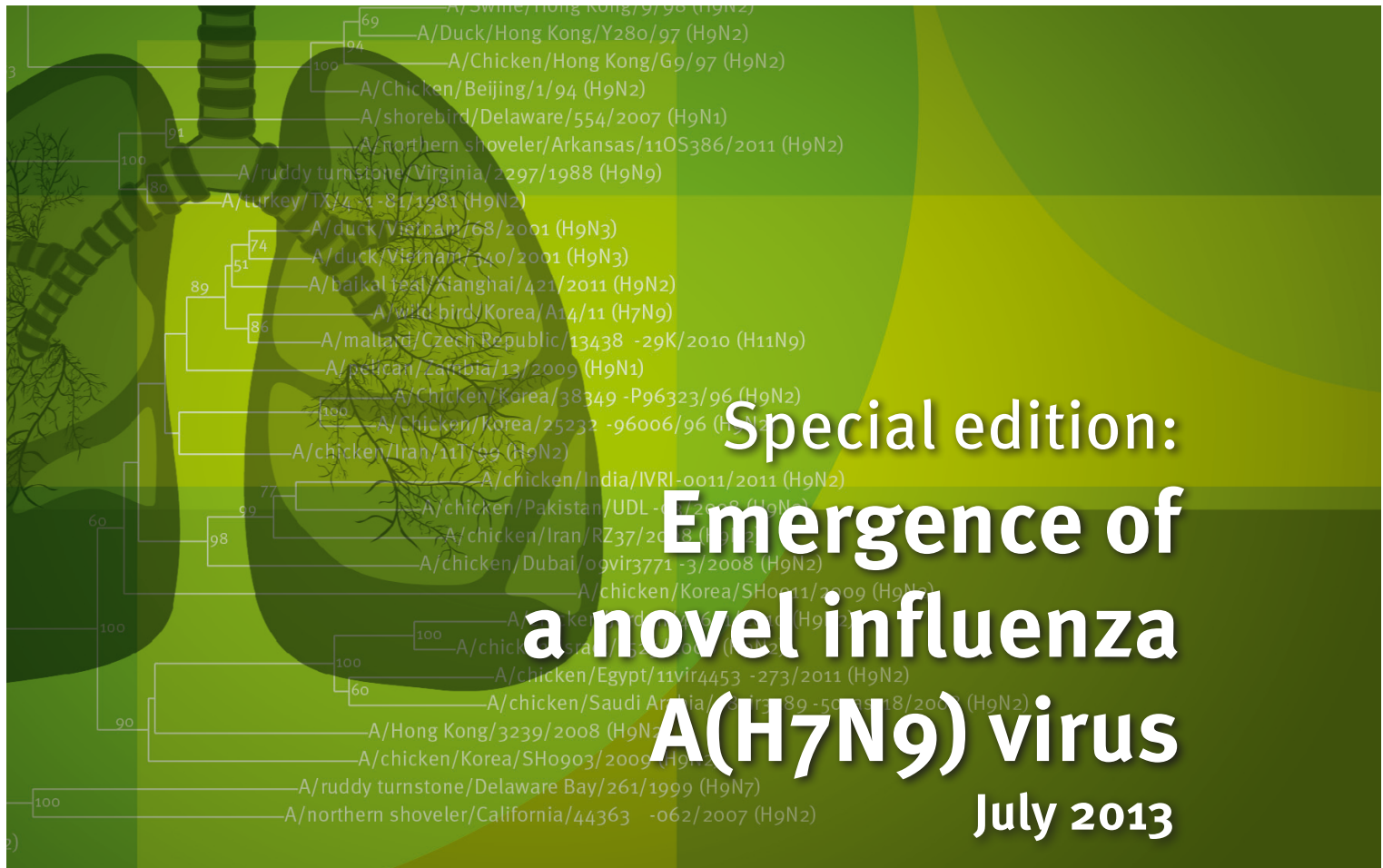


Impact factor **5.49**



Eurosurveillance

Europe's journal on infectious disease epidemiology, prevention and control



Special edition: Emergence of a novel influenza A(H7N9) virus July 2013

- This issue presents papers on early epidemiological and virological aspects concerning the novel reassortant avian influenza A(H7N9) virus causing human disease in China in 2013 and the implications for public health.



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Illustration of lungs, phylogenetic tree

A novel reassortant avian influenza A(H7N9) virus in China – what are the implications for Europe

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Citation style for this article:

Nicoll A, Danielsson N. A novel reassortant avian influenza A(H7N9) virus in China – what are the implications for Europe. *Euro Surveill.* 2013;18(15):pii=20452. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2045>

Article submitted on 10 April 2013 / published on 11 April 2013

As of 10 April 2013, 33 human cases infected with a novel influenza A(H7N9) virus have been laboratory confirmed in Shanghai, Anhui, Jiangsu and Zhejiang provinces in China (Figure 1). This case count came after on 31 March 2013, the Chinese authorities had announced the identification of a novel influenza A virus, an A(H7H9) virus, in three people in Shanghai and Anhui province. Two men in Shanghai, 87 and 27 years old, respectively, had become ill with influenza-like (ILI) symptoms and progressed to severe lower respiratory tract infections within a week in mid to late February, and died from acute respiratory distress syndrome hereafter [1,2]. The two had no epidemiological link and no known exposure to evidently sick animals. One of them was a pork butcher. The third case was a 35-year-old woman from Anhui province, adjacent to Shanghai, who also became ill with ILI with symptom onset on 9 March followed by severe respiratory disease and death.

The detection of these cases was possible because of a well-functioning surveillance system with a laboratory component through which the initially non-subtypeable influenza A viruses were sent to the World Health Organization (WHO) Influenza Collaborating Centre at the Chinese Center for Disease Control and Prevention (CDC) in Beijing for sequencing. Upon laboratory identification of the new viruses, the responsible Chinese authorities notified the cases as required in the International Health Regulations (IHR) to WHO and other member states [3].

Moreover, researchers from the Chinese CDC posted the genetic information of the viruses on the publicly accessible GISAID website [4]. The viruses were not genetically identical, indicating they had been circulating for some time over a wide region [5]. The same type of viruses were reported by Chinese veterinary authorities from 4 April onward in different species of poultry and environmental samples from live bird markets in Shanghai [6]. The sequences of the veterinary and environmental specimens were also posted on the GISAID site by the Chinese national veterinary laboratory in Harbin [3].

Following the detection of the first cases, the Chinese CDC has rapidly made specific polymerase chain reaction (PCR) test kits for the new A(H7N9) viruses available to provincial and local laboratories across China to ensure timely testing of suspected cases. Since then individual human cases are being confirmed and made public daily by the Chinese authorities at provincial level in the four affected provinces. More cases are being detected with onset dates since late March (Figure 2). While this could simply reflect increasing awareness among clinicians and public health authorities and that testing became available more widely, close monitoring is necessary to detect changes in transmission patterns, especially human-to-human transmission and cases appearing in China beyond the four provinces.

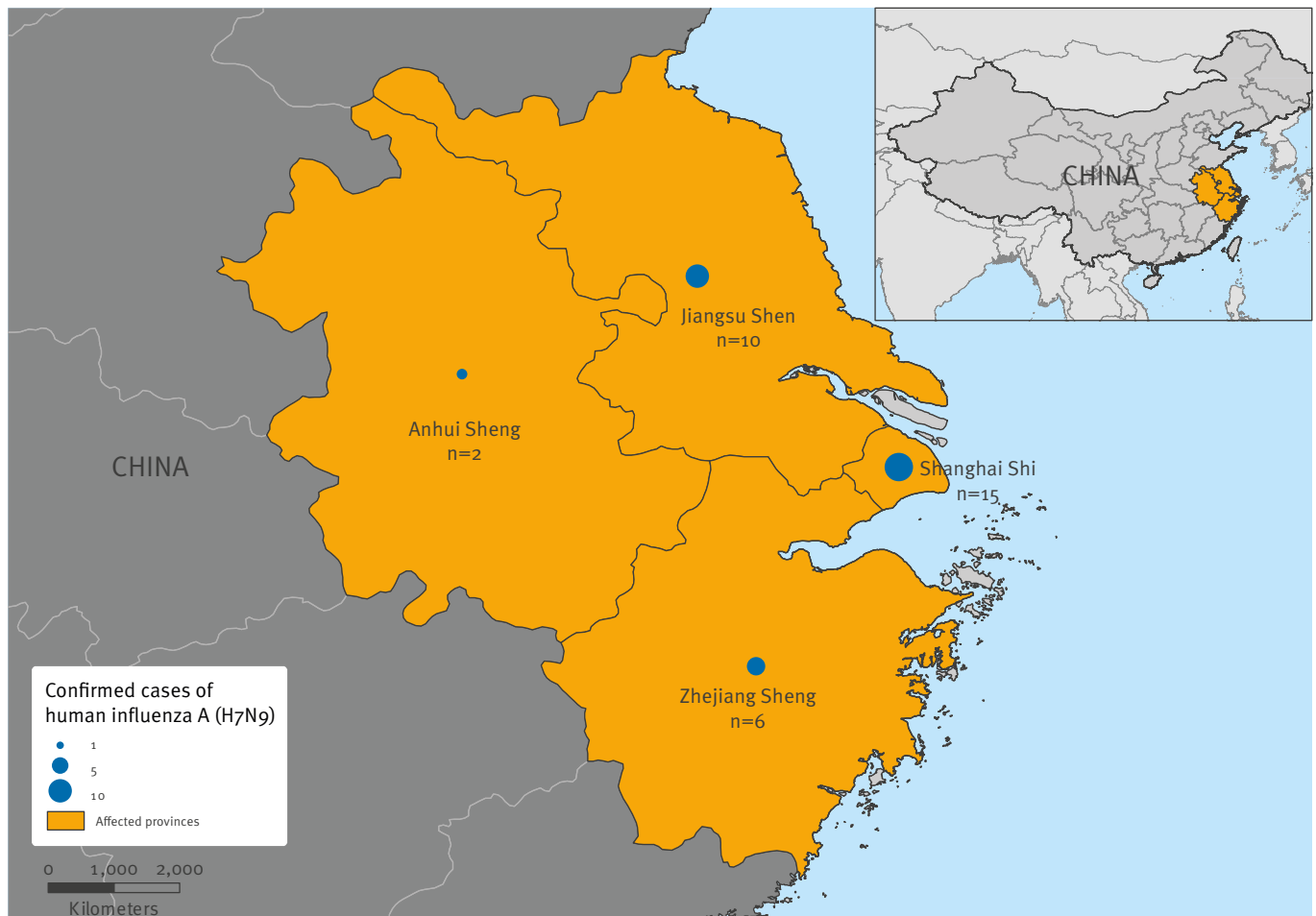
While the novel A(H7N9) virus has been detected in birds and environmental specimens at a bird markets in Shanghai and the other affected provinces, the source of infection in most of the cases still remains to be determined [6]. It is equally unclear how the virus is introduced into the markets. Nevertheless, China has stepped up vigilance and intensified human and animal surveillance [7]. It has also implemented public health measures that include the closure of some live poultry and bird markets and culling of birds [8].

A striking feature is that human cases are sporadic and very few possible clusters have been detected. They are being investigated by the Chinese authorities. So far, there has been no documented sustained human-to-human transmission and there is no clear indication of such transmission even though the virus has genetic markers that are known to be associated with improved replication of avian influenza viruses in mammals [4,5].

When compared with A(H5N1) viruses, animal-to-human transmissibility seems to be higher for influenza A(H7N9). It is noteworthy that the timeframe during which cases have been identified is very different from that of human cases of influenza A(H5N1) detected in China of late. Between January 2010 and March 2013, only seven human A(H5N1) cases were reported, five

FIGURE 1

Laboratory-confirmed cases of human influenza A(H7N9) in China as of 10 April 2013 (n=33)



Source: European Centre for Disease Prevention and Control (ECDC).

of which are known to have died [9]. Few human cases due to infection with avian influenza A(H7) viruses have been described in the literature, possibly because the symptoms are usually mild in humans and of low pathogenicity in poultry [10]. A well described outbreak involving humans was that of a highly pathogenic avian influenza A(H7N7) among poultry in the Netherlands in 2003. It resulted in 86 mild infections, mainly conjunctivitis, among poultry workers, three cases of non-sustained human-to-human transmission among their household contacts, but only one fatality [11,12].

Only careful serological surveys in China can reveal if there were such transmissions and these investigations are underway. Of the detected 33 human A(H7N9) cases as of 10 April, 30 developed severe illness with nine fatalities while three presented with mild symptoms (Figure 2). It can be expected that surveillance activities will lead to detection of additional cases in the coming weeks, but so far no cases have been identified outside the four Chinese Provinces.

A limited number of scenarios that could follow from the emergence of this novel virus are possible. The one that explains the current human and animal epidemiological situation best, based on available clinical and virological analyses, is that of the emergence of a novel reassortant avian influenza virus of low pathogenicity to birds but of significant pathogenicity to humans. This virus has probably spread undetected among poultry in parts of eastern China. When this started is unclear. It only came to light because some people infected through contact with birds or environmental exposure, became severely ill. Even though the viruses were found in poultry and the environment in live bird markets in Shanghai, the species introducing the infection into the markets has not been identified. The various species reported as being infected may have only become infected at the markets.

The speed, transparency and intensity of the work performed in respect to the novel A(H7N9) virus in China and by the Chinese CDC and veterinary authorities is

impressive and deserves full credit [13]. It also has to be acknowledged that there is tremendous value for all those concerned with public health in that the WHO Collaborating Centre for Influenza at the Chinese Center for Disease Control and Prevention has shared the viruses and that the molecular data have been published on the publicly accessible GISAID database. This data sharing platform has been important for scientists to gain important insight into the molecular virus characteristics and the origins of the virus as well as for public health experts to assess the current situation.

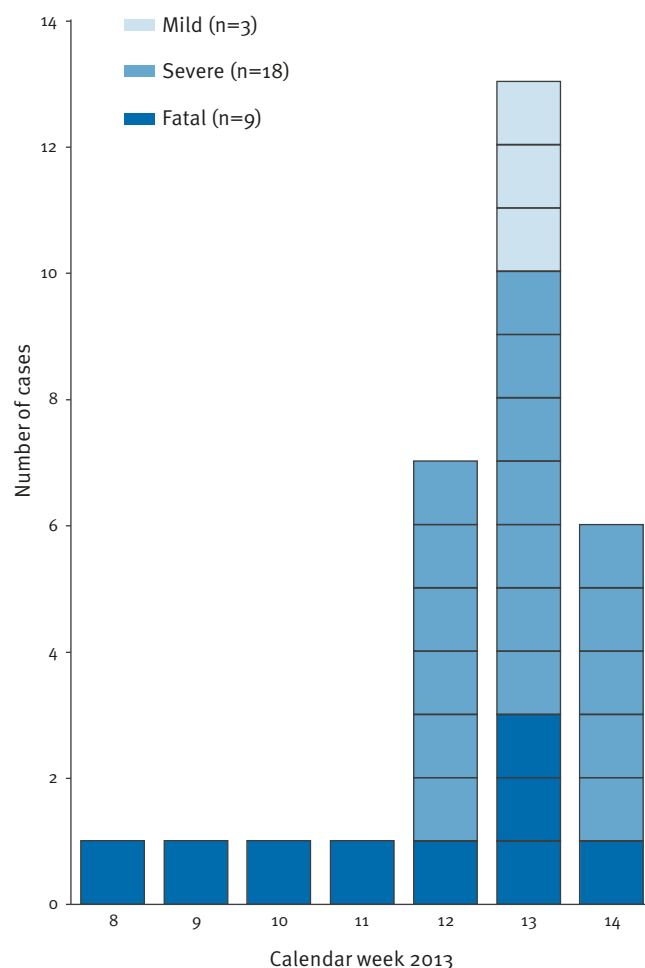
However, the tasks lying ahead, namely analysing, describing and especially controlling the virus cannot be underestimated. The extent of distribution of this A(H7N9) virus in domestic poultry in China and possibly other countries is unclear and surveillance and control of a low pathogenicity avian influenza virus in countries with complex mixes of informal and formal poultry sectors will be challenging. The markers of poultry die-offs seen with high pathogenicity avian influenza A viruses such as H5N1 and H7N7, will not signal the presence of the new A(H7N9) virus. In such situations, animal surveillance on the basis of sampling of live birds, including wild birds, such as done in Hong Kong and in European Union (EU) countries will be essential [14,15].

What are the possible implications of the current situation for Europe and European citizens and which actions should the EU take and which ones have been taken already? The European Centre for Disease Prevention and Control (ECDC) published its first risk assessment on 3 April and is providing updated assessments and short reports on the epidemiology as new information emerges [16]. Several guidance documents on prevention of infections, infection control and case management developed earlier for influenza A(H1N5) by ECDC, WHO and Member States are, with some modifications, applicable to the current situation [16-18]. Visitors to China and other countries where avian influenzas have caused severe human disease of late [9], should avoid visiting bird markets and follow basic hygienic measures. Persons returning from China who develop severe respiratory infection within 10 days should be evaluated and tested for the new virus to rule out such infection [17], though most likely another infection will be detected. Case management and infection control guidelines for A(H5N1) apply in the short term. This will include antiviral treatment given that the Chinese CDC promptly established that the A(H7N9)viruses are susceptible to neuraminidase inhibitors [4,5].

There is a standing procedure in place in Europe to send all non-subtypeable influenza A viruses isolated from humans promptly to the WHO Collaborating Center in London for further analysis. Notwithstanding this, ECDC, the WHO Regional Office for Europe, the WHO Influenza Collaborating Centre, the University of Bonn and the Community Network Reference Laboratories are working in together to make testing for A(H7N9)

FIGURE 2

Laboratory-confirmed cases of human influenza A(H7N9) by week of symptom onset and severity as of 10 April, China March–April 2013 (n=30)



Three patients with unknown dates of symptom onset not included. Source: European Centre for Disease Prevention and Control (ECDC).

possible in all National Influenza Centres in Europe as soon as possible.

Some candidate H7 and H9 vaccines viruses already exist under WHO's strain selection system for the eventuality of an emerging virus [19]. They may not be effective against the new influenza A(H7N9) virus and once the regulatory laboratories have obtained the novel virus, WHO and presumably EU authorities will now need to consider if they wish to proceed with the very early stages of vaccine development as has been done for the candidate H7 and H9 viruses.

Overall, how concerned Europe should be cannot yet be determined. The new virus is a reassortant virus based on an haemagglutinin antigen A(H7) to which

most humans will not have been exposed. Therefore, if human-to-human transmission starts, and that is only an 'if', population immunity cannot be presumed. It would have to be assessed now by determining age-specific sero-reactivity of human sera to this influenza A(H7N9) virus as a priority. Immunity, or lack of it, in the human population are key data required for assessing pandemic risk. As stated above, they needed to come from field investigations in China as well as seroepidemiological studies in Europe based on protocols developed precisely for such situations [20].

At this very moment it cannot be ruled out that there are some human-to-human transmissions causing mild or asymptomatic infections as happened in the Netherlands in 2003. It also remains unclear to what extent the predominance of severe disease may represent a bias because mainly people with severe disease are tested. Investigations of patients' contacts including serological studies, will clarify this point. Such investigations orchestrated by the Chinese CDC are underway.

There will be many other calls for research and it will be important and difficult to prioritise. Fortunately a framework exists for making decisions on priorities. The Influenza Risk Assessment Tool (IRAT) has been developed since 2011 for this purpose by the United States (US) Centers for Disease Control and Prevention with some international partners [21,22]. It looks at 10 parameters bundled into three families: properties of the virus, attributes of the population, ecology and epidemiology. It has already been deployed to inform US decisions on the A(H3N2)v vaccines. It does not predict pandemic risk or make decisions but it informs decisions. Though the IRAT is still being evaluated as a tool it will certainly indicate what should be some of the most important public health research priorities for A(H7N9).

It is also important that the sequence and virological analyses are considered in combination with the epidemiological findings. Despite the virological markers described in the recent report from the WHO Collaborating Centres [5] it should not be seen as inevitable on the longer term that this reassortant A(H7N9) will develop efficient human-to-human transmissibility or become established in Europe, though both should be kept in mind as possibilities. Neither has happened for the highly pathogenic influenza A(H5N1) virus in the decade and a half since its emergence in China in 1996 [23]. Despite multiple detections of the A(H5N1) virus in wild birds and some outbreaks in domestic poultry flocks in Europe, the high levels of biosafety in the EU have not permitted A(H5N1) viruses to become established in European domestic poultry. It is fortunate that the European Commission and the Member States have since 2007 established surveillance for low pathogenicity avian influenza in domestic and wild birds in Europe [14]. The recent events have underlined the importance of this system.

Acknowledgements

The authors acknowledge that some of these analyses have been possible using the virological and genetic molecular data provided in the publicly accessible GISAID database by the WHO Collaborating Centre for Influenza at the Chinese Center for Disease Control and Prevention.

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Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013

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Citation style for this article:

Kageyama T, Fujisaki S, Takashita E, Xu H, Yamada S, Uchida Y, Neumann G, Saito T, Kawaoka Y, Tashiro M. Genetic analysis of novel avian A(H7N9) influenza viruses isolated from patients in China, February to April 2013. *Euro Surveill.* 2013;18(15):pii=20453. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20453>

Article submitted on 08 April 2013 / published on 12 April 2013

Novel influenza viruses of the H7N9 subtype have infected 33 and killed nine people in China as of 10 April 2013. Their haemagglutinin (HA) and neuraminidase genes probably originated from Eurasian avian influenza viruses; the remaining genes are closely related to avian H9N2 influenza viruses. Several characteristic amino acid changes in HA and the PB2 RNA polymerase subunit probably facilitate binding to human-type receptors and efficient replication in mammals, respectively, highlighting the pandemic potential of the novel viruses.

Humans are rarely infected with avian influenza viruses, with the exception of highly pathogenic avian influenza A(H5N1) viruses, which have caused 634 infections and 371 deaths as of 12 March 2013 [1]. A few isolated cases of human infection with viruses of the H7N2, H7N3, and H7N5 subtypes have been reported, but none were fatal [2-11]. In 2003, in the Netherlands, 89 people were infected with an influenza virus of the H7N7 subtype that caused conjunctivitis and one fatality [5,7].

On 19 February 2013, an 87 year-old man in Shanghai developed a respiratory infection and died on 4 March, and on 27 February 2013, a 27 year-old pork seller in a Shanghai market became ill and died on 10 March. A 35 year-old woman in Chuzhou City in Anhui province (west of Shanghai), who had contact with poultry, became ill on 15 March 2013, and remains hospitalised in critical condition. There is no known epidemiological relationship among these three cases. A 38 year-old man in Hangzhou (Zhejiang province, south of

Shanghai) became ill on 7 March 2013 and died on 27 March. All four cases presented with respiratory infections that progressed to severe pneumonia and breathing difficulties.

On 31 March 2013, the Chinese Centre for Disease Control and Prevention announced the isolation in embryonated eggs of avian influenza viruses of the H7N9 subtype (designated A/Shanghai/1/2013, A/Shanghai/2/2013, and A/Anhui/1/2013) from the first three cases. The sequences of the coding regions of all eight viral genes were deposited in the influenza sequence database of the Global Initiative on Sharing All Influenza Data (GISAID) on 31 March (Table 1). On 5 April 2013, the Hangzhou Center for Disease Control and Prevention deposited the haemagglutinin (HA), neuraminidase (NA), and matrix (M) gene sequences of A/Hongzhou/1/2013 virus (Table 1), which was isolated in cell culture from samples obtained from the 38 year-old man.

All four human influenza A(H7N9) viruses are similar at the nucleotide and amino acid levels, suggesting a common ancestor. The HA gene of the novel viruses belongs to the Eurasian lineage of avian influenza viruses and shares ca. 95% identity with the HA genes of low pathogenic avian influenza A(H7N3) viruses isolated in 2011 in Zhejiang province (south of Shanghai) (Figure 1, Table 2). The NA gene of the novel viruses is ca. 96% identical to the low pathogenic avian influenza A(H11N9) viruses isolated in 2010 in the Czech Republic (Figure 1, Table 2).

TABLE 1

Origin of influenza A(H7N9) isolates included in the phylogenetic analysis, China, February–April 2013 (n=7)

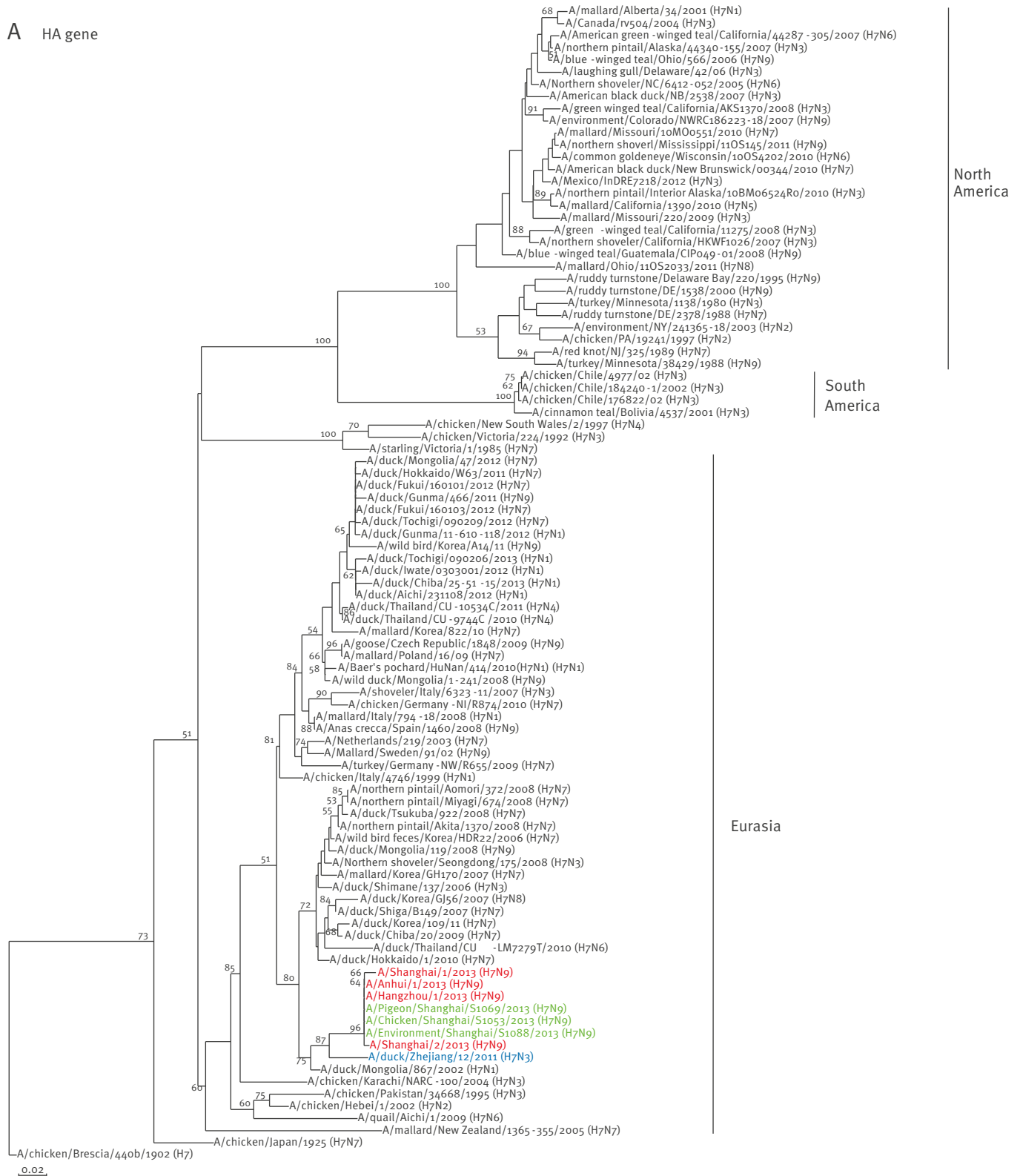
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EPI439489	PB1					
EPI439490	PA					
EPI439486	HA					
EPI439491	NP					
EPI439487	NA					
EPI439493	M					
EPI439494	NS					
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EPI439501	PB1					
EPI439498	PA					
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EPI439496	NP					
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EPI439497	M					
EPI439499	NS					
EPI439504	PB2	A/Anhui/1/2013	2013	-	WHO Chinese National Influenza Center	Lei Yang
EPI439508	PB1					
EPI439503	PA					
EPI439507	HA					
EPI439505	NP					
EPI439509	NA					
EPI439506	M					
EPI439510	NS					
EPI440095	HA	A/Hangzhou/1/2013	2013-03-24	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li,J; Pan,JC; Pu,XY; Yu,XF; Kou,Y; Zhou,YY
EPI440096	NA					
EPI440097	M					
EPI440682	PB2	A/Chicken/Shanghai /S1053/2013	2013-04-03	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Huihui Kong
EPI440683	PB1					
EPI440681	PA					
EPI440685	HA					
EPI440678	NP					
EPI440684	NA					
EPI440680	M					
EPI440679	NS					
EPI440690	PB2	A/Environment/ Shanghai /S1088/2013	2013-04-03	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Huihui Kong
EPI440691	PB1					
EPI440689	PA					
EPI440693	HA					
EPI440686	NP					
EPI440692	NA					
EPI440688	M					
EPI440687	NS					
EPI440698	PB2	A/Pigeon/Shanghai /S1069/2013	2013-04-02	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	Huihui Kong
EPI440699	PB1					
EPI440697	PA					
EPI440701	HA					
EPI440694	NP					
EPI440700	NA					
EPI440696	M					
EPI440695	NS					

We gratefully acknowledge the authors and laboratories for originating and submitting these sequences to the EpiFlu database of the Global Initiative on Sharing All Influenza Data (GISAID); these sequences were the basis for the research presented here.

