ORIGINAL ARTICLE

Human Infection with a Novel Avian-Origin Influenza A (H7N9) Virus

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ABSTRACT

BACKGROUND

Infection of poultry with influenza A subtype H7 viruses occurs worldwide, but the introduction of this subtype to humans in Asia has not been observed previously. In March 2013, three urban residents of Shanghai or Anhui, China, presented with rapidly progressing lower respiratory tract infections and were found to be infected with a novel reassortant avian-origin influenza A (H7N9) virus.

METHODS

We obtained and analyzed clinical, epidemiologic, and virologic data from these patients. Respiratory specimens were tested for influenza and other respiratory viruses by means of real-time reverse-transcriptase–polymerase-chain-reaction assays, viral culturing, and sequence analyses.

RESULTS

A novel reassortant avian-origin influenza A (H7N9) virus was isolated from respiratory specimens obtained from all three patients and was identified as H7N9. Sequencing analyses revealed that all the genes from these three viruses were of avian origin, with six internal genes from avian influenza A (H9N2) viruses. Substitution Q226L (H3 numbering) at the 210-loop in the hemagglutinin (HA) gene was found in the A/Anhui/1/2013 and A/Shanghai/2/2013 virus but not in the A/Shanghai/1/2013 virus. A T160A mutation was identified at the 150-loop in the HA gene of all three viruses. A deletion of five amino acids in the neuraminidase (NA) stalk region was found in all three viruses. All three patients presented with fever, cough, and dyspnea. Two of the patients had a history of recent exposure to poultry. Chest radiography revealed diffuse opacities and consolidation. Complications included acute respiratory distress syndrome and multiorgan failure. All three patients died.

CONCLUSIONS

Novel reassortant H7N9 viruses were associated with severe and fatal respiratory disease in three patients. (Funded by the National Basic Research Program of China and others.)

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This article was published on April 11, 2013, at NEJM.org.

N Engl J Med 2013.
DOI: 10.1056/NEJMoa1304459

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Sporadic human infections with avian influenza A viruses, which usually occur after recent exposure to poultry, have caused a wide spectrum of illness, ranging from conjunctivitis and upper respiratory tract disease to pneumonia and multiorgan failure. Low pathogenic avian influenza A (H7N2, H7N3, H9N2, or H10N7)[1-4] virus infections have caused lower respiratory tract illness that is mild (conjunctivitis or uncomplicated influenza-like illness) to moderate in severity. Most human infections with highly pathogenic avian influenza (HPAI) A (H7) viruses have resulted in conjunctivitis (H7N3) or uncomplicated influenza illness, but one case of fatal acute respiratory distress syndrome (ARDS) was reported in a patient with H7N7 virus infection during an outbreak in the Netherlands.[1,5] In contrast, the cumulative case fatality rate since 2003 for reported cases of HPAI H5N1 virus infection is approximately 60%.[6-8]

The transmission of H7 viruses to mammals has been reported only rarely[9] in Asia. Human infections with N9 subtype viruses had not been documented anywhere in the world. In February and March 2013, three patients were hospitalized with severe lower respiratory tract disease of unknown cause. We report the identification of a novel avian-origin reassortant influenza A (H7N9) virus associated with these infections.

METHODS

SURVEILLANCE, REPORTING, AND DATA COLLECTION

Throat-swab specimens obtained from three adult Chinese patients (two from Shanghai City and one from Anhui Province) who were hospitalized with severe bilateral pneumonia, leukopenia, and lymphocytopenia were sent to Shanghai Public Health Clinical Center, the Shanghai Centers for Disease Control and Prevention (CDC), and the Anhui CDC, respectively. After preliminary detection of respiratory pathogens, the samples were sent to the Chinese National Influenza Center (CNIC) on March 25, 2013.

A standardized surveillance reporting form was used to collect epidemiologic and clinical data, including demographic characteristics; underlying medical conditions; history of seasonal influenza vaccination; recent exposures to swine, poultry, or other animals; recent visits to a live animal market; clinical signs and symptoms; chest radiographic findings; laboratory testing results, including diagnostic testing for influenza and other respiratory viruses; antiviral treatment; clinical complications; and outcomes. A confirmed case of human infection with avian-origin influenza A (H7N9) virus was defined as evidence of pneumonia with H7N9 viral RNA or isolation of H7N9 virus from respiratory specimens at the CNIC.

