

Human infection with a novel reassortment avian influenza A H3N8 virus: an epidemiological investigation study

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Abstract

A four-year-old boy developed recurrent fever and severe pneumonia in April, 2022. High-throughput sequencing on his bronchoalveolar lavage fluid revealed a novel reassortant avian influenza A-H3N8 virus (A/Henan/ZMD-22-2/2022(H3N8)). Both HA and NA genes of the virus were of avian origin, with the HA gene most closely related to H3N2 and H3N8 viruses detected in ducks in Guangdong Province, and NA gene most closely related to wild bird H3N8 influenza viruses detected in the USA and Japan. The six internal genes were acquired from Eurasian lineage H9N2 viruses. Molecular substitutions analysis revealed the haemagglutin retained avian-like receptor binding specificity but PB2 genes possessed sequence changes (E627K) associated with increased virulence and transmissibility in mammalian animal models. The patient developed respiratory failure, liver, renal, coagulation dysfunction and sepsis. Endotracheal intubation and extracorporeal membrane oxygenation were administered. Positive detection of the novel A(H3N8) RNA was obtained from nasopharyngeal swab of a dog, anal swab of a cat, and environmental samples collected in the patient's house. The full-length HA sequence from the dog and cat was identical to sequence from the patient. No influenza like illness developed and no H3N8-RNA was identified from the family members. Serological test revealed neutralizing antibody induced against ZMD-22-2 virus in the patient and three of his family members. Our results suggest a novel triple reassortant H3N8 caused severe human disease. There is some evidence of mammalian adaptation, possible via an intermediary mammalian species, but no evidence of person to person transmission. The potential threat from avian influenzas viruses warrants continuous evaluation and mitigation.

Introduction

In recent years, many subtypes of avian influenza viruses (AIVs) have been found to be infectious to mammals and to pose a threat to the health of humans and other animals^{1,2,3}. So far, 11 subtypes of AIVs (mainly H5N1, H5N6, H6N1, H7N7, H7N9, H9N2, and H10N8) have been identified to cause human infections. While there are relatively frequent spillover infections (cases of single infections) with avian influenza virus in humans or other mammals^{4,5,6,7}, only a small proportion of cases have caused epidemics or pandemics in mammalian hosts⁸. AIV infections in humans can result in a wide spectrum of illness, ranging from conjunctivitis and upper respiratory tract disease to pneumonia and multiorgan failure. Low pathogenic avian influenza A (H7N1, H7N2, H7N3, H9N2, or H10N7) virus infections have caused lower respiratory tract illness that is mild (conjunctivitis or uncomplicated influenza-like illness) to moderate in severity^{9,10,11}. Of great concern are the highly pathogenic avian influenza (HPAI) viruses. A (H7) viruses have resulted in conjunctivitis or uncomplicated influenza illness, with only a single fatal case with H7N7 virus infection during an outbreak in the Netherlands¹². In contrast, HPAI H5N1 and H7N9 virus infections have resulted in case fatality rates of approximately 53% and 34% respectively^{13,14}.

The most ubiquitous hemagglutinin (HA) subtype of influenza A viruses is the H3, with a wide host range shown that includes humans, horses, dogs, cats, seals, poultry, pigs, and wild aquatic birds^{3,15}. An in vitro experiment also demonstrated its potential to establish successful infections in pigs¹⁶. However,

infection of humans with an H3N8 influenza A virus has not been previously reported. Here we report the first human case of infection with H3N8 and describe the epidemiologic, clinical, and genetic features of the case.

Methods

Epidemiological investigations and data collection

In April 2022, as part of hospital surveillance of febrile patients in Zhumadian Central Hospital, Henan province, China, a febrile pediatric patient with recurrent fever of unknown cause was screened. Following the identification of infection with an avian influenza virus, an epidemiological investigation was performed on the patient and his close-contact family members and relatives using a standard questionnaire, which included demographic information, pre-existing underlying diseases and the exposure history before the onset of illnesses. To infer the possible infection source of the patient, we performed field investigation in the residence of the patient and the village on April 13. Nasopharyngeal swabs, anal swabs, faecal and blood samples were collected from companion animals, livestock and poultry; environmental samples that included the surface swabs, drinking water, sewage, and water in a pool close to the family. Throat swabs and blood samples were collected from close contacts of the patient, who were also asked to report for fever ($^{\circ}\text{38°C}$) and influenza-like symptoms for seven days since the interview. According to the regulations and guidelines of the NHFPC of China, data collection on this patient was part of the routine surveillance and outbreak investigation, and was therefore exempt from the oversight by institutional review board. Written informed consent was obtained from the guardian of the patient and all the participating subjects.

