*

Gene segment	Sequence		
	36038, 36226	36038, 36254	36226, 36254
PB2	0	1	1
PB1	0	0	0
PA	1	0	1
HA	0	2 (S181P, H273Y)	2 (S181P, H273Y)
NP	0	0	0
NA	1 (L363I)	4 (S164P, N166S, K186N, L363I)	3 (S164P, N166S, K186N)
MP	0	0	0
NS	0	0	0

\*Sequences compared are A/duck/England/36038/2014, A/duck/England/36226/2014, and A/duck/England/36254/2014. Amino acid residue in parentheses indicate that the number of nucleotide differences correspond to a nonsynonymous change. PB2, polymerase basic 2; PB1, polymerase basic 1; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix; NS, nonstructural.



**Figure 2.** Maximum clade credibility tree of 31 N8 sequences derived from the neuraminidase gene of avian influenza viruses (1,377 nt). Sampling dates and locations are included on the tip labels; where specific dates were unknown, '01' was assigned. Node labels indicate significant posterior probabilities (>0.75). The dates for the most recent common ancestor (MRCA) are indicated at the relevant nodes with 95% highest posterior density (HPD) levels. Sequences relate to H5N8 subtype unless otherwise noted. Sequences reported in this study are in bold.

was also observed. This mutation has also been linked to increased virulence in mice and studies in ducks showed a minor role in pathogenesis. Of interest is the lack of a deletion in the nonstructural 1 protein at amino acid positions 80–84 that is conserved among contemporary H5N1 viruses, possibly decreasing the zoonotic potential of the H5N8 virus. Two mutations in PB2, E627K, and K526R (12), described to be involved with mammalian host adaptation and increased replication in mammalian cells, were not observed. Eight mutations were identified in dkEng14; only 1 (T156A) of the 5 necessary mutations associated with high risk for zoonotic transmission were observed (13,14). The mutations identified in Table 2 are analogous to that of A/mallard duck/Korea/W452/2014 (H5N8), a virus that has been studied for its pathogenic and pandemic potential (15) and was found to result in impaired replication and inefficient contact transmission among ferrets.

## Conclusions

The genome of the H5N8 virus isolated in England suggests that it is still predominantly an avian-adapted virus, without any specific increased affinity for humans. Close

genetic homology among the viral genes of the H5N8 viruses detected in England, the Netherlands, and Germany suggest they share a common ancestor with the recent H5N8 viruses isolated from wild ducks in Japan, a result of reassortment estimated to have occurred in June 2014. Reliable interpretation of the topology of the European and Japanese cluster cannot be made with these similar sequences. Phylogenetic analysis of sequences from more viruses will help to resolve these relationships. Detection of H5N8 (HPAI) viruses in 3 countries in Europe over a short time period in different poultry species without the establishment of clear epidemiologic links implicates a role for wild birds in spreading of viruses. The potential for further dissemination of HPAI (H5N8) viruses in Europe is a threat to poultry. Viral sequence analysis from new outbreaks is recommended to monitor virus evolution, understand risk pathways for introduction, and assess the emergence of mutations that may be relevant for veterinary and public health.

## Acknowledgments

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**Table 2.** Genetic mutations identified in highly pathogenic avian influenza (H5N8) virus isolate A/duck/England/36254/2014 that might result in phenotypic consequences, England, 2014\*

Protein, amino acid position/motif	Phenotypic consequences†
PB1-F2, N66S	Increased virulence, replication efficiency and antiviral response in mice
HA S133A T156A 323–330 (R-X-R/K-R)	Increased pseudovirus binding to $\alpha$ 2,6 Increased virus binding to $\alpha$ 2,6 and increased transmission in guinea pigs Polybasic cleavage motif sequence required for high pathogenicity
M1 N30D T215A	Increased virulence in mice Increased virulence in mice
M2 S31N	Reduced susceptibility to amantadine and rimantadine antiviral drugs
NS1 P42S I101M	Increased virulence in mice Increased virulence in mice

\*Phenotypic consequences may include an influence on viral phenotypic characteristics of importance, adaptation to mammalian species, or altered susceptibility to existing antiviral drugs. H5N1 numbering based on the mature HA protein relative to A/Vietnam/1203/2004. PB, polymerase basic protein; HA, hemagglutinin; M, matrix; NS, nonstructural.

†The mutation to the right of the amino acid position confers the phenotypic consequence described.

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Miss Hanna is a research scientist at the Animal and Plant Health Agency, Weybridge, UK. Her primary research interests are genetic characterization and pathogenesis studies of avian influenza viruses.