ISOLATION OF THE VIRUS

Throat-swab specimens obtained from all three patients were maintained in a viral-transport medium. The specimens were propagated in the allantoic sac and amniotic cavity of 9-to-11-day-old specific pathogen-free embryonated chicken eggs for 48 to 72 hours at 35°C.

RNA EXTRACTION AND REAL-TIME RT-PCR

RNA was extracted from throat-swab samples with the use of the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. Specific real-time reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assays for seasonal influenza viruses (H1, H3, or B), H5N1, severe acute respiratory syndrome coronavirus (SARS-CoV), and novel coronavirus were used. Real-time RT-PCR assays with self-designed specific primer and probe sets for detecting H1 to H16 and N1 to N9 subtypes were then performed to verify the viral subtypes.

GENOME SEQUENCING AND PHYLOGENETIC ANALYSIS

A total of 198 primer sets were used to amplify the full genome for sequencing, with the use of Qiagen OneStep RT-PCR Kit. PCR products were purified from agarose gel with the use of the QIAquick Gel Extraction Kit (Qiagen). We performed the sequencing using an ABI 3730xl automatic DNA analyzer (Life Technologies) and the ABI BigDye Terminator v3.1 cycle sequencing kit (Life Technologies), according to the manufacturer's recommendations. Full genome sequences of the viruses from these patients were deposited in the Global Initiative on Sharing Avian Influenza Data (GISAID) database on March 29, 2013 (accession numbers are provided in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).

We performed multiple sequence alignments

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Table 1. Demographic, Epidemiologic, and Virologic Characteristics and Complications, Treatment, and Clinical Outcomes of Three Patients Infected with Avian-Origin Influenza A (H7N9) Virus.*

Characteristic	Patient 1	Patient 2	Patient 3
Age (yr)	87	27	35
Sex	Male	Male	Female
Occupation	Retired	Butcher	Housewife
Underlying conditions	COPD, hypertension	Hepatitis B	Depression, hepatitis B, obesity
Area of origin	Shanghai	Shanghai	Anhui
Exposure to chicken market in past 7 days	No	Yes	Yes
Date of illness onset	February 18, 2013	February 27, 2013	March 13, 2013
Date of admission	February 25, 2013	March 4, 2013	March 19, 2013
Admission to ICU	None	March 6, 2013	March 20, 2013
Date of specimen collection	February 26, 2013	March 5, 2013	March 20, 2013
Date of laboratory confirmation of virus	March 30, 2013	March 30, 2013	March 30, 2013
Viral isolation	A/Shanghai/1/2013 (H7N9)	A/Shanghai/2/2013 (H7N9)	A/Anhui/1/2013 (H7N9)
Complications			
Septic shock	No	No	Yes
ARDS	Yes	Yes	Yes
Acute renal damage	No	No	Yes
Encephalopathy	Yes	No	Yes
Rhabdomyolysis	No	Yes	Yes
Secondary infections	No	Yes†	Yes†
Oxygen therapy	Mask‡	Mechanical ventilation	Mechanical ventilation
Extracorporeal membrane oxygenation	No	No	Yes
Continuous renal-replacement therapy	No	No	Yes
Antibiotic therapy	Imipenem, moxifloxacin, and vancomycin	Cefoperazone–sulbactam, levofloxacin, and linezolid	Imipenem and vancomycin
Antiviral agent§	Oseltamivir (started on day 7)	Oseltamivir and amantadine (started on day 7)	Oseltamivir (started on day 8)
Glucocorticoid therapy	Yes	Yes	Yes
Intravenous immune globulin therapy	Yes	Yes	Yes
Length of stay in hospital	6 days	6 days	19 days
Date of death	March 4, 2013	March 10, 2013	April 9, 2013

* ARDS denotes acute respiratory distress syndrome, and COPD chronic obstructive pulmonary disease.
† Patients 2 and 3 were infected with carbapenem-resistant *Acinetobacter baumannii*.
‡ This patient refused intubation and mechanical ventilation.
§ Oseltamivir was administered in Patient 1 on February 25, 2013; the viral sample was obtained after two doses of oseltamivir were administered. Oseltamivir was administered in Patient 2 on March 6, 2013, and in Patient 3 on March 21, 2013.

with the ClustalW program using MEGA software, version 5.05. Phylogenetic trees were constructed by means of the neighbor-joining method with the use of MEGA software, version 5.05, to estimate the viral gene relationship with selected influenza A virus strains obtained from GenBank.