Metagenomic analysis and genome assembly

The RNA from the bronchoalveolar lavage fluid of the patient were subject to high-throughput sequencing. Briefly, the Viral RNA was extracted using AllPrep DNA/RNA mini kit (Qiagen, Germany) and sequenced using Illumina Nextseq 550 platform. Sequenced data was assembled with the reference sequences in database using CLC Genomic Workbench v21. The sequence alignment and annotation were performed using CLC Genomic Workbench v21.

Virus detection

All the collected samples were tested for 14 commonly seen respiratory pathogens by Real-time PCR/PCR/RT-PCR, as described previously¹⁷. Briefly, the nucleic acids were extracted from specimens using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. For all influenza virus (IFV) positive samples, a pair of universal full-length primers (MBTuni-12 and MBTuni-13) were used to reverse transcribe and amplify all eight segments of the virus genome¹⁸. Specific test for the novel H3N8 was performed by real-time Reverse Transcriptase-PCR. Sequences of the primers and probes are available on request. Antigen test for nine respiratory pathogens (legionella pneumophila, mycoplasma pneumoniae, Q fever coxiella burnetiiid, chlamydia pneumoniae,

adenovirus, respiratory syncytial virus, influenza B virus and parainfluenza virus) was also performed by using a commercial indirect immunofluorescent assay (Vircell SL, Spain).

Phylogenetic analysis

The reference nucleotide sequences of virus were downloaded for phylogenetic analysis. The maximum likelihood method was performed to generate the virus genome phylogenetic tree. Phylogenetic trees were reconstructed with nucleotide sequences using IQ-TREE (v1.6.12) with GTR+G as the best-fit substitution model and 1,000 bootstrap replicates. MAFFT method was used for sequence alignment.

Pseudovirus based neutralization assay

The novel H3N8 pseudovirus was generated, based on which the pseudotyped virus neutralization assay was measured by the reduction in Luc gene expression and performed in accordance with the method described in previous study¹⁹. Briefly, serially diluted samples were incubated with pseudotyped virus in duplicate for 1 hour at 37°C together with the virus control and cell control wells in sextuplicate.

Thereafter, 2×10^4 freshly trypsinized Huh-7 cells were added to each well of the 96-well plate. After 48 hours of incubation at 37°C with 5% CO₂, the RLU was measured in accordance with the instruction manual provided by PerkinElmer (Waltham, MA). The ED50 (median effective dilution) was calculated using the Reed-Muench method.

Results

Clinical features and laboratory abnormalities of the patient

The patient was a 4-year-old boy living with his father, grandparents, brother and sister, who are otherwise healthy, in Shangcai County, Zhumadian City of Henan province, China (**Supplemental Figure 1**). On April 5, 2022, he developed a high fever of 39.3°C (**Figure 1**), with lethargy and anorexia appearing on April 6, and cough was present on April 8. Although treated with antipyretic drugs and other supportive therapy, his condition did not improve and respiratory symptoms worsened. On April 10, day 6 of his illness, he was admitted to a local hospital (Hospital A) for treatment. On admission, he had fever, shortness of breath, with the blood oxygen saturation values dropping to 80%. On the same day, severe respiratory signs and symptoms appeared, and the patient was transferred to the intensive care unit in hospital B (Zhumadian Central Hospital, Zhumadian city of Henan). On admission, physical examination revealed a body temperature of 39.5°C, abnormal pupillary light reflex, moist rales, and cyanosis of lips, accompanied by lymphadenopathy. His blood pressure was 135/91 mmHg, tachycardic (pulse rate, 150/min), and transcutaneous oxygen saturation level was 80.0 % with mask oxygen inhalation. Blood tests showed leukopenia ($2.63 \times 10^9/L$), hyperglycemia (blood sugar level 20.3 mmol/L), hyponatremia (129 mmol/L) and hypocalcemia (1.9 mmol/L). There was decreased concentrations of total protein (50.2 g/L), albumin (30.9 g/L), total bilirubin (1.9 µmol/L) and elevated concentrations of aspartate aminotransferase (AST, 138 U/L), and adenosine deaminase (ADA, 31.0 U/L), indicating liver dysfunction. Coagulation tests showed a significant prolongation of prothrombin time (PT, > 23.9 s), activated partial

thromboplastin time (APTT, > 170 s), thrombin time (TT, > 160 s), and elevated D-dimer (D-D, 5.11 ug/ml) and fibrin degradation products (FDP, 17.36 ug/ml), indicating a coagulation dysfunction. Myocardial damage was displayed by high level of lactate dehydrogenase (LDH, 265 U/L), creatine kinase (CK, 603 U/L) and creatine kinase BB (CK-BB, 55.0 U/L). A chest computed tomographic (CT) scan showed multiple patchy high-density shadows in both lungs, especially in the lower lobe of the right lung and the left lung (**Figure 2A-C**). Antigen test for nine respiratory pathogens were all negative. The bronchoalveolar lavage fluid was positive for influenza A, while negative for the other 13 commonly seen respiratory pathogens. The same sample was simultaneously subject to high throughput sequencing.

