Human infection with avian influenza A H6N1 virus: an epidemiological analysis


Summary
Background Avian influenza A H6N1 virus is one of the most common viruses isolated from wild and domestic avian species, but human infection with this virus has not been previously reported. We report the clinical presentation, contact, and environmental investigations of a patient infected with this virus, and assess the origin and genetic characteristics of the isolated virus.

Methods A 20-year-old woman with an influenza-like illness presented to a hospital with shortness of breath in May, 2013. An unsubtyped influenza A virus was isolated from her throat-swab specimen and was transferred to the Taiwan Centres for Disease Control (CDC) for identification. The medical records were reviewed to assess the clinical presentation. We did a contact and environmental investigation and collected clinical specimens from the case and symptomatic contacts to test for influenza virus. The genomic sequences of the isolated virus were determined and characterised.

Findings The unsubtyped influenza A virus was identified as the H6N1 subtype, based on sequences of the genes encoding haemagglutinin and neuraminidase. The source of infection was not established. Sequence analyses showed that this human isolate was highly homologous to chicken H6N1 viruses in Taiwan and had been generated through interclade reassortment. Notably, the virus had a G228S substitution in the haemagglutinin protein that might increase its affinity for the human α2-6 linked sialic acid receptor.

Interpretation This is the first report of human infection with a wild avian influenza A H6N1 virus. A unique clade of H6N1 viruses with a G228S substitution in haemagglutinin have circulated persistently in poultry in Taiwan. These viruses continue to evolve and accumulate changes, increasing the potential risk of human-to-human transmission. Our report highlights the continuous need for preparedness for a pandemic of unpredictable and complex avian influenza.

Funding Taiwan Centres for Disease Control.

Introduction Influenza A viruses have two surface glycoproteins: haemagglutinin and neuraminidase. The former binds to sialic acids on target cells to initiate virus infection and the latter cleaves sialic acids to allow virus release. The haemagglutinins of human influenza viruses preferentially bind to α2-6 sialic acid receptors whereas the haemagglutinins of avian influenza viruses bind to α2-3 sialic acid receptors. The functional balance of haemagglutinin and neuraminidase activities has roles in viral pathogenicity, replication efficiency, and transmissibility. Influenza A viruses are classified into various subtypes on the basis of the antigenicity of their haemagglutinin and neuraminidase. In addition to subtypes H1, H2, and H3 of seasonal influenza A viruses, subtypes H5, H7, H9, and H10 have been reported to cause infections in human beings. Influenza A H6N1 virus has been isolated from migratory birds and domestic poultry in many continents. It is thought to be of low pathogenicity for avian species and has caused outbreaks in poultry with slight morbidity and mortality. Infection with H6N1 has been prevalent in domestic chickens in Taiwan since 1972. The virus circulating among Taiwan poultry is of a genetically unique lineage different from that circulating in Hong Kong and southeast China, and has shown the potential for cross-species infection of mammals. Human infection with the wild influenza A H6 subtype has not been reported in the scientific literature. In our study, we detected a human case of infection with the H6N1 virus. We report the clinical presentation, contact and environmental investigations of the infected patient, and assess the origin and genetic characteristics of the isolated virus.

Methods Patient presentation A previously healthy, 20-year-old woman with an influenza-like illness presented to a hospital with shortness of breath. Clinical presentation and use of medical resources during the infection episode were obtained from the patient. The medical records of the patient were reviewed. Close contacts were defined as those who had provided care to, had been living with, or
had potentially been exposed directly to respiratory secretions or body fluids of the patient from the 14th day before illness onset to the 14th day after the patient’s illness subsided. The patient was interviewed to obtain information regarding her occupation, travel history, exposure to poultry within the previous 3 months, and to obtain a list of close contacts. The lists of health-care workers who had close contact with the patient were obtained from the hospitals visited.

Investigations
All the close contacts and health-care workers were interviewed to establish whether they had experienced a fever or respiratory tract infection 7 days before contact to 7 days after contact. All contacts with a fever or respiratory tract infection were interviewed with a structured questionnaire, which queried the symptoms of illness, details of contact with the index patient and poultry exposure. Throat swabs and blood samples were obtained from symptomatic contacts. The household environment of the patient was inspected to assess the potential source of infection.