RESULTS

PATIENTS

Patient 1 was an 87-year-old man with chronic obstructive pulmonary disease (COPD) and hypertension who reported a cough and sputum production at the onset of illness. High fever and dyspnea de-

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veloped 1 week after the onset of illness. He had no known history of exposure to live birds during the 2 weeks before the onset of symptoms.

Patient 2 was a 27-year-old man with a history of hepatitis B virus infection with positive hepatitis B surface antigen who presented to the hospital with high fever and cough. This patient was a butcher who worked at a market where there were transactions involving live birds. He sold pork but had not butchered bird meat before the onset of illness. Both Patient 1 and Patient 2 lived in Min-hang District, Shanghai, and were admitted to the Fifth People's Hospital.

Patient 3 was a 35-year-old woman who lived in Anhui Province. She had a history of depression, hepatitis B virus infection, and obesity. Patient 3 also had high fever and cough at the onset of the illness. She had visited a chicken market 1 week before the onset of symptoms. The demographic and epidemiologic characteristics of the three patients are summarized in Table 1.

DETERMINATION OF CAUSATIVE PATHOGENS

We confirmed, by means of real-time RT-PCR, viral isolation, and full genome sequencing, that all three patients were infected with a novel avian-origin influenza A (H7N9) virus. Original clinical samples obtained from all three patients were confirmed, by means of real-time RT-PCR, to be positive for H7N9 and negative for seasonal influenza viruses (H1, H3 or B), H5N1, SARS-CoV, and HCoV-Erasmus Medical Center (EMC). Influenza viruses A/Shanghai/1/2013 (H7N9), A/Shanghai/2/2013 (H7N9), and A/Anhui/1/2013 (H7N9) were isolated from Patients 1, 2, and 3, respectively. Complete sequences of the three H7N9 influenza viruses showed that they were 97.7 to 100% identical in all eight gene segments (see Table S1 in the Supplementary Appendix). Phylogenetic analysis of all genes of the isolates showed that each gene was of avian origin (Fig. 1, and Fig. S1 in the Supplementary Appendix). The gene encoding hemagglutinin (HA) shared the highest identity with A/duck/Zhejiang/12/2011 (H7N3, subtype ZJ12). The gene encoding neuraminidase (NA) protein was most closely related to A/wild bird/Korea/A14/2011 (H7N9, subtype KO14); however, the HA gene from the H7N9 viruses in our three patients was highly divergent from that in the KO14 virus. All six internal genes shared the highest similarity with A/brambling/Beijing/16/2012-like viruses (H9N2) (Fig. 1). Phylogenetic results indicated that it was a triple reassortant H7N9 virus (Fig. 2).

Figure 1 (facing page). Phylogenetic Trees of Genes of H7N9 Influenza A Viruses.

Shown are phylogenetic trees of full-length hemagglutinin (HA; Panel A) and neuraminidase (NA; Panel B) genes of H7N9 influenza A viruses. The trees were generated by means of the distance-based neighbor-joining method with the use of MEGA software, version 5.05. The reliability of the trees was assessed by means of bootstrap analysis with 1000 replications. Horizontal distances are proportional to the genetic distance. The novel reassortant H7N9 viruses are shown in red. The viruses in green presented the highest similarity with the novel H7N9 viruses. Other H7N9 and human H7 subtype are shown in blue and pink, respectively. Phylogenetic trees of the other six genes are included in the Supplementary Appendix.