All submitters of data may be contacted directly via the GISAID website www.gisaid.org

FIGURE 1

Phylogenetic analysis of the haemagglutinin (A) and neuraminidase (B) genes of the novel influenza A(H7N9) viruses, China, February–April 2013 (n=7)



HA: haemagglutinin; NA: neuraminidase.

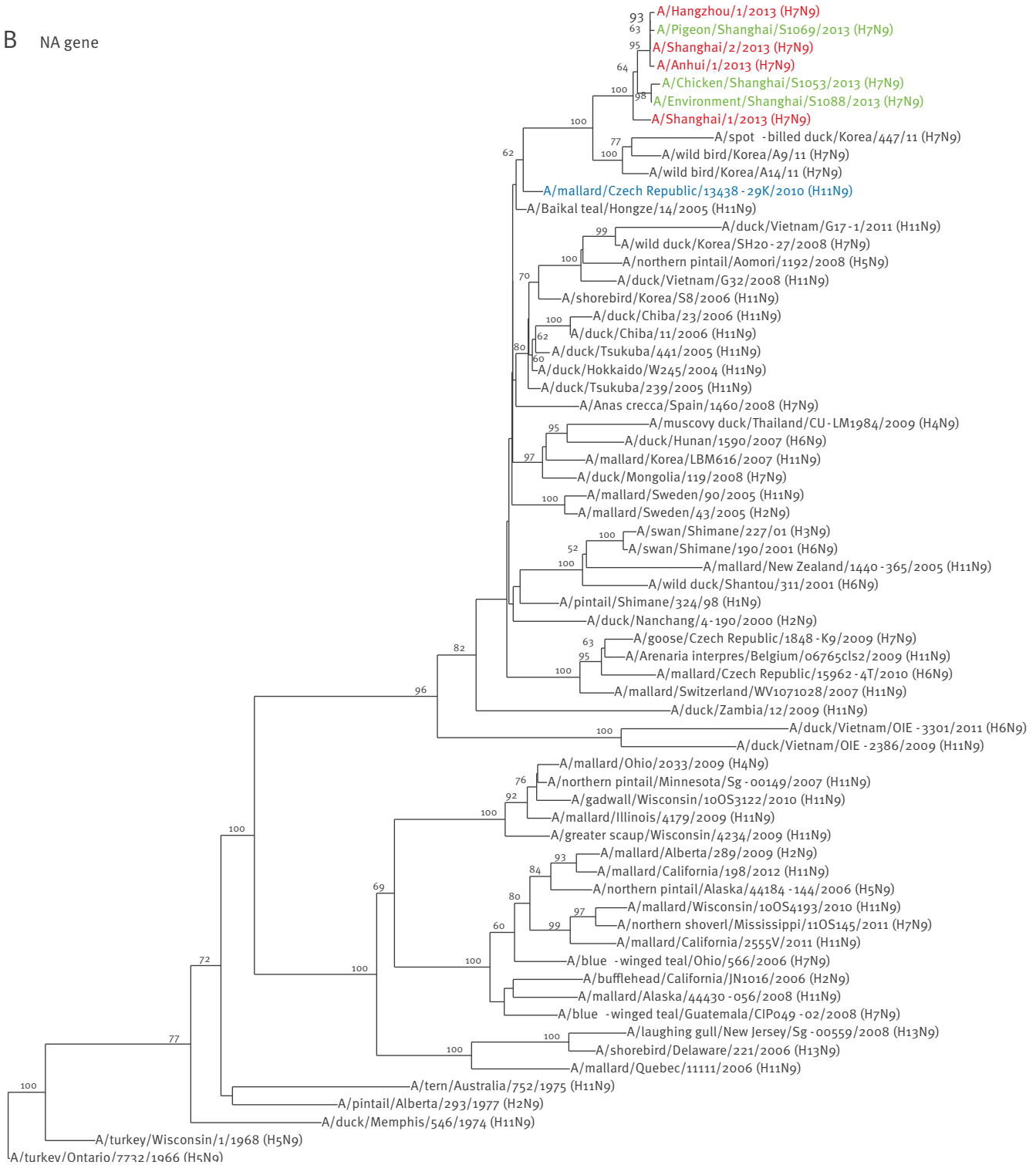
Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

Novel human H7N9 viruses are shown in red; novel H7N9 viruses from birds and the environment are shown in green; viruses with the highest similarities to the novel viruses are shown in blue. The HA clade names, North America, South America, and Eurasia, are based on epidemiological studies of H7 viruses [27,28].

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B NA gene



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TABLE 2

Nucleotide identity of novel influenza A(H7N9) virus genes and their closest relative, China, February–April 2013

Viral gene	Closest influenza virus relative	Nucleotide identity (%)
PB2	A/brambling/Beijing/16/2012(H9N2)	99
PB1	A/chicken/Jiangsu/Q3/2010(H9N2)	98
PA	A/brambling/Beijing/16/2012(H9N2)	99
HA	A/duck/Zhejiang/12/2011(H7N3)	95
NP	A/chicken/Zhejiang/611/2011(H9N2)	98
NA	A/mallard/Czech Republic/13438-29K/2010(H11N9)	96
M	A/chicken/Zhejiang/607/2011(H9N2)	98
NS	A/chicken/Dawang/1/2011(H9N2)	99

HA: haemagglutinin; M: matrix gene; NA: neuraminidase; NP: nucleoprotein; NS: non-structural gene; PA: RNA polymerase acidic subunit; PB1: RNA polymerase basic subunit 1; PB2: RNA polymerase basic subunit 2.

The sequences of the remaining viral genes are closely related (>97% identity) to avian influenza A(H9N2) viruses, which recently circulated in poultry in Shanghai, Zhejiang, Jiangsu, and neighbouring provinces of Shanghai (Table 2, Figure 2). These findings strongly suggest that the novel influenza A(H7N9) viruses are reassortants that acquired their H7 HA and N9 NA genes from avian influenza viruses, and their remaining genes from recent influenza A(H9N2) poultry viruses (Figure 1, Figure 3, Table 2).

At the nucleotide level, A/Shanghai/2/2013, A/Anhui/1/2013, and A/Hangzhou/1/2013 share more than 99% identity and differ by no more than three nucleotides per gene, even though they were isolated in different cities several hundred kilometres apart. On 7 April 2013, the Harbin Veterinary Research Institute deposited the full genome sequences of isolates from a pigeon (A/pigeon/Shanghai/S1069/2013), a chicken (A/chicken/Shanghai/S1053/2013), and an environmental sample (A/environment/Shanghai/S1088/2013) that were collected on 2 and 3 April from a Shanghai market (Table 1). All eight genes of these three isolates are similar to those of A/Shanghai/2/2013 and A/Anhui/1/2013 at the nucleotide level, except for the PB1 gene of A/pigeon/Shanghai/S1069/2013, which belongs to a different lineage than the PB1 of the other H7N9 isolates (Figures 1 and 2).

Interestingly, A/Shanghai/1/2013 and A/Shanghai/2/2013 differ by 52 nucleotides (for example, there are 13 nucleotide and nine amino acid differences in their HA sequences) even though these two cases were identified in the same city and at around the same time. These findings suggest that A/Shanghai/2/2013, A/Anhui/1/2013, A/Hangzhou/1/2013, as well as the viruses from the chicken and the environment, share a closely related source of infection, whereas A/Shanghai/1/2013 and A/pigeon/Shanghai/S1069/2013 are likely to have originated from other sources.

Highly pathogenic avian influenza viruses are characterised by a series of basic amino acids at the HA cleavage site that enable systemic virus spread. The HA cleavage sequence of the novel influenza A(H7N9) viruses possesses a single basic amino acid (EIPKGR*GL; *indicates the cleavage site), suggesting that these viruses are of low pathogenicity in avian species.

The amino acid sequence of the receptor-binding site (RBS) of HA determines preference for human- or avian-type receptors. At this site, A/Shanghai/1/2013 encodes an A138S* mutation (H3 numbering; Figure 4, Table 3), whereas A/Shanghai/2/2013, A/Anhui/1/2013, the two avian isolates, and the virus from the environmental sample encode G186V and Q226L mutations; any of these three mutations could increase the binding of avian H5 and H7 viruses to human-type receptors [12–14]. The finding of mammalian-adapting mutations in the RBS of these novel viruses is cause for concern. The A/Hangzhou/1/2013 isolate encodes isoleucine at position 226, which is found in seasonal influenza A(H3N2) viruses.

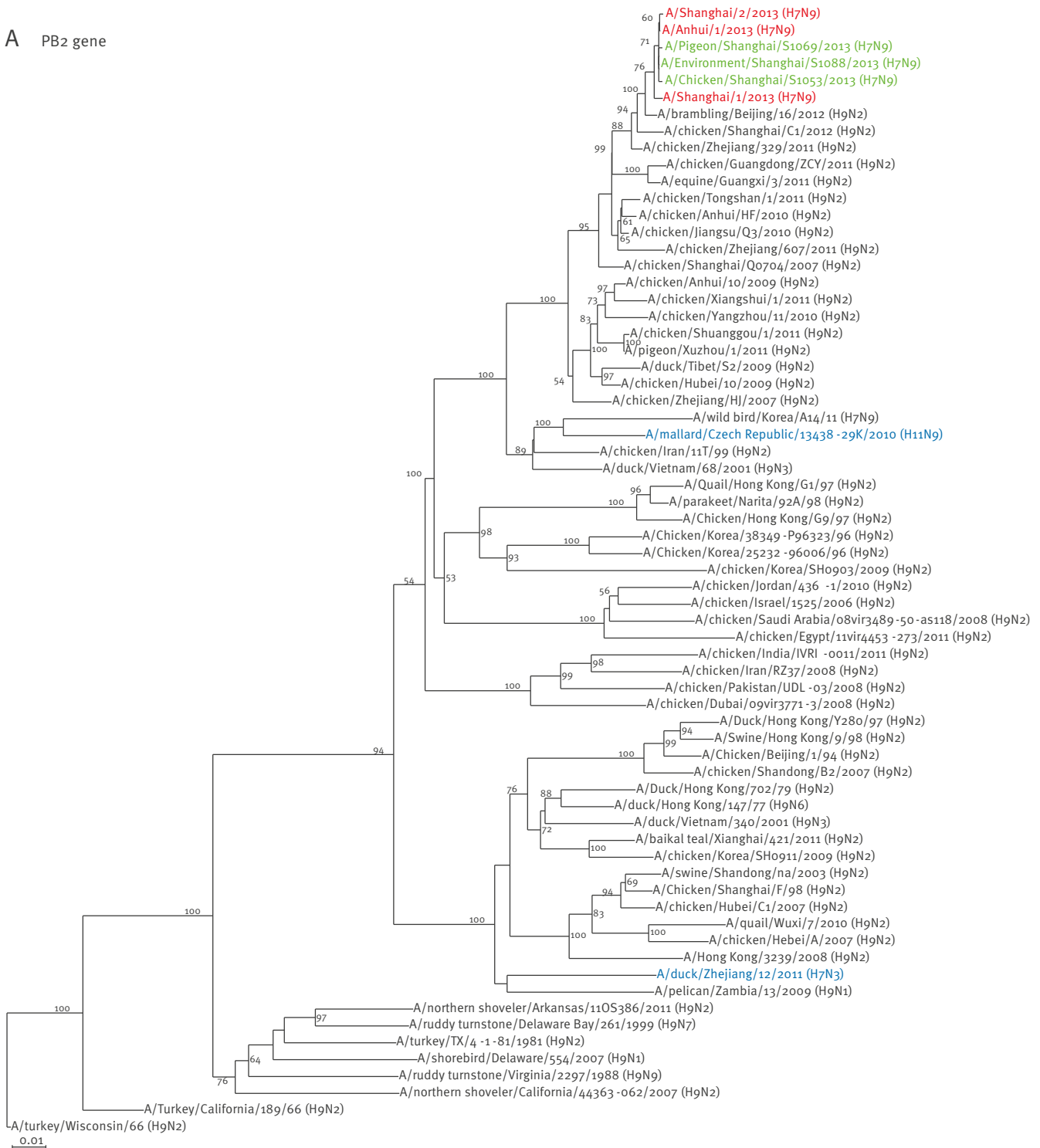
In addition, all seven influenza A(H7N9) viruses possess a T160A substitution (H3 numbering; Table 3) in HA, which is found in recently circulating H7 viruses; this mutation leads to the loss of an *N*-glycosylation site at position 158 (H3 numbering; position 149 in H7 numbering), which results in increased virus binding to human-type receptors [15].

Lysine at position 627 of the polymerase PB2 protein is essential for the efficient replication of avian influenza viruses in mammals [16] and has been detected in highly pathogenic avian influenza A(H5N1) viruses and in the influenza A(H7N7) virus isolated from the fatal case in the Netherlands in 2003 [17]. PB2-627K is rare among avian H9N2 PB2 proteins (i.e. it has been found in only five of 827 isolates). In keeping with this finding, the avian and environmental influenza A(H7N9)

FIGURE 2

Phylogenetic analysis of the six remaining genes of the novel influenza A(H7N9) viruses, China, February–April 2013 (n=7)

A PB2 gene



PB2: RNA polymerase basic subunit 2.

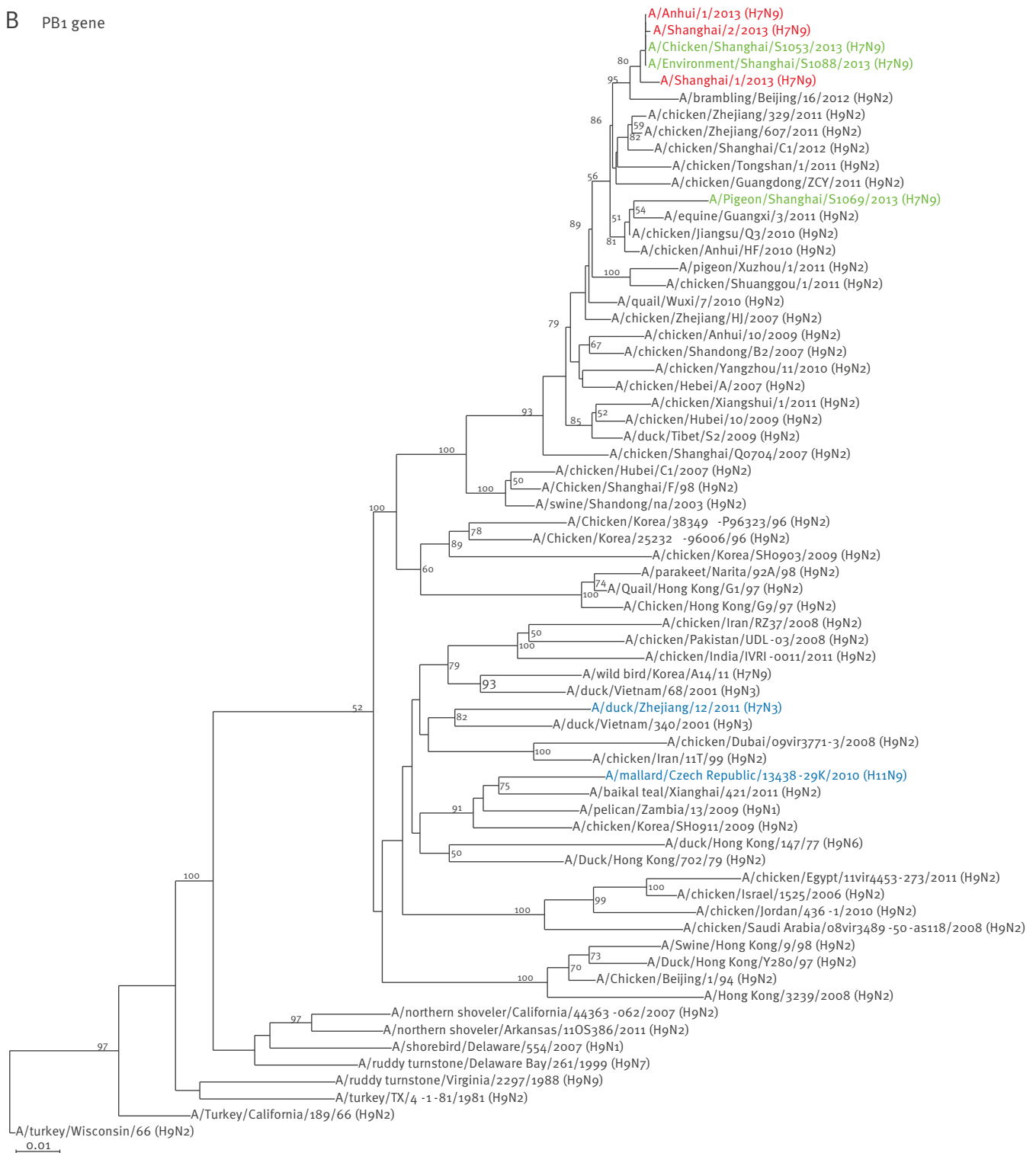
Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

The novel human H7N9 viruses are shown in red; novel H7N9 viruses from birds and the environment are shown in green. Influenza viruses whose HA and NA genes are most closely related to the novel human H7N9 viruses are shown in blue.

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B PB1 gene



PB1: RNA polymerase basic subunit 1.

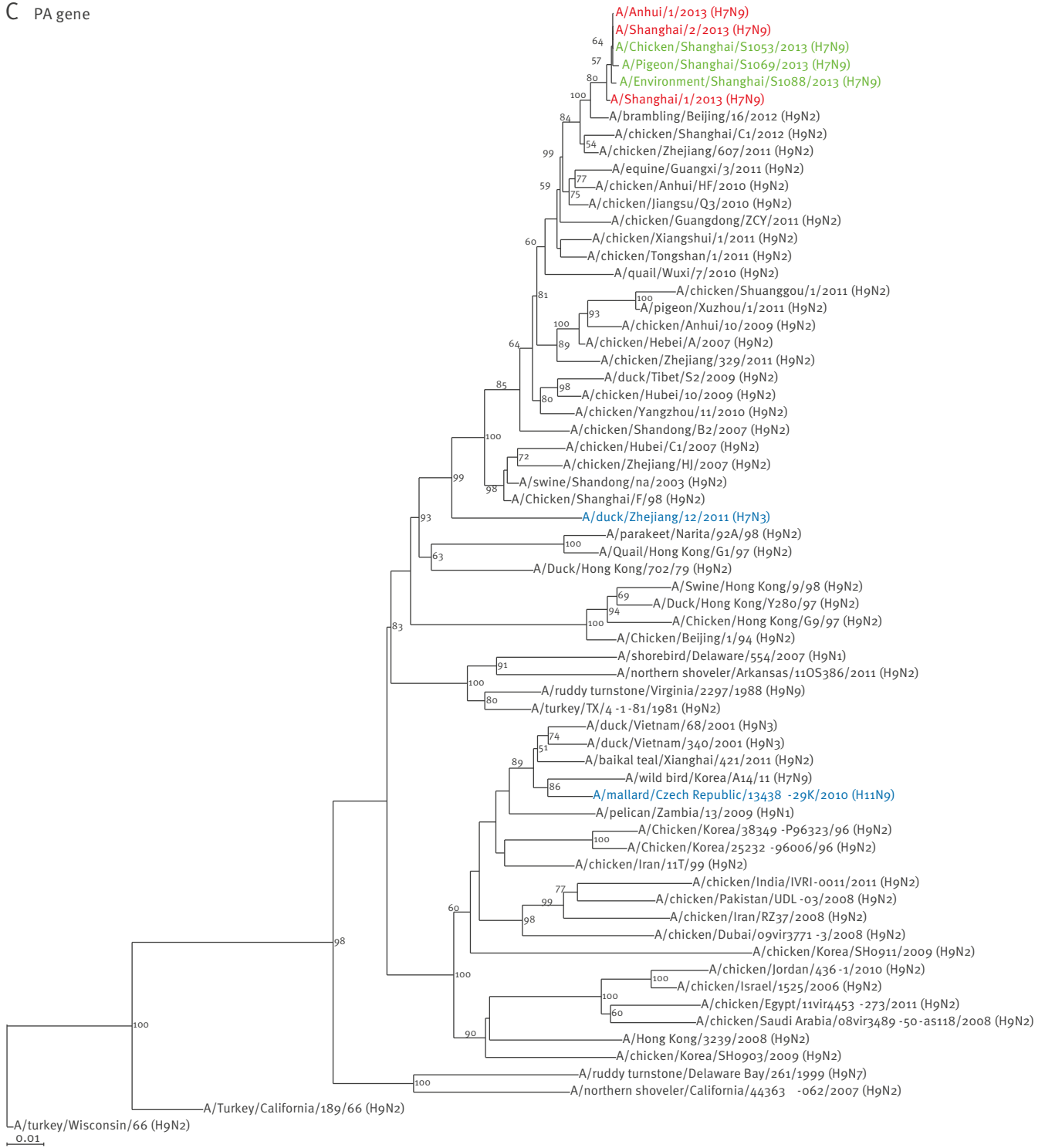
Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

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FIGURE 2

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C PA gene



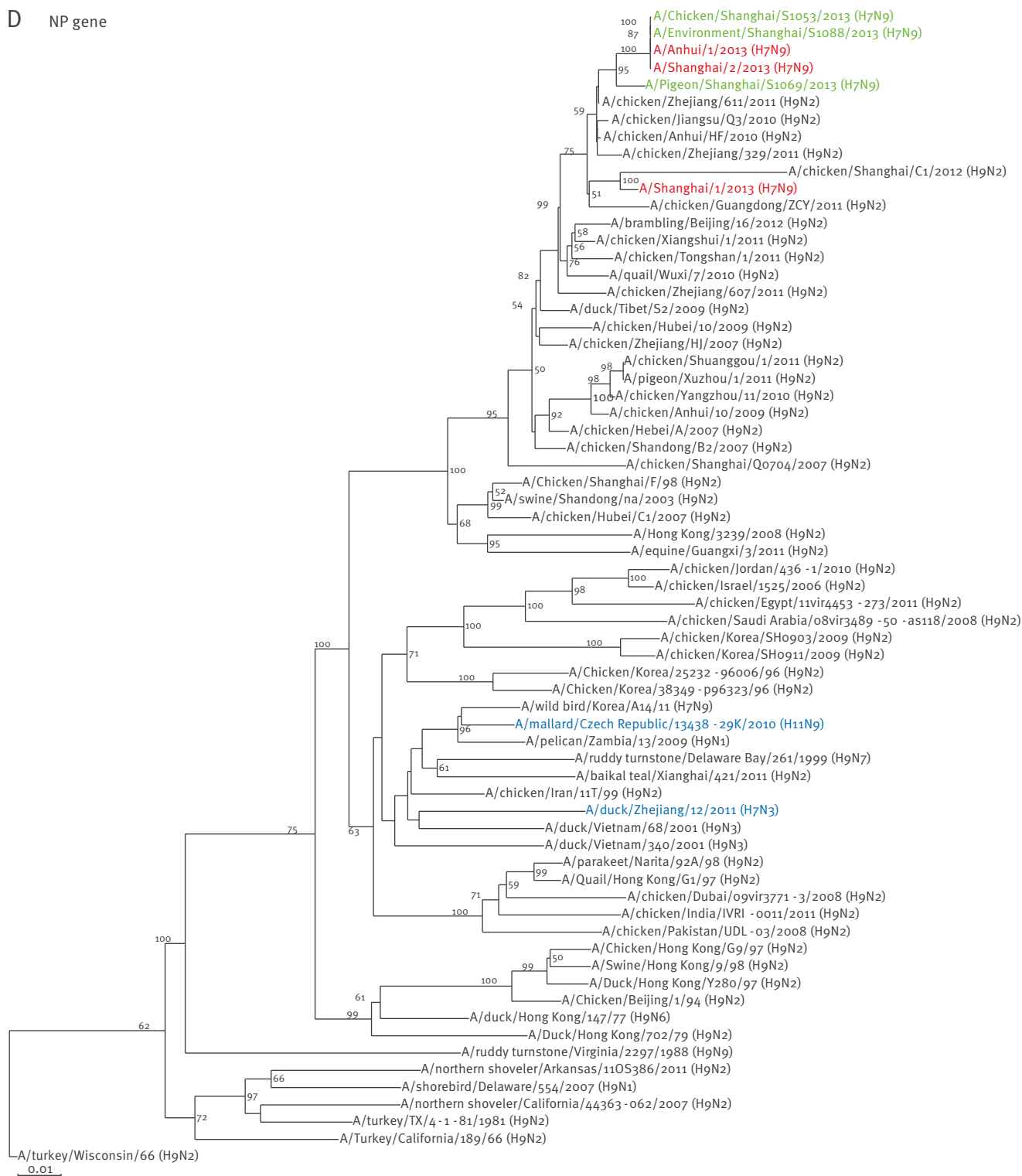
PA: RNA polymerase acidic subunit.

Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

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D NP gene

NP: nucleoprotein.

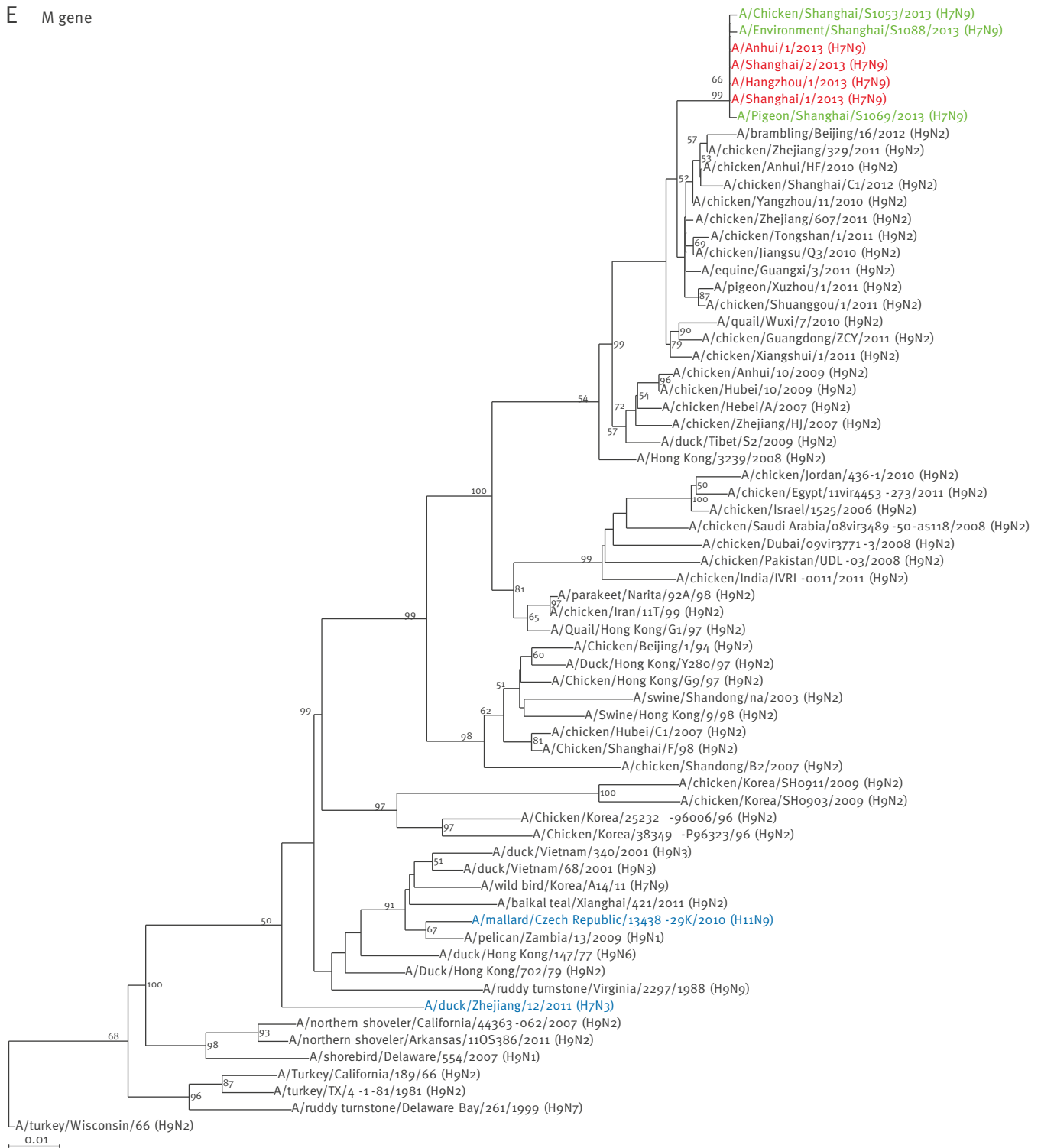
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E M gene



M: matrix gene.