Due to high fever of 41 °C, and exacerbation of respiratory symptoms, the patient was further transferred to a tertiary hospital in Zhengzhou City (Hospital C). Laboratory investigations revealed leukopenia, hyperglycemia, hyponatremia, liver dysfunction, myocardial damage, renal damage, and coagulation dysfunction, which contributed to multiple organ failure and sepsis (**Figure 1**). An inflammatory response characterized by elevated interleukin 6 (IL-6, 13.91 pg/mL), interferon- γ (IFN- γ , 45.55 pg/mL), IL-12P70 (5.63 pg/mL) was observed on day 8 after disease, indicating the development of systemic inflammatory response syndrome. The C-reactive protein (CRP) elevated to a remarkably high level on day-19 after disease, possibly due to bacterial coinfection. The respiratory symptoms exacerbated with laboratory abnormalities including platelet, albumin, Na⁺ remained abnormally low, while γ -glutamyl transferase (GGT), lactate dehydrogenase (LDH), APTT, FDP, D-D, CK and blood urea nitrogen (BUN) elevated above normal until recently (**Supplemental Figure 2**). *Pseudomonas aeruginosa* was determined in bronchoalveolar lavage fluid on day 8 of disease. Supportive care was prescribed on disease onset, in addition to this, extensive treatment, including antiviral therapy (Oseltamivir 30 mg twice a day and interferon α 1b 30 ug twice a day), antibiotic therapy (linezolid, meropenem, and compound sulfamethoxazole), mechanical ventilation via endotracheal intubation, and extracorporeal membrane oxygenation (ECMO) was administered since day 6 of disease onset. (**Supplemental Table 1**). The patient was still hospitalized, and his condition improved with ECMO withdrawn on May 3, 2022.

Detection and identification of avian influenza A H3N8 virus

By high-throughput sequencing on the bronchoalveolar lavage fluid collected on April 10, we obtained the whole genome sequence of an influenza A/H3N8 virus (A/Henan/ZMD-22-2/2022(H3N8); GenBank accession number ON342803-ON342810). Phylogenetic analysis revealed ZMD-22-2 as a novel reassortant H3N8 influenza virus which is distinct from previously reported H3N8 viruses.

The closest related HA gene was in the clade of H3N2 and H3N8 viruses detected in ducks from Guangdong Province (A/duck/Guangdong/F352/2018 (A/H3N2) determined in 2018 with a nucleotide homology of 96.09% (**Figure 3A**). The highest similarity of NA segment was to H3N8 viruses in various species of birds in North America in 2014 and Japan in 2016 (A/northern pintail/Alaska/870/2014 (A/H3N8), with nucleotide homologies of 97.07% (**Figure 3B**). The nucleotide homologies of internal genes were closely related to H9N2 viruses that had been identified throughout China and isolated from humans, ducks or wild birds in recent years (**Supplemental Figure 3**). This indicates that the virus was a

novel reassortant genotype which had undergone complicated mutation and reassortment events (**Figure 4**).

The amino acid sequence at cleavage site in HA protein is PEKQTR/GL, indicate that the virus was a low pathogenic avian influenza (LPAI) virus. Genetic features were evaluated based on a well-accepted correlation between receptor binding characteristics and host specificity of influenza viruses²⁰, i.e., viruses isolated from wild aquatic birds bind strongly to α-2,3 sialyl glycan (SA 2,3) receptor, in contrast, human-adapted influenza viruses recognize and bind SA 2,6 receptors, which predominate in the human respiratory tract. For the novel H3N8 virus, the residue 226 and 228 of HA gene were glutamine (Q) and glycine (G), respectively. The key mutation Q226L and G228S in HA protein which rendered a strong binding to SA 2,6 receptors and increased transmission ability by air, are absent, thus indicating limited ability to bind to cells in the human respiratory tract.

