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Comparative Analysis of Avian Influenza Virus Diversity in Poultry and Humans during a Highly Pathogenic Avian Influenza A (H7N7) Virus Outbreak▽†

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Although increasing data have become available that link human adaptation with specific molecular changes in nonhuman influenza viruses, the molecular changes of these viruses during a large highly pathogenic avian influenza virus (HPAI) outbreak in poultry along with avian-to-human transmission have never been documented. By comprehensive virologic analysis of combined veterinary and human samples obtained during a large HPAI A (H7N7) outbreak in the Netherlands in 2003, we mapped the acquisition of human adaptation markers to identify the public health risk associated with an HPAI outbreak in poultry. Full-length hemagglutinin (HA), neuraminidase (NA), and PB2 sequencing of A (H7N7) viruses obtained from 45 human cases showed amino acid variations at different codons in HA ($n = 20$), NA ($n = 23$), and PB2 ($n = 23$). Identification of the avian sources of human virus infections based on 232 farm sequences demonstrated that for each gene about 50% of the variation was already present in poultry. Polygenic accumulation and farm-to-farm spread of known virulence and human adaptation markers in A (H7N7) virus-infected poultry occurred prior to farm-to-human transmission. These include the independent emergence of HA A143T mutants, accumulation of four NA mutations, and farm-to-farm spread of virus variants harboring mammalian host determinants D701N and S714I in PB2. This implies that HPAI viruses with pandemic potential can emerge directly from poultry. Since the public health risk of an avian influenza virus outbreak in poultry can rapidly change, we recommend virologic monitoring for human adaptation markers among poultry as well as among humans during the course of an outbreak in poultry.

Avian influenza viruses are typically restricted to the intestinal tract of aquatic birds, in which they cause mild or subclinical and unspecific symptoms. Occasionally, however, they acquire the properties to directly infect the respiratory tracts of terrestrial birds and mammals, including humans (49). Introduction of avian influenza in humans could result in a pandemic when the viruses (i) possess a hemagglutinin (HA) subtype antigenically distinct from the currently circulating human influenza virus subtypes, (ii) have the ability to infect and efficiently replicate in humans, and (iii) transmit efficiently from human to human (29, 33, 44). Infection of humans in direct contact with wild or domestic poultry has been observed for influenza A viruses with HA subtypes H5, H7, H9, and H10 over the past 2 decades. Low-pathogenic avian influenza (LPAI) A (H9N2) virus caused human infections in China in 1998 and Hong Kong in 1999 and 2003 (7, 20, 35). LPAI A (H7N2) virus infected humans in the United States in 2002 and 2003 and the United Kingdom in 2007, and LPAI A (H7N3) virus infected humans in the United Kingdom in 2006 (10, 15, 32). Additionally, LPAI A (H10N7) virus caused human infections in Egypt in 2004 (50). Symptoms of these LPAI virus infections were mild, and no fatalities were reported. In contrast, avian influenza viruses of subtypes H5 and H7 are notorious for their abilities to mutate from LPAI viruses to viruses with increased virulence for poultry. These highly pathogenic avian influenza (HPAI) viruses typically emerge after introduction of an LPAI virus from wild birds into large commercial poultry flocks. This was exemplified by LPAI A (H7N1) viruses in Italy in 1999, LPAI A (H7N3) in Canada in 2004 and Chile in 2002, and LPAI A (H7N7) in Spain in 2009 that initiated outbreaks in poultry before mutating into the HPAI phenotype (9, 21, 22, 40). The emergence of a lineage of HPAI A (H5N1) virus in poultry and wild birds in Southeast Asia and its dissemination over a wide geographic region have been of particular concern. This virus now has evolved through drift and reassortment into distinct lineages, with reports of transmission to >500 laboratory-confirmed infected humans and a case fatality rate of 60% (51). The widespread presence of an influenza virus that can be transmitted to humans in regions with limited surveillance constitutes a permanent pandemic threat. So far, however, the prerequisite of efficient human-to-human transmission has not been met. Similarly, the unprecedented number of human infections during the HPAI A (H7N7) outbreak in the Netherlands in 2003 was cause for concern. This outbreak struck a large number of Dutch

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poultry farms and resulted in the culling of 30 million chickens. In addition, 89 virologically confirmed human cases, including 1 death and limited human-to-human transmission, were reported (14, 18, 26).