Enhanced surveillance for H6N1 virus was launched in health-care facilities, including three hospitals and seven primary care clinics within a radius of 5 miles from the patient’s residence. Outpatients and inpatients who had influenza-like illness or pneumonia were eligible to submit throat swabs to the Taiwan Centres for Disease Control (CDC) for mandatory influenza virus isolation and identification.

Procedures
The throat swab specimen was inoculated onto Madin-Darby canine kidney (MDCK) cells. The inoculated cells were incubated at 35°C and examined for cytopathic effect. Influenza A virus was identified by immunofluorescence assay as described elsewhere. The isolated virus was confirmed as influenza A virus (targeting the matrix gene), but tested negatively for H1, and H3 subtypes using real-time RT-PCR assays, as described elsewhere. The clinical specimen and unsubtype influenza isolate of the patient were sent to the Taiwan CDC. The unsubtype influenza A virus was analysed with conventional RT-PCR using universal primer sets. The clinical specimen of the patient was tested later using real-time RT-PCR assay for H6 subtyping, which was confirmed on the basis of the haemagglutinin sequence of the H6N1 virus isolated in this study. The primers and probe sequences were H6-1578F (TGTTGTTGATCATAATCATTCTTG), H6-1706R (GCAATTGCAATTGARCCAT), and H6-1620P (FAM-TATAGTACGGTATCGAGCAGTCTRGT-BHQ). The clinical specimen of the patient was tested for other respiratory viruses, using 13 multiplex real-time RT-PCR reactions that were set up to detect 23 human respiratory viruses: adenovirus, respiratory syncytial virus, coronavirus (229E, OC43, NL63, HKU1, MERS-CoV), metapneumovirus, parainfluenza type 1–4, herpes simplex virus 1 and 2, varicella zoster virus, Epstein–Barr virus, cytomegalovirus, human herpes virus 6 and 7, bocavirus, parvovirus, enterovirus, and rhinovirus. The assay conditions, primers, and probes for each virus have been described elsewhere. For haemagglutinin inhibition (HI) tests, H6N1 virus (A/Taiwan/2/2013 isolated from the patient) was used as the antigen to test for specific antibodies. The HI tests were done in accordance with procedures described elsewhere. For this test we used turkey red blood cells. We report HI titres as the reciprocal of the highest dilution of serum that inhibited virus-induced haemagglutination. Sera that tested negative at a dilution of 1:10 were recorded as a titre of less than 10.

To establish viral nucleotide sequences, conventional RT-PCR was done to amplify each genome segment of the influenza A virus using universal primer sets and the OneStep RT-PCR Kit (Qiagen, Santa Clara, CA, USA). Nucleotide sequences of the amplified PCR products were determined and translated into aminoacid sequences. Multiple sequence alignments, protein translations, and phylogenetic analyses were done based on the nucleotide sequences with MEGA5.2 and BioEdit 7.0 software. Phylogenetic trees were constructed with the neighbour-joining method and 1000 bootstrap replications were done to assess the reliabilities. For each gene segment, the full-length sequences of H6N1 isolated in Taiwan available on the influenza virus resource at the US National Center for Biotechnology Information (NCBI) were selected for the construction of phylogenetic trees. The nucleotide sequences of the influenza viruses included in our study have been submitted to the Global Initiative on Sharing All Influenza Data database (accession numbers EPI459852–EPI459859). In the appendix we list the accession numbers of viruses we assessed.

Role of the funding source
The sponsor had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication. The corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results
The patient developed a fever and non-productive cough on May 5, 2013. She visited the emergency department of hospital A on May 6 and the emergency department of hospital B for a persistent high fever on May 7 and revisited that hospital for shortness of breath during the late evening on that day. Figure 1 shows the dates on which the patient’s throat swabs and serum samples were collected. During her last visit to hospital B, rales were noted over the bilateral lower chest. A radiographic examination of the chest showed a bilateral infiltrate in the lower lung fields (Figure 2). She was admitted with a diagnosis of lower respiratory tract infection in the early morning of May 8.
Two sets of throat swabs were submitted for influenza rapid immunochromatography test on May 7 and 8 and both gave negative results. The throat swab collected on May 8 was inoculated onto MDCK cells for influenza detection. The isolated virus was identified as influenza A by immunofluorescence assay on May 10. The subsequent subtype identification showed negative results for human H1N1, H3N2, and H1N1pdm09 viruses. Both the legionella urinary antigen test, aiming at *Legionella pneumophila* serogroup 1, and the pneumococcal urinary antigen test were negative. The serological test for immunoglobulin M in acute and convalescent sera against *Mycoplasma pneumoniae* and *L pneumophila* was negative. The result of blood culture was negative. Bacterial culture of sputum specimens was not done because of the scarcity of sputum production.

