Viral genome and antiviral drug sensitivity analysis of two patients from a family cluster caused by the influenza A(H7N9) virus in Zhejiang, China, 2013

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SUMMARY

Objectives: In the winter of 2013, people were facing the risk of human-to-human transmission of the re-emerging influenza A(H7N9) virus. We report herein information on the clinical features of two patients from the same family infected with this virus, the genomic sequences of the viruses harbored, and antiviral drug sensitivity.

Methods: Clinical and epidemiological data of two patients from the same family were collected and analyzed. Sequencing was done for the viruses isolated from these two patients and one epidemiologically related chicken, and the sequences of the eight gene segments of the viruses were analyzed phylogenetically. The sensitivity of the viruses to antiviral drug treatment was determined by neuraminidase inhibitor susceptibility test.

Results: The two patients from one family cluster shared the same symptoms but had different outcomes, and had a strong epidemiological link. Three strains, two from these two patients and one from the chicken, were isolated. Genome sequencing and analyses of phylogenetic trees demonstrated that the two viruses were almost identical. We noted the presence of the PB2 E627K amino acid substitution that was not present in isolates from the first wave, as well as two new mutations in the NA gene and six in the PB2 gene. Drug sensitivity testing showed that the new isolates were resistant to oseltamivir but sensitive to peramivir.

Conclusions: The two patients from one family cluster were probable human-to-human transmission cases. The new isolates were sensitive to peramivir but showed reduced sensitivity to oseltamivir.

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1. Introduction

As of July 14, 2014, a total of 451 cases of avian origin influenza A(H7N9) infection had been reported to the World Health Organization (WHO).1 The first epidemic wave of influenza A(H7N9) in China occurred from February to May of 2013, and the second epidemic wave was from October 2013 to April 2014. The virus seems not to cause diseases in poultry and thus they could serve as a reservoir of infection that may lead to frequent sporadic human infections.

In experiments in ferrets, the H7N9 virus replicated well in the upper and lower respiratory tract and was efficiently transmitted by direct contact following intranasal inoculation.2 This led to the conclusion that under appropriate conditions, human-to-human transmission of the H7N9 virus may be possible.

We report a family cluster of two patients infected with the re-emerging virus in the winter of 2013. Information on variation in the viral genome, antiviral drug sensitivity, and the clinical...
presentation of the family cases is provided here. We sought to establish whether the family cluster of cases occurred as a result of possible human-to-human transmission and to determine the effects of neuraminidase inhibitors on these new isolated viruses.

2. Methods

2.1. Data and sample collection

Clinical features including demographic characteristics, laboratory abnormalities, viral kinetics, treatment, clinical outcome, and epidemiological data were obtained from the family cluster of two laboratory-confirmed H7N9 virus-infected patients on December 16, 2013. A standardized form, described previously by Gao et al. and Li et al., was used to collect the data.

All household members who had been in close contact with the two patients were placed under medical observation until 7 days after the patients had been isolated in the hospital. Paired serum samples (the interval between the two samples was at least 3 weeks) were collected and investigated to ascertain potential human-to-human transmission as well as asymptomatic and subclinical infections.

The first sputum specimens were obtained when the patients were hospitalized and had not yet initiated antiviral therapy. Feces of 33 chickens were obtained from epidemiologically linked wet markets (all from Anji County, Zhejiang). The research ethics board of the First Affiliated Hospital, Zhejiang University approved the study design.

2.2. H7N9 laboratory assays

The first sputum specimens of the patients and the excreta specimens of the chickens, delivered in viral transport medium, were tested by real-time reverse-transcriptase PCR (RT-PCR) assay to confirm H7N9 virus infection. RNA was extracted from specimens with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) using the manufacturer’s protocol and were tested by real-time RT-PCR with H7N9-specific primers and probes, as described previously by Gao et al. and Li et al. The specific sequences have been published on the WHO website at http://www.who.int/influenza/gisrs_laboratory/a_h7n9/en/.

Samples were diluted with equal amounts of the virus growth liquid and filtered centrifugally. The filtrate liquid was cultured in Madin–Darby canine kidney (MDCK) cells for virus isolation, including one to three blind passages, as described previously. When most of the cells had positive cytopathic changes, the supernatant of the culture solution was harvested and virus infection was confirmed by RT-PCR. Virus handling was conducted in an enhanced biosafety level 3 containment facility, in accordance with institutional guidelines.