Avian influenza viruses must undergo molecular changes to adapt to a mammalian host (2). This is a complex process that involves adaptation to human host cell factors to facilitate replication and transmission (13, 30, 31, 34, 45). In addition to emergence of the HPAI phenotype by alteration of the HA cleavage site, LPAI virus replication in poultry can induce polygenic molecular changes. Examples are a deletion in the neuraminidase (NA) stalk region, which is associated with a shift in tissue tropism in chickens (43), and receptor binding site (RBS) mutations in HA, which affect host cell binding affinity (13, 48). Collectively, poultry can serve as an intermediate host and facilitate mammalian host adaptation of avian influenza viruses. Although increasing data that link human adaptation with specific molecular changes in the virus have become available, the molecular changes of influenza viruses during a large HPAI outbreak in poultry, along with cross-species transmission events, have never been documented. Such information could help to improve surveillance aimed at the timely detection of emergence of human adaptation markers to direct public health measures in the future. In this study, the 2003 HPAI A (H7N7) outbreak in the Netherlands was used as a model (26). We combined virologic and epidemiologic data collected during the veterinary and medical outbreak control activities in order to identify the most likely source of infection for each person. We then compared viruses from humans and poultry to identify possible human adaptation markers. Precursors of potential pandemic variants with increased public health risk were detected in poultry prior to avian-to-human transmission events.

MATERIALS AND METHODS

HPAI A (H7N7) outbreak. From individuals in contact with HPAI A (H7N7) and suffering from conjunctivitis and/or influenza-like illness, throat, nose, and conjunctiva swabs were collected into virus transport medium; the patients' written consent was obtained and a questionnaire was completed. After diagnostic testing (26), leftovers of clinical specimens were stored in a biobank at −80°C. For all human clinical specimens, the date of sampling, the first day of illness, demographic data, symptoms, and exposure data were stored in the human case finding database (26). The veterinary database contained information on type of poultry, geographical location, date of sampling, date of culling, daily mortality data, and a unique farm identification number (6). For each farm, the probable day of introduction of infection had been estimated previously from daily mortality data based on a back-calculation method (6).

Sequencing of viruses in human clinical samples. All A (H7N7) influenza virus-positive cases for which clinical samples were still available in a biobank ($n = 53$) were used. Prior to direct sequencing of the virus in clinical specimens, the viral load of the specimens was determined by quantitative influenza virus reverse transcription-PCR targeting a 94-bp matrix gene fragment (23). Due to the low viral load in the 53 eye swabs, a direct sequencing experiment was designed that covered the HA receptor binding site and previously reported host range markers PB2 A199S, E627K, D701N, and K702R (16, 18, 27). Therefore, three PCRs targeting HA fragment amino acids (aa) 160 to 239 and PB2 fragments aa 156 to 225 and aa 585 to 720 were developed by using the PSQ assay design package v1.0.6 (Biotage AB) for conventional Sanger sequencing. In addition, pyrosequencing targeting of the four PB2 host range markers was performed. Total RNA was isolated using a High Pure RNA isolation kit (Roche) followed by reverse transcription using SuperScript III (Invitrogen) and cDNA amplification using HotstarTaq Mastermix (Qiagen). All PCR products were Sanger sequenced using an ABI 3730 sequencer, and PB2 host determinant markers were pyrosequenced using the PSQ MA96 platform (Biotage AB). Contig assembly and alignment were done using BioNumerics Package 6.5. Primers and PCR protocols are available upon request.

Sequencing of human virus isolates. Due to insufficient viral load in some clinical specimens, influenza virus culture supernatants of human A (H7N7) viruses were used to complement the data set. Routine culture of clinical specimens obtained from virologic confirmed A (H7N7) cases ($n = 89$) using MDCK-I cells yielded 47 A (H7N7) virus isolates corresponding to 45 human cases (18). Forty-three A (H7N7) virus isolates were obtained from eye swabs, three from throat swabs, and one from postmortem lung tissue from the fatal case. As initial screening of poultry viruses using whole-genome analysis did not reveal substantial differences when comparing nucleoprotein (NP), matrix, NS, PA, and PB1 genes from A (H7N7) viruses sampled early and late in the outbreak (data not shown), we focused on the more-diverse HA, NA, and PB2 genes. Hence, the full-length sequences of the HA, NA, and PB2 gene segments of all 47 human A (H7N7) virus isolates were determined. Total RNA was isolated using a High Pure RNA isolation kit, followed by reverse transcription using ThermoScript (Invitrogen) and HA, NA, and PB2 gene amplification with the LongRange kit (Kapa Biosystems). After sequencing of the amplicons, trace files were assembled and aligned.