In all three viruses, the HA cleavage site possesses only a single amino acid R (arginine), indicating low pathogenic effects in poultry. A T160A mutation was identified at the 150-loop (H3 numbering) in the HA gene of all three viruses. Substitution Q226L at the 210-loop in the HA gene was found in both the A/Anhui/1/2013 and A/Shanghai/2/2013 viruses but not in the A/Shanghai/1/2013 virus (Table 2). Five amino acids were deleted in the stalk region of NA residue 69 to 73. The M2 protein contained the S31N substitution, indicating resistance to amantadine. Other mutations — 89V and E627K in PB2 and 42S in NS1 — were also identified (Table 2). The amino acids in A/Shanghai/1/2013, which differed from those in A/Anhui/1/2013 and A/Shanghai/2/2013, are shown in Table S2 in the Supplementary Appendix. To date, five additional H7N9 viruses have been isolated from five patients. Sequencing analysis indicates that all five viruses are highly similar to both A/Shanghai/2/2013 and A/Anhui/1/2013. Some variability is observed, such as Q226L in HA and R292K in NA.

On the basis of these data, diagnostic tests for the novel reassortant H7N9 viruses have been developed. The specific sequences are available on the website of the World Health Organization (www.who.int/influenza/gisrs_laboratory/a_h7n9/en/).

CLINICAL FEATURES AND OUTCOMES OF THE PATIENTS

The clinical characteristics of the patients are shown in Table S3 in the Supplementary Appen-

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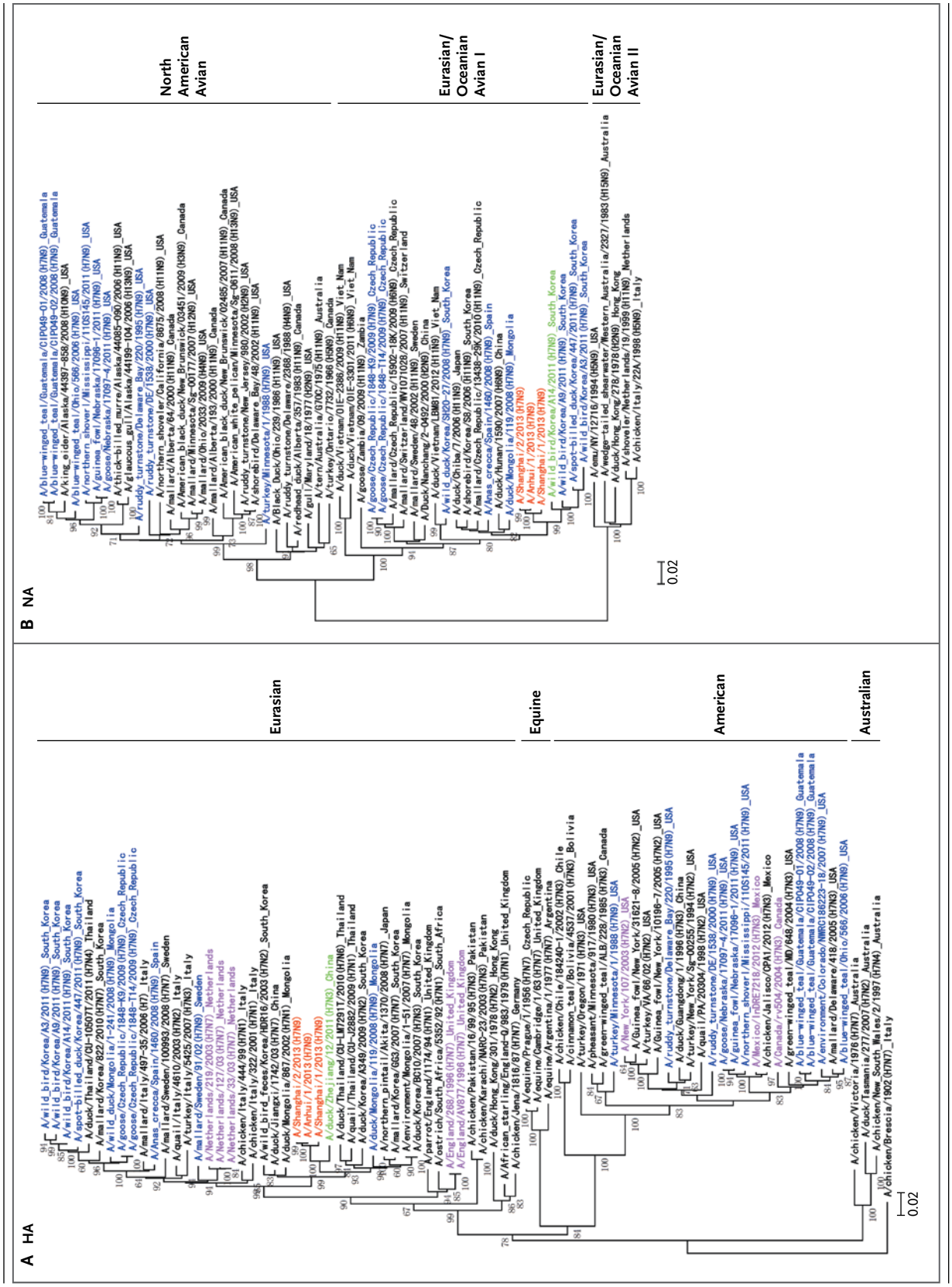

