Journal of Infection (2011) **62**, 394—399

BIAM British Infection Association

www.elsevierhealth.com/journals/jinf

CASE REPORT

Infection of immunocompromised patients by avian H9N2 influenza A virus

Vincent C.C. Cheng [a], Jasper F.W. Chan [a], X. Wen [a,b], W.L. Wu [a,b], T.L. Que [d], H. Chen [a,b,c], K.H. Chan [a], K.Y. Yuen [a,b,c,*]

[a] *Department of Microbiology, Queen Mary Hospital, Hong Kong Special Administrative Region, China*
[b] *State Key Laboratory for Emerging Infectious Diseases, The University of Hong Kong, Hong Kong Special Administrative Region, China*
[c] *Research Center of Infection and Immunology, The University of Hong Kong, Hong Kong Special Administrative Region, China*
[d] *Department of Pathology, Tuen Mun Hospital, Hong Kong Special Administrative Region, China*

Accepted 19 February 2011
Available online 26 February 2011

KEYWORDS
Avian influenza A H9N2;
Bone marrow transplantation;
Immunocompromised host

Summary Avian influenza A (H9N2) virus is transmitted sporadically from avian species to human causing mild diseases in immunocompetent person. We report two cases of human infection in immunocompromised patients in Hong Kong between 2008 and 2009. One patient had uneventful recovery with viral shedding at day 10 after symptom onset despite her underlying acute lymphoblastic leukaemia. The other patient with post-bone marrow transplant chronic graft-versus-host disease and bronhioltis obliterans went into respiratory failure. Genetic analysis revealed that these cases were caused by different genetic variants which are circulating in poultry in this region. Review of literature identified another 9 human cases reported in Southern China since 1988. It is possible that human infection with H9N2 is more common than what has been recognized. Continuous surveillance of H9N2 influenza virus infection in human is warranted.

* Corresponding author. Department of Microbiology, Queen Mary Hospital, Pokfulam Road, Pokfulam, Hong Kong Special Administrative Region, China. Tel.: +852 22553206; fax: +852 28724555.
E-mail address: kyyuen@hkucc.hku.hk (K.Y. Yuen).

0163-4453/$36
doi:10.1016/j.jinf.2011.02.007

Introduction

Since the first documentation of human infection with the highly pathogenic avian influenza A (H5N1) virus in 1997 resulting in 18 hospitalizations and 6 deaths,[1] a series of infection control measures to prevent viral transmission from poultry to human have been implemented in the Hong Kong Special Administrative Region, China. These included a monthly moratorium, or rest day with cleansing of all the poultry stalls in order to interrupt the viral replication cycle and reduce the viral load in the poultry markets.[2] Live ducks and geese, natural reservoirs of avian influenza viruses, were not allowed in the markets because of their potential of asymptomatic viral shedding. Biosecurity measures were tightened in local farms such as using bird-proof nets to prevent the spread of virus from migratory birds to poultry. Active immunization of chickens from local and crossed border farms with H5 vaccines and randomly taken cloacal swabs from chickens for H5 detection by RT-PCR before transferring them to the wet markets were performed.[2] These measures have successfully prevented the occurrence of newly acquired human cases of avian influenza H5N1 in the locality since 1997. However these measures were unable to stop the endemicity of avian influenza A H9N2 in our poultry population.

The H9N2 virus is another major subtype of avian influenza virus which has been prevalent among domestic poultry in Asia since the early 1990s.[3–6] As routine immunization in poultry against this subtype of virus is not a common practice, the persistence of the virus in poultry markets may be the source of the sporadic human infections in southern China since 1998.[7,8] Three human cases of avian influenza A (H9N2) have been reported in Hong Kong between 1999 and 2003.[9,10] All of them were children with uncomplicated influenza-like illness and fully recovered. Serological analysis in a limited number of humans in Southern and Northern China found that about 1% of the tested subjects had antibodies against the H9 subtype haemagglutinin.[11,12] It is unclear whether human infection with H9N2 influenza is in fact more common than that which has been reported. Here, we described 2 cases of avian influenza A (H9N2) infection in immunocompromised patients and reviewed the relevant English and Chinese literatures on culture confirmed cases of human infections of avian influenza A (H9N2).