Data analysis. Human A (H7N7) sequences were compared with HA, NA, and PB2 gene sequences obtained from 184 infected poultry farms that were available from a separate study (1), supplemented by sequences obtained from 47 other poultry farms infected during the outbreak (combined, sequences were obtained from 91% of the infected farms). Each A (H7N7) farm sequence is the consensus sequence of RNA extracted from a pool of five A (H7N7) tracheal tissue samples of poultry. The character data from a concatenated full-length HA, NA, and PB2 gene nucleotide alignment were used to build a maximum parsimony network in BioNumerics using the Fitch method (17) with a greedy tree construction algorithm, followed by random branch swapping to find the optimal network topology. This method was chosen for its ability to link human A (H7N7) viruses to their suspected avian source of infection by using a minimum number of "evolutionary events" based on the simplest, most parsimonious explanation of an observation and free of specific evolutionary assumptions. The faithfulness of the entire network was determined by calculation of the global cophenetic correlation coefficient (42). The significance of branches linking human viruses with poultry viruses was assessed by bootstrap analysis (1,000 iterations) and permutation resampling (200 resampling cycles) (38). Finally, the constructed transmission network was validated by comparing the first day of human illness with the estimated day of introduction of A (H7N7) virus into a poultry farm for all farm-human links.

After validation of the transmission network, potential human adaptation markers were explored using the combined avian and human A (H7N7) sequence data set following categorization based on the following characteristics: first, the observed mutations were labeled poultry induced or human induced, depending on the host in which they were first identified. Subsequently, positively selected sites within the human data set were detected using the single-likelihood ancestor counting (SLAC), the random effect likelihood (REL), and the fixed effect likelihood (FEL) methods of the Datamonkey website (36). Finally, HA and PB2 mutations were categorized based on the location in the gene segments and literature, while NA mutations were analyzed phenotypically for their effects on susceptibility to the antiviral drug oseltamivir carboxylate as described previously (24, 37).

Nucleotide sequence accession numbers. GISAID accession numbers assigned to our sequences included the following: human A (H7N7) sequence, EPI_ISL_90869-90912; poultry A (H7N7) sequences, EPI_ISL_68268-68352 and EPI_ISL_82373-82472 (1), supplemented by EPI_ISL_83984-84031.

RESULTS

Direct sequencing of A (H7N7) virus fragments from clinical specimens. Direct sequencing of virus in the 53 influenza A (H7N7) virus eye swabs, which had an average virus load of ~3,000 genomic viral RNA copies/ml, was successful for 44 human clinical samples, yielding HA receptor binding site fragments ($n = 43$) and sequences covering previously reported host determinant markers in PB2 at residue 199 ($n = 43$) and the residues 627, 701, and 702 ($n = 39$). Sequence comparison with influenza A/Netherlands/33/03 (H7N7) virus obtained from the first human conjunctivitis case demonstrated the absence of PB2 host determinant markers A199S, E627K,

D701N, and K702R but the presence of an amino acid variation in the HA receptor binding site (HA V223I [$n = 1$] and HA subpopulation V223A [$n = 1$]) (Table 1).

Sequencing of A (H7N7) virus isolates. To expand the search for human adaptation markers beyond these known positions, influenza virus isolates were utilized to obtain sequences for the entire HA, NA, and PB2 genes corresponding with 45 human cases. Compared with A/Netherlands/33/03 (H7N7) virus, amino acid variations were observed at 20 different HA codons, 23 NA codons, and 23 PB2 codons.

Detection of sources of human A (H7N7) virus infection. To discriminate mutations that emerged in poultry from mutations that were detected in viruses from humans only, the data set was supplemented with 232 veterinary A (H7N7) HA, NA, and PB2 sequences, representing 231 poultry farms, to allow identification of sources of human A (H7N7) virus infection. The high level of genetic diversity observed in avian influenza A (H7N7) viruses isolated during the 2003 HPAI outbreak in the Netherlands (1) was used to build a maximum parsimony transmission network to identify chains of transmission and probable sources of infection of the sequenced human A (H7N7) cases. Since initial analysis with 231 poultry farms did not identify clustering of human A (H7N7) viruses with avian A (H7N7) viruses containing a deletion in the NA stalk region ($n = 17$), these were removed from the data set. Removal of these sequences from the data set did not affect the results of the study. Moreover, as a result of observed nucleotide variations in the NA stalk region, the final transmission network was constructed with concatenated HA, NA, and PB2 sequences from avian A (H7N7) viruses containing full-length NA sequences, representing 214 poultry farms, to maximize the network resolution (Fig. 1). The global cophenetic correlation of the transmission network was 99%, and the branches connecting human cases ($n = 32$) with farm sequences were supported through a bootstrap analysis and permutation resampling by 100%.