The mutation of E627K was identified in PB2 gene. This mutation had been associated with increased virulence in mice and was reported to be associated with improved replication of avian influenza virus in mammals^{20, 21, 22}. Besides, the N30D and T215A mutation in M gene and P42S mutation in NS gene were also observed, which mutations were associated with increased virulence in animal models^{23, 24}.

Epidemiological investigations

Epidemiological investigation revealed the family had been raising 6 chickens in the back yard, which however, had been killed before the investigation was performed. The patient had intensive contact with a domesticated dog and a cat kept in the household before his illness onset, by feeding and playing with the dog and the cat. Five close-contact family members (grandfather, grandmother, father, eight-year-old brother, and six-year-old sister) who lived together with the patient, and the aunt who had close contact with the patient while he was ill were interviewed. None of them had bred livestock, or reported recent visit to poultry market, the grandfather and brother reported infrequent contact to the dog and cat, although less frequently than the patient. No other significant exposures, i.e., visiting poultry market or exposure to febrile patients were reported. There was a pond 27-meters distant from the household of the patient, which wild ducks inhabited, with frequent congregation with the domesticated poultry in the backyard (**Supplemental Figure 1**).

The molecular test on the 63 animal and environmental samples yielded 10 positive results for the novel H3N8, all from the patient's household, including one nasopharyngeal swab of the dog; one anal swab of the cat; surface swabs of the boarding kennels of cat (1), dog (1), and chicken (1); drinking water (1) and food (1) of the dog; swab of the dining table (1), waste bin (1) and cabinet (1). No positive result was obtained in other 53 animal or environmental samples collected in the household (13) or the village (40).

No obvious illness was observed for the animals, although the dog was less active than usual and labored breathing as recalled by the family. Biochemistry test revealed elevated level of creatine kinase (CK, 323 U/L), lactate dehydrogenase (LDH, 1.29 g/mL) of the dog, Decreased LDH (0.96 g/mL) in the cat

(**Supplemental Table 2**). Nucleotide sequence of full-length HA from the dog and cat were identical to sequence from the patient.

Detection for the novel A(H3N8), IFV, as well as 13 common respiratory pathogens were all negative for throat swabs collected from the 6 close contacts. The follow-up of the close contacts revealed influenza like illness developed until recently. Serological test on serum of the patient collected on April 10 revealed neutralizing antibody (Nab) titer of 1:121 against ZMD-22-2 virus by using a pseudotyped virus-based assay. Positive Nab was also determined from the grandparents of the patient (1:339 and 1:179, respectively) (**Supplemental Table 3**).

Discussion

H3N8 virus is a common subtype of virus detected in wild and domestic ducks²⁵, and it has attracted a high attention because of its cross species transmission from avian to mammals^{3, 26, 27}. This represented the first report on human infection with H3N8, since its first identification in Florida in 1963²⁸. Although the current H3N8 subtype viruses could infect mammalian hosts, the whole genome sequence showed that it is still avian-like virus. The reassortant among different influenza viruses is considered the main mechanism for the emergence of novel virus, such as H5N1, H7N9, and H10N8 which can adapt to mammals and gain the ability to infect humans. In a likewise manner, a multiple reassortant from different origins was displayed for this novel H3N8. Genetic evolution of the HA revealed a very close relationship with H3 in ducks that circulated in Guangdong, and the NA gene was derived from birds in both North America and Japan lineages. Still, a high similarity of internal gene with the widely circulating H9N2 in China was demonstrated. The pattern of the Chinese sub-lineage mixing with North America and Japanese influenza viruses remained obscure. It's speculated that a frequent exchange of influenza virus might have occurred due to wild bird migration, or international poultry trade.

The field investigation revealed a natural environment that was suitable for the congregating and contact among migratory birds with the local backyard poultry, which can promote the transmission of AIV. Geographically, the village was located in the East Asian-Australian migratory birds' flyway and it is one of potential stopovers for migratory birds (**Supplemental Fig. 1**), rendering a logical postulation of this mixture between wild birds and poultry.

