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Pathogenicity of an H5N1 avian influenza virus isolated in Vietnam in 2012 and reliability of conjunctival samples for diagnosis of infection

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ABSTRACT

The continued spread of highly pathogenic avian influenza virus (HPAIV) subtype H5N1 among poultry in Vietnam poses a potential threat to animals and public health. To evaluate the pathogenicity of a 2012 H5N1 HPAIV isolate and to assess the utility of conjunctival swabs for viral detection and isolation in surveillance, an experimental infection with HPAIV subtype H5N1 was carried out in domestic ducks. Ducks were infected with $10^{7.2}$ $TCID_{50}$ of A/duck/Vietnam/QB1207/2012 (H5N1), which was isolated from a moribund domestic duck. In the infected ducks, clinical signs of disease, including neurological disorder, were observed. Ducks started to die at 3 days-post-infection (dpi), and the study mortality reached 67%. Viruses were recovered from oropharyngeal and conjunctival swabs until 7 dpi and from cloacal swabs until 4 dpi. In the ducks that died or were sacrificed on 3, 5, or 6 dpi, viruses were recovered from lung, brain, heart, pancreas and intestine, among which the highest virus titers were in the lung, brain or heart. Results of virus titration were confirmed by real-time RT-PCR. Genetic and phylogenetic analysis of the HA gene revealed that the isolate belongs to clade 2.3.2.1 similarly to the H5N1 viruses isolated in Vietnam in 2012. The present study demonstrated that this recent HPAI H5N1 virus of clade 2.3.2.1 could replicate efficiently in the systemic organs, including the brain, and cause severe disease with neurological symptoms in domestic ducks. Therefore, this HPAI H5N1 virus seems to retain the neurotrophic feature and has further developed properties of shedding virus from the oropharynx and conjunctiva in addition to the cloaca, potentially posing a higher risk of virus spread through cross-contact and/or environmental transmission. Continued surveillance and diagnostic programs using conjunctival swabs in the field would further verify the apparent reliability of conjunctival samples for the detection of AIV.

1. Introduction

Highly pathogenic avian influenza viruses (HPAIVs) of H5N1 subtype have caused a serious problem for the poultry industry worldwide. The first case of H5N1 HPAIV infection was reported in 1996 at a goose farm in Guangdong province in China (Xu et al., 1999). Since then, H5N1 HPAIV infections have spread in poultry in Asia, Europe and Africa (Monne et al., 2008; Salzberg et al., 2007; Smith et al., 2006; Starick et al., 2008). HPAIVs not only continue to threaten animal health but also pose concerns for zoonotic infection and public health. To date, there have been 628 cases of human infection with HPAI H5N1 viruses, which resulted in 374 deaths (WHO, 2013). Thus, HPAIVs continue to be a high priority for both veterinary and public health perspectives around the world.

Domestic ducks and other wild aquatic birds are considered natural reservoirs for AIV, and it is known that these birds can carry various subtypes of AIV with little, or perhaps no impact on their health (Alexander, 2000; Kida et al., 1980; Kuiken, 2013). However, Asian strains of HPAI H5N1 viruses have shown a broad profile of pathogenicity to domestic ducks, ranging from the complete absence of clinical signs to severe neurological dysfunction and death. Interestingly, the 1997–2001 HPAI H5N1 viruses caused either no symptoms or mild disease associated with the respiratory track in domestic ducks (Chen et al., 2004; Perkins and Swayne,

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2002; Shortridge et al., 1998; Sturm-Ramirez et al., 2004). However, since 2002, the pathobiology of HPAIV H5N1 viruses in domestic ducks has changed to cause a systemic infection, which results in wide variation in lesions and symptoms, proceeding to death (Lee et al., 2005; Nguyen et al., 2005; Sturm-Ramirez et al., 2004). On the other hand, some H5N1 viruses have induced neurological signs in domestic ducks without causing mortality (Kishida et al., 2005). The variation in pathogenicity of H5N1 viruses in domestic ducks may highlight the importance of characterizing the pathogenicity of new H5N1 isolates to monitor the pathobiological changes of these H5N1 viruses in the nature.

We recently found that an H5N1 HPAIV was recovered from the conjunctival swab of a whooper swan with neurological signs captured in Japan. The viral titer in the conjunctival sample from this swan was higher than those in cloacal and tracheal samples, suggesting the possibility of viral shedding from conjunctiva at high titers in wild birds infected with H5N1 viruses (Bui et al., 2013). Experimental infection with HPAIVs has previously shown that a common clinical sign in ducks is cloudy eyes (Hulse-Post et al., 2005; Sturm-Ramirez et al., 2004, 2005). In addition, it was reported that symptoms in human cases of H5N1 infection involved conjunctivitis during the outbreak in Hong Kong (Chan, 2002; Tam, 2002). These findings raise a question of whether an ocular tropism may be a general feature of recent H5N1 viruses. In this study, an H5N1 virus (clade 2.3.2.1) that was recently isolated from a diseased domestic duck in Vietnam was used to experimentally infect domestic ducks for the first time in order to assess and evaluate viral pathogenicity and virus shedding in ducks.