The genetic diversity observed at 214 poultry farms, represented by 149 distinct avian A (H7N7) virus sequences in the transmission network, illustrates the emergence of multiple avian A (H7N7) virus clusters and chains of transmission during the outbreak. Human A (H7N7) viruses are interspersed with avian viruses, reflecting the virus diversity in poultry, allowing identification of probable sources of human A (H7N7) virus infection. In addition to the three human secondary cases, 22% (10/45) of the human A (H7N7) viruses were identical to their presumed avian source of infection, while 71% (32/45) of the human A (H7N7) viruses displayed genetic variation from their avian source. Thirty of these 32 human A (H7N7) viruses are at the tips of the network, illustrating that the majority of human cases were dead-end A (H7N7) virus infections. One A (H7N7)-positive culler infected both his wife and daughter, and another culler infected his father (Fig. 1A). Of the probable avian sources of the remaining 42 human A (H7N7) virus infections, 35 (83%) persons had their first symptoms after the estimated date of introduction of virus on the farm (back-calculated from mortality data) (5). The avian source of infection of three different clusters of human cases ($n = 7$) did fit molecularly, but infection chronology was reversed (Fig. 1A, yellow dots). As 9% ($n = 24$) of the infected poultry farms were missing in our analysis, the farms of infection origin of these human clusters could be missed. Further-

TABLE 1. Amino acid substitutions found in human A (H7N7) viruses compared to their avian source of infection[a]

Source of virus	HA gene		NA gene		PB2 gene	
	Fragment sequenced (aa)	Mutation(s) detected	Fragment sequenced (aa)	Mutations detected	Fragment(s) sequenced	Mutation(s) detected
Isolate ($n = 48$) from human conjunctivitis cases	1–562	R65K, A156S, V223A, V223I, D264G, E279K, E287G, R504K, E505K, D515N	1–471	A14T, V19A, Q44K, N57S, N67S, V115I, M174V, V263I, E267V, I275T, N284I, N338S, V426I, V456I	1–759	V89 M, T117P, V139I, V167I, C409R, M467L, I562T, D567N, K586R, P620Q, N711K
Clinical specimen ($n = 44$) from human conjunctivitis cases	160–239	V223A, V223I			156–225, 585–720	N711K
Isolate ($n = 1$) from fatal human case (13)	1–562	K416R	1–471		1–759	E627K

[a] Underlined mutations are associated with human adaptation, based on the literature or selection pressure analysis.