The blood sample obtained on May 8 showed normal blood counts except for the leucocyte count, which was 10 040 cells per μL (reference range 3600–10 000), with 75·6% segmented leucocytes, 17·2% lymphocytes, and 6·7% monocytes. The C-reactive protein concentration was 981 nmol/L (reference range <28·5) and the aspartate aminotransferase concentration was 13 U/L (reference range 15–37). Her creatine kinase concentration was 126·6 U/L (reference range 38–176).

75 mg oseltamivir twice a day and 500 mg levofloxacin once a day were given empirically during her admission to hospital between May 8 and May 11. The symptoms lessened from May 9. She declined to take any drugs after discharge. The patient remained healthy, and no pulmonary abnormalities were identified at follow-up. A follow-up chest radiographic study on May 17 showed complete resolution of pulmonary infiltrates (figure 2).

The patient did not have a travel history for 3 months before the infection. She worked in a delicatessen before the infection, serving as a clerk; she was not involved in cooking raw animal products. The delicatessen did not market live animals. We identified 36 close contacts: three family members including her mother who provided care during her admission to hospital, her boyfriend who visited her on May 12 with close physical contact, five neighbouring family members whom she visited three times in early May, eight colleagues in the delicatessen, seven health-care workers in hospital A, and 12 health-care workers in hospital B. Of the 36 close

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**Figure 1:** Chronology of patients

Timelines of the patient with H6N1 infection and contacts. The timepoints of illness onset, contact time, and specimen collection are shown by symbols explained in the key.

**Figure 2:** Chest radiograph of the index patient

Chest radiograph taken on May 8 (A), the fourth day of illness, shows increased lung marking and infiltrate in the bilateral lower lung fields. Radiograph taken on May 17 (B) shows the clear lung fields.
contacts, six developed fever or respiratory tract infection (table 1). They reported no recent contact with poultry. All the health-care workers reported using a surgical mask when in contact with the patient, except for her attending physician at hospital B, who had contact with a series of patients with respiratory tract infections in April and May, including the index patient, without appropriate respiratory tract precautions and had developed a respiratory tract infection in mid-May. Throat swab and blood samples were obtained from symptomatic contacts on May 24 or 25 (figure 1). Additional blood samples were collected on June 8.

Table 1: Clinical presentation and serological response of the index patient and her symptomatic contacts

<table>
<thead>
<tr>
<th>Age (years), sex</th>
<th>Date of illness</th>
<th>Symptoms</th>
<th>Haemagglutinin inhibition titres*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>First serum†</td>
</tr>
<tr>
<td>Patient</td>
<td>20, female</td>
<td>May 5</td>
<td>Fever, cough, sore throat, shortness of breath</td>
</tr>
<tr>
<td>Brother</td>
<td>17, male</td>
<td>Apr 24</td>
<td>Rhinorhoea, sore throat</td>
</tr>
<tr>
<td>Neighbour</td>
<td>83, female</td>
<td>Early May</td>
<td>Cough, sore throat, headache</td>
</tr>
<tr>
<td>Nurse in hospital A</td>
<td>34, female</td>
<td>May 8</td>
<td>Fever, rhinorhoea, cough, sore throat, tinnitus, muscle ache</td>
</tr>
<tr>
<td>Mother</td>
<td>45, female</td>
<td>May 11</td>
<td>Fever, cough, sore throat</td>
</tr>
<tr>
<td>Boyfriend</td>
<td>31, male</td>
<td>May 14</td>
<td>Fever, rhinorhoea, sore throat</td>
</tr>
<tr>
<td>Physician in hospital B</td>
<td>40, male</td>
<td>Mid May</td>
<td>Sore throat, muscle ache</td>
</tr>
</tbody>
</table>

*Established by use of turkey red blood cells. Titres are reported as the reciprocal of the highest dilution of serum that inhibited virus-induced haemagglutination.†Obtained on May 24 except for her boyfriend, who received the first serological test on May 25. ‡Obtained on June 8. §Exact date of illness onset not available. ¶The registered nurse’s two children developed influenza-like illnesses 1 day before her illness. She declined to have her children tested for influenza infection.