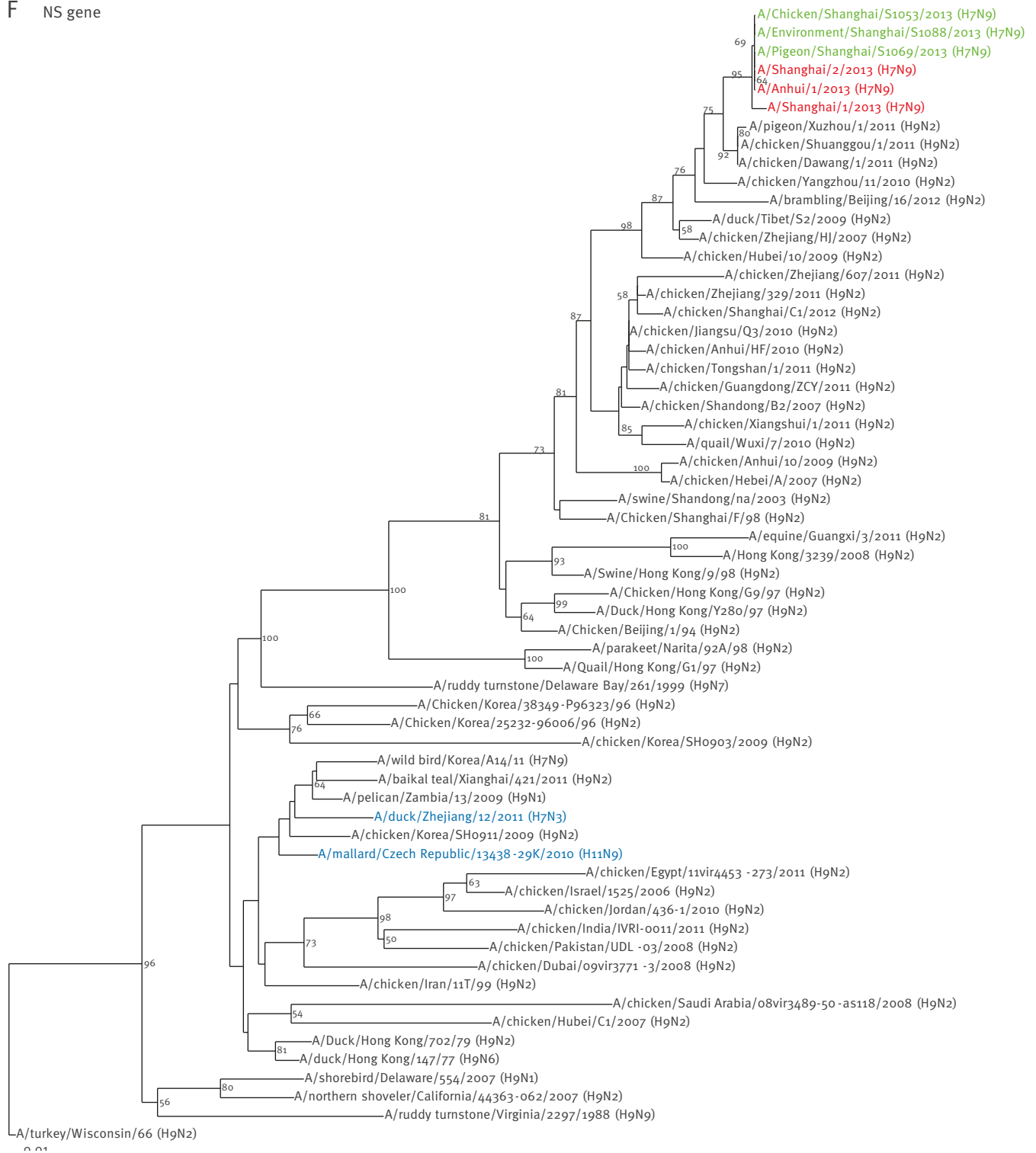
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Phylogenetic analysis of the six remaining genes of the novel influenza A(H7N9) viruses, China, February–April 2013 (n=7)

F NS gene



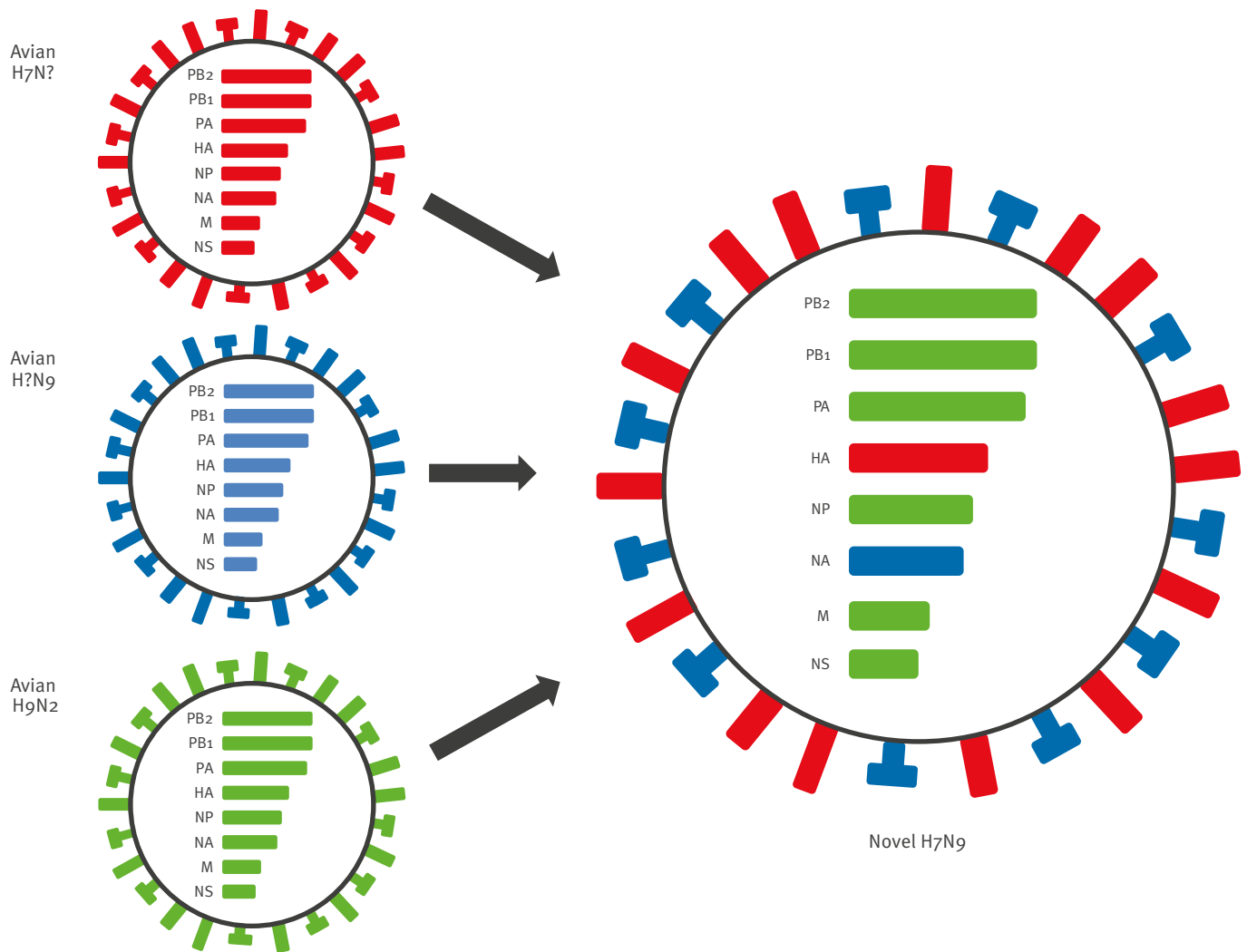
NS: non-structural gene.

Multiple alignments were constructed by using the CLUSTAL W algorithm. Genetic distances were calculated by using the Kimura's 2-parameter method [26], and phylogenetic trees were constructed by using the neighbour-joining method with bootstrap analyses of 1,000 replicates in CLUSTAL W. Numbers next to nodes indicate bootstrap value percentages (>50%).

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FIGURE 3

Schematic diagram of novel influenza A(H7N9) virus generation



HA: haemagglutinin; NA: neuraminidase.

The novel influenza A(H7N9) viruses are likely to have acquired their HA gene from an avian H7 virus of unknown NA subtype, their NA gene from an avian N9 virus of unknown HA subtype, and their remaining six viral segments from avian H9N2 viruses circulating in poultry.

viruses analysed here encode PB2-627E. By contrast, all four human H7N9 viruses analysed here encode PB2-627K (Table 3).

Antiviral compounds are the first line of defense against novel influenza viruses until vaccines become available. All seven novel influenza A(H7N9) viruses sequenced to date encode the S31N substitution in the viral ion channel M2 (encoded by the M segment) (Table 3), which confers resistance to ion channel inhibitors [18,19]. Based on the sequences of their NA proteins, all H7N9 viruses analysed here, with the exception of A/Shanghai/1/2013, should be sensitive to neuraminidase inhibitors (Table 3). However, the R294K mutation in the NA protein of A/Shanghai/1/2013 is known to

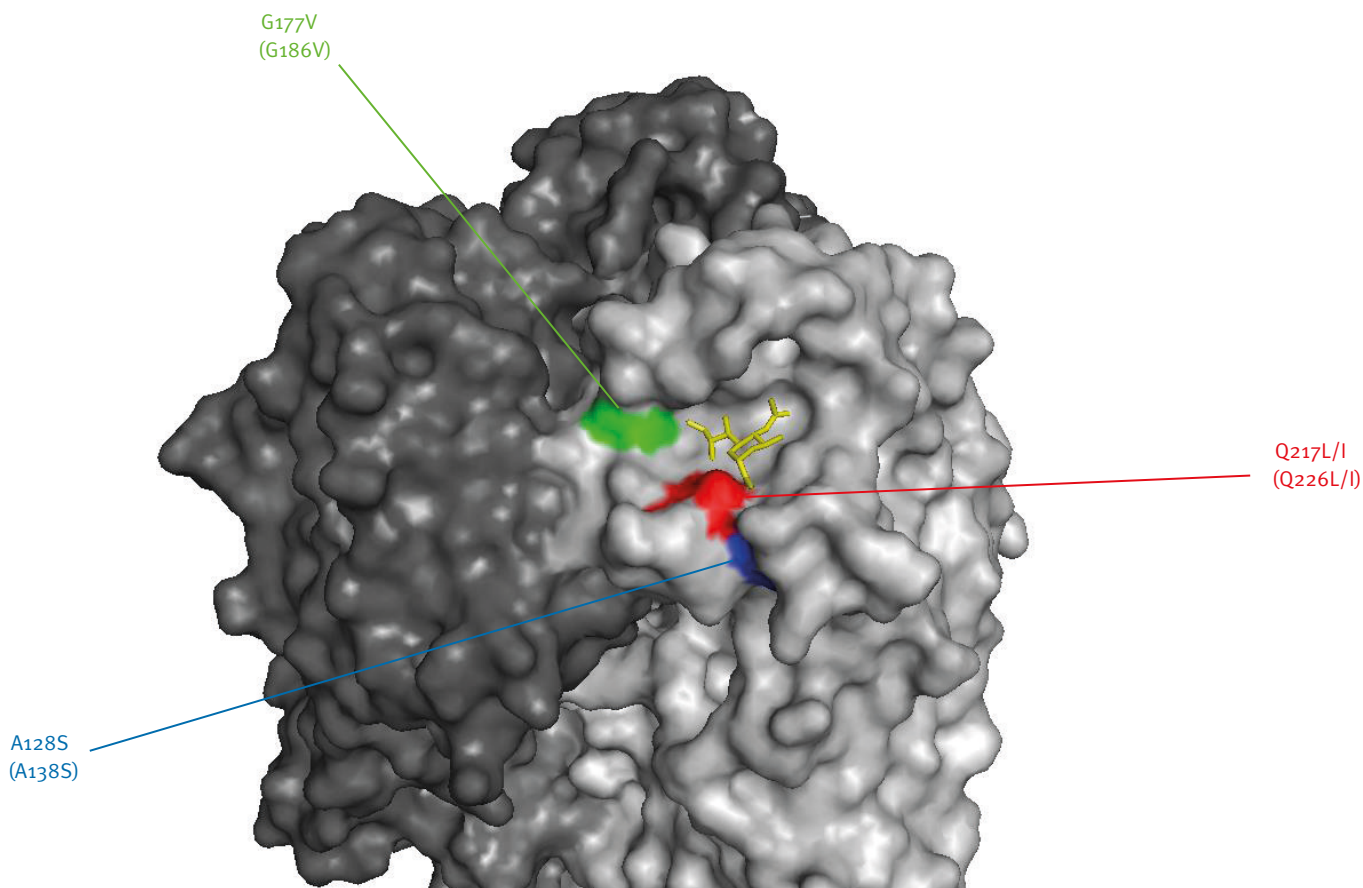
confer resistance to NA inhibitors in N2 and N9 subtype viruses [20], and is therefore of great concern.

All H7N9 viruses encode a deletion at positions 69–73 of the NA stalk region (Table 3), which is reported to occur upon virus adaptation to terrestrial birds. This finding suggests that the novel H7N9 viruses (or their ancestor) may have circulated in terrestrial birds before infecting humans. Moreover, this deletion is associated with increased virulence in mammals [21].

The influenza A virus PB1-F2 protein (encoded by the PB1 segment) is also associated with virulence. The available sequences indicate that the H7N9 PB1 genes of all of the human viruses encode a full-length PB1-F2 of 90 amino acids, but lack the N66S mutation that is

FIGURE 4

Amino acid changes in the three novel influenza A(H7N9) viruses that may affect their receptor-binding properties, China, February–April 2013 (n=7)



H7 numbering
(H3 numbering)

Shown is the three-dimensional structure of three monomers (light and dark gray) of the influenza A(H7N7) virus (A/Netherlands/219/2003) haemagglutinin (accession code 4DJ8). Also shown is the part of 6'-sialyl-N-acetylglucosamine (a sialyloligosaccharide) to which human viruses bind preferentially (yellow). Indicated are amino acid changes in the H7N9 virus haemagglutinin protein at positions known to increase binding to human-type receptors.

associated with the increased pathogenicity of the 1918 pandemic virus and the highly pathogenic avian influenza A(H5N1) viruses [22]. Interestingly, the pigeon isolate encodes a truncated PB1-F2 of only 25 amino acids; the significance of this truncation is unknown.

The NS1 protein (encoded by the NS segment) is an interferon antagonist with several functions in the viral life cycle. All available H7N9 NS1 sequences lack the C-terminal PDZ domain-binding motif; the lack of the PDZ domain-binding motif may attenuate these viruses in mammals [23].

Other amino acids in the NS1 and matrix (M1; encoded by the M segment) proteins of the novel viruses are also associated with increased virulence (Table 3) [24,25]. However, these amino acids are found in many

avian influenza viruses, and therefore, their significance for the biological properties of the novel influenza A(H7N9) viruses is currently unclear.

In conclusion, we here present a biological evaluation of the sequences of the avian influenza A(H7N9) viruses that caused fatal human infections in China. These viruses possess several characteristic features of mammalian influenza viruses, which are likely to contribute to their ability to infect humans and raise concerns regarding their pandemic potential.

*Authors' correction:

The mutation A138S was erroneously written as S138A in the original publication. This mistake was corrected on 13 April 2013

TABLE 3

Selected characteristic amino acids of the three novel influenza A(H7N9) viruses, China, February–April 2013 (n=7)

Viral protein	Amino acid position	Shanghai/1/2013	Shanghai/2/2013	Anhui/1/2013	Hangzhou/1/2013	Chicken/Shanghai/S1053/2013	Environment/Shanghai/S1088/2013	Pigeon/Shanghai/S1069/2013	Human influenza viruses	Avian influenza viruses	Comments	Reference(s)
PB2	627	K	K	K	Nd	E	E	E	K	E	E627K: Mammalian host adaptation	16
	128/138 ^a	S	A	A	A	A	A	A	A	A ^b	S138A: Increased virus binding to human-type receptors	13
	151/160 ^a	A	A	A	A	A	A	A	K	A^b	T160A: Loss of N-glycosylation and increased virus binding to human-type receptors	15
	177/186 ^a	G	V	V	V	V	V	V	G	G ^b	G186V: Increased virus binding to human-type receptors	14
	217/226 ^a	Q	L	L	I	L	L	L	I	Q ^b	Q226L: Increased virus binding to human-type receptors	12
NA	69–73 ^c	Deletion	Deletion	Deletion	Deletion	Deletion	Deletion	Deletion	No deletion	No deletion	Deletion of amino acids 69–73: Increased virulence in mice	21
	289/294/292 ^d	K	R	R	R	R	R	R	R	R	R294K: Reduced susceptibility to oseltamivir and zanamivir	20
M1	30	D	D	D	D	D	D	D	D(S)	D	N30D: Increased virulence in mice (most influenza A viruses encode 30D)	24
	215	A	A	A	A	A	A	A	A	A	T215A: Increased virulence in mice (most avian influenza A viruses encode 215A)	24
M2	31	N	N	N	N	N	N	N	S/N	S/(N)	S31N: Reduced susceptibility to amantadine and rimantadine	18,19
	42	S	S	S	Nd	S	S	S	S	S/A	P42S: Increased virulence in mice (most avian influenza A viruses encode 42S)	25
NS1	218–230	Deletion	Deletion	Deletion	Nd	Deletion	Deletion	Deletion	No deletion ^e	No deletion/Deletion	Lack of PDZ domain binding motif: Decreased virulence in mice	23

Substitutions of particular concern are shown in bold.

Nd: not determined.

^a H7/H3 numbering.^b H7 virus.^c N9 numbering.^d H7N9/avian N9/N2 numbering.^e Influenza A(H1N1)pdm09 viruses from the 2009 influenza pandemic have the deletion.

Acknowledgements

We are grateful to Dr. Shu Yuelong, Chinese National Influenza Center, Chinese Center for Disease Control and Prevention, Beijing, China, for his rapid publication of the entire gene sequence data of A(H7N9) viruses isolated from human cases in China, and also for his information sharing and advice to this study. We also thank Susan Watson for scientific editing. This work was supported by Grants-in-Aid for Pandemic Influenza Research (TK, SF, HX, and MT) and Grant-in-Aid for Specially Promoted Research (MT) from the Ministry of Health, Labour and Welfare, Japan, by the NIAID-funded Center for Research on Influenza Pathogenesis (CRIP, HHSN266200700010C)(YK), by a Grant-in-Aid for Specially Promoted Research, by the Japan Initiative for Global Research Network on Infectious Diseases from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (YK), and by ERATO, Japan (YK).

Authors contributions

Designed the analyses: TK, SF, ET, SY, GN, YK, MT. Analysed and interpreted data: TK, SF, ET, HX, SY, YU, GN, YK, MT. Drafted the article: TK, SF. Revised the article: ET, GN, TS, YK, MT.

Conflict of interest

None declared.

This manuscript was accepted on 11 April 2013
and published online on 12 April 2013

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Surveillance of avian influenza A(H7N9) virus infection in humans and detection of the first imported human case in Taiwan, 3 April to 10 May 2013

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Citation style for this article:

Lo YC, Chen WC, Huang WT, Lin YC, Liu MC, Kuo HW, Chuang JH, Yang JR, Liu MT, Wu HS, Yang CH, Chou JH, Chang FY. Surveillance of avian influenza A(H7N9) virus infection in humans and detection of the first imported human case in Taiwan, 3 April to 10 May 2013. *Euro Surveill.* 2013;18(20):pii=20479. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20479>

Article submitted on 03 May 2013 published on 16 May 2013

On 3 April 2013, suspected and confirmed cases of influenza A(H7N9) virus infection became notifiable in the primary care sector in Taiwan, and detection of the virus became part of the surveillance of severe community-acquired pneumonia. On 24 April, the first imported case, reported through both surveillance systems, was confirmed in a man returning from China by sequencing from endotracheal aspirates after two negative throat swabs. Three of 139 contacts were ill and tested influenza A(H7N9)-negative.

The Taiwan Centers for Disease Control (TCDC) listed avian influenza A(H7N9) virus infection in humans as a nationally notifiable disease on 3 April 2013 [1], after the Chinese authorities had on 31 March 2013 announced the identification of two male influenza cases in Shanghai and one female case in Anhui with severe respiratory disease caused by an avian influenza A(H7N9) virus that had not previously been detected in humans or animals [2]. The viruses had genetic markers known to be associated with adaptation to mammalian hosts and respiratory transmission of avian influenza viruses, raising concerns about their pandemic potential [2]. The probability of introduction of this virus into Taiwan is considered high because of geographic proximity and more than 90,000 personal or business travels from Shanghai and Anhui to Taiwan per month. This report summarises Taiwan's surveillance for avian influenza A(H7N9) virus infection in humans in the period from 3 April to 10 May 2013.

Influenza surveillance in Taiwan

The National Influenza Surveillance System (NISS) in Taiwan consists of virological surveillance by sentinel primary care physicians, syndromic surveillance of influenza-like illness in emergency and outpatient departments, and surveillance of influenza with complications reported through the National Notifiable Disease Surveillance System. These surveillance activities have been described [3,4]. On 3 April 2013, the TCDC added human infection with avian influenza

A(H7N9) virus into the National Notifiable Disease Surveillance System to detect suspected and confirmed cases in the primary care sector. Before 3 April 2013, specimens positive for untypeable influenza A submitted through NISS were routinely tested for influenza A(H5) by realtime RT-PCR. Since 3 April 2013, such specimens have in addition been routinely tested by RT-PCR for influenza A(H7). The TCDC has also conducted surveillance of severe community-acquired pneumonia (CAP) of unknown aetiology since 2010. We focused on these two surveillance activities in this report.

Surveillance of influenza A(H7N9) virus infection in the primary care sector

The maximal incubation period of influenza A(H7N9) was defined as seven days in the period from 3 to 25 April and was revised as 10 days on 26 April based on a recent study [5]. Contacts were defined as those who had provided care to, had been in the same place with, or had directly exposed to respiratory secretions or body fluids of a case since the day before illness onset of the case.

A suspected influenza A(H7N9) case was defined as a person with onset of pneumonia or fever (≥ 38 °C) with cough within the maximal incubation period of at least one the following exposures: (i) contact with a confirmed case; (ii) travel to provinces or cities in China where human infections with the avian influenza A(H7N9) virus have been reported; (iii) exposure to human, animal or environmental specimens or laboratory samples that are suspected or confirmed to contain the influenza A(H7N9) virus. A case was confirmed if tested positive for the influenza A(H7N9) virus by RT-PCR and/or culture at TCDC.

Physicians were required to report suspected cases to their local health departments within 24 h of identification and to submit nasopharyngeal or oropharyngeal swabs of all suspected cases to TCDC for influenza

testing. Local public health professionals verified case characteristics including presenting symptoms, dates of illness onset, underlying medical conditions, and exposure to poultry based on the physicians' reports and interviews with the patients or their parents.

Contact persons were identified through interviews with patients and their family and through hospital records. All contacts were interviewed for dates and mode of the exposure as well as and protective measures, and followed up daily for fever and respiratory symptoms during the maximal incubation period after last exposure.

Surveillance of influenza A(H7N9) virus in severe pneumonia of unknown aetiology

Surveillance of severe CAP of unknown aetiology has been established in Taiwan since 2010. Physicians from 29 hospitals (including 13 tertiary referral hospitals) were requested to submit respiratory specimens from CAP patients with respiratory failure for whom no aetiological pathogen had been identified through general clinical investigations. Submitted specimens were tested for viruses using a specifically designed multiplex PCR panel targeting influenza A(H1N1), A(H3N2) and B viruses, parainfluenza viruses 1–3, adenovirus, respiratory syncytial virus (A and B), human bocavirus, human coronavirus (229E, NL63, OC43, and HKU1), enterovirus, rhinovirus, human metapneumovirus, parvovirus B19, and viruses of the human Herpesviridae. Since 3 April, influenza A(H7) virus has been incorporated into the multiplex PCR panel as a supplementary target for all cases of severe CAP of unknown aetiology. Retrospective testing of influenza A(H7) virus was also conducted on stored samples from cases of severe CAP of unknown aetiology reported from 1 January to 2 April 2013.

Laboratory testing of influenza A(H7N9) virus

Viral culture was performed on respiratory specimens using Madin Darby canine kidney cells. The RT-PCR for influenza A and B viruses and subtyping of human influenza A(H1N1) and A(H3N2) have been described before [6]. Subtyping of influenza A(H7N9) viruses was conducted with the protocol provided by the World Health Organization Collaborating Center for Reference and Research on Influenza [7].

Case description

In the period from 3 April to 10 May, TCDC was notified of 358 suspected human cases of avian influenza A(H7N9) virus infection and 41 cases of severe CAP of unknown aetiology, including one confirmed case reported through both of the surveillance systems. Of the 357 suspected cases that tested negative for influenza A(H7), 49 tested positive for influenza A(H1N1), 29 tested positive for influenza A(H3N2), and five tested positive for influenza B. Of the 88 cases of severe CAP of unknown aetiology reported in the period from 1 January to 10 May, 47 cases were negative in all tests,

16 were positive for influenza virus (13A(H1N1), two A(H3N2), and one A(H7N9)), and 25 were positive for other viruses (details not presented because the review of the medical records is still outstanding). None of the specimens submitted through other NISS surveillance activities from 3 April to 10 May tested positive for influenza A(H7) viruses.

The confirmed case occurred in a man in his 50s who returned from Jiangsu Province, China on 9 April. The clinical course has been described in details elsewhere [8]. The patient experienced fever and general malaise without respiratory symptoms on 12 April, first sought medical attention on 16 April because of high fever (40 °C) and mild sore throat, and was reported as a suspected influenza A(H7N9) case on 16 April. A throat swab collected on 16 April tested negative for influenza A(H7N9) virus by RT-PCR. Right lower lobe interstitial pneumonia developed on 18 April and progressed to bilateral lower lung consolidation and respiratory failure on 20 April. The patient was reported to TCDC on 21 April as severe pneumonia of unknown aetiology and a throat swab was collected and submitted to TCDC on the same day for testing by RT-PCR; this sample was negative for influenza A(H7N9) virus. Endotracheal aspirates collected on 20 April tested positive for influenza A on 22 April and were subtyped as influenza A(H7N9) in the evening of 23 April at a university research laboratory. On 24 April, influenza A(H7N9) virus infection was confirmed by positive influenza A(H7N9) RT-PCR and sequencing at the TCDC National Influenza Center on endotracheal aspirates collected in the late evening of 23 April. As of 10 May, the patient had made a good recovery; mechanical ventilation had been removed.

All of 139 contact persons of this case, including three family contacts, 26 casual contacts (colleagues and friends), and 110 healthcare workers, were followed up for 10 days after last exposure. Three healthcare workers at the intensive care unit experienced respiratory symptoms within two to three days after providing routine nursing care to the patient, using N95 respirators, goggles, gloves and protective clothing. Throat swabs collected from all three symptomatic contacts on April 24 tested negative for influenza A(H7N9) virus by RT-PCR. Further epidemiological and laboratory investigations of this confirmed case and close contacts are ongoing.

Discussion

This first human influenza A(H7N9) case outside China provided important lessons on public health surveillance and detection of human influenza A(H7N9) cases. Firstly, influenza A(H7N9) RT-PCR was negative on two throat swabs collected on Day 4 and Day 9 after illness onset, but was positive on endotracheal aspirates collected on Day 8 after onset. The findings are consistent with a recent study based on four cases, that indicated sputum specimens were more likely to test influenza A(H7N9)-positive than throat swabs [9]. As a result, TCDC revised the sampling guidance on 26

April to include sputum, endotracheal aspirates and other lower airway specimens, in addition to pharyngeal swabs, as recommended specimens for collection in suspected reported influenza A(H7N9) cases with productive cough, pneumonia or other complications. TCDC also recommended that physicians submit follow-up respiratory specimens in suspected influenza A(H7N9) cases with progressive disease after initially negative test results.

Secondly, the patient presented with fever but no cough. Although the presenting symptoms did not meet our case definitions, his clinician decided to report the case based on recent travel in eastern China and fever with sore throat, and the reporting was accepted by our surveillance system. The case presentation was different from that of the first three influenza A(H7N9) cases reported in China, all of whom presented with fever and cough [2]. However, adult and paediatric influenza A(H7N9) cases that presented without cough have been reported [10,11]. This illustrates possible limitations of current case definitions using fever and cough as one of the clinical criteria. Although inclusion of respiratory symptoms other than cough might improve sensitivity of the case definitions, broader clinical criteria might not necessarily lead to strengthened case confirmation, if testing on pharyngeal specimens at an early stage is not sensitive for influenza A(H7N9) virus detection. Alternatively, as exemplified by this case, physicians should be allowed to report suspected cases that do not fully meet the case definitions.

Further studies that characterise influenza A(H7N9) virus infection in humans will provide evidence for public health practices of case detection. For example, because a recent study showed maximal intervals of 10 days between poultry exposure and illness onset in influenza A(H7N9) cases [5], TCDC revised case definitions on 26 April to extend the maximal incubation period to 10 days. Studies that examine viral positivity at different anatomic sites and shedding over the disease course in comparison with seasonal influenza, such as previous studies on pandemic influenza A(H1N1)pdm09, could provide guidance for laboratory testing and monitoring of influenza A(H7N9) cases [12-14].