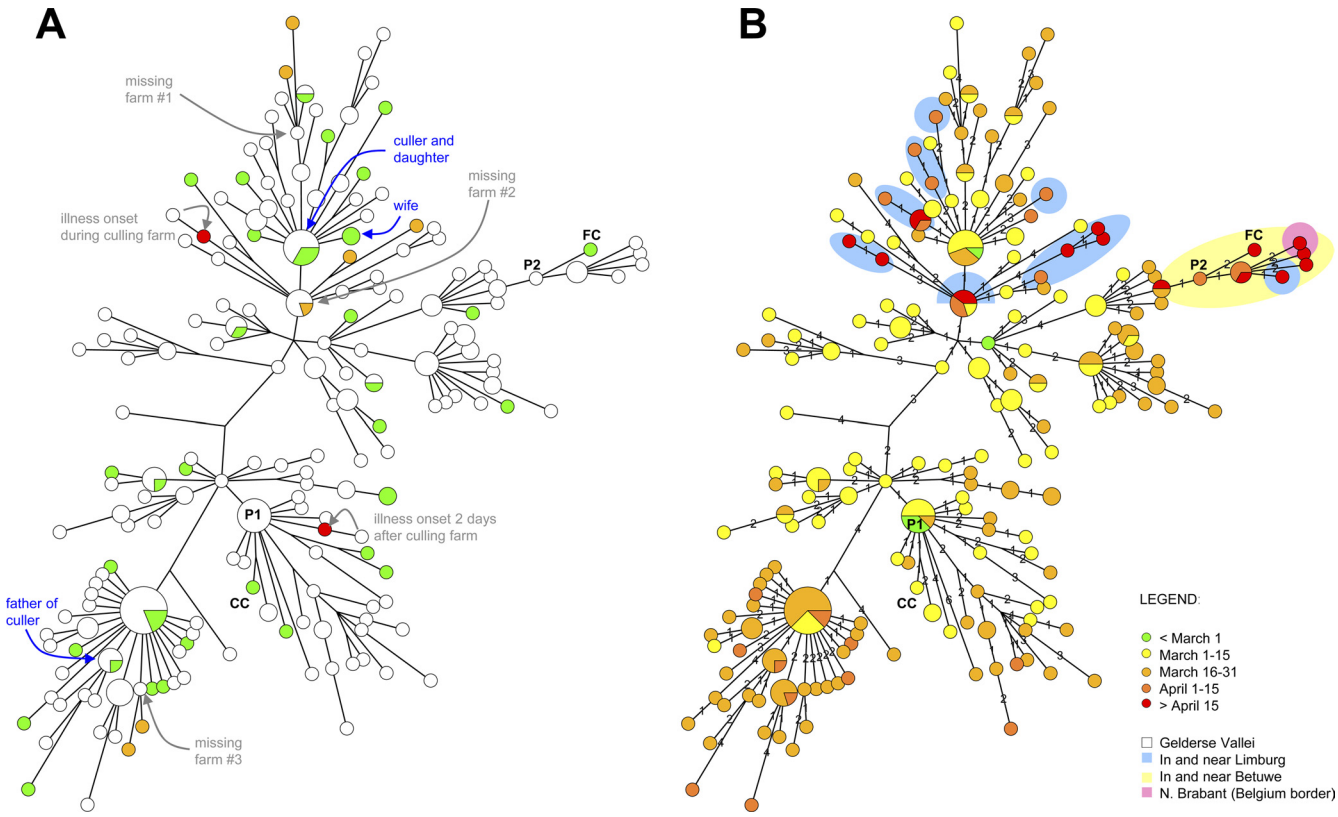

FIG. 1. Transmission network built with concatenated full-length HA, NA, and PB2 gene segments of the A (H7N7) virus outbreak, illustrating the position of 45 human A (H7N7) virus infections (colored) in relation to 215 A (H7N7) virus-infected farms (white) (A) and the nucleotide differences between the A (H7N7) sequences supplemented by information on sample period and farm location (B). (A) Human A (H7N7) virus infections in agreement with epidemiologic data are shown in green. The positions of the three secondary human cases within the network are indicated in blue. Human infections ($n = 7$) with onset of illness before their avian sources were estimated to be infected are indicators for missing poultry data (yellow). Two human viruses within the transmission network could not have caused human-to-avian transmissions based on infection dates (red). (B) After notification of the A (H7N7) outbreak on 28 February 2003, multiple poultry A (H7N7) virus clusters and chains of transmission emerged that spread over time (colored circles) and location (colored areas). The locations of human cases refer to the locations of their suspected avian source of infection. The nucleotide changes between specific A (H7N7) virus strains are shown on the branches. CC, A/Netherlands/033/03, FC, A/Netherlands/219/03; P1, A/chicken/Netherlands/01/03; P2, A/chicken/Netherlands/03010132/03.

more, two human cases with unique virus sequences were found within the transmission network (Fig. 1A, red dots). Although these could represent human-to-avian transmissions, the infection chronology demonstrated avian-to-human transmission. A phylogenetic tree constructed with neighbor joining and representative A (H7N7) virus isolates demonstrated identical A (H7N7) virus relations (see Fig. S1 in the supplemental material).

Acquisition of human adaptation markers in humans. The observed A (H7N7) virus diversity among viruses in poultry could mask the occurrence of possible human adaptation during infection of humans when comparing grouped human and avian sequences. Therefore, we assessed which differences were observed between A (H7N7) sequences from human influenza isolates with those from their most likely source, based on the transmission network. A total of 37 amino acid substitutions were identified between human A (H7N7) viruses and their avian source of infection (HA [$n = 11$], NA [$n = 14$], and PB2 [$n = 12$]) (Table 1). Three of these mutations (HA V223A, HA V223I, and PB2 N711K) were detected in both the virus isolate and the corresponding human clinical specimen, while the presence of the remaining 34 mutations could not be confirmed by direct sequence analysis because there were no leftover clinical specimens in our biobank, the viral load was too low, or the site was not covered by direct sequencing.

Selection pressure analysis, using the combined avian and human sequence data set to determine whether the potential human adaptation markers presented in Table 1 emerged under (host) selection pressure, only recognized HA codon 223 as a positive selection site (REL method; Bayes factor, 1.72E+08). Comparison of human A (H7N7) virus sequences with previously identified host-specific residues (11, 28, 30) detected PB2 mutation D567N in addition to the human adaptation marker PB2 E627K, which was identified in the fatal human case.