Figure 3: Phylogenetic relatedness of the M6N1 viruses isolated in Taiwan

Phylogenetic relatedness of the full-length haemagglutinin (A) and neuraminidase (B) genes. The tree was constructed using the neighbour-joining method of MEGA 5.2; the reliability of the trees was assessed by bootstrap analysis with 1000 replications. The H6N1 viruses isolated from chickens in Taiwan comprise a unique lineage and are divided into two clades: 228S with the serine residue at 228 and 228G with the glycine residue at 228. These colours are used in the phylogenetic trees of the other segments. The A/Taiwan/2/2013 in green is the virus isolated from the index patient and the other viruses are from avian species. Phylogenetic trees of the other six segments are shown in the appendix.
From June 10 to Aug 13, 2013, 125 cases of influenza-like illness or pneumonia were reported to the Taiwan CDC through the enhanced surveillance, and all tested negative for H6N1. Of the 125 cases, eleven (9.2%) tested positive for H1N1pdm and 23 (19.2%) for H3N2.

The unsubtyped influenza A isolate and the corresponding throat swab specimen of the case were sent to Taiwan CDC on May 20. The virus isolate was identified as H6N1 (named A/Taiwan/2/2013; TW02/13 for short) on the basis of the sequences of viral haemagglutinin and neuraminidase genes. The original throat swab specimen taken on May 8 was examined later and was identified to be positive for H6N1 virus by use of real-time RT-PCR assay. The samples of throat swabs obtained from the six symptomatic contacts all tested negative for influenza virus with real-time RT-PCR assays.

A family neighbouring the patient bred a flock of 60 poultry (including ducks, chicken, and geese) on the riverbank near the patient’s residence. The patient reported no contact with the poultry in the past year because the riverbank was low-lying land and was not readily accessible to other residents. Eight fresh avian stool samples collected on May 24 tested negative for influenza virus with real-time RT-PCR.

To establish whether the case was coinfected with other respiratory tract-related pathogens, the throat swab specimen obtained on May 8 was assessed further with several real-time RT-PCR/PCR reactions targeting 23 human viruses. All tests were negative.

The paired serum specimens of the case and the six symptomatic contacts were assessed with the HI test (table 1). The first serum sample of the case had an HI titre of 40, and the second had a titre of 80. All samples from the six symptomatic contacts had HI titres of 10 or less.