It's notable that H3N8 virus was also detected from the domesticated cat and dog that were in close contact with the patient. Both dogs and cats are known to be susceptible to human influenza and avian influenza strains. Dogs are particularly susceptible to influenza A viruses, including H3N2, H3N8, H5N1, and H6N1. In Asia, respiratory disease caused by influenza virus H3N2 was documented in dogs, and fatal infection with the highly pathogenic avian influenza virus (HPAIV) H5N1 has also been reported²⁹. A number of single cases of H5N1 HPAI infections in cats have also been reported in different parts of the world, mainly associated with recent avian outbreaks³⁰. Here we observed the H3N8 infected dog developed mild clinical signs, moreover, laboratory abnormality of elevated LDH was observed, possibly indicating a systematic infection. The infected dog excreted virus not only via the respiratory tract but

also possibly via the digestive tract as evidenced by positive detection of H3N8 specific RNA in the drinking water. Therefore, both the respiratory and gastrointestinal routes of infection may cause horizontal transmission among dog, cat and the human being. However, it is not possible to infer the direction of transmission, since both dogs and cats are naturally susceptible to influenza virus strains from other hosts, including birds and mammals. Under current situation, both cat and dog are semidomesticated and may highly likely come in contact with wild birds, ducks in the nearby pond, on the other hand, frequently exposed to human and poultry. Spillover of H3N8 virus into dog and cat further to human, or the otherwise manner can both occur, and it's impossible to determine mammalian adaptation happened first in dog, cat or human. The potential ability of cat and dog to be a "mixing vessel" of diverse origin influenza strains into novel reassortant might be indicated. Unfortunately, the lack of poultry specimens from the household of the patient, and the unsuccessful sampling of the duck in the neighboring pond means we cannot firmly establish the original zoonotic source of infection nor determine the genetic sequence of the original virus.

There was no evidence of infection in other family members and it is unlikely an outbreaks or epidemics could be induced, due to the biological and ecological barriers of the novel genotype. In line with the epidemiological findings, the genetic characterization revealed H3N8 is still avian-like influenza of low pathogenic, and has not gained transmissibility from person to person. On the other hand, the presence of mutations, mainly E627K in PB2 gene and P42S in NS gene, indicated the capacity of this novel influenza A virus subtype to cross the species barrier, acquire some mammalian adaptations and cause human severe disease.

The study was subject to a major limitation that no virus isolation was performed due to the biosafety concern, thus the antibody test was performed on a pseudovirus system, and the cross reaction with other H3 influenza cannot be excluded. The biological features of the novel H3N8 need to be further investigated by experimental approaches. Moreover, based on only one case, our understanding of the clinical aspects of H3N8 infection is rather limited. A full understanding of the clinical spectrum might be proposed based on a large-scale population surveillance.

As of preparing the manuscript, an equine influenza (EIV) outbreak caused by H3N8 virus was reported in America³¹. Although not evolved from AIV, it might be transmitted from horses to dogs or birds. It is therefore important to strengthen the surveillance of H3N8 infections among various animal species, based on which a close monitoring of the viral evolution and timely identification of new human cases could be achieved.

Declarations

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Contributors

The author contributions are as follows. YL, PTB, PH, XBL, LQF and WL conceived, designed and supervised the study. YL, SFW, JZ, HYL, RC, LG, ZHS, LZG, SQ, and XCL treated the patient. YL, PTB, XAZ, HF, SFW, JZ, HYL, RC, LG, ZHS, LZG, SQ, XCL, and XY gathered data and conducted the epidemiological investigation. XAZ, YHW, SL and QQC did the laboratory tests. PTB, XAZ, HF, YHW, HHG, and XBL performed the data analyses. PTB, XAZ, HF, PH, XBL, LQF and WL wrote the drafts of the manuscript and interpreted the findings. All authors read the manuscript, provided feedback, and approved the final version.

Potential conflicts of interest: The authors declare no conflicts of interest.