During the A (H7N7) virus outbreak, the neuraminidase inhibitor oseltamivir was used for antiviral treatment and prophylaxis of A (H7N7) virus infection. Human influenza virus isolates ($n = 15$) with and without NA mutations compared to their avian source of infection were screened for emergence of oseltamivir resistance, but no resistance or reduced susceptibility was observed (mean 50% inhibitory concentration [IC_{50}], 0.46 nM; range, 0.12 to 0.70 nM). Moreover, one human A

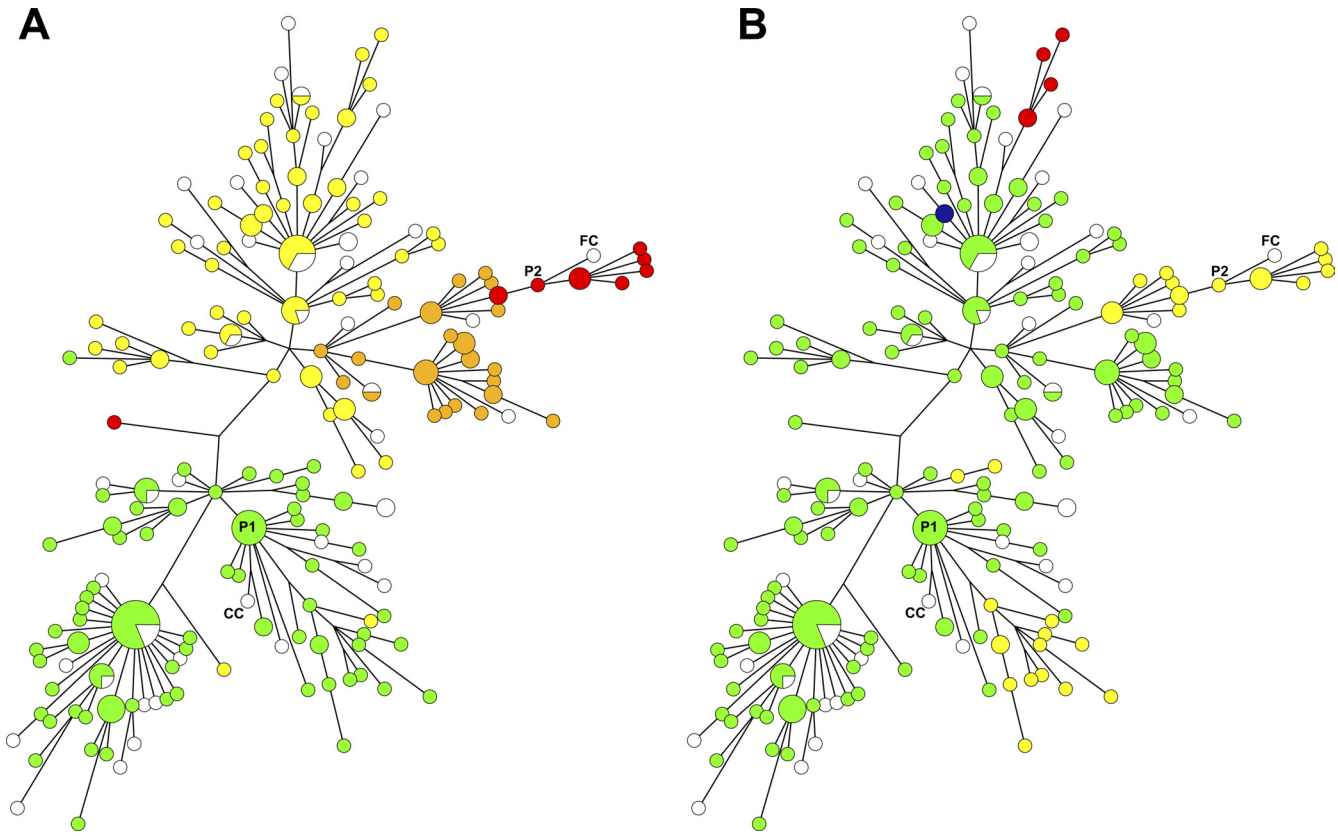

FIG. 2. The accumulation of virulence and human adaptation markers in poultry farms (green) per gene segment, illustrating the accumulation of NA mutations T442A and P458S (yellow), T442A, P458S plus A346V (orange) and T442A, P458S, A346V plus N308S (red) (A) and independent emergence of HA A143T variants (yellow) and emergence of PB2 D701N (red) and PB2 S714I (blue) (B). Human viruses are represented by open circles. CC, A/Netherlands/033/03; FC, A/Netherlands/219/03; P1, A/chicken/Netherlands/01/03; P2, A/chicken/Netherlands/03010132/03.