Genome sequencing was done for two human isolates and one chicken isolate. All eight gene segments of the patient and chicken isolates together with the first wave isolates from the same county were deposited in GenBank; these sequences along with the other virus sequence data available in GenBank were characterized and analyzed phylogenetically. The sequences were analyzed using BioEdit version 7.0.9.0 DNA analysis software. Phylogenetic trees were constructed using MEGA5 software version 5.05, applying the neighbor-joining method with bootstrap analysis (1000 replicates). The sequence data obtained in this study have been deposited in GenBank under accession numbers KM879319-KM879326 (chicken isolate).

Drug sensitivities of oseltamivir (Roche Diagnostics GmbH, Mannheim, Germany) and peramivir (Nanxin Pharmaceuticals, Guangzhou, China) were assessed by neuraminidase inhibitor (NAI) susceptibility test using the method described by Gubareva et al. The IC50 values (drug concentrations required to inhibit NA activity by 50%) were used to characterize NAI susceptibility. To interpret the NAI susceptibility, the WHO criteria based on the fold change of IC50 values compared with reference IC50 values were applied. For influenza A viruses, normal inhibition was defined as a <10-fold increase, reduced as a 10–100-fold increase, and highly reduced as a >100-fold increase.

3. Results

3.1. Epidemiology, clinical features, and laboratory abnormalities

The index patient was a man aged 57 years with a history of hypertension for more than 10 years. He lived in a semi-urban area of Anji County in Zhejiang Province. There was a wet market where several kinds of live poultry were sold located about 1000 m from his district of residence. He was responsible for purchasing food for the whole family every day. He returned to his hometown in a rural area and cleaned the chicken coop on November 16, 2013; a fever developed on November 21, following an incubation period of 5 days.

The second patient was a 31-year-old male, the son-in-law of the index patient. He was a businessman and lived in the same county as the index patient, but not in the same district of residence, and came into occasional contact with the index patient. He fell sick on November 30. The second patient denied a history of exposure to poultry or any animal and had not been to a wet market during the 2 weeks prior to the onset of illness. He had come into close contact with the index patient during the period November 20 to November 26, including being in the same room, talking with the index patient, and providing unprotected bedside care to him; the time density of contact was from 0.3 to 18 h per day. During this exposure, the index patient had a continuous productive cough. The incubation period for the secondary case ranged from 4 to 10 days. Epidemiological data and detailed exposure and timeline information are available in the Supplementary Material (Figure S1 and Tables S1 and S2). The youngest daughter of the index patient – the wife of the second patient, who was in her first trimester of pregnancy – provided unprotected care to the index patient and the second patient, one after the other, until the second patient was isolated in a negative-pressure ward. She had the same density of contact time with the index patient as the second patient and did not have any symptoms. Hemagglutination inhibition antibodies against A(H7N9) virus were detected in paired serum samples from this daughter (three serum samples were collected because we needed to calculate the exposure time again when the second patient developed symptoms); the remaining household members were negative.

The abnormal clinical and laboratory findings are given in the Supplementary Material (Table S3). Both patients had a fever, productive cough, and shortness of breath, and the second patient had diarrhea. The two patients also had elevated levels of creatine kinase, lactate dehydrogenase, and C-reactive protein. Both had lymphopenia. We noted that the pneumonia began in one segment of the left lung and spread to other segments and even the contralateral lung in both patients (Supplementary Material, Figure S2). The condition of the index patient deteriorated further until he became dependent on extracorporeal membrane oxygenation (ECMO). Oseltamivir was initiated 4 days after the onset of illness and at that time this patient already had involvement of both lungs. He became negative for the virus after 15 days of therapy.

The second patient received oseltamivir therapy once at an outpatient clinic. When he was admitted, he received peramivir in order to prevent the delayed viral clearance observed in the index
patient. At that time, the second patient had pneumonia in two segments of the left lung. His symptoms were controlled rapidly. Five days later he became negative for the virus and 11 days later was discharged having made a full recovery. There was no evidence of any bacterial co-infection in either patient, however the index patient had a continuous secondary infection during the hospitalization (see Supplementary Material, Table S4).

3.2. Laboratory investigations

Strains isolated from these two patients were named A/ Zhejiang/DTID-ZJU17/2013 (ZJU17) and A/Zhejiang/DTID-ZJU18/2013 (ZJU18). Five out of 33 chickens tested positive for the H7N9 virus. We selected one strain, named A/chicken/Zhejiang/DTID-ZJU06/2013 (chicken/ZJU06), for analysis together with the strains isolated from humans. The sequences of all eight genes of ZJU17 and ZJU18 were almost identical (similarity ranging from 99.8% to 100%), but were slightly different from the sequences from the chicken (similarity ranging from 94.4% to 99.9%) (see Supplementary Material, Table S5). Further phylogenetic analysis showed that the eight genes of ZJU17 and ZJU18 belonged to the same clade (Supplementary Material, Figure S3).