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Figures

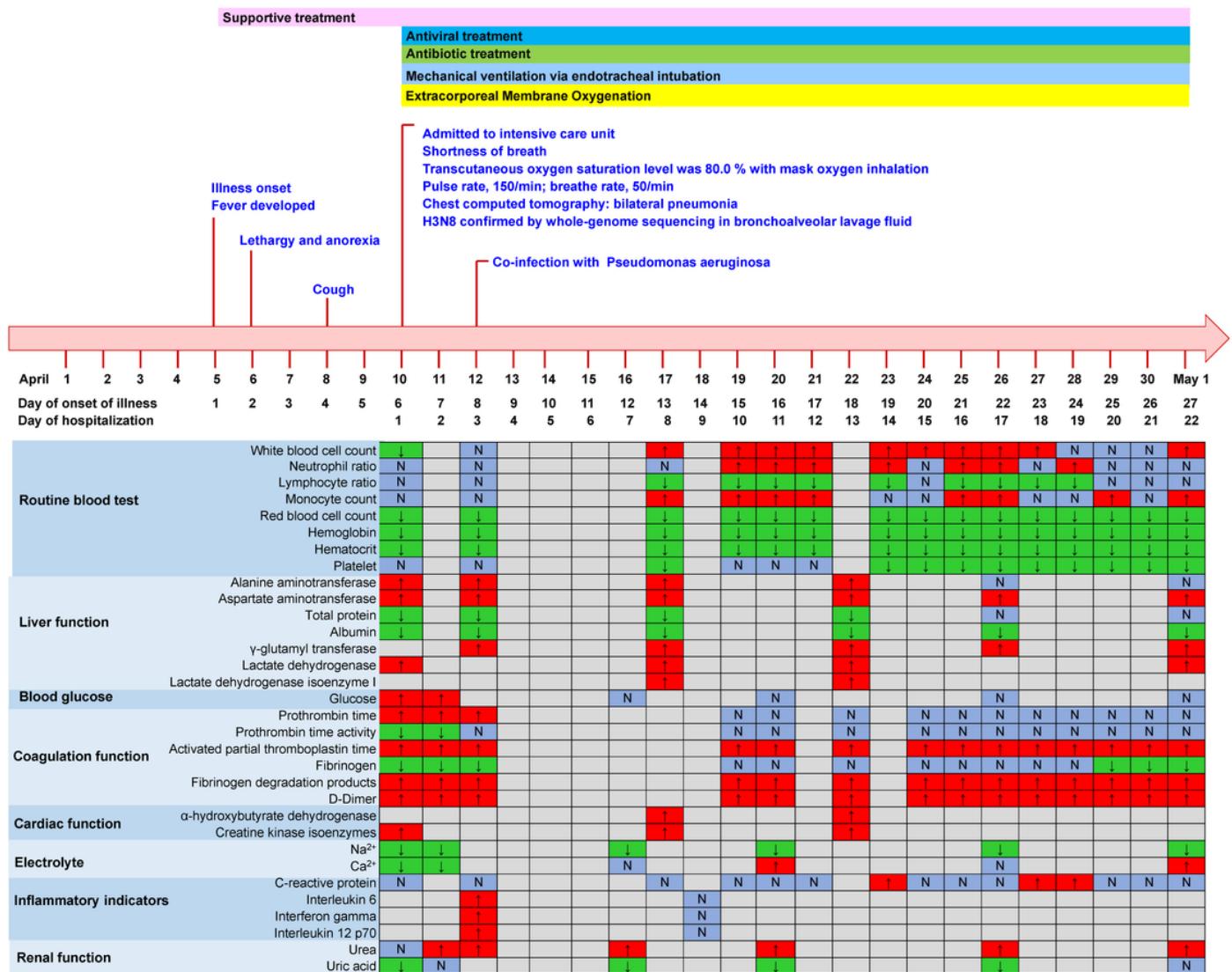


Figure 1

Timeline of the clinical course of the patient, identification of causative pathogen and laboratory indicators during hospitalization. ↑ = above normal range. ↓ = below normal range. N = normal range. + = positive. - = negative.