(H7N7) isolate with NA mutations N67S, V115I, and I275T displayed 4-fold-enhanced sensitivity against oseltamivir compared with the mean (IC_{50}, 0.12 nM).

Detection of potential human adaptation markers in poultry. Based on a selection of positions that are potentially relevant for human adaptation of the avian virus, we detected variations in PB2 codons 292, 567, 701, and 714 of poultry A (H7N7) viruses that were previously identified to be host-specific residues (11, 28, 30). Mutation I292V is not associated with host adaptation but represents natural variation within avian sequences (28). Mutation D567Y is not a known human adaptation marker but is present in A/swine/Shizuoka/120/97 (H3N2) (GenBank). Mutation D701N was previously associated with efficient influenza virus replication and transmission in mammalian species, similar to PB2 E627K (12, 19). This mutation did emerge and subsequently spread to four poultry farms. PB2 mutation S714I is associated with increased pathogenicity for mammals and emerged in a chicken farm before it spread to one of the cullers and another chicken farm (19).

We also tracked the accumulation of mutations leading up to the fatal human infection, previously described by de Wit and colleagues (13). Their study identified substitutions HA A143T and PB2 E627K, attributable to increased pathogenicity in the mouse model, in addition to four replication-enhancing NA substitutions. Mapping of markers in farm sequence data shows the emergence and spread of these mutations during the A (H7N7) virus outbreak in poultry (Fig. 2). HA substitution A143T emerged during the outbreak at three different chicken farms and spread to subsequent farms. For two of these farms, this mutation emerged in the first week after the outbreak was identified and authorities were notified. In addition to the virus isolated from the fatal A (H7N7) case, a 49-year-old owner of a layer farm who developed conjunctivitis without fever appeared to be infected with virus variant HA A143T. He was infected by an A (H7N7) virus identical to the virus variant HA A143T obtained from his 17,500-layer hen farm, except for one silent PB2 nucleotide substitution. The accumulation of NA mutations T442A and P458S (73 farms) supplemented by NA A346V (30 farms) and NA N308S (11 farms) in poultry eventually gave rise to an A (H7N7) variant with enhanced NA activity that was transmitted to the fatal human case (13). The PB2 E627K mutation detected in the virus isolated from the fatal A (H7N7) case was not detected in poultry.

DISCUSSION

In contrast to the ongoing outbreak of HPAI A (H5N1), in which human infections have occurred as isolated cases or in a small cluster ($n < 8$), the HPAI A (H7N7) outbreak that struck the Netherlands in 2003 infected at least 89 humans, including

three secondary cases and one death (18, 46). The culling of >30 million birds combined with control measures did not prevent A (H7N7) spread to a total of 255 poultry farms in the Netherlands and 9 farms in the neighboring countries of Belgium ($n = 8$) and Germany ($n = 1$) (8, 47). By comprehensive virologic analysis of combined veterinary and human A (H7N7) samples obtained during this large avian influenza outbreak, this study provides unique knowledge on the acquisition of human adaptation markers and the public health risk associated with an HPAI virus outbreak in poultry. We demonstrated the polygenic accumulation and farm-to-farm spread of known virulence and human adaptation markers in A (H7N7)-infected poultry farms following farm-to-human transmission. These include the independent emergence of HA mutants with increased replication kinetics, accumulation of NA mutations facilitating efficient release of virus particles from the host cell, and farm-to-farm spread of virus variants harboring mammalian host determinant D701N and S714I in PB2 (13, 44). The emergence of PB2 E627K detected in the virus obtained from the fatal case could not be assigned to the avian source of infection and might have emerged in the human host. Similarly, PB2 mutation D567N and HA V223I/A in the RBS were detected in human A (H7N7) viruses only.

Sequence analysis of A (H7N7) viruses obtained from human cases was performed on all available clinical samples and virus isolates. For 26 cases, both a clinical sample and virus isolate were present, allowing comparison of direct sequencing results with full-length sequences obtained from virus isolates (Table 1). This demonstrated an absence of virus adaptation to MDCK-I cells within the regions sequenced. Moreover, HA RBS sequences were preserved during virus isolation using MDCK-I cells. Since the RBS is considered to be one of the most variable regions of the influenza virus and prone to host cell adaptation, we reasoned that molecular variation shown in Table 1 is the result of human adaptation. We cannot, however, fully exclude that some variation was MDCK culture induced. Nonetheless, human adaptation markers PB2 S714I and HA V223I/A were confirmed by direct sequencing.