Method

RT-PCR assay

Total nucleic acid was extracted from 250 μl of nasopharyngeal aspirate (NPA) using NucliSens easyMAG instrument (bioMerieux, NC, US) according to the manufacturer's instructions. RT-PCR targeting the M gene of influenza A and subtype was performed as previously described.[13] In the conventional 1-step RT-PCR assay for H9, a forward primer 5′-TGR TGT ATG CCC CAC ATG AA-3′ and a reverse primer 5′-TTG CTC CAC ACA GAG CAC AAT-3′ was used to amplify the haemagglutinin gene and generated a fragment of 432 bp. 5 μL of extracted nucleic acid was reversely transcribed and amplified in a 50 μL reaction containing 1× RT-PCR buffer (Invitrogen), 0.5 mM of $MgSO_4$, 0.8 μM of each primer and 1 μL of Super II RT/Tag mix (Invitrogen). The reactions were incubated at 45 °C for 60 min and then at 95 °C for 3 min. This was followed by thermal-cycling for 45 cycles for 1 min each at 95 °C, 50 °C and 72 °C. The RT-PCR products were analyzed in 2% agarose gel by standard gel electrophoresis procedures and the bands were visualized by staining with ethidium bromide (0.5 μL/mL). Positive and negative controls were included in each run with strict adherence to measure against amplicon carryover.

Microneutralization assay

Serial dilutions of sera were tested on a 96-well tissue culture plate for neutralizing antibody titre by the microneutralization assay inside a type II Biosafety Cabinet in a Biosafety Containment level III facility. The 2009 influenza A (H9N2) virus strain A/HK/464419/09 (H9N2) was used. The 50% tissue culture infectious dose ($TCID_{50}$) of the virus was determined by titration in Madin—Darby canine kidney (MDCK) cells and was calculated by the method of Reed and Muench. One hundred of $TCID_{50}$ was used as the inoculum in 100 μL of cell culture medium.[9] Two-fold dilutions of the sera (starting from 1 in 10) were made in Eagle's minimum essential medium (MEM). Each 50 μL of virus inoculum was mixed with equal volume of diluted sera. After 2 h of incubation at 37 °C in a 5% CO_2 humidified atmosphere, the mixture was added to the seeded microtitre plate with a confluent layer of MDCK cells and was incubated in a 5% CO_2 at 37 °C. After 1 h of infection, the virus-sera mixture was removed, and Eagle's MEM with 2 μg/mL exogenous L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK-treated trypsin, Sigma Immunochemical Co., St. Louis, Mo.) was added to each well. A duplicate of each serum dilution was performed on the same microtitre plate. The cells were examined for cytopathic effect (CPE) at 72—96 h. The microneutralization titre was taken as the highest serum dilution at which the percentage of CPE is less than or equal to 50%.

Virus isolation

MDCK cell monolayers in culture tubes were inoculated with 200 μl of the combined swab-viral transport medium suspension and the cells were maintained in serum-free MEM (MEM, Gibco, N.Y., USA) containing tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (2 μg/ml) (Sigma, St. Louis, MO), and incubated at 33 °C for 7 days. They were examined daily for CPE, and immunofluoresence for influenza A nucleoprotein was done on fixed cell smears when CPE appeared or at the end of the incubation period.

Phylogenetic analysis

The haemagglutinin gene of both H9N2 isolates from MDCK culture was sequenced and analyzed. Viral RNA extraction from cell culture lysate, cDNA synthesis and direct PCR sequencing were performed as previously described.[4] Neighbour-joining tree was constructed using PAUP* 4.0(2).