Conclusions

This first imported human influenza A(H7N9) case in Taiwan was reported through both the National Notifiable Disease Surveillance and severe CAP surveillance systems. Laboratory confirmation was achieved through astute pursuit of laboratory diagnoses by physicians, testing a deep endotracheal sample despite two earlier negative throat swabs and absence of cough as the initial presentation. A flexible surveillance system allows for timely revision of case definitions and sampling guidance. Sensitivity in case detection is likely to improve with collection of sputum, endotracheal aspirates, or other lower airway specimens in addition to pharyngeal swabs. Retrospective

testing of severe CAP cases since January 2013 did not demonstrate any earlier influenza A(H7N9) cases. Preliminary results of contact investigations indicated no evidence of person-to-person transmission. We recommend rapid communication and dissemination of results of epidemiological and virological studies to ensure evidence-based surveillance and detection of influenza A(H7N9) virus infection.

Authors' contributions

Yi-Chun Lo, Wan-Chin Chen, Wan-Ting Huang, Yung-Ching Lin, and Ming-Chih Liu prepared the first draft of this manuscript. Hung-Wei Kuo, and Jen-Hsiang Chuang provided the surveillance data. Ji-Rong Yang, Ming-Tsan Liu, and Ho-Sheng Wu provided the virological data. Chin-Hui Yang, Jih-Haw Chou, Feng-Yee Chang interpreted the surveillance and virological data. All authors reviewed and revised the first and final drafts of this manuscript.

Conflict of interest

None declared.

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Preliminary inferences on the age-specific seriousness of human disease caused by avian influenza A(H7N9) infections in China, March to April 2013

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Citation style for this article:

Cowling BJ, Freeman G, Wong JY, Wu P, Liao Q, Lau EH, Wu JT, Fielding R, Leung GM. Preliminary inferences on the age-specific seriousness of human disease caused by avian influenza A(H7N9) infections in China, March to April 2013. *Euro Surveill.* 2013;18(19):pii=20475. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20475>

Article submitted on 25 April 2013 / published on 9 May 2013

Between 31 March and 21 April 2013, 102 laboratory-confirmed influenza A(H7N9) infections have been reported in six provinces of China. Using survey data on age-specific rates of exposure to live poultry in China, we estimated that risk of serious illness after infection is 5.1 times higher in persons 65 years and older versus younger ages. Our results suggest that many unidentified mild influenza A(H7N9) infections may have occurred, with a lower bound of 210–550 infections to date.

Introduction

In recent weeks, increasing numbers of avian influenza A(H7N9) virus infections have been identified in humans in China [1,2]. Laboratory-confirmed cases of influenza A(H7N9) infection have typically suffered serious illness [3,4], and there is a notable excess of confirmed cases in the elderly [3,5]. In the present analysis, we compared the incidence of serious influenza A(H7N9) infections with data on age-specific patterns in exposure to domestic poultry and live poultry markets to estimate the relative seriousness of influenza A(H7N9) and obtain a lower bound on the number of human infections to date.

Methods

Poultry exposures in China

We obtained unpublished data on poultry exposures in Shenzhen, a city in Guangdong province on the border with Hong Kong, and in Xiuning, a rural county in Anhui province in eastern China. In each location, a two-stage household-based cluster survey was conducted to assess poultry exposures based on average annual visits to poultry wet markets (Shenzhen, $n=2,058$), and ownership of backyard poultry (Xiuning, $n=2,892$). Trained investigators conducted each face-to-face interview with selected households, and every family member who met the inclusion criteria (aged at least five years, and resident in the study area for at least three months) was interviewed. Poultry wet

markets were defined as places where small animals and poultry may be purchased alive or slaughtered just before purchase. The surveys were conducted from July to September 2007.

Data on poultry exposures in urban and semi-rural areas of Guangzhou, the capital of Guangdong province in Southern China, were obtained through face-to-face interviews, from January through March 2006 [6]. Households were selected for interview through stratified cluster sampling in the ten urban districts ($n=1,363$) and two satellite towns ($n=187$) of Guangzhou. One adult per selected household was interviewed. We assessed household exposures to retail and domestic poultry in both urban and semi-rural locations based on average annual visits to poultry wet markets to purchase live poultry, and ownership of backyard poultry [6].

Avian influenza A(H7N9) cases

Information on laboratory-confirmed human infections with influenza A(H5N1) and A(H7N9) was obtained from official notifications, including age, geographic location, and seriousness of disease (mild/serious). The definition for an influenza A(H7N9) case is given elsewhere [3]. A serious case was defined as a laboratory-confirmed influenza A(H7N9) case that required hospital admission for medical reasons, i.e. with a complication such as pneumonia, rather than merely for isolation. Cases defined as serious included all fatal laboratory-confirmed cases. The age-specific populations of provinces in China were obtained from the 2010 population census of the People's Republic of China [7].

Statistical analysis

We specified a model for the observed number of serious influenza A(H7N9) infections under the assumption that the risk of infection was directly proportional to the risk of exposure, while the seriousness of infection varied by age. Specifically, we modelled X_{ij} , the number

of serious influenza A(H7N9) infections in age group i and area j , as following a Poisson distribution with mean $A_{ij} \times p_{ij} \times r_i$, where A_{ij} is the population of persons in age group i ($i=1$ for 0–14 years, 2 for 15–24 years, 3 for 25–34 years, 4 for 35–44 years, 5 for 45–54 years, 6 for 55–64 years, 7 for ≥ 65 years) and area j (1 for Anhui-urban, 2 for Beijing-urban, 3 for Henan-rural, 4 for Jiangsu-urban, 5 for Jiangsu-rural, 6 for Shanghai-urban, 7 for Zhejiang-urban, 8 for Zhejiang-rural), p_{ij} represents the incidence rate of infection by age and area over the time period covered by our analysis, and r_i represents the age-specific risk of serious illness if infected. For urban areas ($\delta_j=1$) and rural areas ($\delta_j=0$), we specified $p_{ij}=\delta_j \times U_i \times \theta_j + (1-\delta_j) \times V_i \times \theta_j$, where U_i and V_i represent the age-specific rates of exposure in urban and rural areas, respectively, while θ_j represents the area-specific risk of infection. In our main analysis, we modelled the risk of serious illness conditional on infection as r_i taking value r_{old} for age ≥ 65 years and r_{young} for age < 65 years. We explored other parameterisations for r_i such as $r_i=r_7 \times \exp(\beta \times (i-7))$ in sensitivity analyses.

We used a Bayesian inferential framework to fit the model to observed data on X_{ij} , A_{ij} and δ_j , incorporating U_{ij} , and V_{ij} as parameters with strong prior distributions from the survey data to retain uncertainty (as is standard in Bayesian evidence synthesis [8]), and r_{old} as a parameter with a strong prior based on observed mild and serious influenza A(H7N9) cases. We estimated θ_j and r_{young} using independent uninformative uniform priors on the positive real line for each θ_j and on the (0,1) interval for r_{young} . Models were fitted with the Hamiltonian Monte Carlo sampler NUTS [9] using the Stan modelling language in R version 3.0.0 (R Foundation for Statistical Computing, Vienna, Austria). Convergence of the simulations was assessed using the potential scale reduction statistic [10].

After fitting the models, posterior estimates of the model parameters were used to estimate $q_{ij}=A_{ij} \times p_{ij}$ as the total number of influenza A(H7N9) infections for each age group i and area j . This estimate can be

FIGURE 1

Geographical location of officially announced serious cases of influenza A(H7N9) virus infection in mainland China, 31 March–21 April 2013 (n=98)

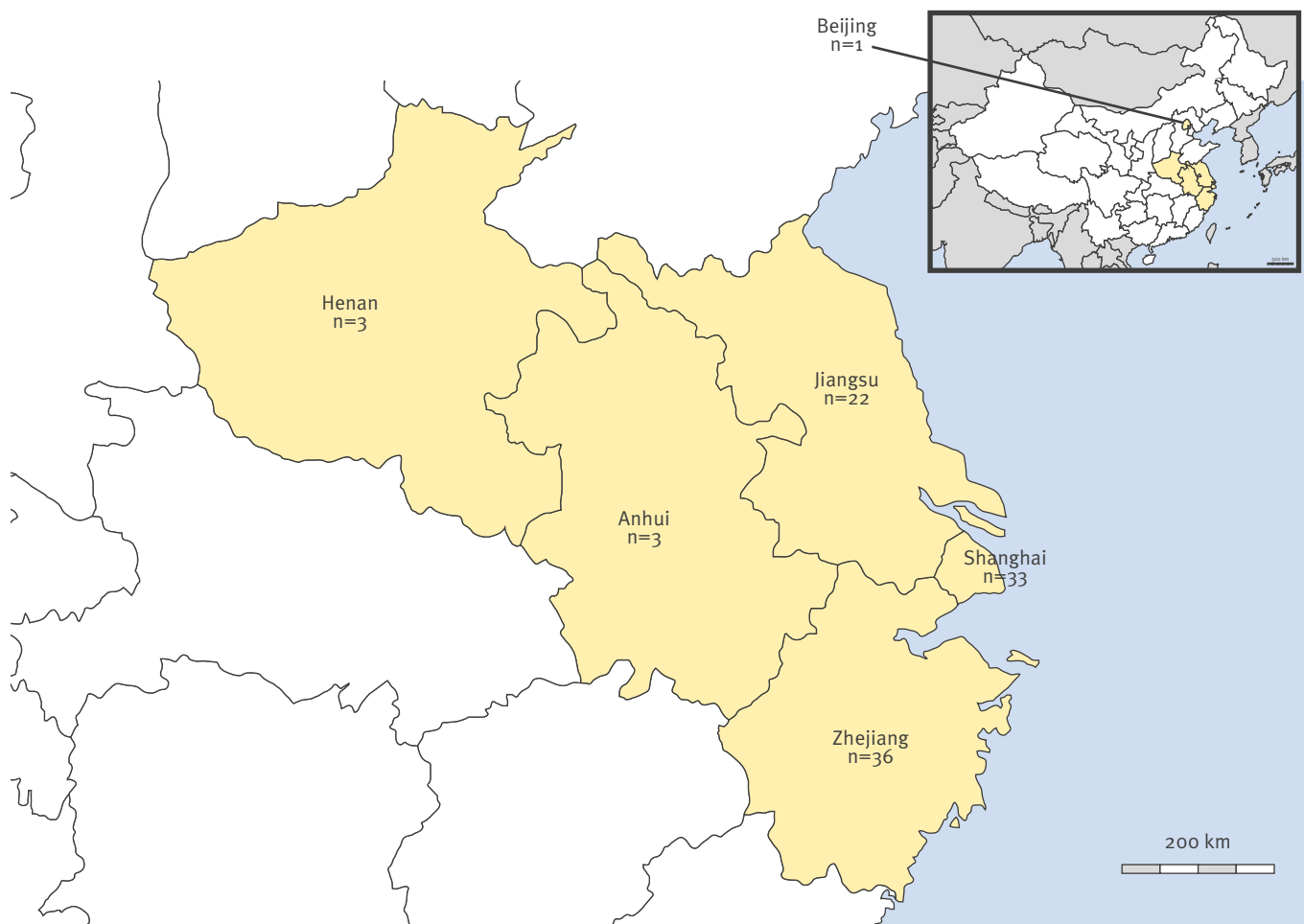
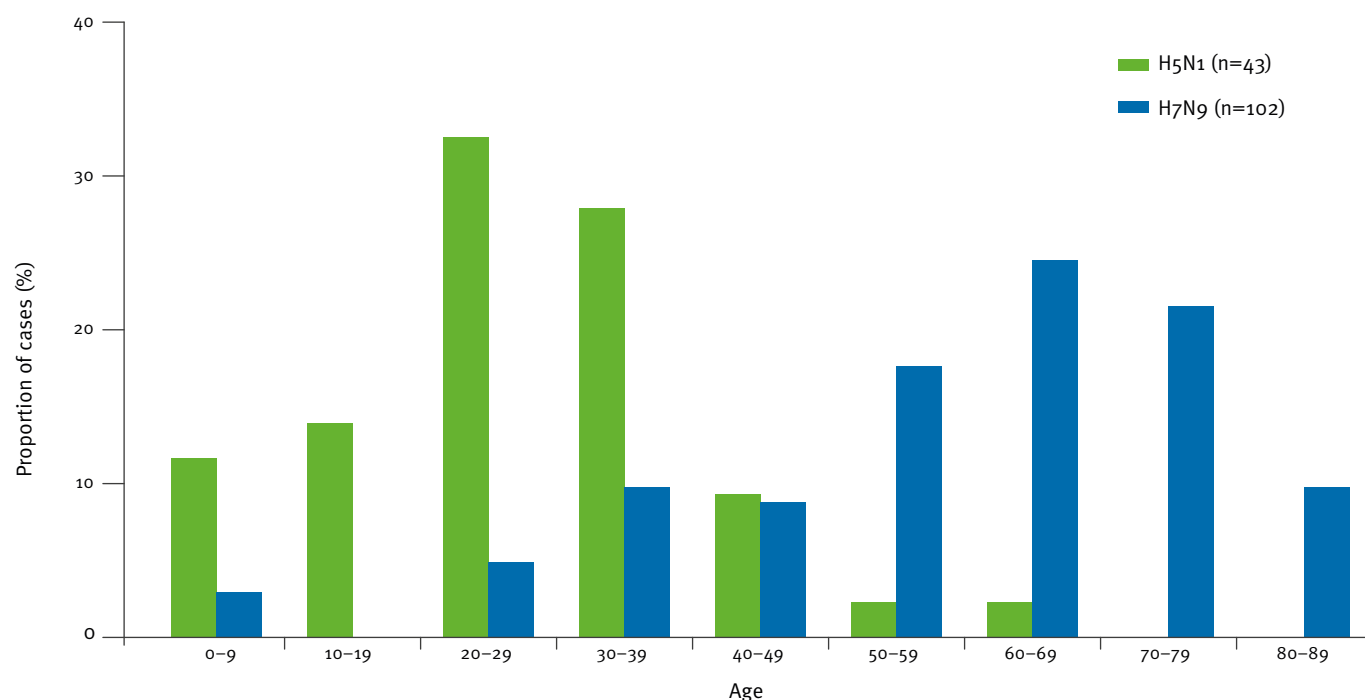


FIGURE 2

Age distribution of laboratory-confirmed human infections with avian influenza A(H5N1) in 2003–2013 (n=43) and A(H7N9) notified between 31 March 2013 through 21 April 2013 (n=102), mainland China

**TABLE**

Serious influenza A(H7N9) cases reported in six provinces of mainland China, and corresponding population denominators, 31 March–21 April 2013 (n=98)^a

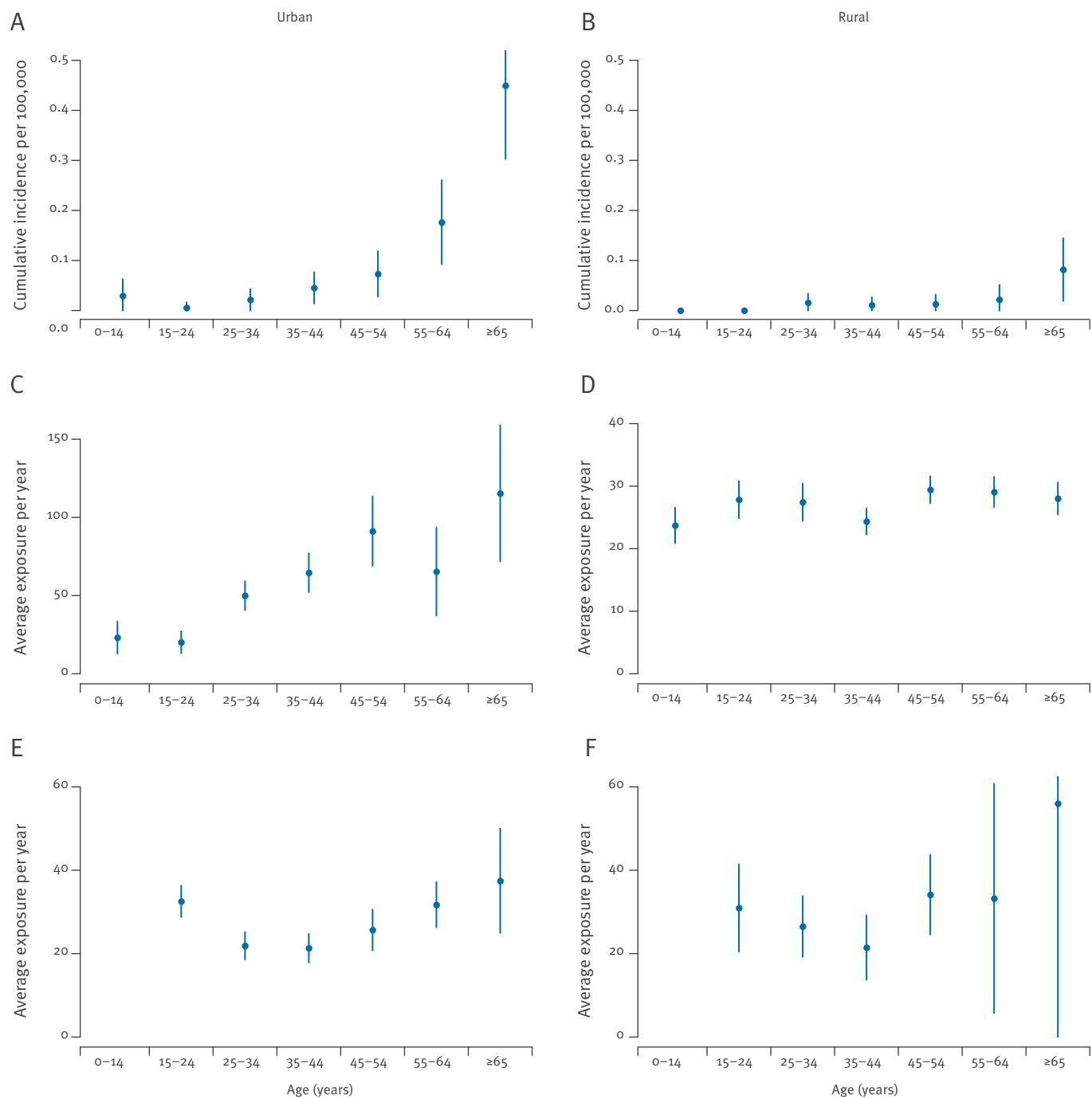
Province-type	Age group (years)						
	0-14	15-24	25-34	35-44	45-54	55-64	≥65
Number of serious influenza A(H7N9) cases							
Anhui-urban	0	0	0	1	0	2	0
Beijing-urban	1	0	0	0	0	0	0
Henan-rural	0	0	1	1	0	0	1
Jiangsu-urban	0	1	3	1	3	4	6
Jiangsu-rural	0	0	1	0	1	0	2
Shanghai-urban	2	0	1	1	5	5	19
Zhejiang-urban	0	0	0	5	2	6	11
Zhejiang-rural	0	0	0	1	1	3	7
Population size ^b							
Anhui-urban	1,617,392	2,299,994	1,965,849	2,512,466	1,671,583	1,135,834	979,469
Beijing-urban	1,311,411	2,968,261	3,513,686	2,657,513	2,278,771	1,485,603	1,347,970
Henan-rural	13,341,020	9,674,352	6,711,837	9,040,458	7,264,034	6,516,703	5,261,768
Jiangsu-urban	3,390,036	6,004,427	5,389,879	5,658,879	4,150,560	3,042,830	2,529,855
Jiangsu-rural	4,421,789	4,517,515	3,459,924	5,406,568	4,789,994	4,443,849	4,249,814
Shanghai-urban	1,483,687	2,821,598	3,660,496	2,797,231	2,809,896	2,267,794	1,800,140
Zhejiang-urban	2,449,320	4,004,494	4,044,383	4,062,503	2,768,791	1,707,271	1,349,532
Zhejiang-rural	2,888,769	2,448,074	2,676,907	3,835,297	3,477,935	2,840,965	2,708,735

^a The four mild cases among the total of 102 cases are not shown in this Table.

^b Population sizes obtained from the 2010 population census of the People's Republic of China, published on the official website of National Bureau of Statistics of China [7].

FIGURE 3

Comparison of age-specific cumulative incidence of serious illness associated with laboratory-confirmed influenza A(H7N9) virus infection, 31 March–21 April 2013, and age-specific poultry exposures, 2006 and 2007, China



Panels A and B show cumulative incidence and 95% confidence intervals of serious influenza A(H7N9) cases in (A) urban and (B) rural populations, based on 98 serious cases reported by 21 April 2013. Panels C to F show rates of exposures to retail and domestic poultry in (C) urban Shenzhen in 2007, (D) rural Xiuning in 2007, (E) urban Guangzhou in 2006, and (F) semi-rural Guangzhou in 2006.

regarded as a lower bound on the number of influenza A(H7N9) infections because it relies on complete ascertainment of all serious influenza A(H7N9) cases, and complete ascertainment of all influenza A(H7N9) infections in people aged 65 years and older. We also estimated $\beta_{age}=r_{old}/r_{young}$, the relative risk of serious illness conditional on infection in those aged 65 years and older compared with those younger than 65 years.

Results

Between 31 March and 21 April 2013, 102 laboratory-confirmed human influenza A(H7N9) cases were officially announced in six provinces of China. The affected areas were the cities and provinces around the city of Shanghai on the eastern coast of mainland China (Figure 1).

The age distribution of influenza A(H7N9) cases was very different to the age distribution of the 43 influenza A(H5N1) cases reported between 2003 and 2013 in mainland China (Figure 2). In particular, 56% of the influenza A(H7N9) cases were persons aged 60 years or older, whereas the majority of influenza A(H5N1) cases were young adults aged 20 to 39 years. In the eight affected areas, there were a total of 98 serious influenza A(H7N9) cases in a total population of 206 million persons (Table). The cumulative number of serious influenza A(H7N9) cases increased substantially with age particularly in urban locations (Figure 3).

We fitted the model described above to data on the incidence rates of serious influenza A(H7N9) cases in the six provinces, along with poultry exposures in urban and rural locations (Figure 2). In the age group of at least 65 years there were 46 serious and one mild infection, so we used a beta(47,2) distribution for the parameter r_{old} .

Based on the exposure data from Shenzhen and Xiuning to reflect exposures in affected urban and rural areas, we obtained the estimate $\beta_{age}=5.06$ (95% credibility interval (CI): 2.99–8.15), corresponding to a 5.06-fold increase in the risk of serious illness for those aged 65 years and older versus those younger than 65 years. The estimated values of p_{ij} and the observed values of A_{ij} were then used to estimate that there have been at least 323 (95% CI: 214–475) total influenza A(H7N9) infections in the population, including those reported. When we used the exposure data from Guangzhou to reflect exposures in affected urban and rural areas, we estimated $\beta_{age}=5.95$ (95% CI: 3.37–10.00), and an estimated minimum number of 352 (95% CI: 225–541) total influenza A(H7N9) infections in adults (because we did not have exposure data for children in Guangzhou).

In sensitivity analyses, results were similar using alternative simple parameterisations for the effect of age. For example when we used $r_i=r_7 \times \exp(\beta \times (i-7))$, we obtained an estimated 1.83-fold (95% CI: 1.56–2.18) increase in the risk of serious illness for every ten-year increase in age, and an estimate of at least 334 (95% CI: 239–461) total influenza A(H7N9) infections in the

population. The small sample size did not allow us to examine more complex functional forms for r_i . All analyses reported above were based on data available until April 25; we repeated the analyses based on data available until May 6 and the relationship between age and seriousness of disease was essentially the same.

Discussion

Our results suggest that the seriousness of influenza A(H7N9) infections increases with age. Previous reports also identified increases with older age in the seriousness of seasonal influenza [11] and H1N1pdm09 [12,13], although this may partly be due to the role of secondary bacterial pneumonia, whereas many of the influenza A(H7N9) deaths have been associated with primary viral pneumonia [4]. However, the age distribution of serious human infections with avian influenza A(H5N1) is very different (Figure 1). The patterns of exposure to avian influenza A(H5N1) and A(H7N9) viruses by age may not be identical because of the high degree of pathogenicity of influenza A(H5N1) in poultry compared with the absence of disease in poultry with influenza A(H7N9) infections [4], at least before to the national influenza A(H5N1) vaccination programme in poultry was introduced in 2006–07. Exposures to sick or dead poultry would be more frequent in farms and backyards, compared to live poultry markets. In addition, healthcare seeking behaviours may also have changed over the past 10 years. There are various potential explanations for an increased risk of serious illness for influenza A(H5N1) infections in young adults compared to other ages, and these hypotheses deserve further investigation [14].

We estimated that a minimum of 210–550 influenza A(H7N9) infections have occurred by 21 April 2013, assuming that almost all influenza A(H7N9) infections are serious in the elderly and that all serious infections have been identified. This estimate is therefore a lower bound on the number of total influenza A(H7N9) infections, and for these two reasons the real figure may be substantially higher. There could be some under-ascertainment of serious influenza A(H7N9) infections through failure to seek care or failure to be tested early enough in the course of disease to permit identification of the influenza infection [5]. Our estimate is also dependent on the assumption that age-specific patterns of exposure to retail and domestic poultry in affected areas of China in 2011 are similar to the patterns measured in Guangzhou, Shenzhen and Xiuning in 2006 and 2007. We are not aware of data on age-specific patterns in poultry exposures from eastern China other than our unpublished data from Xiuning, and future collection of such data from across China (and across South-east Asia) in urban and rural settings would be extremely useful.

Our estimates are limited by the lack of data on exposures in affected urban and rural areas. In particular, the higher risk for infection in males compared to females could be due to variation in sex-specific rates

of exposure by region [5]. Without data on such differences, we did not include sex in our models. Most confirmed cases report exposure to live poultry [3] and this remains the most likely source of infection for the majority of influenza A(H7N9) cases. However, the exposure distributions used in our analysis may not fully capture the age-specific risk profile, if there are other sources of infection apart from retail and domestic poultry. As of April 25, we are not aware of provinces in China with laboratory-confirmed A(H7N9) cases in poultry but not in humans. Finally, no published information is available on population levels of immunity to influenza A(H7N9), although preliminary investigations suggest very low antibody levels against influenza A(H7N9) virus in all ages, and we assumed there was no heterogeneity in immunity by age. If older persons had some degree of immunity against influenza A(H7N9) through potential past exposures to avian influenza viruses, this would imply an even higher number of undetected infections in adults based on our method.

In conclusion, we estimated a lower bound for the number of influenza A(H7N9) infections based on the possible age distribution of exposures and varying seriousness of infection by age. More accurate estimates of the risk of influenza A(H7N9) infection and the age-specific seriousness of infection could be provided by detailed seroepidemiological studies in affected areas [15].

Acknowledgements

We thank Vicky Fang, Angel Li, Michael Ni and Hoi Wa Wong for technical assistance.

This research was supported by the Harvard Center for Communicable Disease Dynamics from the National Institute of General Medical Sciences (grant no. U54 GM088558), the Research Fund for the Control of Infectious Disease, Food and Health Bureau, Government of the Hong Kong Special Administrative Region, and the Area of Excellence Scheme of the Hong Kong University Grants Committee (grant no. AoE/M-12/06). The funding bodies had no role in study design, data collection and analysis, preparation of the manuscript, or the decision to publish.

Potential conflicts of interest

BJC reports receipt of research funding from MedImmune Inc., and consults for Crucell NV. GML has received speaker honoraria from HSBC and CLSA. The authors report no other potential conflicts of interest.

Authors' contributions

Designed the study: BJC. Collected, synthesised and analysed data: BJC, GF, JYW, PW, QL, RF. Wrote the first draft: BJC. Interpreted the results and revised the article: GF, JYW, QL, PW, JTW, EHYL, RF, GML. All authors read and approved the final manuscript.

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Epidemiological link between exposure to poultry and all influenza A(H7N9) confirmed cases in Huzhou city, China, March to May 2013

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Citation style for this article:

Han J, Jin M, Zhang P, Liu J, Wang L, Wen D, Wu X, Liu G, Zou Y, Lv X, Dong X, Shao B, Gu S, Zhou D, Leng Q, Zhang C, Lan K. Epidemiological link between exposure to poultry and all influenza A(H7N9) confirmed cases in Huzhou city, China, March to May 2013. *Euro Surveill*. 2013;18(20):pii=20481. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20481>

Article submitted on 05 May 2013 / published on 16 May 2013

We analysed the association between influenza A(H7N9) confirmed cases and exposure to poultry in Huzhou city, China. All cases (n=12) had a history of direct exposure to poultry or live poultry markets. We detected A(H7N9)-positive poultry samples from each site that was epidemiologically associated with cases. None of the cases' close contacts tested positive. After closure of the markets, no new cases were identified, suggesting an epidemiological link between poultry exposure and A(H7N9) virus infection.

Background

Since February 2013, a novel avian influenza A(H7N9) virus has led to an outbreak in the Yangtze River Delta Region and elsewhere in China [1,2]. As of 10 May 2013, it has resulted in 129 cases, including 31 deaths. Sporadic human infections by several H7 subtypes of influenza A viruses (e.g. H7N2, H7N3 and H7N7) had been reported previously in several countries in Europe and North America [3]. Apart from an influenza A(H7N7) outbreak in the Netherlands in 2003, infections with these H7 subtypes usually result in a mild, self-limiting illness [3]. In contrast, in the current influenza A(H7N9) outbreak, infection with the virus has resulted in severe and fatal respiratory disease [2,4] – the first time human infections have been seen for this virus [1]. The origin of the virus has been demonstrated to be associated with a reassortant event between three earlier avian influenza viruses [1,5]. Its genome comprises a haemagglutinin (HA) fragment from A(H7N3), a neuraminidase (NA) fragment from an earlier A(H7N9) virus and six internal genomic fragments from A(H9N2).