On the basis of the analysis of full-length gene sequences, all gene segments of TW02/13, except PB1, had high homology (>96% nucleotide identity) with the corresponding sequences of A/chicken/Taiwan/A2837/2013 H6N1 (A2837/13) isolated from chickens in Taiwan in 2013. The PB1 gene of TW02/13 shared greatest nucleotide identity (95·7%) with the older A/chicken/Taiwan/PF3/02 H6N1 (appendix). Phylogenetic analyses of the eight gene segments showed that TW02/13 belonged to an H6N1 lineage that has circulated in chickens in Taiwan since 1997. This lineage comprises all H6N1 viruses isolated in Taiwan and is separated from the viruses in Hong Kong and other regions, suggesting that all segments of TW02/13 originated from viruses in chickens in Taiwan (figure 3 and appendix). Gene segment constellations of human TW02/13 isolate and chicken A2837/13 viruses were analysed and are summarised in figure 4 based on the phylogenetic topologies of the eight gene segments from viruses of this lineage. The PB2, PA, NP, NA, M, and NS segments of TW02/13 and A2837/13 were all descended from A/chicken/Taiwan/A342/05 H6N1 (A342/05). The PB1 gene of TW02/13 was evolutionarily related to A2837/13 and could be traced back to the older A/chicken/Taiwan/PF3/02 H6N1 (PF3/02). The HA gene of TW02/13 and A2837/13 clustered with A/chicken/Taiwan/PF3/02 (0204/05) virus, providing the PB2, PA, NP, NA, M, and NS segments. PF3/02=A/chicken/Taiwan/PF3/02. 0204/05=A/chicken/Taiwan/0204/05. A342/05=A/chicken/Taiwan/A342/05. TW02/13=A/Taiwan/2/2013. A2837/13=A/chicken/Taiwan/A2837/2013.
antiviral resistance, and pathogenesis are analysed and listed in table 2. The haemagglutinin cleavage site of TW02/13 did not contain additional basic aminoacids (PQIATR/G), suggesting low pathogenicity in poultry species. Analysis of the receptor binding site of the haemagglutinin protein showed a G228S substitution (H3 numbering) that is also evident in some avian H6N1 viruses in Taiwan. Based on the phylogenetic topology of HA genes, these G228S-carrying viruses formed a clade (designated as 228S in figure 3). The H6N1 viruses isolated from chickens in Taiwan after 2005 had the G228S substitution, suggesting that the 228S clade viruses have become endemic and predominant in Taiwan (figure 3). The G228S substitution has been reported to contribute to the specificity of haemagglutinin for the human α2-6 sialic acid receptor, thereby enabling the replication and transmission of the virus within and among human beings. Notably, most of the H6N1 viruses isolated in Taiwan after 2005 exhibited the G228S substitution and all haemagglutinin sequences among human beings. These reports suggest that H6N1 virus is not adapted for human infection and is poorly immunogenic in human beings, consistent with the low HI titres of the case in our report.

The index patient showed alleviated symptoms after receiving oseltamivir within 3 days of illness. Based on the molecular analysis that H6N1 is susceptible to oseltamivir, we speculate that giving oseltamivir halted the propagation of the lower respiratory tract infection and prevented a severe outcome for the patient.

The investigation of the transmission pathways that caused the patient’s infection did not reach a clear conclusion. Her close contacts’ respiratory tract illnesses and the lack of known poultry exposure for the index patient raise the question of human-to-human transmission. However, there was no laboratory evidence of human-to-human transmission. To our knowledge, no study has shown that H6N1 virus is able to transmit among mammals through aerosols. Because understanding the transmissibility of a novel influenza virus is of major importance to public health, further field and laboratory investigations are warranted to elucidate the mode of transmission of the virus.

In Taiwan, some chicken H6N1 viruses have acquired a G228S substitution in haemagglutinin since 2000 (figure 3). The G228S substitution has been reported to contribute to the specificity of haemagglutinin for the human α2-6 sialic acid receptor, thereby enabling the replication and transmission of the virus within and among human beings. Notably, most of the H6N1 viruses isolated in Taiwan after 2005 exhibited the G228S substitution and all haemagglutinin sequences of H6N1 with the G228S substitution in the NCBI database were obtained from isolates in Taiwan, suggesting that a unique H6N1 lineage with the human-adapted G228S substitution has become endemic and predominant in poultry in Taiwan. The human H6N1
TW02/13 virus had an identical 14-residue deletion pattern of neuraminidase with avian H6N1 isolates in Taiwan. These avian H6N1 isolates with 14-residue deletion had become predominant strains in Taiwan since 2001. Deletion in the neuraminidase stalk seemed to be associated with adaptation and virulence of aquatic viruses in chickens; it should be carefully monitored and assessed for a possible relation between NA deletion and human adaptation. These viruses continue to evolve and accumulate changes, increasing the potential risk of avian-to-human transmission. Regarding the origin of human H6N1 TW02/13 virus, Yuan and colleagues analysed our submitted sequences and speculated that the virus was derived from the reassortment of H5N2 and H6N1 viruses with G228S in its haemagglutinin and a unique group of H6N1 viruses with G228S have circulated persistently in poultry in Taiwan since 2000.