Although popular phylogenetic analysis uses parametric techniques of maximum likelihood and Bayesian Markov chain Monte Carlo methods, such results can become inconsistent when evolutionary rates vary. Especially when using short time scales, e.g., during local virus outbreaks, the combination of rapidly evolving viruses with nonevolving variants challenges the molecular clock model, resulting in statistically inconsistent results (25). Because maximum parsimony analysis does not assume a specific distribution and is best suited for analyzing sequences that are quite similar, this simple approach is the method of choice for analyzing defined virus outbreaks, such as the HPAI A (H7N7) virus outbreak. By addition of statistical parameters, like the global cophenetic correlation coefficient, bootstrap, and permutation resampling support, the potential avian sources of human A (H7N7) virus infection could be identified with high significance (38, 42).

Genetic variation was not associated with a diagnostic delay, as the difference between the first day of illness and day of consultation was, on average, 1.3 days for patients infected with either identical viruses ($n = 14$), infected with viruses with synonymous mutations ($n = 8$), or infected with viruses with nonsynonymous mutations ($n = 23$). Furthermore, one A (H7N7) virus obtained from a culler was detected in his daughter (100% identical) and wife (1 synonymous substitution, 2 days later). From the wife and a culler, influenza A (H7N7) virus isolates from eye and throat swabs were available for sequence analysis. Sequence comparison showed no variation between viruses obtained from eye or throat. The latter can be explained by replication-independent spread of virus from the eye to the throat (3, 4). The above illustrates limited A (H7N7) virus adaptation during human infection, probably hampered by suboptimal virus replication in the human eye combined with oseltamivir treatment. However, the prolonged 24-day A (H7N7) course of illness of the veterinarian that eventually died could have facilitated virus adaptation to humans by means of the mutation PB2 E627K. The phenotype of virus variant PB2 E627K underlines the importance of the viral RNA replication complex (NP, PA, PB1, and PB2) in human adaptation of avian influenza viruses (13, 16). Available PA sequences from the A (H7N7) outbreak revealed amino acid variation of PA codon 666 (13). Although NP and PB1 amino acid sequences were identical, variation (L62P) was observed within the full-length PB1-F2 proteins. Interestingly, this PB1-F2 variant was present in the A (H7N7) virus obtained from the culler and it subsequently spread to both his wife and daughter. However, experiments are needed to characterize the effect of PB1-F2 sequence variation on viral pathogenesis.

The HPAI A (H7N7) virus outbreak was most probably initiated by the introduction of an LPAI virus in poultry, following multiple weeks of silent replication and spread until it was identified and authorities were notified at the first poultry farm on 28 February 2003 (1, 18). The HPAI A (H7N7) virus subsequently infected 255 poultry farms in the Netherlands and 8 poultry farms in Belgium (47). Of interest is the long farm-to-farm transmission distance of the A (H7N7) variant harboring the four NA substitutions N308S, A346V, T442A, and P458S, which spread on average >10 km (Fig. 1B). These variants initiated an A (H7N7) outbreak in Belgium before reemerging in a hobby farm in the southern part of the Netherlands (47). A similar observation was made by Shi and colleagues, who associated NA mutations with rapid geographical spread of avian influenza A (H9N2) virus in China (41). The exceptional level of human exposure resulting from active culling and screening activities over a wide geographical region during 2.5 months resulted in 89 human A (H7N7) cases. Our results demonstrate that viruses obtained from human A (H7N7) conjunctivitis cases reflect the virus diversity generated in poultry. Of greater concern, we observed the polygenic accumulation and spread of known virulence and human adaptation markers in A (H7N7) virus-infected poultry farms following farm-to-human transmission. This implies that HPAI viruses with pandemic potential can emerge directly from poultry without the need to adapt in the human host. The dynamics of emerging virus variants in poultry with increased virulence and enhanced transmission characteristics provide a challenge during culling activities. Since the public health risk of an avian influenza virus outbreak in poultry can rapidly change, we recommend virologic monitoring of poultry in addition to humans during the course of an outbreak in poultry.

Although the emergence and spread of viruses with multiple potential human adaptation mutations were detected in poultry, the impact of detecting specific mutations for public health

risk assessment is uncertain. Nonetheless, this study suggests that the effective control of influenza virus in poultry can prevent avian influenza viruses from acquiring all their prerequisites to become human tropic, in addition to the reduction of human exposure (39). Increased awareness by veterinary and medical authorities is needed to design more efficient surveillance, diagnostic algorithms, and prepandemic planning strategies.

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