Two recent studies have provided compelling evidence that the novel A(H7N9) viruses from patients have a close genetic relationship with isolates from

poultry [6,7], suggesting that the A(H7N9) virus may have spread to humans from poultry. However, preliminary epidemiological data showed that 18 of 77 confirmed cases did not have a history of contact with poultry [2]. Therefore, it remains to be determined whether there is a direct epidemiological link between exposure to poultry and human A(H7N9) virus infection.

Huzhou city, located in northern Zhejiang Province, China, is the geographical centre of the Yangtze River Delta (Figure 1). As of 10 May, 12 confirmed A(H7N9) cases have been reported in Huzhou city, accounting for about 9% (12/129) of all cases in China. There are two natural wetlands that provide habitats for over a 160 kinds of wild birds and, until the markets were closed, there had been an active live poultry business in Huzhou city. Therefore, we performed a detailed epidemiological study of the links between the confirmed cases and prior exposure to poultry.

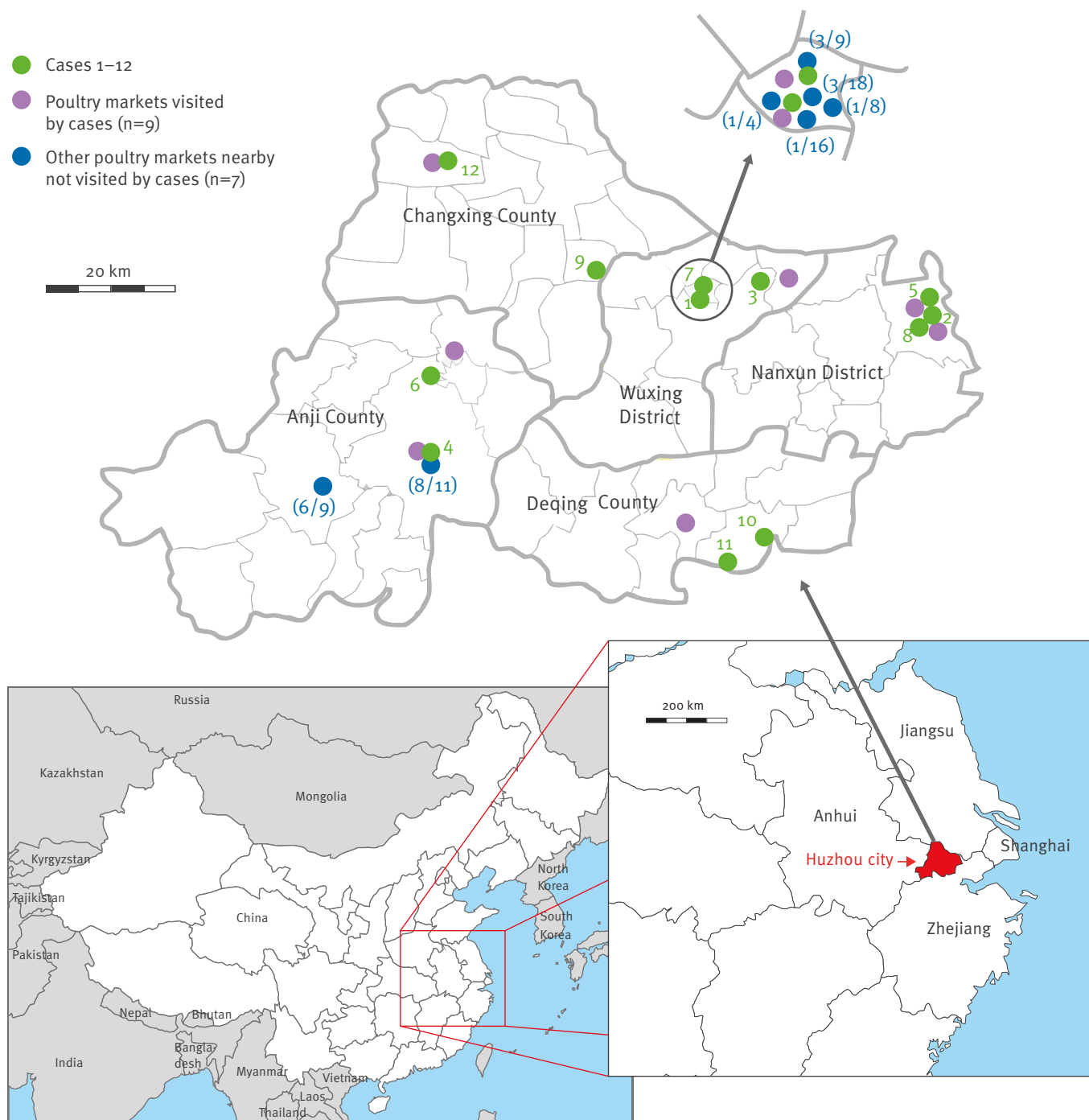
Data collection

A total of 12 persons were identified as influenza A(H7N9) confirmed cases, according to the definition in the national guidelines [8]. The infection was laboratory confirmed by real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis [9].

A close contact was defined as a person who came within two metres of a confirmed case without the use of effective personal protective equipment (e.g. masks and gloves) during the presumed infectious period. The close contacts included, among others, the cases' families and clinical staff (doctors and nurses) who had been in contact with the cases. All close contacts were traced and quarantined for seven days after their most recent exposure to a confirmed case.

FIGURE 1

Distribution of the influenza A(H7N9) confirmed cases and live poultry markets in Huzhou city, China, March–May 2013



For markets that the cases did not visit, the numbers of the samples positive for influenza A(H7N9) virus are shown (number of positive/number of total samples). The results for the markets that the cases visited are shown in Table 1.

In our investigation, a ‘visit’ included only occasions in which a case either bought poultry, or had been close to (within a distance of two metres) or touched live poultry booths at the market.

Information on cases' demographic characteristics, dates of symptom onset, exposure to poultry and/or other animals and/or visits to a live poultry market during the 10 days before symptom onset, as well as clinical signs and symptoms were collected using a standardised questionnaire and an open interview with the cases or their relatives when the cases were admitted to hospital. In our investigation, a 'visit' included only occasions in which a case either bought poultry, or had been close to or touched live poultry booths at a market.

To determine the source of the influenza A(H7N9) virus, we collected poultry faeces, waste (swab samples from culling benches) and sewage from the nine live poultry markets visited by the cases, for detection of A(H7N9) viral RNA by real-time RT-PCR.

In addition, samples from several surrounding live poultry markets (n=7) not visited by cases were also collected.

Data analysis

Demographic and clinical characteristics of influenza A(H7N9) cases in Huzhou city

As of 10 May 2013, 12 influenza A(H7N9) cases (four were male and eight female) were confirmed in Huzhou city (Table 1). As of 30 April, two had died, four had recovered fully, two were recovering and the other four remained critically ill (Figure 2). The median age was 60 years (range: 32–81) and most (n=9) were aged over 50 years.

The first case developed symptoms on 29 March 2013; the infection was laboratory confirmed on 4 April [6]. In fact, another patient (Case 2) became ill earlier, on 12 March, but the infection was not laboratory confirmed until 8 April. The last two patients (Cases 11 and 12) both became ill on 17 April and were laboratory confirmed on 25 and 26 April, respectively. The initial symptoms were fever (axillary temperature greater than 37.5 °C) (n=7), cough (n=4), myalgia (n=4), chills

TABLE 1

Demographic and exposure information of influenza A(H7N9) confirmed cases in Huzhou city, China, March–May 2013 (n=12)

Case number	Sex	Age (years)	Visits to live poultry markets ^a during 10 days before symptom onset		Testing for A(H7N9) viral RNA by real-time RT-PCR in markets visited by cases			Testing for A(H7N9) viral RNA by real-time RT-PCR in close contacts of cases	
			Date of last visit (2013)	Number of visits	Number of markets	Number of samples	Number of positive samples	Number of close contacts	Number who were positive
1	Male	64	NA	10	1	21	6	55	0
2	Female	50	NA	4	1	2	2	68	0
3	Female	54	NA	1	1	12	2	26	0
4	Female	61	31 March	1	1	17	5	26	0
5	Female	64	4 Apr	4	1 ^b	19	7	57	0
6	Female	66	30 March	4	1	18	3	35	0
7	Male	41	8 April	0 ^c	1	18	5	6	0
8	Female	66	3 April	1	1 ^b	19	7	4	0
9	Female	81	None	0	NA ^d	10	2	53	0
10	Male	32	NA	6	1	6 ^e	2	9	0
11	Female	60	None	0	NA ^f	6	2	8	0
12	Male	38	NA	2	1	6	2	22	0
Total	–	–	–	33	9	135	38	339	0

NA: not available; RT-PCR: reverse transcription-polymerase chain reaction.

^a In our investigation, a 'visit' included only occasions in which a case either bought poultry, or had been close to (within a distance of two metres) or touched live poultry booths at a market.

^b Cases 5 and 8 visited, on separate occasions, the same live poultry market.

^c Although this case did not purchase poultry, he took part in a government campaign of culling poultry at a live poultry market to limit the transmission of the novel influenza A(H7N9) virus, for about three hours on 8 April 2013.

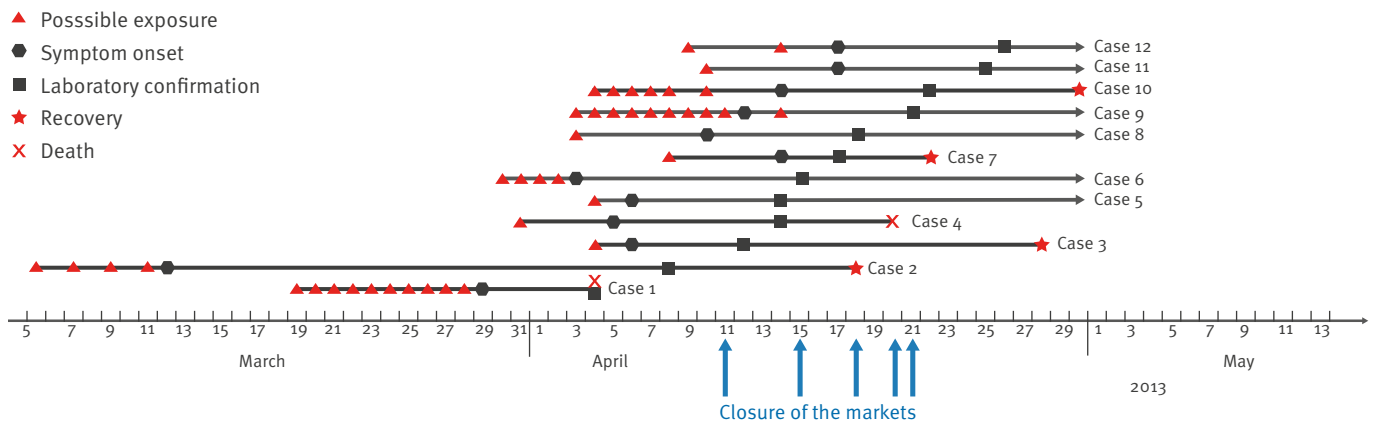
^d This case did not visit a live poultry market. She raised chickens in a courtyard with her neighbour. Because the case slaughtered all her chickens, we collected 10 samples from five chickens raised by her neighbour.

^e Pigeon-related samples. All other samples in the study were chicken-related samples.

^f The case's husband purchased four live chickens from a market on 8 April 2013 and raised them at home. On 10 April, because the chickens developed an acute illness, the case gave them antibiotics. We collected chicken faeces from her house.

FIGURE 2

Timeline of laboratory-confirmed influenza A(H7N9) cases in Huzhou city, China, March–May 2013 (n=12)



(n=2), weakness (n=2), nasal obstruction and runny nose (n=1), expectoration (n=1), pruritic body rash (n=1), chest tightness (n=1) and nausea (n=1). Of the 12 cases, nine developed severe pneumonia and pulmonary dysfunction 2–10 days after symptom onset.

Of the 12 cases, 10 had chronic underlying conditions such as hypertension, bronchitis or heart disease, before infection. Three cases had low counts of white blood cells (between $1.7 \times 10^9/L$ and $3.5 \times 10^9/L$); in another two, the count was high ($12.7 \times 10^9/L$ and $13.4 \times 10^9/L$), while the others were within the normal reference range ($4\text{--}10 \times 10^9/L$). All but one case (with 3.4 mg/L) had high levels of high-sensitivity C-reactive protein (between 18.4 mg/L and $>200 \text{ mg/L}$ (i.e. exceeding the detection range); normal reference range: $0\text{--}10 \text{ mg/L}$).

All cases had a history of exposure to poultry before symptom onset

Nine of the 12 cases had visited nearby live poultry markets at least once (range: 1–10 times) during the 10 days before symptom onset (Table 1). Of these nine cases, four (Cases 4, 5, 6, and 8) had had direct contact with live poultry during this time. Although three patients had not visited poultry markets, they all had a history of direct contact with live poultry during the 10 days before symptom onset. Case 7 was exposed to live poultry as part of a government campaign to cull poultry at live poultry markets. Case 9 and her neighbour had purchased 12 chickens from a chicken vendor and had raised them in the same courtyard for about 20 days. Case 9 killed her seven chickens when she found that one of them had become ill. For Case 11, her husband purchased four live chickens from a market on 8 April and raised them at home. On 10 April, because the chickens developed an acute illness, the patient gave them antibiotics.

Influenza A(H7N9) viral RNA was detected in all poultry markets visited by cases

In total, nine live poultry markets were epidemiologically associated with the patients (Table 1, Figure 1). Therefore, we collected poultry faeces, waste and sewage from these markets, to test for the presence of A(H7N9) viral RNA. We also collected throat and anal swabs and faeces from the chickens raised by the neighbour of Case 9 and chicken faeces from the house of Case 11. Of the 135 samples obtained, 38 samples were positive. Of particular note, A(H7N9) viral RNA was detected in samples from all nine markets, as well as those from the courtyard of Case 9 and the house of Case 11.

In addition, we expanded our surveillance to seven other nearby live poultry markets that the cases had not visited. Of 75 samples tested, 23 were positive for A(H7N9) viral RNA.

We also collected throat swabs from the close contacts (n=339) of the 12 patients. Among 339 samples, none tested positive for A(H7N9) viral RNA, indicating no human-to-human transmission of the virus.

Discussion

Previous studies have suggested that several mutations in the HA might be involved in the acquisition of the ability of the A(H7N9) virus to infect humans [5-7,10], and genetic evidence indicates that poultry is the reservoir of the virus [6,7]. However, preliminary observations that not all patients have had a history of exposure to poultry raise the controversial issue of the source and transmission route of the A(H7N9) virus [2].

Our results provide epidemiological evidence to support the hypothesis that A(H7N9) virus-infected poultry are a transmission source. A total of 139 live poultry

TABLE 2

Effect of closure of live poultry markets in the five regions of Huzhou city, China, March–May 2013

Region	Date of symptom onset (2013)		Date of market closure (2013)	Number of markets closed	Number of confirmed influenza A(H7N9) cases ^a	
	First case	Last case			Before market closure	After market closure
Wuxing District	29 March	14 April	11 April	32	3	0
Nanxun District	12 March	10 April	15 April	30	3	0
Deqing County	14 April	17 April	21 April	19	2	0
Changxing County	12 April	17 April	20 April	38	2	0
Anji County	3 April	15 April	18 April	20	2	0
Total	–	–	–	139	12	0

^a In order to exclude people who were infected by the virus but did not develop symptoms before market closure, case numbers were counted seven days after closure of the corresponding market.

markets (including those tested) in the five districts or counties in Huzhou city were closed sequentially, from 11 April to 21 April (Table 2). As of 15 May, no new cases have been identified in Huzhou city ($p < 0.01$). Although based on small case numbers, our findings support the view that poultry are a crucial transmission source and also indicate that closing live poultry markets in affected areas is an effective strategy to stop the outbreak.

With respect to the absence of reported poultry exposures in some patients ($n=18$) in a previous study [2], we can suggest two possible explanations, arising from our findings: (i) some patients may have forgotten some details of their exposure history by the time the epidemiological investigation was carried out; or (ii) some patients may have been unable to provide timely and reliable information due to their serious clinical conditions. It may therefore be possible that patients with no documented exposure may have in fact been exposed to poultry.

We tested 339 throat swabs from the cases' close contacts, but none tested positive for the A(H7N9) viral RNA, suggesting that these patients did not spread the virus to their close contacts. Although throat swabs may not be as often positive as deep sputum samples [7,11], we did not collect sputum samples from these close contacts because they had no obvious symptoms. Most patients ($n=9$) were aged 50 years or older, consistent with the nationwide data (78/107) [4]. Distinct from the nationwide data, however, two thirds (8/12) of the cases in Huzhou city were female (nationwide data: 32/106). This could possibly be due to the fact that in Huzhou city, housewives are mainly responsible for buying food, such as meat or vegetables, in local markets. It should also be borne in mind that most of the cases ($n=10$) had chronic underlying conditions.

Whether an individual's health status is associated with susceptibility to A(H7N9) virus infection remains to be proved.

Although an earlier study found that some live poultry markets tested positive, only a few poultry vendors ($n=4$) were found to be infected with the virus [2]. Why most vendors remained infection-free despite extremely frequent exposure to infected poultry is also unclear. Whether there is some pre-existing cross-reactive immunity, which enhances the susceptibility of patients to A(H7N9) virus infection [4] or prevents poultry vendors from infection needs to be determined.

Acknowledgements

This work was supported by grants from the China National Mega-projects for Infectious Diseases (2012ZX10004211-002 and 2013ZX10004101-005) to KL and the Li Ka-Shing Foundation to QL.

Authors' contributions

KL, CZ, JH and MJ designed and supervised the study. JH, PZ, MJ, JL, LW, DW, GL, XL, YZ, XD, BS, and SG performed the epidemiological investigation, sample collection, and laboratory confirmation of H7N9 infection. KL, CZ, JH, QL and MJ analysed and discussed the results. CZ wrote the paper, and KL and QL revised the paper. All authors have seen and approved the final version.

Conflict of interest

None declared.

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A comparison of rapid point-of-care tests for the detection of avian influenza A(H7N9) virus, 2013

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Citation style for this article:

Baas C, Barr IG, Fouchier RA, Kelso A, Hurt AC. A comparison of rapid point-of-care tests for the detection of avian influenza A(H7N9) virus, 2013. *Euro Surveill.* 2013;18(21):pii=20487. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20487>

Article submitted on 21 May 2013 / published on 23 May 2013

Six antigen detection-based rapid influenza point-of-care tests were compared for their ability to detect avian influenza A(H7N9) virus. The sensitivity of at least four tests, standardised by viral infectivity (TCID₅₀) or RNA copy number, was lower for the influenza A(H7N9) virus than for seasonal A(H3N2), A(H1N1)pdm09 or other recent avian A(H7) viruses. Comparing detection limits of A(H7N9) virus with Ct values of A(H7N9) clinical specimens suggests the tests would not have detected most clinical specimens.

Human infections with influenza viruses derived directly from wild birds or poultry are relatively rare, although since 2003, over 600 human infections with influenza A(H5N1) viruses have been detected, many of which were fatal [1]. During the same period, a small number of influenza A(H7) virus infections worldwide have also occurred in humans upon contact with infected poultry, generally resulting in mild symptoms such as conjunctivitis with occasional respiratory involvement and one death [2-4]. In contrast, China announced in March 2013 human infections with a novel reassortant avian influenza A(H7N9) virus which caused severe pneumonia resulting in a number of deaths [5]. Cases have occurred predominantly in men over 60 years of age living in urban areas, and most cases had a history of recent contact with poultry or poultry products [5]. By 16 May 2013, 131 human cases of influenza A(H7N9) virus infection, in 10 provinces and municipalities in eastern China, had been reported to the World Health Organization (WHO), of which 32 had resulted in death [6]. To date there have not been any reports of sustained human-to-human transmission of the influenza A(H7N9) virus, but the rapid emergence of the virus has led to significant concerns that it could in the future acquire human transmissibility and spread globally, causing the next influenza pandemic.

Rapid testing and diagnosis of possible human influenza A(H7N9) virus infections is an important diagnostic and public health task. An accurate diagnosis will allow the timely administration of antiviral therapy

[7,8] and may also enable the quarantining of infected cases to prevent further spread of the virus. Real-time PCR is now considered the gold standard laboratory-based assay for the detection of influenza virus infections due to its high sensitivity and specificity [6] and, although such assays have already been developed for the detection of influenza A(H7N9) virus [6], they require a high level of laboratory expertise and may not be available in all places where cases occur.

Point-of-care tests (POCTs) based on antigen detection, however, are simple to use and are designed for use in a medical clinic or outpatient setting, enabling the rapid testing of patient specimens within 15 minutes [9]. POCTs have mostly been licensed for detection of seasonal human influenza viruses, for which they generally have good specificity but low sensitivity [10]. Recently however, some POCTs have been specifically developed to utilise automated readers which have resulted in improved sensitivity. For public health purposes, it is important to determine whether the new or existing POCTs can detect the novel influenza A(H7N9) virus, particularly as previous studies have found that some POCTs had poorer sensitivity in detecting avian influenza strains compared to circulating human seasonal influenza strains [9]. If POCTs could reliably detect influenza A(H7N9) virus at clinically relevant levels, they would be a useful adjunct to real-time PCR in the detection of possible human cases, especially where technical resources are limited.

We evaluated six widely available POCTs that are based on detection of the nucleoprotein antigen (Table 1) for their ability to detect the avian influenza A(H7N9) virus A/Anhui/01/2013 [5], compared with three other low pathogenic avian influenza A(H7) viruses (A/Northern Shoveller/Egypt-EMC/1/2012, A/Mallard/Netherlands/4/2010 and A/Mallard/Lithuania-EMC/2/2010), two human seasonal influenza A(H3N2) (A/Sydney/506/2013 and A/Victoria/361/2011) and two influenza A(H1N1)pdm09 viruses (A/Auckland/1/2009 and A/Brisbane/292/2010).

TABLE 1

Details of influenza point-of-care tests evaluated in this study

Point-of-care test	Manufacturer	Specimen type approved	Proportion of virus sample following addition of diluent ^a	Format	Time (minutes)	Analysis of result
SD Bioline Influenza Ag/A/B/A(H1N1)Pandemic	Standard Diagnostics, Korea	NPS, NS, NA, NPA	50% (S:100 µl + D:100 µl)	Test strip	10–15	Eye
Binax Now Influenza A & B Card	Alere, Unites States	NW, NA, NPS, NS	100% (S:100 µl)	Card	15	Eye
Clearview Exact Influenza A & B	Inverness Medical, Australia	NS	29% (S: 50 µl ^b + D:120 µl)	Test strip	15	Eye
BD Veritor System for rapid detection of Flu A+B	Becton, Dickinson, Unites States	NS, NPS	11% (S: 50 µl ^b + D: 400 µl)	Cartridge	10	Automated reader
BD Directigen EZ Flu A+B	Becton, Dickinson, Unites States	NW, NA, NPS, TS	83% (S: 300 µl + D: 60 µl)	Cartridge	15	Eye
Sofia Influenza A+B FIA	Quidel, Unites States	NS, NPS, NPA, NW	46% (S: 260 µl + D: 300)	Cartridge	15	Automated reader

D: diluent; NA, nasal aspirate; NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; NS, nasal swab; NW, nasal wash; S: specimen.

^a Dilution of specimen in kit diluent is presented as a percentage, where volumes of specimen (S) and diluent (D) are shown in parentheses

^b Because the kit is not approved for testing of wash or aspirate samples, the specimen was absorbed by the swab provided after at least a 15 second immersion in the virus sample. The volume taken up by the swab was found to be approximately 50 µl.

Methods

All viruses were cultured in Madin-Darby Canine Kidney (MDCK) cells at a low multiplicity of infection for at least one passage before testing. All viruses were harvested at near full cytopathic effect (CPE), supernatant was centrifuged at low speed to remove cell debris, and viruses were frozen at -70°C prior to testing. A mean tissue culture infectious dose 50 (TCID₅₀) per mL was determined for each virus, based on at least three independent assays. Viruses were standardised to an infectivity titre of 1x10⁶ TCID₅₀/mL and then diluted in phosphate-buffered saline (PBS) in half-log₁₀ dilutions. Real-time RT-PCR analysis was conducted on each virus dilution to determine a cycle threshold (Ct) value and RNA copy number, using an Applied Biosystems 7500 Fast cycler and the real-time RT-PCR primer and probe set recommended by the United States Centers for Disease Prevention and Control (US CDC) for the detection of influenza A matrix genes (version 4 April 2006). RNA copy number was calculated using a standard curve of RNA standards (10-fold dilutions) of known copy number prepared from a pGEMT-A/California/7/2009 matrix plasmid using the Riboprobe In Vitro Transcription System (Promega, United States).

Each virus dilution was then tested in each POCT according to the manufacturer's instructions and a limit of detection (LOD), based on either the TCID₅₀/mL or the RNA copy number/µL, was determined. Standardising viruses by viral infectivity (TCID₅₀/mL)

is the most widely used method for the evaluation of POCTs, however it does not account for defective viral particles which may react in these antigen-detection assays. Therefore comparison of the LOD based on both TCID₅₀/mL and RNA copy number/µL (which accounts for both infective and defective viruses) can be informative. Half-log₁₀ dilutions of influenza A/Anhui/01/2013 virus were prepared in duplicate and both sets tested with the six POCTs. The number of available test kits was not sufficient to conduct duplicate testing of the other seven viruses. The duplicate sets of influenza A/Anhui/01/2013 virus concentrations gave highly comparable LOD data, therefore data for only the first set is presented. Four of the kits were read by eye, while two POCTs (Veritor and Sofia) utilised a mechanical reader (Table 1).

Results

Based on the TCID₅₀/mL, the LOD of five of the six POCTs for the A/Anhui/01/2013 influenza A(H7N9) virus ranged from 1x10⁵ to 1x10^{5.5} TCID₅₀/mL, with the Sofia and Directigen EZ detecting virus at the lower limit. The Clearview POCT was unable to detect the influenza A(H7N9) virus at any of the concentrations tested (1x10⁶ TCID₅₀/mL or lower) (Table 2). In comparison, the LOD of the POCTs for the other influenza A(H7) viruses tested was generally better than that seen with the A/Anhui/01/2013 virus, with some tests detecting virus levels as low as 1x10² TCID₅₀/mL. Seasonal influenza A viruses were also more easily detected by most POCTs

TABLE 2TCID₅₀ limit of detection of the influenza point-of-care tests evaluated in this study

Influenza virus origin/ subtype	Designation	Limit of detection (log ₁₀ TCID ₅₀ /mL)					
		SD Bioline	Binax Now	Clearview	Veritor	Directigen EZ	Sofia
Human A(H7N9)	A/Anhui/01/2013	5.5	5.5	>6 ^a	5.5	5	5
Avian A(H7)	A/Northern Shoveller Egypt-EMC/1/2012	5	4	>6 ^a	4	4	3
	A/Mallard/Netherlands/4/2010	5	4	>6 ^a	4	4	2.5
	A/Mallard/Lithuania-EMC/2/2010	4	3	4.5	2.5	2.5	2
Human A(H1N1)pdm09	A/Auckland/1/2009	4.5	3.5	5	3	3	2.5
	A/Brisbane/292/2010	4	3	4.5	3	2.5	2
Human A(H3N2)	A/Sydney/506/2013	5	4	5	4	4	3
	A/Victoria/361/2011	4	3.5	4.5	3	3	2.5

TCID₅₀: tissue culture infectious dose 50.^a >6, the virus was not detected at any of the concentrations tested.**TABLE 3**

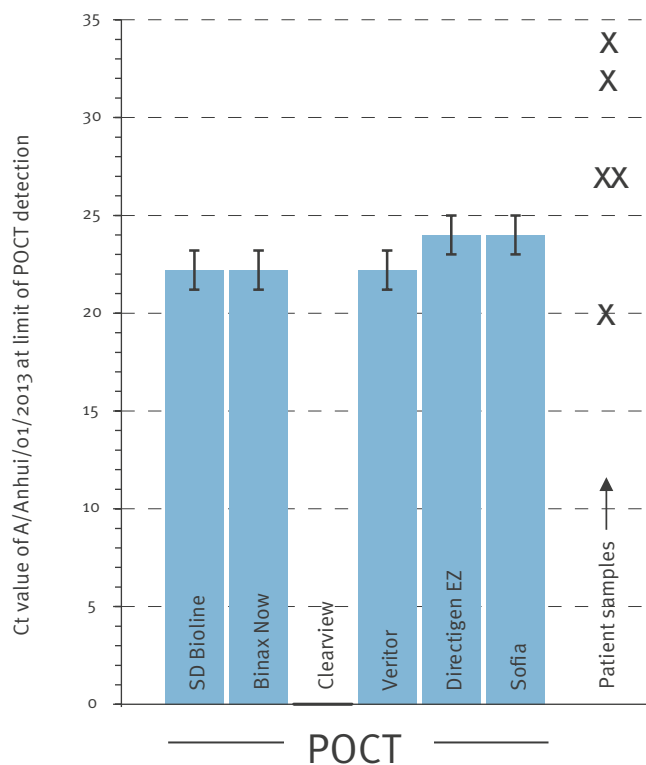
RNA copy number and Ct value limit of detection of the influenza point-of-care tests evaluated in this study

Influenza virus origin/ subtype	Designation	Limit of detection (RNA copies/μL [Ct value])						RNA copies/ μL [Ct value] of 1x10 ^{4.5} TCID ₅₀ /mL concentrations
		SD Bioline	Binax Now	Clearview	Veritor	Directigen EZ	Sofia	
Human A(H7N9)	A/Anhui/01/2013	5.0x10 ⁵ [22.2]	5.0x10 ⁵ [22.2]	>1.5x10 ⁶ ^a [<20.4]	5.0x10 ⁵ [22.2]	1.6x10 ⁵ [24.0]	1.6x10 ⁵ [24.0]	3.6x10 ⁴ [26.2]
Avian A(H7)	A/Northern Shoveller/ Egypt-EMC/1/2012	2.3x10 ⁶ [19.7]	2.3x10 ⁵ [23.3]	>2.9x10 ⁷ ^a [<15.8]	2.3x10 ⁵ [23.3]	2.3x10 ⁵ [23.3]	2.1x10 ⁴ [27.2]	4.1x10 ⁵ [22.5]
	A/Mallard/ Netherlands/4/2010	6.3x10 ⁵ [21.6]	5.6x10 ⁴ [25.7]	>1.1x10 ⁷ ^a [<17.2]	5.6x10 ⁴ [25.7]	5.6x10 ⁴ [25.7]	6.0x10 ² [31.8]	1.3x10 ⁵ [24.2]
	A/Mallard/ Lithuania-EMC/2/2010	7.4x10 ⁵ [21.7]	6.6x10 ⁴ [25.5]	1.5x10 ⁶ [20.5]	1.5x10 ⁴ [27.6]	1.5x10 ⁴ [27.6]	8.8x10 ³ [28.6]	1.5x10 ⁶ [20.5]
Human A(H1N1) pdm09	A/Auckland/1/2009	1.2x10 ⁶ [20.8]	8.9x10 ⁴ [25.0]	4.5x10 ⁶ [18.8]	5.6x10 ⁴ [25.3]	5.6x10 ⁴ [25.3]	4.6x10 ³ [28.2]	1.2x10 ⁶ [20.8]
	A/Brisbane/292/2010	2.7x10 ⁶ [19.5]	3.2x10 ⁵ [23.0]	4.5x10 ⁶ [19.0]	3.2x10 ⁵ [23.0]	5.7x10 ⁴ [25.5]	1.3x10 ⁴ [26.5]	4.5x10 ⁶ [19.0]
Human A(H3N2)	A/Sydney/506/2013	2.6x10 ⁶ [19.7]	2.2x10 ⁵ [23.5]	2.6x10 ⁶ [19.7]	2.2x10 ⁵ [23.5]	2.2x10 ⁵ [23.5]	6.3x10 ³ [26.8]	4.9x10 ⁵ [22.2]
	A/Victoria/361/2011	5.9x10 ⁵ [21.9]	1.1x10 ⁵ [24.3]	1.1x10 ⁶ [21.0]	5.7x10 ⁴ [26.0]	5.7x10 ⁴ [26.0]	7.9x10 ³ [27.8]	1.1x10 ⁶ [21.0]

Ct: cycle threshold; TCID₅₀: tissue culture infectious dose 50.^a The virus was not detected at any of the concentrations tested.