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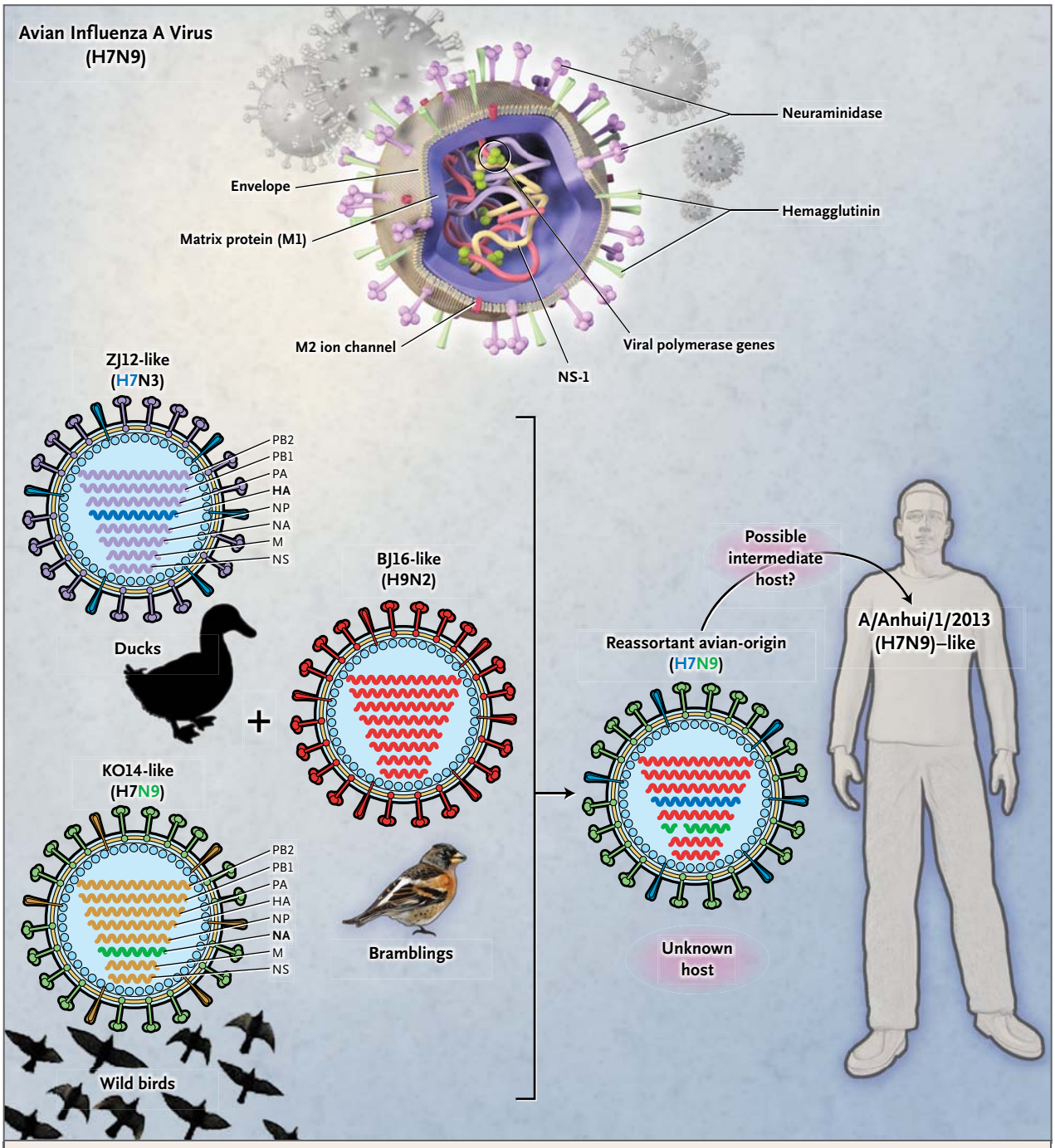