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# Genetic Characterization of Highly Pathogenic Avian Influenza (H5N8) Virus Among Domestic Ducks, England, November 2014

## Technical Appendix: Methods for genetic characterization of HPAI H5N8 virus among domestic ducks in England

### Methods

#### Sample Preparation

Twenty oropharyngeal swabs, 20 cloacal swabs and two carcasses were collected from each of the three sheds. Tissue samples collected from each carcass were pooled per shed; brain, lung and trachea, intestines and mixed viscera (liver, heart, spleen and kidney) for testing by rRT-PCR. Individual swabs were pooled in groups of five per shed, together with the pooled tissues for virus isolation.

#### Next Generation Sequencing

Double stranded cDNA (cDNA synthesis system, Roche, UK) was generated from RNA according to the manufacturer's instructions. This was quantified using the fluorescent PicoGreen reagent and 1ng was used as a template for the preparation of the sequencing library (NexteraXT, Illumina, Cambridge, UK). Sequencing libraries were run on a MiSeq instrument (Illumina, Cambridge, UK) with 2x75 base paired end reads.

#### Phylogenetic Sequence Analysis

Phylogenetic analysis was implemented using Bayesian Markov chain Monte Carlo simulation in the BEAST package v.1.7. The analysis used uncorrelated lognormal relaxed molecular clocks and a constant-population coalescent process prior over the phylogenies was selected. The HKY substitution model with a rate variation and a proportion of invariant sites (G+I) were used.

**Technical Appendix Table.** Authors and originating and submitting laboratories of the sequences used for phylogenetic analysis in this study from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu database\*

Segment, ID no.	Country of origin	Subtype	Collection date (YYYY-Mon-DD)	Isolate name	Originating and submitting laboratory	Authors
HA, EPI548493 NA, EPI548495	Japan	H5N8	2014-Nov-18	A/duck/Chiba/26-372-61/2014	National Institute of Animal Health	Takehiko Saito
HA, EPI548485, NA, EPI548487	Japan	H5N8	2014-Nov -18	A/duck/Chiba/26-372-48/2014	National Institute of Animal Health	Takehiko Saito
HA, EPI548623, NA, EPI548626	Netherlands	H5N8	2014-Nov -15	A/chicken/Netherlands/14015531/2014	Central Veterinary Institute	Rene Heutink; Frank Harders, Sylvia Verschuren-Pritz, , Alex Bossers, Guus Bouwstra Koch, Rene Ruth
HA, EPI547678, NA, EPI547683	Netherlands	H5N8	2014-Nov -14	A/chicken/Netherlands/14015526/2014	Central Veterinary Institute	Heutink, Frank Harders, Sylvia Verschuren-Pritz, Alex Bossers, Guus Koch, Ruth Bouwstra, Elke Starick
HA, EPI544756, NA, EPI544759	Germany	H5N8	2014-Nov -04	A/turkey/Germany/MV-R2472/2014	Friedrich-Loeffler-Institut	
HA, EPI517161, NA, EPI517163	Japan	H5N8	2014-Apr-13	A/chicken/Kumamoto/1-7-2014	National Institute of Animal Health, National Agriculture and Food Research Organization	K. Kanehira, N. Takemae, Y. Uchida, R. Tunekuni, H. Hikono, T. Saito
HA, EPI543010, NA, EPI543012	China	H5N8	2014-Jan-22	A/duck/Beijing/CT01/2014	Institute of Microbiology, Chinese Academy of Sciences	Di Liu
HA, EPI543002, NA, EPI543004	China	H5N8	2014-Jan-20	A/duck/Beijing/FS01/2014	Institute of Microbiology, Chinese Academy of Sciences	Di Liu
HA, EPI530054, NA, EPI530056	China	H5N6	2014-Jan-10	A/duck/Jiangxi/95/2014	BGI Shenzhen	Xu Bing, Zhang Tao, Li Xiaowen,
HA, EPI507673, NA, EPI507675	China	H5N8	2013-Nov-18	A/mallard duck/Shanghai/SH-9/2013	Institute of Military Veterinary, Academy of Military Medical Sciences, Institute of Laboratory Animal Sciences, Chinese Academy	S. Fan, X. Gao, Y. Ying, J. Guo, W. Sun, T. Wang, Z. Ren, Z. Yu, Y. Li, Y. Zhao, S. Yang, Y. Gao, X. Xia
HA, EPI542617, NA, EPI542627	China	H5N8	2013-Nov-10	A/duck/Beijing/FS01/2013	Institute of Microbiology, Chinese Academy of Sciences	Di Liu
NA, EPI339175	Germany	H6N8	2010-unknown-unknown	A/mallard/Germany-BY/R1353/2010	Friedrich-Loeffler-Institut	Elke Starick

\*<http://platform.gisaid.org/epi3/frontend#5412a6>