FIGURE

Mean Ct limit of detection for influenza A/Anhui/01/2013 in point-of-care tests compared with Ct values reported for four influenza A(H7N9) cases confirmed by RT-PCR



Ct: cycle threshold; TCID₅₀: tissue culture infectious dose 50; POCT: point-of-care test.

The Ct values of the A/Anhui/01/2013 concentrations containing 1×10^6 , $1 \times 10^{5.5}$, 1×10^5 , $1 \times 10^{4.5}$ TCID₅₀/mL were 20.4, 22.2, 24.0 and 26.2 respectively, while the RNA copy number was $1.5 \times 10^6/\mu\text{L}$, $5.0 \times 10^5/\mu\text{L}$, $1.6 \times 10^5/\mu\text{L}$, and $3.6 \times 10^4/\mu\text{L}$ respectively. Error bars indicate the standard deviation based on triplicate real-time RT-PCR analysis of the virus concentration at the POCT limit of detection. Influenza A matrix gene Ct values for the cases were 27, 32-34, 20 and 27 for the four patients, respectively, and were taken from the published article by Chen et al. [11]. RT-PCR efficiencies and therefore Ct values may differ slightly between the assay used here and that used by Chen et al. Because the Clearview POCT did not detect the influenza A/Anhui/01/2013 virus at any concentration tested, a Ct value of the limit of detection could not be determined for this kit.

than the influenza A(H7N9) virus, with the Sofia kit performing best: LOD ranging from 1×10^2 to 1×10^3 TCID₅₀/mL for the human influenza A(H3N2) and A(H1N1) pdm09 viruses.

Comparison of POCT LODs based on RNA copy number/ μL showed similar results to those based on TCID₅₀/mL for four of the kits (Binax Now, Clearview, Veritor and Sofia). These POCTs were less sensitive for the detection of the influenza A(H7N9) virus compared to the seasonal or other influenza A(H7) viruses (Table 3). However, for the SD Bioline and the Directigen EZ tests,

comparison of the LODs based on RNA copy number/ μL showed that influenza A(H7N9) was detected at a similar sensitivity to the other viruses (Table 3).

LODs based on RNA copy number/ μL or Ct also allowed an estimate of the expected performance of the POCTs in detecting influenza A(H7N9) virus in clinical samples (Figure). Comparison of the published Ct values of clinical samples from patients with confirmed influenza A(H7N9) infection [11] suggested that five of the six POCTs would have detected only one of the four influenza A(H7N9)-positive clinical specimens, with the other three specimens being outside the LOD of these assays (Figure).

Discussion

For all viruses tested, the Sofia POCT, which uses an automated reader, had the highest sensitivity. The BD Veritor test, which also uses an automated reader, had comparable sensitivity to the BD Directigen EZ and the Binax Now tests, both of which are read by eye. The Clearview and SD Bioline POCTs demonstrated the poorest sensitivity.

It is important to note that both the Clearview and the BD Veritor tests are only approved for analysis of swab specimens, therefore the test method used here may not have been appropriate. Similarly, all POCT assays may perform better using a particular specimen type, which was not tested here. The collection of the virus sample used for the Clearview and the BD Veritor POCTs (dipping the swab into liquid and waiting at least 15 seconds for absorption) resulted in a sample volume of approximately 50 μL which, when combined with the recommended diluent volume, resulted in the lowest concentrations of virus used in this evaluation (Table 1).

Other limitations of this study include the use of only a single influenza A(H7N9) isolate A/Anhui/01/2013 (although this virus is genetically closely related to other human influenza A(H7N9) viruses for which sequences have been reported) and the fact that clinical specimens were not available for analysis. It is also important to note that these POCTs have not been primarily designed or licensed to detect influenza A(H7N9) viruses or other avian-derived viruses.

Nevertheless, this study does demonstrate that the sensitivity of at least four of the six evaluated POCTs is lower for the novel influenza A(H7N9) virus than for seasonal influenza viruses and the other avian influenza A(H7) viruses tested. Comparison with published Ct values for clinical specimens from influenza A(H7N9) patients suggested that these POCTs may not detect the majority of influenza A(H7N9) cases, particularly if samples are taken late in the course of disease. Therefore RT-PCR remains the diagnostic test of choice for the testing of suspected influenza A(H7N9) influenza cases.

Acknowledgements

The authors are grateful to Dr Yuelong Shu and Dr Dayan Wang, WHO Collaborating Centre for Reference and Research on Influenza, Chinese Center for Disease Control and Prevention, Beijing, China, for providing the A/Anhui/1/2013 A(H7N9) virus. We are grateful to Heidi Peck, WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, for preparing the plasmid used for RNA quantitation. The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health and Ageing. RF was financed through NIAID-NIH contract HHSN266200700010C.

Conflict of interest

None declared

Authors' contributions

Designed the study: CB, IB, AH. Analysed and interpreted the data: CB, RF, AK, IB and AH. Drafted the article: CB and AH. Revised the article: CB, RF, AK, IB and AH.

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Guiding outbreak management by the use of influenza A(H7Nx) virus sequence analysis

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Citation style for this article:

Jonges M, Meijer A, Fouchier RA, Koch G, Li J, Pan JC, Chen H, Shu YL, Koopmans MP. Guiding outbreak management by the use of influenza A(H7Nx) virus sequence analysis. *Euro Surveill.* 2013;18(16):pii=20460. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20460>

Article submitted on 15 April 2013 / published on 18 April 2013

The recently identified human infections with avian influenza A(H7N9) viruses in China raise important questions regarding possible source and risk to humans. Sequence comparison with an influenza A(H7N7) outbreak in the Netherlands in 2003 and an A(H7N1) epidemic in Italy in 1999–2000 suggests that widespread circulation of A(H7N9) viruses must have occurred in China. The emergence of human adaptation marker PB2 E627K in human A(H7N9) cases parallels that of the fatal A(H7N7) human case in the Netherlands.

Background

Since 31 March 2013, Chinese health authorities have been reporting human cases of avian influenza A(H7N9) virus infections. This novel reassortant influenza virus, carrying six internal gene segments of poultry A(H9N2) viruses, supplemented with a haemagglutinin (HA) subtype 7 and a neuraminidase (NA) subtype 9 originating from wild birds [1,2], has caused infections in at least 82 persons, of whom 17 have died, as of 17 April 2013. The human infections occurred in eastern China in four provinces (Henan, Anhui, Jiangsu, and Zhejiang) and two municipalities (Shanghai and Beijing). Currently, the source of the human infections is unclear. However, in response to the detection of the influenza A(H7N9) virus among chickens, pigeons, ducks and environmental samples from some animal markets, as reported to the World Organisation for Animal Health (OIE), Chinese authorities have suspended live poultry trade and implemented the immediate closure of poultry markets, launched road inspections for transport of poultry, and have culled birds in an effort to deal with the issue. The outbreak raises important questions regarding possible source and risk to humans, and these will be addressed through case investigations. Here, we compare some findings from the first two weeks of the outbreak with those from a large highly pathogenic avian influenza (HPAI) A(H7N7) virus outbreak in the Netherlands in 2003 and from a low

pathogenic avian influenza (LPAI) A(H7N1) epidemic in Italy in 1999–2000 [3-5] and discuss issues related to diagnosis and the use of molecular surveillance to monitor the outbreak.

Influenza A(H7N7) outbreak in the Netherlands in 2003

Exactly 10 years ago, the Netherlands was struck by an HPAI A(H7N7) virus outbreak that resulted in the infection of poultry on 255 farms and the subsequent culling of about 30 million chickens. A total of 453 exposed persons had mild symptoms and were investigated, of whom 89 were laboratory-confirmed as having an A(H7N7) virus infection [6,7].

Diagnosis of influenza A(H7Nx) virus infection

During the HPAI A(H7N7) virus outbreak in the Netherlands, almost all human cases had mild symptoms, particularly conjunctivitis, but one veterinarian died after an episode of severe influenza-like illness complicated by acute respiratory distress syndrome (ARDS) [7]. Diagnosis was based on virus detection by reverse transcription polymerase chain reaction (RT-PCR) from eye swabs, or combined nose and throat swabs. An important observation was that the sensitivity of eye swab-based diagnostics was much higher than that of diagnostics based on combined nose and throat swabs [6,7]. Similarly, in later sporadic infections of humans with H7 influenza A viruses, ocular symptoms were observed, probably caused by a preference of H7 influenza viruses for receptors in the eye [8]. Studies have shown that H7 influenza viruses may use the ocular mucosa as portal of entry for systemic infection and that this is strain dependent [9,10]. Such symptoms have not been described for the cases of A(H7N9) virus infection in China in 2013, but it may be important to actively monitor for conjunctivitis in the outbreak investigation, as it may increase the success of case finding, particularly for mild cases.

TABLE, PANEL A

Origin of the sequences of influenza A(H7Nx) viruses used for the comparative analysis

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI439488	PB2	China	2013-Jan-01	A/Shanghai/1/2013		WHO Chinese National Influenza Center	
EPI439486	HA	China	2013-Jan-01	A/Shanghai/1/2013		WHO Chinese National Influenza Center	
EPI439487	NA	China	2013-Jan-01	A/Shanghai/1/2013		WHO Chinese National Influenza Center	
EPI439495	PB2	China	2013-Jan-01	A/Shanghai/2/2013		WHO Chinese National Influenza Center	
EPI439500	NA	China	2013-Jan-01	A/Shanghai/2/2013		WHO Chinese National Influenza Center	
EPI439502	HA	China	2013-Jan-01	A/Shanghai/2/2013		WHO Chinese National Influenza Center	
EPI439504	PB2	China	2013-Jan-01	A/Anhui/1/2013		WHO Chinese National Influenza Center	
EPI439507	HA	China	2013-Jan-01	A/Anhui/1/2013		WHO Chinese National Influenza Center	
EPI439509	NA	China	2013-Jan-01	A/Anhui/1/2013		WHO Chinese National Influenza Center	
EPI441601	PB2	China	2013-Mar-24	A/Hangzhou/1/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li, J; Pan, J; Pu, XY; Yu, XF; Kou, Y; Zhou, YY
EPI440095	HA	China	2013-Mar-24	A/Hangzhou/1/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li, J; Pan, J; Pu, XY; Yu, XF; Kou, Y; Zhou, YY
EPI440096	NA	China	2013-Mar-24	A/Hangzhou/1/2013	Hangzhou Center for Disease Control and Prevention	Hangzhou Center for Disease Control and Prevention	Li, J; Pan, J; Pu, XY; Yu, XF; Kou, Y; Zhou, YY
EPI440682	PB2	China	2013-Apr-03	A/Chicken/Shanghai/S1053/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440684	NA	China	2013-Apr-03	A/Chicken/Shanghai/S1053/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440685	HA	China	2013-Apr-03	A/Chicken/Shanghai/S1053/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440690	PB2	China	2013-Apr-03	A/Environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440692	NA	China	2013-Apr-03	A/Environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440693	HA	China	2013-Apr-03	A/Environment/Shanghai/S1088/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440698	PB2	China	2013-Apr-02	A/Pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440700	NA	China	2013-Apr-02	A/Pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	
EPI440701	HA	China	2013-Apr-02	A/Pigeon/Shanghai/S1069/2013	Harbin Veterinary Research Institute	Harbin Veterinary Research Institute	

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)s EpiFlu Database, on which this research is based.

TABLE, PANEL B

Origin of the sequences of influenza A(H7Nx) viruses used for the comparative analysis

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI238407	HA	Italy	1999-Jan-01	A/chicken/Italy/1067/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238409	NA	Italy	1999-Jan-01	A/chicken/Italy/1067/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238414	PB2	Italy	1999-Jan-01	A/chicken/Italy/1067/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI63315	HA	Italy	1999-Jan-01	A/chicken/Italy/1082/1999	Other Database Import	
EPI63320	NA	Italy	1999-Jan-01	A/chicken/Italy/1082/1999	Other Database Import	
EPI63332	PB2	Italy	1999-Jan-01	A/chicken/Italy/1082/1999	Other Database Import	
EPI63258	HA	Italy	1999-Dec-21	A/chicken/Italy/4746/1999	Other Database Import	
EPI63263	NA	Italy	1999-Dec-21	A/chicken/Italy/4746/1999	Other Database Import	
EPI63275	PB2	Italy	1999-Dec-21	A/chicken/Italy/4746/1999	Other Database Import	
EPI69063	HA	Italy	1999-Dec-22	A/chicken/Italy/4789/1999	Other Database Import	
EPI69068	NA	Italy	1999-Dec-22	A/chicken/Italy/4789/1999	Other Database Import	
EPI69080	PB2	Italy	1999-Dec-22	A/chicken/Italy/4789/1999	Other Database Import	
EPI407331	PB2	Italy	1999-Jan-01	A/chicken/Italy/5093/99	Other Database Import	
EPI407338	HA	Italy	1999-Jan-01	A/chicken/Italy/5093/99	Other Database Import	
EPI407342	NA	Italy	1999-Jan-01	A/chicken/Italy/5093/99	Other Database Import	
EPI69044	HA	Italy	1999-Dec-30	A/quail/Italy/4992/1999	Other Database Import	
EPI69049	NA	Italy	1999-Dec-30	A/quail/Italy/4992/1999	Other Database Import	
EPI69061	PB2	Italy	1999-Dec-30	A/quail/Italy/4992/1999	Other Database Import	
EPI69215	HA	Italy	1999-Apr-10	A/turkey/Italy/1265/99	Other Database Import	
EPI69220	NA	Italy	1999-Apr-10	A/turkey/Italy/1265/99	Other Database Import	
EPI69232	PB2	Italy	1999-Apr-10	A/turkey/Italy/1265/99	Other Database Import	
EPI69177	HA	Italy	1999-Jul-12	A/turkey/Italy/2715/99	Other Database Import	
EPI69182	NA	Italy	1999-Jul-12	A/turkey/Italy/2715/99	Other Database Import	
EPI69194	PB2	Italy	1999-Jul-12	A/turkey/Italy/2715/99	Other Database Import	
EPI238618	HA	Italy	1999-Jan-01	A/turkey/Italy/2732/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238619	NA	Italy	1999-Jan-01	A/turkey/Italy/2732/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238624	PB2	Italy	1999-Jan-01	A/turkey/Italy/2732/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI68484	HA	Italy	1999-Sep-03	A/turkey/Italy/3185/99	Other Database Import	
EPI68489	NA	Italy	1999-Sep-03	A/turkey/Italy/3185/99	Other Database Import	
EPI68501	PB2	Italy	1999-Sep-03	A/turkey/Italy/3185/99	Other Database Import	
EPI238625	HA	Italy	1999-Jan-01	A/turkey/Italy/3283/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238627	NA	Italy	1999-Jan-01	A/turkey/Italy/3283/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238632	PB2	Italy	1999-Jan-01	A/turkey/Italy/3283/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)s EpiFlu Database, on which this research is based.

TABLE, PANEL C

Origin of the sequences of influenza A(H7Nx) viruses used for the comparative analysis

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI68503	HA	Italy	1999-Sep-23	A/turkey/Italy/3488/1999	Other Database Import	
EPI68508	NA	Italy	1999-Sep-23	A/turkey/Italy/3488/1999	Other Database Import	
EPI68520	PB2	Italy	1999-Sep-23	A/turkey/Italy/3488/1999	Other Database Import	
EPI69139	HA	Italy	1999-Sep-23	A/turkey/Italy/3489/99	Other Database Import	
EPI69144	NA	Italy	1999-Sep-23	A/turkey/Italy/3489/99	Other Database Import	
EPI69156	PB2	Italy	1999-Sep-23	A/turkey/Italy/3489/99	Other Database Import	
EPI69158	HA	Italy	1999-Sep-27	A/turkey/Italy/3560/99	Other Database Import	
EPI69163	NA	Italy	1999-Sep-27	A/turkey/Italy/3560/99	Other Database Import	
EPI69175	PB2	Italy	1999-Sep-27	A/turkey/Italy/3560/99	Other Database Import	
EPI238633	HA	Italy	1999-Jan-01	A/turkey/Italy/3675/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238635	NA	Italy	1999-Jan-01	A/turkey/Italy/3675/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238640	PB2	Italy	1999-Jan-01	A/turkey/Italy/3675/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI42171	PB2	Italy	1999-Jan-01	A/turkey/Italy/4169/99	Other Database Import	
EPI89837	NA	Italy	1999-Jan-01	A/turkey/Italy/4169/99	Other Database Import	
EPI90388	HA	Italy	1999-Jan-01	A/turkey/Italy/4169/99	Other Database Import	
EPI69082	HA	Italy	1999-Nov-22	A/turkey/Italy/4294/99	Other Database Import	
EPI69087	NA	Italy	1999-Nov-22	A/turkey/Italy/4294/99	Other Database Import	
EPI69099	PB2	Italy	1999-Nov-22	A/turkey/Italy/4294/99	Other Database Import	
EPI68465	HA	Italy	1999-Nov-22	A/turkey/Italy/4295/1999	Other Database Import	
EPI68470	NA	Italy	1999-Nov-22	A/turkey/Italy/4295/1999	Other Database Import	
EPI68482	PB2	Italy	1999-Nov-22	A/turkey/Italy/4295/1999	Other Database Import	
EPI69125	NA	Italy	1999-Nov-22	A/turkey/Italy/4301/1999	Other Database Import	
EPI69137	PB2	Italy	1999-Nov-22	A/turkey/Italy/4301/1999	Other Database Import	
EPI69120	HA	Italy	1999-Nov-22	A/turkey/Italy/4301/1999	Other Database Import	
EPI238648	HA	Italy	1999-Jan-01	A/turkey/Italy/4482/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238650	NA	Italy	1999-Jan-01	A/turkey/Italy/4482/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238655	PB2	Italy	1999-Jan-01	A/turkey/Italy/4482/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238656	HA	Italy	1999-Jan-01	A/turkey/Italy/4580/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238658	NA	Italy	1999-Jan-01	A/turkey/Italy/4580/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238663	PB2	Italy	1999-Jan-01	A/turkey/Italy/4580/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI69101	HA	Italy	1999-Dec-14	A/turkey/Italy/4617/1999	Other Database Import	
EPI69106	NA	Italy	1999-Dec-14	A/turkey/Italy/4617/1999	Other Database Import	
EPI69118	PB2	Italy	1999-Dec-14	A/turkey/Italy/4617/1999	Other Database Import	

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TABLE, PANEL D

Origin of the sequences of influenza A(H7Nx) viruses used for the comparative analysis

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Authors
EPI69025	HA	Italy	1999-Dec-16	A/turkey/Italy/4644/99	Other Database Import	
EPI69030	NA	Italy	1999-Dec-16	A/turkey/Italy/4644/99	Other Database Import	
EPI69042	PB2	Italy	1999-Dec-16	A/turkey/Italy/4644/99	Other Database Import	
EPI68427	HA	Italy	1999-Dec-21	A/turkey/Italy/4708/1999	Other Database Import	
EPI68432	NA	Italy	1999-Dec-21	A/turkey/Italy/4708/1999	Other Database Import	
EPI68444	PB2	Italy	1999-Dec-21	A/turkey/Italy/4708/1999	Other Database Import	
EPI238664	HA	Italy	1999-Jan-01	A/turkey/Italy/977/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238666	NA	Italy	1999-Jan-01	A/turkey/Italy/977/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.
EPI238671	PB2	Italy	1999-Jan-01	A/turkey/Italy/977/1999	Other Database Import	Kim, L.M.; Scott, M.A.; Suarez, D.L.; Spackman, E.; Swayne, D.E.; Afonso, C.L.

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)'s EpiFlu Database, on which this research is based.

Serological surveillance is important to rule out infection in patients sampled too late for direct virus detection and to assess the extent of transmission. This may be a problem since serological responses in persons with confirmed influenza A(H7Nx) virus infection have been difficult to detect, making assessment of A(H7N9) virus exposure using serosurveys challenging [11,12]. However, determining the kinetics of the antibody response in confirmed cases of influenza A(H7N9) virus infection will provide important information that can inform public health action.

Comparative analysis based on virus sequencing

Detecting the novel virus in animals is challenging as the A(H7N9) virus is a LPAI virus that is expected to cause few or no signs of disease in poultry, allowing silent spread among poultry flocks. The sharing of influenza A(H7N9) virus sequence data by both Chinese veterinary and public health institutes through the Global Initiative on Sharing All Influenza Data (GISAID) allows comparison with the sequences obtained during the Dutch outbreak. We therefore performed a comparative analysis using HA, NA and PB2 (subunit of the influenza virus RNA polymerase complex) fragment sequences from Chinese A(H7N9) viruses in 2013, Dutch A(H7N7) viruses in 2003 and sequences from a well-described LPAI A(H7N1) epidemic in Italy in 1999–2000 [5]. Providers of sequences downloaded from GISAID, listed with accession numbers, are acknowledged in the Table.

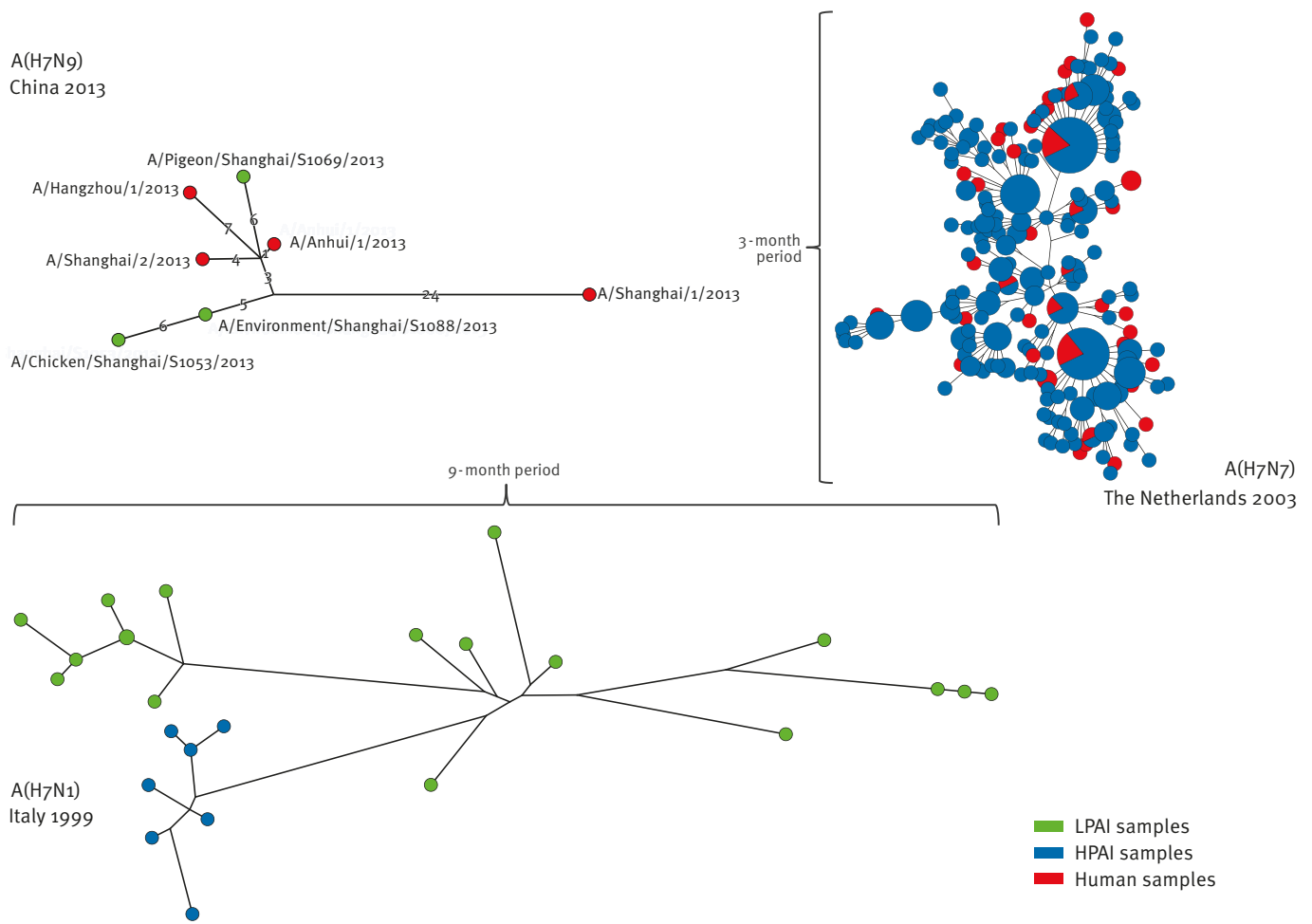
Sequence analysis of the Dutch viruses detected in poultry and in humans showed rapid diversification of the outbreak strain into multiple lineages (Figure). On the basis of the combined epidemiological and laboratory analyses, we demonstrated that sequences from humans were positioned mostly at ends of the branches of minimal spanning trees, confirming that humans were probably not involved in onward transmission [3].

In the current study, we compared the sequence diversity observed during the Dutch A(H7N7) outbreak and Italian A(H7N1) epidemic with the initial A(H7N9) virus sequences from the current outbreak in China. The maximum genetic distance generated during the three months of the Dutch HPAI A(H7N7) outbreak in concatenated HA, NA and PB2 segments of A(H7N7) viruses was 25 nucleotide substitutions. For the Italian LPAI A(H7N1) epidemic, the distance generated during a nine-month period was 66 nucleotide substitutions. For the A(H7N9) outbreak strains, this genetic distance is 35 substitutions, or 21 substitutions when the outlier strain A/Shanghai/1/2013 is ignored (Figure).

All (n=7) NA sequences of the A(H7N9) viruses are characterised by a deletion in the stalk region, associated with adaptation to gallinaceous hosts [1,2,13]. Similar deletions in the NA stalk were also observed during the A(H7N7) outbreak in the Netherlands and the A(H7N1) epidemic in Italy [5]. Given the degree of

FIGURE

Genetic diversity of three influenza A(H7Nx) virus outbreaks expressed by minimum spanning trees



HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza.

The minimum spanning trees were constructed using concatenated haemagglutinin, neuraminidase and PB2 (subunit of the influenza virus RNA polymerase complex) nucleotide sequences in BioNumerics software version 6.6.4. The scaling of the branches, representing nucleotide substitutions, is equal for the three outbreaks.

sequence diversity present in initial A(H7N9) virus sequences, compared with that of the Dutch HPAI A(H7N7) and Italian LPAI A(H7N1) outbreak strains, and the large geographical area affected, the data are suggestive of (silent) spread and adaptation in domestic animals before the novel A(H7N9) virus was identified in humans.