Figure 2. Hypothetical Host and Lineage Origins of the Gene Segments of the Novel Reassortant Human Influenza A (H7N9) Viruses.
The colors of the gene segments in the ovals indicate their origin. BJ16 denotes A/brambling/Beijing/16/2012, KO14 A/wild bird/Korea/A14/2011, and ZJ12 A/duck/Zhejiang/12/2011.

dix. Fever and cough were the most common symptoms. The white-cell count was normal or slightly decreased. Elevated levels of aspartate aminotransferase, creatine kinase, and lactate dehydrogenase were observed in all the patients. Bilateral ground-glass opacities and consolidation were detected on chest radiography (Fig. 3).

Several complications of the illness were ob-

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Table 2. Molecular Analysis of Three of the 2013 H7N9 Viruses.*

Gene	Sites†	Position	A/Shanghai/1/2013	A/Shanghai/2/2013	A/Anhui/1/2013
HA	Cleavage site		PEIPKGR*G	PEIPKGR*G	PEIPKGR*G
	RBS positions (H3 numbering), altered receptor specificity				
	Q226L	226	Q	L	L
	G228S	228	G	G	G
	Glycosylation motifs		30NGTK, 46NATE, 249NDTV, 421NWTR, 493NNTY (conserved in H7 HA viruses)	30NGTK, 46NATE, 249NDTV, 421NWTR, 493NNTY (conserved in H7 HA viruses)	30NGTK, 46NATE, 249NDTV, 421NWTR, 493NNTY (conserved in H7 HA viruses)
NA	Stalk		69–73 deletion	69–73 deletion	69–73 deletion
	Antiviral resistance R294K (oseltamivir)	294	K	R	R
PB2	Enhanced polymerase activity and increased virulence in mice				
	L89V	89	V	V	V
	E627K	627	K	K	K
PB1	H5 virus transmissible among ferrets				
	H99Y	99	H	H	H
	I368V	368	I	V	V
PB1-F2	Full length		90 aa	90 aa	90 aa
M1	Increased virulence in mice				
	N30D	30	D	D	D
	T215A	215	A	A	A
M2	Antiviral resistance S31N (amantadine)	31	N	N	N
NS1	Increased virulence in mice P42S	42	S	S	S

* Single letters refer to the amino acid (aa) found in the noted gene at a specific site.
† Sites are numbered from M, the start codon.

served. All the patients had ARDS. Patient 3 had septic shock and acute renal damage. Carbapenem-resistant *Acinetobacter baumannii* was cultured from lower respiratory tract specimens obtained from two of the patients after the initiation of mechanical ventilation. Combination antibiotic therapy, glucocorticoids, and intravenous immunoglobulin were administered in all three patients. Antiviral therapy was initiated 6 to 7 days after the onset of illness (Table 1).

Patient 1 declined admission to the intensive care unit (ICU) and intubation. He died from refractory hypoxemia 13 days after the onset of illness. Patient 2 was admitted to the ICU and intubated 48 hours after admission owing to progressive dyspnea. He died from refractory hypoxemia after 4 days in the ICU. ARDS and septic shock developed in Patient 3 on day 6 after the onset of illness. She was admitted to the ICU, and extracorporeal membrane oxygenation was initiated. She died on April 9.