Analysis of the critical amino acids related to mammalian adaptation and drug resistance showed that both strains were the same as strains ZJU01 and ZJU02 that were isolated during the first wave in 2013 in the same county. Analysis of the H7 receptor-binding site showed a G186V substitution (H3 numbering) and a C226L substitution (H3 numbering) in both the human and chicken isolates, which have been associated with an increasing binding affinity for the α(2–6)-linked sialic acid receptor. In NA, no R289K (N9 numbering) substitution associated with neuraminidase inhibitors was found in ZJU17 and ZJU18. However, we noted an E627K substitution in the PB2 protein in both ZJU17 and ZJU18, but no D701N substitution, which has been associated with enhanced transmission in guinea pigs, as was reported in ZJU01 during the first wave. Two amino acid mutations (75 and 335) in the NA protein and six amino acid mutations (191, 511, 535, 570, 647, and 702) in PB2 were noted, and these mutations have not been found in previously reported H7N9 viruses (Table 1).

Table 1

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Province also showed the E627K mutation.\textsuperscript{10} The presence of the PB2 E627K mutation compared with the first wave in the same district may be further evidence that the family cluster of two patients involved probable human-to-human transmission. We noted another six amino acid mutations in the PB2 protein. We cannot rule out the possibility that these particular mutations also increased the capacity of the virus for human adaptability.

NALs are the first-line therapeutic options against H7N9 virus. Previous research has identified three oseltamivir-resistant strains: A/shanghai/1/2013,\textsuperscript{3} A/Taiwan/S02076/2013, and A/Taiwan/T02081/2013. They all had the R294K substitution, which reduces the effectiveness of oseltamivir by over 100,000-fold using protein- and virus-based assays.\textsuperscript{11} However, crystal structure analysis of the neuraminidase N9 also showed that the R294K substitution would interfere with its binding to sialic acid and reduce viral fitness. This explains why oseltamivir-resistant viruses are not dominant human-infecting viruses. Another explanation is that the virus exists in the human body with a ‘quasispecies’ formation, and oseltamivir resistance is from the selective pressure during oseltamivir therapy.

In real-life clinical practice, we found delayed clearance of the virus after oseltamivir therapy. In addition to drug resistance, another possible explanation is the delayed initiation of antiviral treatment. The analysis of 111 cases of influenza A(H7N9) virus infection showed antiviral drug therapy was initiated at a median of 7 days after the onset of illness.\textsuperscript{12} Glucocorticoid usage is another possibility.\textsuperscript{13} However, in our drug sensitivity testing, we noted the reduced sensitivity of the virus to oseltamivir, which was identical to the phenomenon of delayed viral clearance during oseltamivir therapy. Selective pressure from antiviral therapy cannot explain this phenomenon, as we isolated the virus from the sputum specimens collected before antiviral therapy. Also, we found two amino acid mutations in the NA protein of the new isolates when comparing them with ZJU01 isolated during the first wave in the same district, which was sensitive to oseltamivir on in vitro testing. We cannot rule out the possibility that these mutations are related to the drug resistance of the H7N9 virus. Further work is needed to investigate the function of these two mutations. We cannot ignore quasispecies formation of the virus either. For the sensitivity limit, using routine sequencing methods, we could not identify the existing resistance site. Of note, because the number of virus strains studied here was small, these may not represent the features of all re-emerging A(H7N9).

Our study has several limitations. First, we cannot completely rule out environmental transmission for these two patients because we did not analyze environmental samples. Secondly, we cannot explain why the symptoms and outcomes of this family cluster of two patients were so different, although we think that these two patients were infected by the same virus strain. Besides the virulence of the virus, the potential bacterial co-infection and the different antiviral drug treatment regimens used in these two patients may have played an important role. Also, we cannot exclude the possibility that during spread among humans, the virulence of the virus is decreased. Thirdly, we cannot explain why with the same exposure density, the daughter of the index patient did not fall sick. Finally, we do not know the significance of the two amino acid mutations in the NA protein and the six amino acid mutations in the PB2 protein. Further crystal structure and function characterization is needed.

In conclusion, although current data do not indicate that A(H7N9) is readily transmissible among humans, we should always be alert to the human-to-human property of this virus. Close monitoring of the variation among more A(H7N9) viruses and the antiviral drug sensitivity are required.

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Conflict of interest: All authors have no reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2014.10.029.

References