Human adaptation markers

The majority of the Dutch human cases of A(H7N7) virus infection had mild symptoms, with the exception of one fatal case who was diagnosed with an A(H7N7) virus with the mammalian adaptation marker PB2 E627K. This mutation most probably occurred during infection of this case and was associated with high virulence [14]. Remarkably, the PB2 segments of the four

available human virus genome sequences from China all carry this E627K substitution, which is absent in the virus isolates obtained from birds and the environment [2]. In addition, three of the four infections with the virus with PB2 E627K were fatal. There are two plausible explanations for this observation:

1. the mammalian adaptation markers are selected during replication in humans following exposure to viruses that do not have this mutation, which are circulating in animals;
2. the mammalian adaptation markers result from virus replication in animals from which humans become infected.

The relatively protracted disease course in the current outbreak of A(H7N9) virus infection, with relatively mild symptoms at first, followed by exacerbation in the course of a week or longer, is suggestive of the first hypothesis, similar to the outbreak in the Netherlands. In this scenario, an important difference in the A(H7N9) observations from the Netherlands is the frequency of finding the PB2 E627K mutation in humans (4/4 A(H7N9) sequenced patient strains compared with 1/61 sequenced A(H7N7) patient strains). Therefore, an outstanding question is whether the A(H7N9) viruses are more readily mutating in humans or milder cases are being missed. Contact investigations have found no mild cases and only one asymptomatic case), but in order to address this issue, more enhanced testing of persons exposed to a similar source is needed, using the most sensitive tests available on the optimal clinical specimen type obtained at the right time.

Although human infections with H7 influenza viruses have occurred repeatedly over the last decades without evidence of sustained human-to-human transmission, the absence of sustained human-to-human transmission of A(H7N9) viruses does not come with any guarantee. Five of seven A(H7N9) virus strains obtained from humans (n=2), birds (n=2) and the environment (n=1) have a mutation in HA, Q226L, that is associated with binding to alpha(2,6)-linked sialic acids, the virus receptors in the human upper respiratory tract [2]. This Q226L substitution in combination with G228S has been associated with human receptor preference for influenza viruses that caused the pandemics of 1957 and 1968 and with airborne transmission of A(H5N1) virus [15,16]. For H7 viruses, it has recently been demonstrated that these mutations also increased human receptor-binding affinity [17]. In combination with the PB2 E627K mutation, the A(H7N9) virus thus contains two well-known mammalian adaptation markers.

Conclusion

Comparative analysis of the first virological findings from the current outbreak of influenza A(H7N9) virus infection in China with those from other influenza A(H7Nx) outbreaks suggests that widespread circulation must have occurred, resulting in major genetic diversification. Such diversification is of concern, given that several markers associated with increased risk for public health are already present. Enhanced monitoring of avian and mammalian animal reservoirs is of utmost importance as the public health risk of these A(H7N9) viruses may change following limited additional modification.

Acknowledgements

This work was supported by the Dutch Ministry of Economic Affairs, Agriculture, and Innovation, Castellum Project.

We acknowledge the authors, originating and submitting laboratories of the sequences from the Global Initiative on Sharing All Influenza Data (GISAID)'s EpiFlu Database (www.gisaid.org), on which this research is based.

Authors' contributions

Yue-Long Shu, Hualan Chen, Jun Li, Jing-Cao Pan, Ron A.M. Fouchier and Guus Koch improved the manuscript following writing by Marcel Jonges, Adam Meijer and Marion Koopmans. All authors were directly involved in the generation, sharing and analysis of influenza sequence data.

Conflict of interest

None declared.

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Specific detection by real-time reverse-transcription PCR assays of a novel avian influenza A(H7N9) strain associated with human spillover infections in China

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Citation style for this article:

Corman VM, Eickmann M, Landt O, Bleicker T, Brünink S, Eschbach-Bludau M, Matrosovich M, Becker S, Drosten C. Specific detection by real-time reverse-transcription reaction assays of a novel avian influenza A(H7N9) strain associated with human spillover infections in China. *Euro Surveill.* 2013;18(16):pii=20461. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20461>

Article submitted on 12 April 2013 / published on 18 April 2013

In response to a recent outbreak in China, detection assays for a novel avian influenza A(H7N9) virus need to be implemented in a large number of public health laboratories. Here we present real-time reverse-transcription polymerase chain reaction (RT-PCR) assays for specific detection of this virus, along with clinical validation data and biologically-safe positive controls.

Background

An avian influenza A(H7N9) virus has emerged in south eastern China in March 2013 [1]. As of 16 April 2013, the Chinese authorities have reported 63 laboratory-confirmed human cases, 14 of whom have died [2]. While epidemiological data suggest no direct human-to-human transmission, there is huge concern that the presence of mutations typical for mammalian-adapted influenza A viruses such as E627K in the polymerase basic protein 2 (PB2) gene might indicate a certain propensity of the virus to further adapt to humans [1,3]. Even in absence of proven human-to-human transmission, the emergence of the avian influenza A(H7N9) virus in humans constitutes a test scenario for pandemic preparedness.

The rapid deployment of diagnostic methodology is among the top priorities in laboratory-based pandemic response. While capacities and responsibilities are in place in many countries, the actual provision of test technology involves major challenges, including the necessity to provide validation data for new test protocols, as well as the need for qualified and safe biological materials suitable as positive controls. In particular, positive controls based on in-vitro transcribed RNA containing only small fragments of the viral genome can be shipped without biosafety concerns. We already started using this option for the wide distribution of diagnostic tests during the severe acute respiratory syndrome (SARS) epidemic in 2003, and made use of it several times thereafter [4-6]. In response to the emergence of HCoV-EMC in 2012 we

provided validated protocols along with positive controls through a European Union (EU) research network. This strategy enabled implementation of diagnostic capacity across the EU within only a few weeks [7,8]. In this report we present diagnostic methods for detection of the emerging influenza A(H7N9) virus from clinical specimens.

Methods

Clinical samples and influenza cell culture supernatants

Respiratory swabs, sputum, and endotracheal aspirates were obtained during 2012 and 2013 from hospitalised patients of the University of Bonn Medical Centre and the University of Marburg Medical Centre. Cell culture supernatants from typed influenza viruses were obtained from the German Society for Promotion of Quality Assurance in Medical Laboratories (INSTAND) proficiency testing panels. RNA was extracted from the samples as described earlier by using a viral RNA mini kit (Qiagen) [8].

Template for design of assays

The first three published genome sequences of the 2013 influenza A(H7N9) epidemic from the GISAID EpiFlu database, as listed in Table 1, served as the template for assay design. An influenza A/Mallard/Sweden/91/2002 (H7N9) strain [9], provided by Ron Fouchier, Rotterdam, to author M.M. was used for initial validation experiments.

Real-time reverse-transcription polymerase chain reaction targets

In order to design highly specific real-time reverse-transcription polymerase chain reaction (RT-PCR) targets that would not cross-react with human influenza viruses, we chose the haemagglutinin (HA) and neuraminidase (NA) genes of avian influenza A(H7N9) as targets for amplification.

TABLE 1

Origin of the haemagglutinin and neuraminidase sequences of emerging influenza A(H7N9) virus used for assay design, April 2013

Segment ID	Segment	Country	Collection date	Isolate name	Submitting laboratory	Submitter/author
EPI439507	HA	China	2013	A/Anhui/1/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439486	HA	China	2013	A/Shanghai/1/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439502	HA	China	2013	A/Shanghai/2/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439509	NA	China	2013	A/Anhui/1/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439487	NA	China	2013	A/Shanghai/1/2013	WHO Chinese National Influenza Center	Lei Yang
EPI439500	NA	China	2013	A/Shanghai/2/2013	WHO Chinese National Influenza Center	Lei Yang

HA: haemagglutinin; ID: identity; NA: neuraminidase; WHO: World Health Organization.

We gratefully acknowledge the authors and laboratories for originating and submitting these sequences to the EpiFlu database of the Global Initiative on Sharing All Influenza Data (GISAID); these sequences were the basis for the research presented here.

All submitters of data may be contacted directly via the GISAID website www.gisaid.org.

Because no isolates of the emerging influenza A(H7N9) lineage were available from China, we selected an influenza A/Mallard/Sweden/91/2002 (H7N9) strain whose HA and NA genes were closely related [1,9]. The finding of annealing sites for primers and probes was guided by an alignment of three available sequences from the 2013 emerging influenza A(H7N9) lineage, and the influenza A/Mallard/Sweden/91/2002 (H7N9) sequence. Thermodynamically suitable primers and probes were selected to minimise the number of nucleotide mismatches at their binding sites to the emerging A(H7N9) sequences as well as the A/Mallard/Sweden/91/2002 (H7N9) sequence. The NA gene fragment of A/Mallard/Sweden/91/2002 (A7N9) had to be sequenced for this purpose.

The final test layout included two adjacent regions in the HA gene, termed HA(I) and HA(II), which were respectively targeted by primers and probes of two RT-PCR assays. The two HA regions were included in one control RNA construct derived from the influenza A/Mallard/Sweden/91/2002 (H7N9) strain (Figure 1A). A region was also chosen for amplification of the NA gene, constituting the target of a third RT-PCR assay (NA(I)). A respective control RNA for this NA gene region, derived from the influenza A/Mallard/Sweden/91/2002 (H7N9) strain was also constructed (Figure 1B). In each of the three regions targeted by the RT-PCR assays, mutations in the oligonucleotide binding sites between the emerging influenza A(H7N9) lineage sequences and the influenza A/Mallard/Sweden/91/2002(H7N9) strain sequence were minimal, enabling the use of influenza A/Mallard/Sweden/91/2002 (H7N9)-derived RNAs as

positive controls for all RT-PCR assays (Figure 1C and D).

Real-time reverse-transcription polymerase chain reaction

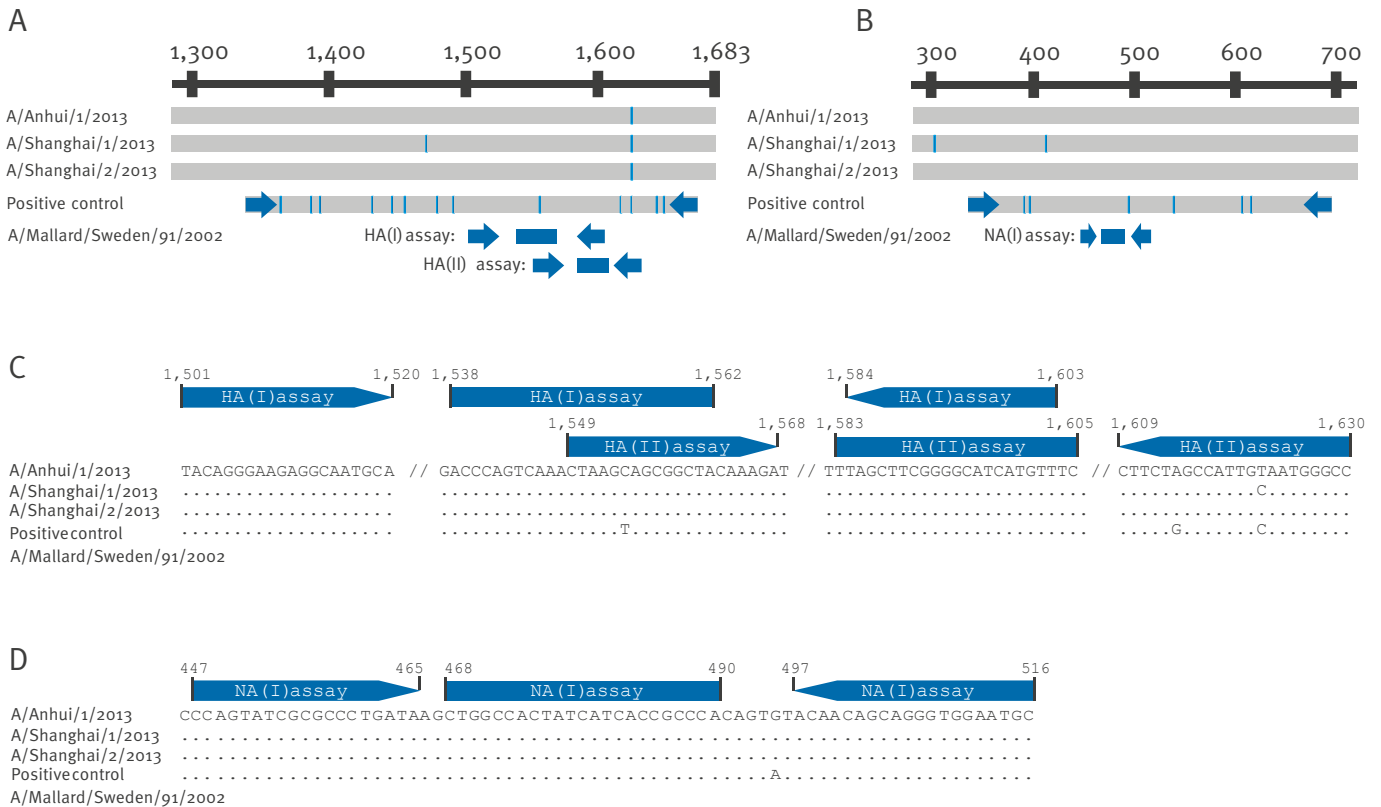
All three assays had the same conditions but the primer and probe sequences varied (Table 2). A 25- μ l reaction was set up containing 5 μ l of RNA, 12.5 μ l of 2 X reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen; containing 0.4 mM of each deoxyribonucleotide triphosphates (dNTP) and 3.2 mM magnesium sulfate), 1 μ l of reverse transcriptase/Taq mixture from the kit, 0.4 μ l of a 50 mM magnesium sulfate solution (Invitrogen – not provided with the kit), 1 μ g of non-acetylated bovine serum albumin (Roche), 400 nM concentrations of each of the primers, as well as 200 nM of the probe. All oligonucleotides were synthesised and provided by Tib-Molbiol, Berlin, where stock solutions from the original synthesis lots are kept. Thermal cycling consisted of 55°C for 15 min, followed by 95°C for 3 min and then 45 cycles of 95°C for 15 s, 58°C for 25 s.

In-vitro transcribed RNA controls

Using influenza A/Mallard/Sweden/91/2002 (H7N9) strain RNA as a template, a reverse-transcription PCR fragment encompassing both HA regions respectively targeted by the two HA(I) and (II) assays as well as additional flanking nucleotides was generated using primers IVT_HA-FWD and IVT_HA-REV. Likewise a reverse-transcription PCR fragment comprising the region of the NA gene targeted by the NA(I) assay was amplified with primers IVT_NA-FWD and IVT_NA-REV (Table 2). The HA and NA reverse-transcription PCR

FIGURE 1

Target sequence regions used for real-time reverse-transcription polymerase chain reaction assays to detect the emerging influenza A(H7N9) virus, March 2013



RT-PCR: real-time reverse-transcription polymerase chain reaction.

Each panel of the Figure shows partial alignments of three available sequences of the emerging influenza A(H7N9) lineage, which are designated as A/Anhui/1/2013, A/Shanghai/1/2013 and A/Shanghai/2/2013. Also aligned are the corresponding partial sequences of the influenza A/Mallard/Sweden/91/2002 (H7N9) strain, which serve to generate positive control templates for the RT-PCR assays. The Figure shows the regions of the haemagglutinin (HA) (panel A and C) and the neuraminidase (NA) genetic sequences (panel B and D) targeted by primers, represented by blue arrows, and probes, as blue bars, of the different PCR assays. Two regions are targeted for the HA gene, resulting in two separate RT-PCR assays, HA(I) and HA(II). One region of the NA gene is targeted by one NA(I) RT-PCR assay. Numbers in panels A to D represent genome positions according to the A/Anhui/1/2013 genome sequence. Grey horizontal bars in panels A and B represent the sequences, while blue vertical lines represent sequence variations between any of the listed strains in the alignment. Panels C and D show detailed alignments of target sequences of the three assays. In Panel C, nucleotides in the aligned sequences between the binding sites were omitted (marked with //). In the alignments, dots represent identity to the A/Anhui/1/2013 sequence, and all nucleotide substitutions are specified.

fragments are thereafter referred to as ‘peri-amplicon fragments’. These PCR products were ligated into pCR 4 plasmid vectors and cloned in *Escherichia coli* by means of a pCR 4-TOPO TA cloning reagent set (Invitrogen). Plasmids were examined for correct orientation of inserts by PCR, purified, and re-amplified with plasmid-specific primers from the reagent set to reduce the plasmid background in subsequent in vitro transcription. Products were transcribed into RNA with the MegaScript T7 in vitro transcription reagent set (Ambion). After DNase I digestion, RNA transcripts were purified with Qiagen RNeasy columns and quantified photometrically. The RNAs derived from the peri-amplicon fragments were used as positive control for the performance of the RT-PCR assays (Figure 1). All

transcript dilutions were carried out in nuclease-free water containing 10 µg/mL carrier RNA (Qiagen).

Results

Sensitivity of the real-time reverse-transcription polymerase chain reaction assays

Sensitivity tests employed quantified, in-vitro transcribed RNA derived from the peri-amplicon fragments of the combined HA(I/II) assays, as well as the NA(I) assay. Transcripts were generated and tested in serial tenfold dilution experiments. To obtain a statistically robust assessment of limits of detection (LODs), transcripts were tested in multiple parallel reactions containing RNA copy numbers above and below the pre-determined end point dilution detection limits of

TABLE 2

Primers and probes for assays used to screen for the emerging influenza A(H7N9) virus, April 2013

Assay/target	Oligonucleotide ID ^a	Sequence (5'–3') ^{b,c}	Polarity
RT-PCR/HA(I)	HA7_1_2013rtF	TACAGGGAAGAGGCAATGCA	+
	HA7_1_2013rtP	FAM-ACCCAGTCAAATAAGCAGCGGCTA-TAMRA	+
	HA7_1_2013rtR	AACATGATGCCCGAAGCTA	-
RT-PCR/HA(II)	HA7_2_2013rtF	CTGAGCAGCGGCTACAAAGA	+
	HA7_2_2013rtP	FAM-TTAGCTTCGGGGCATCATGTTTC-BBQ	+
	HA7_2_2013rtR	GKCCCATRCAATGGCTAGAAG	-
RT-PCR/NA(I)	NA9_2013rtF	CCAGTATCGCGCCTGATA	+
	NA9_2013rtP	FAM-CTGGCCACTATCATCACCGCCA-TAMRA	+
	NA9_2013rtR	GCATTCCACCCTGCTGTTGT	-
Sequencing/HA-IVT	IVT_HA-FWD	CAATTGATCTGGCTGATTGAGA	+
	IVT_HA-REV	GTGCACCGCATGTTCCATTC	-
Sequencing/NA-IVT	IVT_NA-FWD	CAAGAGAACCCTATGTTTCATGC	+
	IVT_NA-REV	GTTGTGGCATAACATTCAGATTC	-
Synthetic control	Fragment I	AGTATCACATCTTTGTAGCCGCTGCTTAGTTGACT GGGTC AATCTGTATTCTATTTGCATTGCCTCTCCCTGTATTGCTGTGA	-
	Fragment II	ACAAAGATGTGATACTTTGGTTTAGCTTCGGGGCA TCATGTTTCATACTTCTAGCCATTGTAATGGGC	+
	Primer F	TCACAGCAAATACAGGGAAGAG	+

BBQ: blackberry quencher; FAM: 6-carboxyfluorescein; HA: haemagglutinin gene; ID: identity; NA: neuraminidase gene; RT-PCR: real-time reverse-transcription polymerase chain reaction; TAMRA: 6-carboxy-N,N,N,N'-tetramethylrhodamine.

HA(I) and (II) correspond to two regions of the HA gene targeted by two respective RT-PCR assays. HA-IVT is a sequence encompassing both of the HA(I) and HA(II) regions, as well as additional flanking nucleotides. NA(I) corresponds to a region of the NA gene targeted by another RT-PCR assay. The NA-IVT sequence comprises the region of the NA gene targeted by the NA (I) RT-PCR assay, as well as flanking nucleotides. The synthetic control is a nucleotide sequence construct presenting 100% identity to a region of the HA sequence of an emerging influenza A(H7N9) virus strain, encompassing HA(I) and HA(II). It is synthesised by PCR fusion of Fragments I and II oligonucleotides using primers F and HA7_2_2013rtR.

^a For the RT-PCR assays, the last letter of the oligonucleotide ID, is either 'F' for forward primer, 'P' for probe, or 'R' for reverse primer. For the sequencing assays, 'FWD' indicates the forward primer and 'REV' the reverse primer.

^b When present, dye labels are indicated.

^c Within the oligonucleotide sequences, a degenerate site with G/T is designated as a K and a site with G/A is designated as R.

each assay. The results in terms of the fractions of positive reactions at each concentration were subjected to probit regression analysis.

Detection probabilities of >95% were achieved at RNA concentrations 7.0 and 7.8 copies per reaction with the HA(I) and NA(I) assays, respectively (Figure 2). Probit analysis is not shown for the HA(II) assay because this assay is not proposed as a first line test; however, sensitivity of this assay was highly comparable to that of HA(I).

Because the peri-amplicon HA(I) and HA(II) oligonucleotide binding sites each presented with a small number of mismatches to the primers and probes designed for the RT-PCR assays (Figure 1), the sequence of the combined peri-amplicon region of the assays was synthesised in-vitro by PCR fusion of oligonucleotides fragment I and II using primers F and HA7_2_2013rtR to match the A/Anhui/1/2013 sequence (region

1491–1629) 100% (Table 1). The fragment was cloned in *E. coli* (GenExpress) and transcribed into RNA to be used for parallel testing of the HA assays. For both assays a concentration of five copies of RNA per reaction returned positive in nine of 10 replicates (none were positive with 0 copies per reaction, and all with 50 copies).

Specificity of the assays

To exclude non-specific reactivity of oligonucleotides among each other, all formulations were tested 45 times in parallel with assays containing water and no other nucleic acid except the provided oligonucleotides. In none of these reactions was any positive signal detected. Cross-reactivity with known hetero-specific human influenza A viruses as well as other human respiratory viruses was excluded by testing virus positive clinical specimens and high-titre cell culture materials as summarised in Table 3.

To obtain a clinically relevant figure of assay specificity, all assays were applied on original clinical samples in which other respiratory viruses had already been detected during routine screening at Bonn and Marburg University Medical Centers (Table 3). These samples were prepared using the Qiagen Viral RNA kit, a formulation widely used to extract RNA in clinical laboratories. Of note, the tested panel included samples containing human influenza A viruses. In total, none of the 121 original clinical samples containing a wide range of respiratory viruses gave any detection signal with either assay, while positive controls were detected. It was concluded that the assay could be applied reliably for clinical samples.

During our validation studies, World Health Organization (WHO) released RT-PCR protocols targeting other regions of the HA and NA genes (http://www.who.int/influenza/gisrs_laboratory/a_h7n9/en/ on 9 April 2013). Due to the lack of sequence agreement between the A/Anhui/1/2013 (H7N9) and A/Mallard/Sweden/91/2002 (H7N9) we were not able to evaluate the sensitivity of those assays. However, we included them for specificity testing running a panel of clinical samples as listed in Table 3. No false-positive amplifications were encountered while a full validation of these assays would require access to the A/Anhui/2013 (H7N9) viral RNA or to generate a longer synthetic gene.

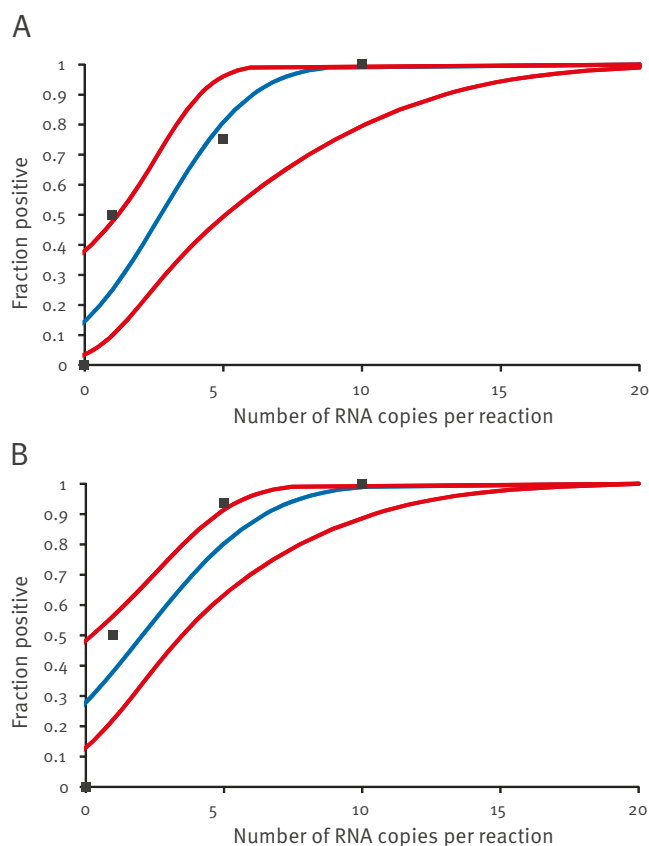
Conclusions

Medical laboratories often use conserved target genes such as the matrix gene for the detection of influenza. In cases of suspected human infection with the emerging influenza A (H7N9) strain, however, laboratories need to make sure their diagnostics do not return false positive results due to cross-reactivity with ubiquitous human influenza A viruses. Such cross-reactivity is likely to occur with matrix gene assays, and will thus pose a risk of misleading interpretations of test data. The here-provided protocols provide high specificity for influenza A(H7N9) while detecting minute quantities of virus due to high analytical sensitivity.

In cases of positive detection of influenza A(H7N9), laboratories would want to achieve confirmation by sequence analysis of the amplified fragment. The two primer pairs IVT_HA-FWD, IVT_HA-REV and IVT_NA-FWD, IVT_NA-REV enable sequence confirmation in the HA and NA genes, respectively. It is important to note that the provided in-vitro transcribed RNA controls contain mutations to be discriminated from the emerging influenza A(H7N9) lineage RNA, making it possible to discriminate true virus detections from possible laboratory contaminations. Control material is available from the authors through the European Virus Archive (www.european-virus-archive.com).

FIGURE 2

Probit regression analyses to determine the sensitivity of the real-time reverse-transcription polymerase chain reaction assays developed to detect the emerging influenza A(H7N9) virus, April 2013



HA: haemagglutinin; LOD: limit of detection; NA: neuraminidase.

The y-axis shows fractional hit-rates (positive reactions per reactions performed), the x-axis shows input RNA copies per reaction. Squares are experimental data points resulting from replicate testing of given concentrations in parallel assays. The blue regression line is a probit curve (dose-response rule). The outer red lines are 95% confidence intervals.

A. HA (I) assay; technical LOD = 7.013 RNA copies/reaction, at 95% hit rate; 95% CI: 4.812–15.41 RNA copies/reaction.

B. NA (I) assay; technical LOD = 7.754 RNA copies/reaction, at 95% hit rate; 95% CI: 5.741–12.739 RNA copies/reaction.

TABLE 3

Known respiratory viruses used for testing the specificity of the assays developed to detect the emerging influenza A(H7N9) virus, April 2013

Virus	Number of samples tested in the assays (HA(I); HA(II), NA) ^a	Number of samples tested in the WHO assays (H7 and N9) ^b
Clinical samples with known virus		
Pandemic influenza A(H1N1)pdm09	12	10
Influenza A(H3N2)	17	13
Influenza B	21	16
Human coronavirus		
hCoV-HKU1	3	-
hCoV-OC43	4	-
hCoV-NL63	4	-
hCoV-229E	4	-
hCoV-EMC	1	-
Human rhinovirus	5	4
Human respiratory syncytial virus	16	15
Human parainfluenza virus		
Parainfluenza 1 virus	1	1
Parainfluenza 2 virus	3	2
Parainfluenza 3 virus	4	2
Parainfluenza 4 virus	3	-
Human metapneumovirus	4	2
Human enterovirus	2	-
Human adenovirus	4	-
Human parechovirus	2	-
Subtotal	110	65
Cell culture supernatants		
Influenza A(H1N1) (older than 2009)	2	2
Influenza A(H5N1)	6	6
Influenza A(H3N2)	3	3
Subtotal	11	11
Total	121	76

WHO: World Health Organization.

^a HA(I); HA(II) and NA(I) were respective target regions of the haemagglutinin and neuraminidase genes of the emerging influenza influenza A(H7N9) virus for real-time reverse-transcription polymerase chain reaction assays developed in this study.

^b Assays published online on 9 April 2013 at http://www.who.int/influenza/gisrs_laboratory/a_h7n9/en/.

Oligonucleotides as well as the synthetic positive plasmid control (DNA) can be ordered from stock at Tib-Molbiol, Berlin (www.tib-molbiol.de). In-vitro transcribed control RNA for the HA(I), HA(II) and NA(I) assays can be acquired from author C. D. through the European Virus Archive platform (www.european-virus-archive.com). Further information and assay updates can be retrieved at www.virology-bonn.de.

Acknowledgements

We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu Database used for assay design (www.gisaid.org).

The development and provision of these assays was done by a European research project on emerging diseases detection and response, EMPERIE, <http://www.emperie.eu/emp/>, contract No 223498 and the European Union Seventh Framework Programme [FP7/2007-2013] under Grant Agreement n°278433-PREDEMICS (<http://predemics.biomedtrain.eu/cms/>).