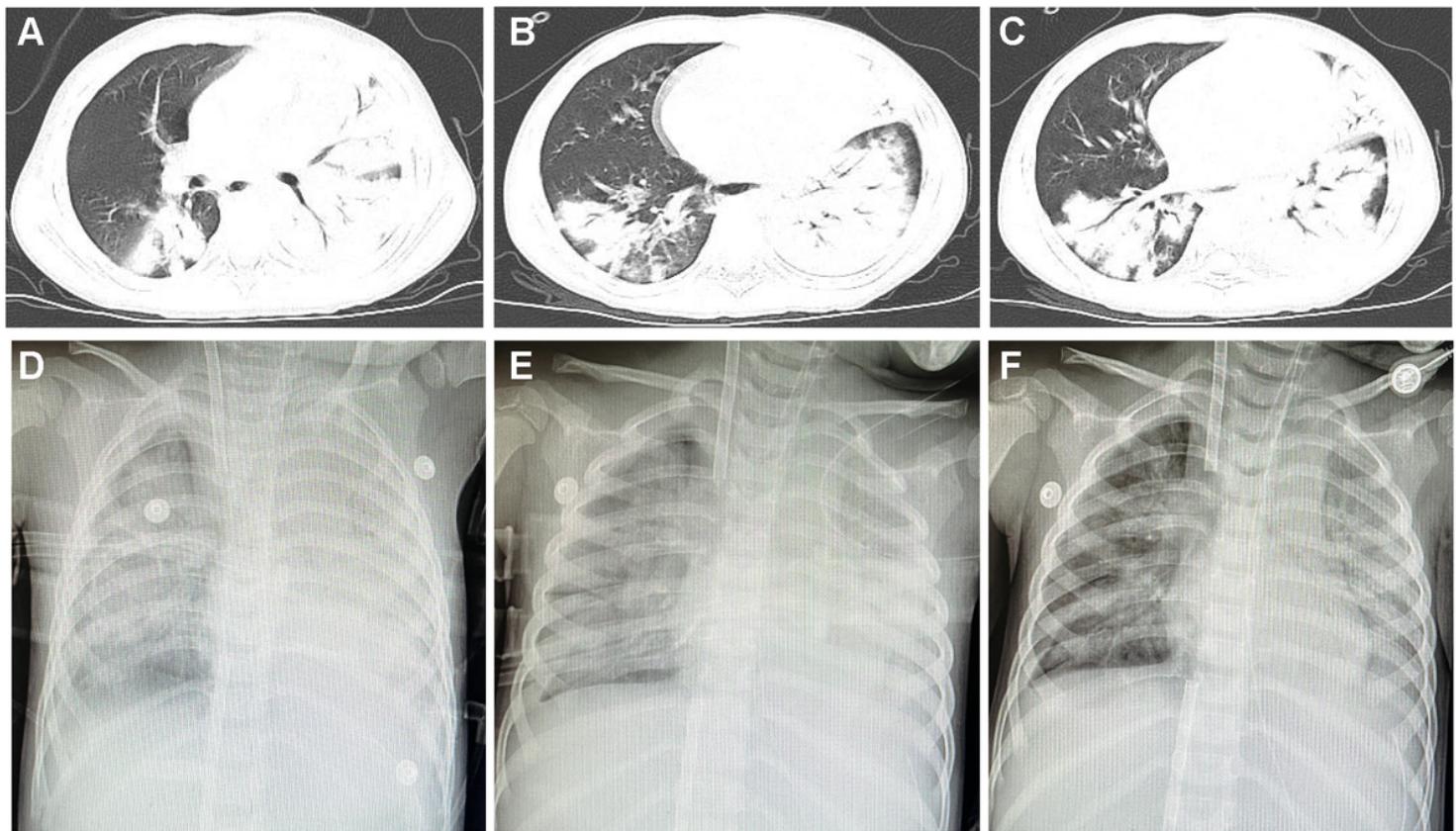
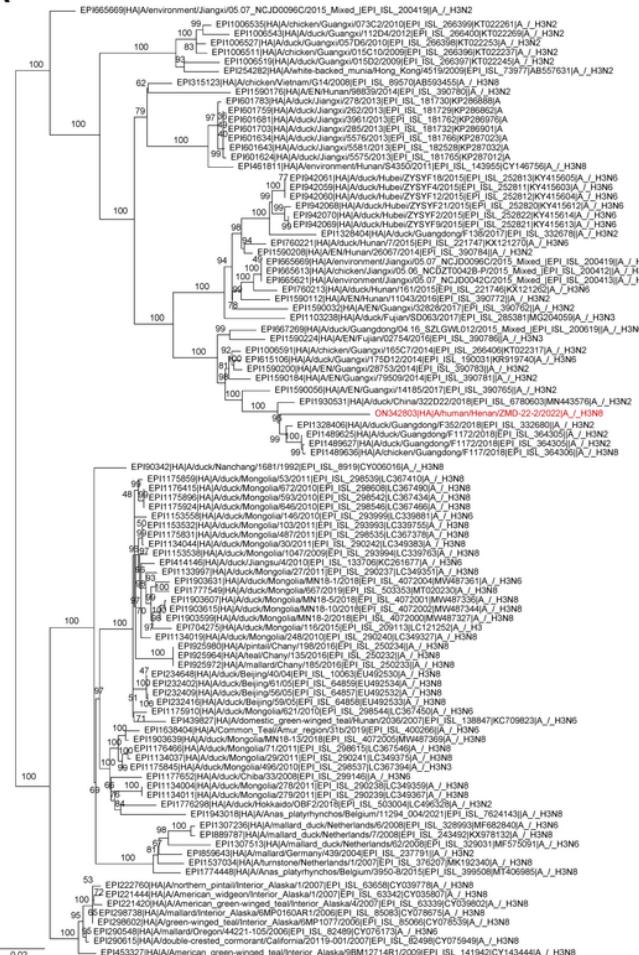


Figure 2

Imaging of the patient's chest. Computed tomographic (CT) scan obtained on day 6 of illness (A–C). CT scan showed multiple patchy high-density shadows in both lungs, especially in the lower lobe of the right lung and the left lung. Chest radiographs showed patchy high-density shadows in both lungs on day 7 of illness (D). After treatment 2 days, the pulmonary shadows were absorbed slightly (E–F).