DISCUSSION

We have identified a novel reassortant influenza A (H7N9) virus that is associated with severe human infection. Currently, only 25 H7N9 viruses are available in GenBank. The H7N9 viruses we identified in the three patients were of avian origin, but only the NA gene was closely related to that from another H7N9 virus (KO14). The HA gene was similar to that of an H7N3 virus (ZJ12) from a nearby region (Zhejiang Province) in China. All the internal gene segments were closely related to those from avian H9N2 viruses, particularly a virus isolated from a brambling in Bei-

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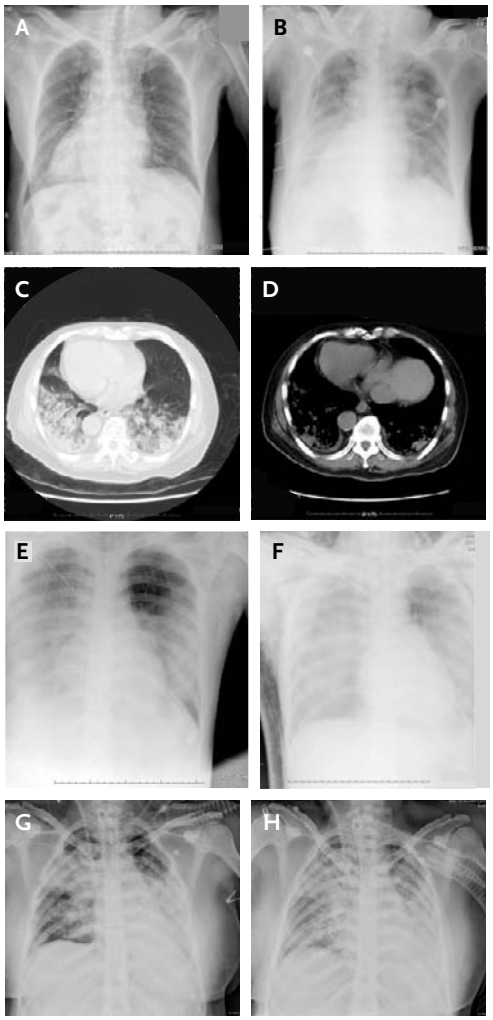

Figure 3. Chest Radiographs.

A chest radiograph (patient's heart is on the right side) of Patient 1 is shown in Panels A and B . Mild ground-glass opacity was observed on day 6 (Panel A). Bilateral ground-glass opacity and consolidation were clearly seen on day 9 (Panel B). A computed tomographic scan of the chest of Patient 1, obtained on day 7 (the day of admission), is shown in Panels C and D. Substantial bilateral ground-glass opacity and consolidation can be seen. Chest radiographs of Patient 2, obtained on day 7 and day 13 after the onset of illness are shown in Panels E and F, respectively. Bilateral ground-glass opacity and consolidation can be seen on day 7, and white lungs on day 13. Chest radiographs of Patient 3 on day 7 and day 13 after the onset of illness are shown in Panels G and H. Bilateral ground-glass opacity and consolidation can be seen on both day 7 and day 13.

jing (BJ16) (Fig. 1, and Fig. S1 in the Supplementary Appendix). Thus, the human H7N9 viruses are the product of reassortment of viruses that are of avian-origin only. In addition, the phylogenetic trees showed that A/Shanghai/1/2013 is phylogenetically distinct from A/Anhui/1/2013 and A/Shanghai/2/2013 across all gene segments, which suggests that there have been at least two introductions into humans (Fig. 1, and Fig. S1 in the Supplementary Appendix). Currently, there are no data to suggest that this reassortment occurred in a mammalian host, and the similarity of the human viruses to avian viruses may be stronger support for direct avian transmission of this virus. However, influenza surveillance of birds, swine, and humans is limited in China and nearby countries, making it difficult to resolve this question.

Although human infections with avian-origin H7 avian influenza viruses have been observed before,[1,2,5,10,11] infection of humans with an N9 subtype influenza virus has not been reported previously. Human H7 influenza infections are generally mild, causing conjunctivitis or modest respiratory symptoms, although a fatal case was reported before this H7N9 outbreak.[5] All three cases of H7N9 infection reported here were virulent, with the patients' conditions deteriorating rapidly with the development of severe pneumonia and ARDS, and ultimately resulted in death. All the patients had preexisting medical conditions, and two had a history of direct contact with poultry. Two patients presented with rhabdomyolysis, which has rarely been reported in patients infected with H1N1 or H5N1[12] influenza viruses. Encephalopathy, which is normally more common in pediatric patients with influenza,[13] was observed in two patients.