In conclusion, to our knowledge we report the first human patient infected with an influenza A H6N1 virus belonging to a unique lineage in Taiwan and with a G228S substitution in haemagglutinin. The occurrence of a human case of H6N1 infection shows the unpredictability of influenza viruses in human populations. Further epidemiological and laboratory investigations are warranted to elucidate the potential threat posed by the emerging virus. Our report highlights the need for influenza pandemic preparedness, including intensive surveillance for ever evolving avian influenza viruses.

Conflicts of interest
We declare that we have no conflicts of interest.

Acknowledgments
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References


The expanding list of zoonotic influenza viruses

In *The Lancet Respiratory Medicine*, Sung-Hsi Wei and colleagues describe a case of lower respiratory tract illness associated with an influenza virus that has not previously been isolated from human beings. The authors show, by virus isolation and sequencing, that this virus is novel and closely related to viruses circulating in poultry in the same country. They have also done an extensive workup of other possible respiratory pathogens—all testing negative. Combined with detection of antibodies to the new virus, the authors have convincingly shown that this is not a chance finding but probably the cause of the illness. This result adds another animal virus to the growing list of influenza viruses that might infect human beings, along with avian influenza viruses of subtypes H5N1, H7N2, H7N3, H7N7, H7N9, H9N2, and H10N7, and swine influenza viruses of subtypes H1N1, H1N2, and H3N2. Furthermore, experimental human infections with H4N8 viruses have shown that these viruses might replicate within human beings. Although the total number of recognised human cases is less than 1000 worldwide, each person infected with an animal influenza virus is judged a threat to public health, because the influenza pandemics of the past 100 years have all emerged from animals.

Science falls short of predicting which animal influenza viruses might lead to a pandemic, and it is clear that many factors might affect this emergence. Several types of wild aquatic birds are the reservoir hosts for avian influenza viruses, and the presence of avian influenza in these reservoir species is affected by population dynamics such as the density of these animals and their migration patterns. Places that are popular rest sites for migratory birds are potential hot spots for transmission, particularly when new variants arise, as has been shown when the avian influenza viruses of subtype H5N1 spread from Asia to Europe and Africa.

In birds, avian influenza viruses circulate mostly in a form that is not associated with substantial illness but is associated with enteric infection, the so-called low-pathogenic type. Therefore, there are no warning signs of the potential presence of the virus and exposure is via contact with bird droppings. Indirect contact, such as swimming in contaminated waters or eating food that is grown in or with contaminated water is hypothetically possible, but has not been associated with increased risk of infection. The low pathogenic nature of most infections is also evident when poultry held for meat and egg production are infected through exposure to wild birds. A different situation is evident with avian influenza viruses in which one of the spikes on the surface of viral particles, the haemagglutinin, has mutations, changing the infection from localised enteric to generalised systemic infection (highly pathogenic avian influenza virus). This has only been recorded for viruses with haemagglutinin subtypes H5 and H7, which is the reason why veterinary disease surveillance focuses on these virus subtypes.

The H5N1 viruses that have been cause for concern since 1996 are an exception to this rule, since they circulate among wild birds in this highly pathogenic genotype. Here, introductions of the virus into domestic poultry flocks causes mortality in birds, and human infections have been recorded that are also associated with severe illness. Although the pathogenicity for poultry does not affect the pathogenesis of human infection, the risk to exposed people is thought to be higher because of generally higher viral load and the handling of diseased animals with highly pathogenic avian influenza virus. Again, however, the recent outbreak of influenza low-pathogenic type H7N9 in
eastern China underscores the unpredictable nature of influenza viruses: first signs of this virus were human cases with severe disease. So far, 135 cases have been notified, of which 44 have died because of severe respiratory disease and multiorgan failure.

Unexpectedly, the haemagglutinin of this virus lacks the pathogenicity marker for poultry, and studies have confirmed that it causes few symptoms in poultry. As a consequence, finding the source of this infection is not easy, and, so far, it remains unclear where the main reservoir resides, although contact with poultry in live bird markets is a recorded risk factor. For pandemic circulation, an essential additional feature is the ability to transmit efficiently from person to person. Research into the genetic factors governing transmissibility is under substantial debate, but work to enhance into the genetic factors governing transmissibility to transmit efficiently from person to person. Research into the genetic factors governing transmissibility is under substantial debate, but work to enhance into the genetic factors governing transmissibility

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