2. Materials and methods

2.1. Virus

A/duck/Vietnam/QB1207/2012 (H5N1) was used in this study. The virus was isolated in late 2012 from a moribund domestic duck in Quang Binh province of North Central Vietnam. Upon capture, the duck was found to show symptoms including neurological signs. The viral isolate was propagated in 10-day-old embryonated chicken eggs at 37 °C for 48 h. The allantoic fluid (AF) of the eggs was then harvested, and aliquots of the AF were stored at −80 °C until use.

2.2. Sequencing and phylogenetic analysis

Total RNA was extracted from the AFs using ISOGEN II (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's instructions. The RNA was transcribed into cDNA using the Uni12 primer (5′-agcraaagcagg-3′) and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) at 42 °C for 60 min followed by 70 °C for 10 min. The cDNA samples were used as template for PCR to amplify the full length HA gene using the primer sets described by Hoffmann et al. (2001). The PCR products obtained were separated by 1% agarose gel electrophoresis and purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified products were used as a template for sequencing reactions using a BigDye terminator ver. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions and analyzed with the ABI PRISM 3500 Genetic Analyzer (Applied Biosystems). The primer sets described above and walking primers we designed were used to obtain the full-length sequence of the HA gene.

The nucleotide sequence of the HA gene was analyzed by GENETYX ver. 10 software (GENETYX Corp., Tokyo, Japan) and compared with other available sequences using BLAST homology searches (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html). The HA nucleotide sequence of A/duck/Vietnam/QB1207/2012 (H5N1) and that of other strains available in GenBank were aligned by Clustal W (Thompson et al., 1994) and evolutionary distances were calculated using the Tamura-Nei model. A phylogenetic tree of the HA gene was constructed with Mega 5.1 software (Tamura et al., 2011) using the Maximum Likelihood method supported by 500 bootstrap replicates.

2.3. Ducks

Four-week-old male domestic ducks were purchased from a local farm in Hanoi, which has been confirmed to be free from AIV by the National Institute of Veterinary Research (NIVR) in Vietnam. Serum was collected from each duck prior to the infection study to confirm that all the ducks were serologically negative to H5N1 virus by using an H5-specific hemagglutination inhibition (HI) test, which was performed as described below. In addition, oropharyngeal, cloacal and conjunctival swabs were collected from the ducks prior to the viral inoculation. All samples were confirmed to be AIV-free by real-time reverse transcription-polymerase chain reaction (RRT-PCR), which detects the matrix (M) gene of influenza A virus, using the method described below.

2.4. Duck HPAI H5N1 virus infection study

The duck infection study was conducted in compliance with the institutional rules for the care and use of laboratory animals, and using a protocol approved by the relevant committee at NIVR in Vietnam.

A total of 12 ducks received intranasal inoculation of AF containing $10^{7.2}$ $TCID_{50}$ of A/duck/Vietnam/QB1207/2012 (H5N1) in 200 μl. Two uninfected ducks served as a control group. Following the viral infection, the ducks were checked daily for clinical signs of disease. Swab samples of the conjunctiva, cloaca, and oropharynx were collected daily from the ducks for virus recovery and viral gene detection. Two ducks were collected as mortalities or euthanized on each of 3, 5 and 7 days post infection (dpi), and brain, lung, kidney, spleen, intestine, heart and pancreas were sampled for the detection of viral genes and measurement of virus titer. Similarly, these organs were collected from additional ducks found dead. The remaining ducks were monitored for clinical signs, and swab samples were collected daily from those ducks until 16 dpi. On 17 dpi, sera were collected from the surviving ducks and checked for the presence of H5N1 specific antibody. For the evaluation of immune response in the ducks, antibodies specific to the H5N1 virus were detected by HI test following the WHO Manual on Animal Influenza Diagnosis and Surveillance using the sera collected from the ducks.

Cloacal, oropharyngeal and conjunctival swabs taken from ducks were kept in virus transport medium (VTM), which consists of minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with antibiotics and antimycotics including penicillin G (final concentration of 1000 U/ml), streptomycin (1 mg/ml), gentamycin (100 μg/ml), and amphotericin B (10 μg/ml). All the samples were kept at 4 °C overnight and stored at −80 °C until use.

2.5. Virus titration

Madin–Darby canine kidney (MDCK) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells were seeded onto 96-well tissue culture plates to evaluate viral titers. Upon virus inoculation, the cells were washed twice with the DMEM and the medium was replaced with virus growth medium according to the WHO Manual on Animal Influenza Diagnosis and Surveillance (http://www.who.int/csr/resources/publications/influenza/