A



B



Figure 3

Phylogenetic trees of genes of the A (H3N8) influenza virus. Phylogenetic trees for the full-length HA (A) and NA (B) genes of H3 and N8 subtype influenza viruses. A/Henan/ZMD-22-2/2022(H3N8) virus was indicated with red color.

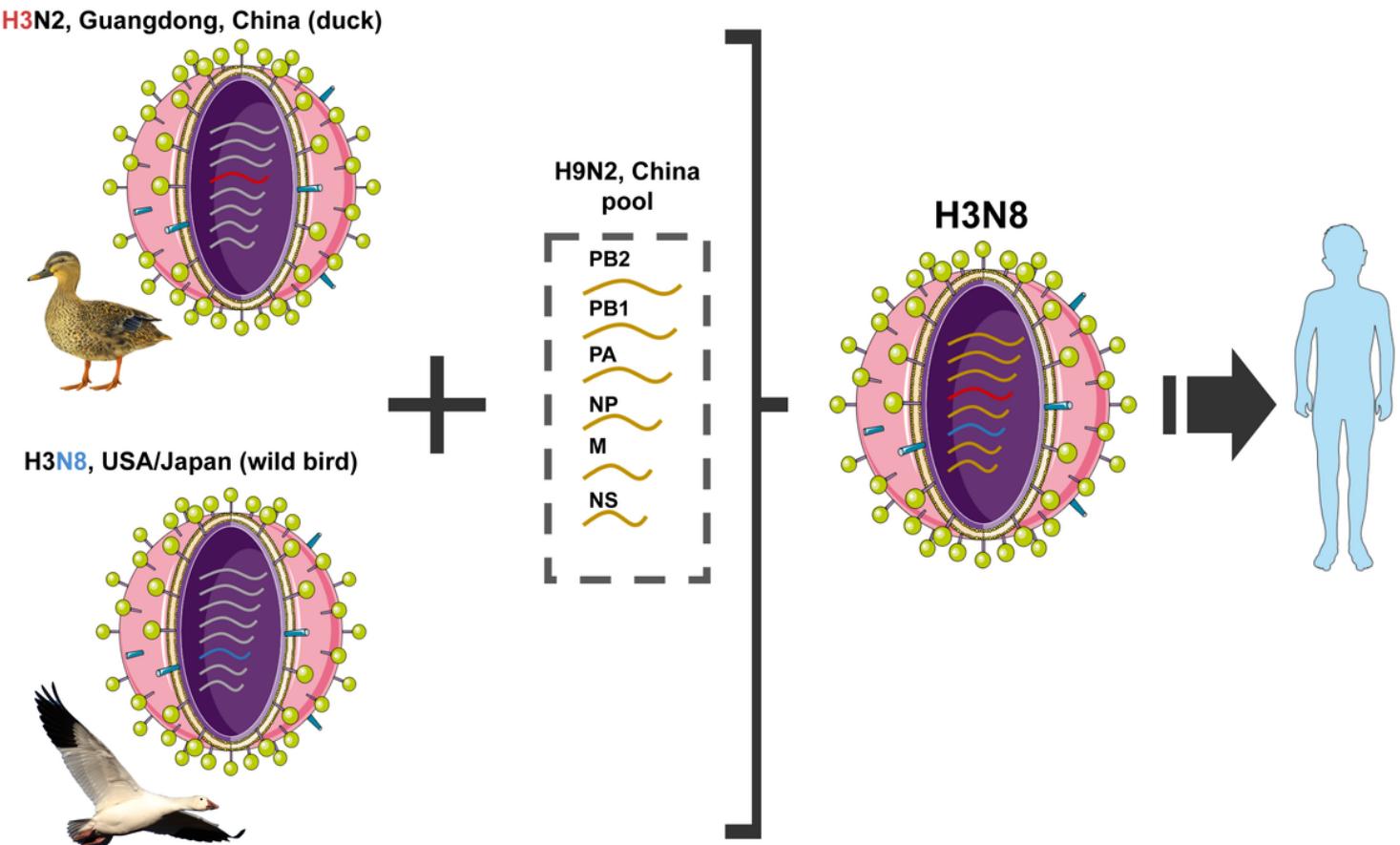


Figure 4

Hypothetical reassortment route and segment origins of the A (H3N8) influenza virus. The colors of the segments indicate their origin.

Supplementary Files

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