Authors C.D., M.M., M.E. and S.B. has received support from the German Centre for Infection Research (DZIF) that included full funding of the position of author V.M.C. at the Institute of Virology, Bonn. The European Virus Archive platform (www.european-virus-archive.com), through which in-vitro transcribed control RNA for the HA(I), HA(II) and NA(I) assays can be acquired from author C. D is funded by the European Commission under contract number 228292.

Conflict of interest

None declared.

Authors' contributions

Authors VMC, ME, OL, MM, S Becker and CD designed the study and analysed data. VMC, ME, OL, TB, S Brünink, and MEB did experiments. VMC, ME, OL, MM, S Becker and CD wrote and revised the article.

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Outbreak with a novel avian influenza A(H7N9) virus in China - scenarios and triggers for assessing risks and planning responses in the European Union, May 2013

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Citation style for this article:

Schenk C, Plachouras D, Danielsson N, Nicoll A, Robesyn E, Coulombier D. Outbreak with a novel avian influenza A(H7N9) virus in China - scenarios and triggers for assessing risks and planning responses in the European Union, May 2013. *Euro Surveill.* 2013;18(20):pii=20482. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20>

Article submitted on 30 April 2013 / published on 16 May 2013

As part of the risk assessment and strategic planning related to the emergence of avian influenza A(H7N9) in China the European Centre for Disease Prevention and Control (ECDC) has considered two major scenarios. The current situation is the one of a zoonotic epidemic (Scenario A) in which the virus might be transmitted sporadically to humans in close contact with an animal reservoir. The second scenario is the movement towards efficient human to human transmission (a pandemic Scenario B). We identified epidemiological events within the different scenarios that would trigger a new risk assessment and a review of the response activities to implement in the European Union (EU). Further, we identified the surveillance activities needed to detect these events. The EU should prepare for importation of isolated human cases infected in the affected area, though this event would not change the level of public health risk. Awareness among clinicians and local public health authorities, combined with nationally available testing, will be crucial. A 'one health' surveillance strategy is needed to detect extension of the infection towards Europe. The emergence of a novel reassortant influenza A(H7N9) underlines that pandemic preparedness remains important for Europe.

Introduction

On 31 March 2013, human cases of infection with a novel avian influenza A(H7N9) were reported in eastern China [1,2]. The first two cases in Shanghai had been detected through astute clinicians alerting the public health authorities. The isolated viruses were of an un-subtypeable influenza A strain that was determined to be a novel reassortant strain by the World Health Organization (WHO) Collaborating Centre in the Chinese Center for Disease Control and Prevention in Beijing. A similar virus was identified in a third human case in Anhui province and subsequently in poultry in live bird markets in Shanghai [1,3,4]. The emergence of a novel reassortant avian influenza virus causing disease among humans is a significant threat for public health. Molecular analysis of this avian origin virus

genome identified markers associated with mammalian adaptation. However, there are difficulties in interpreting the significance of molecular data from the limited number of virus sequences posted to date and without linked information on the clinical and epidemiological behaviour of the viruses in humans [2]. There is also a particular lack of data on both the geographic spread and the distribution of the viruses among avian species in China [5].

The European Centre for Disease Prevention and Control (ECDC) systematically gathers, analyses and interprets epidemic intelligence data to fulfil its mandate for risk assessment and developing guidance for Europe. For the emergence of influenza A(H7N9) virus, we used scenario analysis as a tool for assessing risks, anticipating possible developments and prioritising preparedness activities. The aim of this paper is to identify the critical events that should inform preparedness, define surveillance priorities and be the basis for risk management options at the European level and in the European Union (EU) Member States.

Scenario analysis

The scenario analysis method was first developed after the Second World War as part of game analysis. In public health, scenario analysis is a tool for strategic planning and for preparing for future events [6]. Subsequently, the significance of a given event can be estimated based on a set of assumptions and premises [7,8].

One important lesson from the influenza A(H1N1)pdm09 pandemic in 2009 was the need for flexible planning based on a range of scenarios, which are refined as more data becomes available [9-11]. Determining the behaviour of a novel reassortant strain of an influenza virus at the early stages of its appearance is challenging. Predicting its future behaviour is impossible. The objective of the analysis in this context is to consider the most likely scenarios for how the underlying patterns of infection and transmission could evolve, and

to identify the key events (triggers) that would prompt a re-assessment of the situation and the strategic planning.

The underlying epidemiological patterns were estimated based on the documented behaviours of avian influenza viruses, their genetic propensity to adapt to a variety of hosts and their ability to cause a broad spectrum of clinical disease in humans [12,13]. Some avian influenza virus subtypes cause sporadic human infections of variable severity. Efficient person-to-person transmission as a result of genetic evolution of the virus would result in a pandemic. Between these two situations, there is a theoretical phase with viruses in transition [14]. However, that phase has never been observed before a pandemic. In this theoretical transition phase, variable epidemiological patterns might be observed with different animal sources, different groups of affected humans, variable clinical severity and variety of cluster size and geographical expansion. In this situation, risk assessments have to be speculative, but can draw upon tools like the international Influenza Risk Assessment Tool (IRAT) [15,16].

Based on the above spectrum of possible human influenza infections, two scenarios were elaborated. Results from the genetic analysis of the isolated strains, the current epidemiology of the influenza A(H7N9) viruses in humans and the very limited knowledge of its epidemiology and behaviour in animals were taken into account [17]. Subsequently, we examined various possible developments from the current epidemiological situation (Table). We categorised the events in human versus animal health related, starting with the current situation and ordered them within each category according to significance. For this, a simple scale was used to estimate the significance of each possible development, based on the likely impact on public health in the EU as perceived by the authors. For each event we described the applicable scenario and the method to detect the event.

Scenarios and triggers

As of 16 May 2013, there are 131 laboratory-confirmed cases, including 32 deaths, with influenza A(H7N9) infection. Cases have been reported from eight provinces (Anhui, Fujian, Henan, Hunan, Jiangsu, Jiangxi, Shandong, and Zhejiang) and two municipalities (Shanghai and Beijing) in mainland China. In addition, one travel-related case is reported by Taiwan [18,19].

Scenario A, the zoonotic scenario, is consistent with the current situation, as of May 2013, in which the novel influenza A(H7N9) virus is distributed in poultry populations in an unknown area of eastern China [5]. The virus has a low pathogenicity for domestic poultry, though there is a possibility of change to high pathogenicity for poultry [5]. Whether it circulates in other animal reservoirs is yet to be determined, for example whether the virus is being transmitted from a wild bird reservoir to poultry in multiple locations or

if the virus has spread to the affected areas through poultry-to-poultry transmission. The transmissibility from poultry to humans is overall low, but higher than for influenza A(H5N1) and therefore resulting in occasional human infections [20]. Epidemiological and virological investigations are expected to accrue evidence over time for the exposure of cases to an animal source. Human-to-human transmissibility seems to be very low [21]. Small clusters occur, but are uncommon in this scenario where most human infections are sporadic and the clinical spectrum of disease is still unclear [20,22]. In some ways influenza A(H7N9) resembles the influenza A(H5N1) zoonotic epidemic, but critical differences from influenza A(H5N1) include the occurrence of some mild or asymptomatic influenza A(H7N9) cases, the absence of pathogenicity for birds at present, the somewhat higher transmissibility of influenza A(H7N9) to human and age and sex distributions among humans which are older and more male-orientated than for influenza A(H5N1). From a European perspective, travellers from the affected area might be infected and diagnosed after arriving in Europe without any change in scenario [23]. Spread of the virus to European poultry might eventually take place either through (illegal) imported birds or migratory birds and failure of biosecurity arrangements in Europe [24]. In that case, human infections might occur mainly in an occupational setting. In Europe, this is the basis for statutory surveillance for low pathogenic avian influenza viruses in poultry and wild bird surveillance [25].

Scenario B, the pandemic scenario entails the emergence of sustained human-to-human transmission resulting in a pandemic [26]. The case-fatality could be low like that of swine-origin influenza A(H1N1)pdm09 in 2009 or of higher magnitude akin to that of influenza A(H1N1) in 1918 [27,28]. Should this scenario occur, the influenza A(H7N9) viruses were detected early in the course of adaptation and would have become increasingly transmissible between humans. An exponential increase in the number of cases and clusters as well as in cluster size would then result [29]. In this scenario, if a substantial proportion of infections were to be mild or asymptomatic, this would also facilitate the spread of the virus. Because spread would occur through human-to-human transmission rather than selective common exposure, all age groups would be exposed. Due to possible pre-existing population immunity, certain risk groups might emerge and be predominantly affected as occurred with influenza A(H1N1)pdm09 [30].

Between these two scenarios, in the theoretical transition phase, multiple variants could be observed based on the dimensions of transmissibility, susceptibility and severity.

The critical events or triggers that we have identified and their likely significance or impact for Europe are listed in the Table. For each event is indicated to which scenario it could apply and which surveillance activity could detect the event.

TABLE
Critical epidemiological events (triggers) for Europe in the context of the emergence of influenza A(H7N9) in China

Event		Public health significance/ impact for Europe	Scenario	How to detect event by public health authorities in Europe
Human health				
1.	Clusters of <4 cases, isolated in time and place ^a	Low: no or limited human to human transmission, as seen with influenza A(H5N1)	Zoonotic	- Epidemic intelligence ^b [38]
2.	Locally acquired human infections taking place within neighbouring provinces to affected area in China ^a	Low: indicator of increased testing or spread in bird populations	Zoonotic	- Epidemic intelligence
3.	Imported case in person returning from affected area to Europe	Low, but with high communication impact	Zoonotic	- Awareness among clinicians and public health authorities in Europe - Human surveillance ^c (case finding algorithm, laboratory capacity and case definition)
4.	Locally acquired human infections in Chinese provinces not next to affected area, or in neighbouring countries of China	Medium, indicating either:		- Epidemic intelligence
		- increased testing or spread in bird populations	Zoonotic	
5.	Locally acquired human infections in countries distant from China (excluding Europe)	High, indicating either:		- Epidemic intelligence
		- wide spread in bird populations	Zoonotic	
6.	Locally acquired human infections in Europe	High, indicating either:		- Awareness among clinicians and public health authorities in Europe - Human surveillance ^c (case finding algorithm, laboratory capacity and case definition) - European veterinary surveillance and link to human occupational surveillance - Case investigation
		- spread of virus in bird population in Europe	Zoonotic	
7.	Multiple or larger clusters of human infections	High: increasing risk of efficient human-to-human transmission	Transition	- Epidemic intelligence/human surveillance ^c (EU/EEA) - Case investigations (EU/EEA) - Cluster investigations (EU/EEA)
		- or increasing human-to-human transmission	Transition	
8.	Continuous chains of human transmission	High: sustained human-to-human transmission	Pandemic	- Epidemic intelligence/human surveillance ^c (EU/EEA) - Case investigations (EU/EEA) - Cluster investigations (EU/EEA)
9.	Apparently decreased severity/case-fatality ratio	High: compromises detection of cases, resulting in increased risk of spread	Any scenario	- Epidemiological evaluation
10.	Primary resistance to neuraminidase inhibitors	High: compromises antiviral treatment	Any scenario	- Monitoring through EU and global (WHO) reference laboratory networks [39,40]
Animal health				
11.	Isolation of virus from other animals than poultry in affected areas (e.g. migratory birds, swine)	Medium: change in exposure risk	Zoonotic	- Veterinary surveillance by national authorities, OIE and FAO
12.	Isolation of virus from wild birds in Europe	Medium: indicating risk for spread to domestic birds in the EU	Zoonotic	- Wild bird surveillance by national authorities, OIE and FAO
13.	Isolation of virus from domestic birds in Europe	High: indicating risk for occupational exposure	Zoonotic	- European veterinary surveillance and link to human occupational surveillance

EEA: European Economic Area; EU: European Union; FAO: Food and Agriculture Organization of the United Nations; OIE: World Organisation for Animal Health; WHO: World Health Organization.

^a Currently only the first two events have been observed in China.

^b Epidemic intelligence activities, including monitoring of notifications through International Health Regulations (IHR) and Early Warning and Response System (EWRS).

^c Human surveillance: severe acute respiratory illness and/or influenza-like-illness and/or seroepidemiology (consortium for the standardization of influenza seroepidemiology (CONWISE) surveys), depending on the epidemiological situation and clinical picture.

Discussion

The emergence of a novel influenza virus infection in humans in China triggered the production of a rapid risk assessment by ECDC, which has subsequently been updated in the light of further developments. The risk of exposure may be limited to a few provinces in eastern China, but the virus may also be more widespread in poultry [5]. Recommendations for European citizens living in or visiting the affected areas have appeared in the rapid risk assessment [17]. An important consideration is that the zoonotic scenario (A) may develop slowly, not progressing towards transition scenarios. ECDC will closely monitor the epidemiological and veterinary situation and report this through updates of its risk assessment and epidemiological updates on its website. In this analysis, thirteen critical epidemiologic events within the different scenarios, summarised in a table, have been identified of which a number would have a high impact for EU. Therefore it is essential to remain alert and capable of timely detecting the occurrence of these critical events, by monitoring of the clinical spectrum of disease and the epidemiological, virological and animal health situation, internationally and in the EU. Currently only the first two events in the table, both with low significance and applicable to scenario A, have been observed in China. Two triggers with a high impact on public health in Europe (increasing resistance to treatment and an apparent decrease in severity) can appear independently of any scenario.

The final column in the table indicates particular priorities for surveillance. It stresses the importance of awareness among hospital clinicians and of surveillance among local public health authorities in Europe. Epidemic intelligence, which also serves for the detection of other threats, plays a key role in detecting events outside Europe. It shows how crucial veterinary and human surveillance is in countries outside Europe, along with transparency and adherence to the International Health Regulations and the procedures of the World Organisation for Animal Health (OIE) [31]. From the activities needed to detect the events, one can deduce the institutional partners with whom to collaborate on national and international level.

The importation into Europe of a human case is likely, given the high volume of international travel between Europe and China and the higher potential for animal to human transmission of influenza A(H7N9) than that of influenza A(H5N1). The likelihood for importation of cases into Europe might increase if the affected area expands. However, if influenza A(H7N9) behaves similar to influenza A(H5N1), transmission to humans is expected to decline during the summer in China and the first European imported cases may not occur in the near future. Even though the significance of the event is ranked as low, EU Member States need to be prepared to manage such cases. Some Member States have already started with this. Following consultation with Member States, ECDC has now published an interim case-finding strategy and a case definition [32].

Local accurate testing is crucial for this and together with the WHO Regional Office for Europe and the Community Network Reference Laboratory (CNRL), ECDC is facilitating the availability of accurate testing in National Influenza Centres or their equivalents in all EU and European Economic Area (EEA) countries [33]. It is important that physicians and clinical laboratories receive all relevant guidance. Also, guidance on managing contacts (prophylaxis) needs to be established and distributed prior to the event and guidance for case management and use of antivirals will be especially important given the severity of influenza A(H7N9) disease in the majority of the cases.

The probability of the appearance of influenza A(H7N9) in wild birds in Europe is difficult to comment upon as the distribution of the virus in the wild bird population in China has not been determined [5]. In this context, it will be essential to sustain the current EU wild bird surveillance for avian influenza after validating the serological and virological tests for influenza A(H7N9) [25]. The risk of spread of infection to domestic birds in the EU is also difficult to comment upon. Importation of live birds from the Far East is prohibited, but cannot be ruled-out. A more likely scenario is that the virus spreads via the mixing of migratory birds, which might allow for westward extension of the virus. This may be a long term event, as it took influenza A(H5N1) nearly a decade to spread in wild birds from China to the EU [34]. Although some flocks of poultry were infected with influenza A(H5N1), rapid detection, stringent action and high levels of biosafety stamped out the infection and the influenza A(H5N1) has never become established in EU poultry the way it has in domestic birds in countries with more informal poultry sectors [25]. An important distinction is that influenza A(H7N9) is currently a low pathogenic avian influenza virus for birds and will not produce the characteristic 'die-offs' signal which trigger testing of poultry flocks. Hence, the statutory low pathogenicity surveillance will become more important for human health. The mandate of public health agencies will not cover animal surveillance and the current collaboration with animal health agencies will need to be intensified under the one health surveillance strategy with greater emphasis on occupational surveillance. In the event of influenza A(H7N9) being detected in domestic animals in the EU, it will be especially important for national public health and animal health authorities to collaborate intensively to ensure timely exchange of surveillance data and early recognition of potential human cases. Occupational guidance to prevent human infections from poultry should build on that for influenza A(H5N1).

Though the risk of person-to-person transmission of influenza A(H7N9) resulting in disease seems to be low at present, the infection of a human with influenza A(H7N9) by transmission within Europe will be a critical event with high significance. Agreed guidance for the assessment of human-to-human transmission will be necessary using the consortium for the standardization

of influenza seroepidemiology (CONSISE) protocols and their national counterparts established for other respiratory infections [35,36]. In addition, epidemiological studies need to be prepared and agreed between countries to identify risk factors among hospitalised cases in the EU. This should again build on routine severe disease surveillance and the CONSISE protocols [37]. The appearance of expanding clusters or chains of transmission, and eventually sustained human-to-human transmission would be another highly significant critical event. Finally, the appearance of influenza A(H7N9) indicates that revising pandemic plans and preparedness in light of the 2009 experience and the anticipated new guidance from WHO should remain a priority for Europe.

In Scenario A, a zoonotic epidemic, the production of a manufactured human vaccine is not of highest priority, though candidate viruses and reagents are being developed by the WHO guided strain selection system as they were previously for other zoonotic viruses of pandemic potential, A(H7) and A(H9) viruses. Decisions on whether to progress to the development of clinical lots to allow early clinical trials, for example for determining dosage and efficacy, will be a matter of judgment informed by tools like the IRAT [15,16]. Relevant CONSISE studies will again be essential in order to determine background protection in the European population [35,36].

Conclusions

The confirmation of novel avian influenza virus infections in humans is a significant threat for public health because of the potential for the virus to develop into a pandemic strain [26] and demonstrates the importance of pandemic preparedness. Developing and examining possible outbreak scenarios and identifying critical events are essential exercises to assess risks. The currently most probable scenario is one of sporadic human infections caused by exposure to birds but with a yet undetermined animal reservoir. Neither importation of human cases into the EU nor limited person-to-person transmission in the currently affected areas [29] would be of significance or change the scenario. Events of medium significance include increasing geographical spread of human infections within China and neighbouring countries, isolation of viruses in animals other than domestic birds or detection of virus in wild birds in Europe. Highly significant events include: transmission in countries distant from China, isolation of viruses from domestic birds in Europe, locally acquired infections in Europe and sustained human-to-human transmission. Epidemic intelligence is crucial for detecting trigger events. Public health authorities and clinicians need to be aware of surveillance guidance and laboratory testing needs to be made available. A comprehensive human and veterinary surveillance strategy is needed to detect extension of the infection towards Europe.

Authors' contributions

All authors were involved in the development of the scenarios and identification of the critical events. Cindy Schenk, Diamantis Plachouras, Niklas Danielsson, Emmanuel Robesyn drafted the manuscript, which was critically reviewed by Angus Nicoll and Denis Couliboumbier.

Conflict of interest

None declared.

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Virus-host interactions and the unusual age and sex distribution of human cases of influenza A(H7N9) in China, April 2013

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Citation style for this article:

Skowronski DM, Janjua NZ, Kwindt TL, De Serres G. Virus-host interactions and the unusual age and sex distribution of human cases of influenza A(H7N9) in China, April 2013. *Euro Surveill.* 2013;18(17):pii=20465. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20465>

Article submitted on 24 April 2013 / published on 25 April 2013

To the editor:

Over the past two weeks, *Eurosurveillance* has published several timely papers related to the emergence of a new influenza A(H7N9) virus affecting humans in China [1-3]. Genetic studies by Kageyama et al. [1] and Jongens et al. [2] assessed evidence in the genome for virus origin, adaptation and virulence, and a paper by Corman et al. [3] described real-time reverse-transcription PCR assays for specific virus diagnosis. While these are important aspects of novel virus characterisation and detection, the accrual of over 100 human cases now also affords opportunity to consider evolving epidemiologic patterns as part of population risk assessment.

Perhaps the most intriguing impression to date from available surveillance findings has been the unexpected age/sex distribution of reported influenza A(H7N9) cases. The age range spans from 2 to 91 years but two thirds of influenza A(H7N9) cases have been 50 years of age or older and two thirds have been

male (Table) [4,5]. Illness severity, with a substantial case fatality of 20%, shows a similar age/sex profile (Table) [4,5]. Unlike the pattern observed for influenza A(H5N1), children, both boys and girls and notably the school-aged, are under-represented among influenza A(H7N9) detections. Among the first 100 adult influenza A(H7N9) cases, men and women were equally represented in the youngest age category 20–34 years, but men were 2–3-fold more frequent than women in older age groups (Table). Furthermore, compared with women 20–34 years of age, women 50–64 and 65–79 years were each twice as frequent among influenza A(H7N9) detections. Conversely, men 50–64 and 65–79 years are each 4–5-fold more frequent among influenza A(H7N9) detections than men 20–34 years of age. While being careful not to over-interpret early surveillance data, what hypotheses might be invoked to explain that pattern?

Disease occurrence is the result of the classic interaction triad of agent–host–environment. Environmental

TABLE

Human cases of influenza A(H7N9) and deaths by age group and sex, China, as of 23 April 2013 (n=109)^a

	Age (years)										
	<2	2–4	5–9	10–14	15–19	20–34	35–49	50–64	65–79	≥80	Unknown
Total cases	0	3	1	0	0	9	16	30	36	12	2
Female	0	0	1	0	0	4	5	9	9	4	0
Male	0	2	0	0	0	5	11	21	27	8	0
Unknown	0	1	0	0	0	0	0	0	0	0	2
Deaths	0	0	0	0	0	1	3	6	7	3	1
Female	0	0	0	0	0	0	1	2	2	0	0
Male	0	0	0	0	0	1	2	4	5	3	0
Unknown	0	0	0	0	0	0	0	0	0	0	1

^a Data sources include the Chinese Center for Disease Control and Prevention and the World Health Organization.

factors such as differences in poultry exposure due to socio-cultural behaviours and host factors such as healthcare-seeking behaviour or underlying comorbid conditions have been postulated to explain these early influenza A(H7N9) surveillance signals [6,7]. However, hypotheses should also include the additional perspective of agent (i.e. virus)–host interactions. Immunological profiles by age likely reflect accumulated lifetime opportunities for influenza virus exposure, leaving intricate imprints that may positively or negatively modulate subsequent risk. We have illustrated this immunological complexity at the population level for influenza, showing variation in age-specific cross-reactive antibody levels to previously emerging influenza A(H1N1)pdm09 virus [8] and more recently to the emerging (swine-origin) influenza A(H3N2)v, probably reflecting complex cohort effects based on differential prime/boost exposures to influenza variants by age [9].

That pre-existing immunity can differentially modulate the infection process for novel pathogens may be relevant in understanding the differing age distributions of the emerging influenza A(H5N1) versus A(H7N9) viruses [6,7]. Anti-neuraminidase (N1) antibodies induced by cumulative influenza A(H1N1) lifetime exposures may have a role in mitigating risk and severity of influenza A(H5N1) infection [10–13] in older individuals accounting for its more youthful profile to date [4–7]. In contrast, for influenza A(H7N9) we may anticipate that anti-N9 antibodies would be less prevalent overall in the population. Other population immunological effects of the 2009 influenza A(H1N1) pandemic, which affected predominantly young people, such as cross-reactive T-cell responses to generally conserved internal virus proteins [14] or memory B cell responses to shared epitopes within group 1 (i.e. H1, H5) versus group 2 (i.e. H3, H7) subtypes [15] may also need to be considered as factors that influence influenza A(H5N1) and A(H7N9) age profiles.

At this stage, we should also stay open to the possibility that pre-existing cross-reactive antibodies may actually facilitate the viral infection process, a phenomenon best recognised for dengue through the mechanism of antibody dependent enhancement (ADE) [16,17]. ADE is thought to occur when low-levels of weakly heterotypic, cross-reactive but not cross-protective, antibodies generated by past exposure to virus antigen, e.g. through prior infection or immunisation, form bridging complexes to facilitate uptake and replication of related but non-identical variants [16–18]. The possibility of ADE in influenza has long been and remains the subject of intense interest among experts [19,20], for which there may recently be indirect evidence. Early during the 2009 influenza pandemic, we described a potentially important interaction between seasonal and novel emerging influenza virus, notably an approximate doubling of the likelihood of medically-attended pandemic influenza A(H1N1) illness among people previously administered seasonal influenza

vaccine that contained virus antigenically related but distant from the emerging influenza A(H1N1)pdm09 strain [18]. In a follow-up experiment, vaccinated ferrets showed higher lung virus titres and greater illness severity after influenza A(H1N1)pdm09 challenge than influenza-naïve animals [21]. In swine, disease exacerbation has also been observed following heterologous challenge [22–24]. ADE was one of the proposed (but unproven) hypotheses to explain the unexpected findings from Canada during the 2009 pandemic [18]. The possible relevance of weakly cross-reactive antibodies in facilitating infection due to other emerging influenza viruses with pandemic potential may therefore warrant further consideration.

In that regard, older Chinese men may not only have a greater likelihood of current poultry/bird exposure, to explain their disproportionate representation among influenza A(H7N9) cases, but also a greater total sum of lifetime avian influenza exposures potentially contributing to cross-reactive H7 antibody. Few serosurveys to assess H7 antibodies in the population of China are available in the English language, and none has yet been sufficiently powered to compare this by age or sex [25–28]. In a serosurvey conducted 20 years ago in central China (Nanchang), 25% of 100 samples collected from women who raised pigs were found by ELISA to have antibodies to purified H7 antigen [25]. In a more recent serosurvey conducted in 2006–08 in northern China, 5–10% of ca. 1,000 farmer families and poultry workers aged 5–87 years had detectable but low-level antibodies (titre of at least 1:20 but not exceeding 1:40) to influenza (H7N1) in a modified haemagglutination inhibition (HI) assay using horse erythrocytes [26]. In 2011, none of >1,500 duck-related workers in Beijing aged 14–71 years had influenza (H7N2) or (H5N1) titres exceeding 1:40 by modified HI, although seropositivity to influenza (H9N2) was more prevalent, particularly among adults older than 50 years of age in whom the rate of seropositivity was four-fold higher than among younger participants [28].

Although the detection of antibodies to H7 subtype viruses has proved challenging even among culture-confirmed cases [29–35], serosurveys to compare cross-reactive antibodies and neutralising effects by multiple assays and by age group could be important, not only to inform possible protection, but also to explore patterns of enhanced risk in influenza A(H7N9) affected areas and more broadly elsewhere to inform risk assessment. Certain immunological effects, including ADE as it pertains to influenza, may yet be speculative. At this early stage of trying to understand the unexpected epidemiological patterns of an emerging pathogen, however, it is prudent for the global scientific and public health community to consider all possibilities within the full virus–host–environment paradigm.

Authors' contributions

All authors contributed to the writing, review and final approval of this letter.

Conflict of interest

GDS has received research grants from GlaxoSmithKline (GSK) and Sanofi Pasteur and participated in an ad hoc GSK advisory board meeting for an unrelated issue for which travel expenses were reimbursed. No other authors have competing interests to declare.

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<http://www.ivz.si>

ROMANIA

Info Epidemiologia
Centrul pentru Prevenirea si Controlul Bolilor Transmisibile, National Centre of Communicable Diseases Prevention and Control, Institute of Public Health, Bucharest
Sporadic, print only. In Romanian.
Sporadic, print only. In Romanian.
http://www.insp.gov.ro/cnscbt/index.php?option=com_docman&Itemid=12

SPAIN

Boletín Epidemiológico Semanal
Centro Nacional de Epidemiología, Instituto de Salud Carlos III, Madrid
Fortnightly, print and online. In Spanish.
<http://revista.isciii.es>

SWEDEN

Smittskyddsinstitutets nyhetsbrev
Smittskyddsinstitutet, Stockholm
Weekly, online. In Swedish.
<http://www.smittskyddsinstitutet.se>

UNITED KINGDOM

England and Wales
Health Protection Report
Health Protection Agency, London
Weekly, online only. In English.
<http://www.hpa.org.uk/hpr>

Northern Ireland
Communicable Diseases Monthly Report
Communicable Disease Surveillance Centre, Northern Ireland, Belfast
Monthly, print and online. In English.
<http://www.cdscni.org.uk/publications>

Scotland
Health Protection Scotland Weekly Report
Health Protection Scotland, Glasgow
Weekly, print and online. In English.
<http://www.hps.scot.nhs.uk/ewr/>

OTHER JOURNALS

EpiNorth journal
Norwegian Institute of Public Health, Folkehelseinstituttet, Oslo, Norway
Published four times a year in English and Russian.
<http://www.epinorth.org>

European Union

“Europa” is the official portal of the European Union. It provides up-to-date coverage of main events and information on activities and institutions of the European Union.
<http://europa.eu>

European Commission - Public Health

The website of European Commission Directorate General for Health and Consumer Protection (DG SANCO).
<http://ec.europa.eu/health/>

Health-EU Portal

The Health-EU Portal (the official public health portal of the European Union) includes a wide range of information and data on health-related issues and activities at both European and international level.
<http://ec.europa.eu/health-eu/>

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ISSN 1025 496X
4,500 copies
Graphic design © ECDC, Stockholm



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