The affinity of the influenza virus to different sialyl-sugar structures is an important determinant of range and pathogenicity in the viral host.[14,15] Human influenza viruses preferentially bind to α2,6 sialyl glycan, whereas most avian viruses bind to α2,3 sialyl glycan.[16,17] Q226L in the HA protein, which was first reported in H7 field viruses, as well as H5 subtypes, was expected to bind strongly to α-2,6 human-like receptors. A laboratory-produced Q226L mutation at the 210-loop of HA has been shown to change the receptor binding of avian origin to a human-

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type receptor binding and might increase the ability of the virus to be transmitted by air, as reported previously.[18,19] Moreover, the lack of a glycosylation site on the 150-loop might decrease the affinity to α-2,3 avian-like receptors. The effects of these mutations require further study.

A deletion of five amino acids in the viral NA stalk has been observed in the novel reassortant H7N9 viruses. A similar deletion in the H5N1 avian virus has been shown to be responsible for the change in viral tropism to the respiratory tract[20] or to enhance viral replication,[21] and it has been suggested that this deletion may be associated with adaptation and transmission in domestic poultry.[22,23] Since April 4, it has been reported that H7N9 viruses similar to those isolated from the three patients described here have been isolated from pigeons and chickens, indicating that the novel H7N9 viruses might currently be circulating in poultry. Moreover, the E627K substitution in the PB2 gene has been associated with increased virulence in mice and was reported to be associated with improved replication of avian influenza viruses in mammals.[24,25] A combination of these substitutions may contribute to the human infection and severe disease. Other possible virulence molecular markers are shown in Table 2. The potential virulence mutations are described on the basis of previous studies in animals, but the pathogenesis in humans remains unknown.

The difference between the two Shanghai viruses and the similarity between the Shanghai/2 and Anhui/1 viruses argue against human-to-human transmission in these cases, and no close contacts of the patients have tested positive for these viruses. However, limited human-to-human transmission was observed in the H7 outbreak in the Netherlands in 2003[10]; therefore, the pandemic potential of these novel avian-origin viruses should not be underestimated.

Currently there is no vaccine available for these novel viruses, and it is not known whether the current candidate H7 vaccine viruses, of which three are North American viruses and the other three are avian viruses from 2000 in the Netherlands, may be effective. The influenza H7N9 A/Anhui/1/2013 strain has been proposed to be one of the candidate vaccine strains since it grows to a very high titer in eggs. Heightened protective measures should be taken when dealing with these viruses, and increased surveillance and analyses of these viruses are needed.

Severe avian influenza A (H7N9) infections, characterized by high fever and severe respiratory symptoms, may pose a serious human health risk. We are concerned by the sudden emergence of these infections and the potential threat to the human population. An understanding of the source and mode of transmission of these infections, further surveillance, and appropriate counter measures are urgently required.

The contents of this article are solely the responsibility of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention in China or other organizations.

Supported by a grant (2011CB504704) from the National Basic Research Program (973) of China, grants (81070005/H0104 and 81030032/H19) from the National Natural Science Foundation of China, grants from the China National Mega-projects for Infectious Diseases (2012ZX10004-211 to Dr. Yuan and 2013ZX10004-101 to Dr. Dexin Li), and the Chinese National Influenza Center–Centers for Disease Control and Prevention (CDC) collaborative project 5U51IP000334-03 from the CDC China-U.S. Collaborative Program on Emerging and Re-emerging Infectious Diseases.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank Prof. Chen Wang in Beijing Hospital, Ministry of Health, for his advice in clinical study, data analysis, and preparation of the manuscript; Dr. Yunde Hou (Chinese Center for Disease Control and Prevention) and Yumei Wen (Fudan University) for their suggestions and discussion; the staff at the National Health and Family Planning Commission for help with coordination; and personnel at the Chinese National Influenza Surveillance Network and National Sci-Tech Key Project of Infectious Disease Surveillance Laboratory Network, China.

APPENDIX

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