

Published in final edited form as:

Virus Res. 2014 January 22; 179: 125–132. doi:10.1016/j.virusres.2013.10.023.

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

Vuong N. Bui^{a,b}, Tung D. Dao^b, Tham T. H. Nguyen^b, Lien T. Nguyen^b, Anh N. Bui^b, Dai Q. Trinh^{a,b}, Nga T. Pham^b, Kenjiro Inui^c, Jonathan Runstadler^d, Haruko Ogawa^{a,*}, Khong V. Nguyen^b, and Kunitoshi Imai^a

^aObihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080 8555, Japan

^bNational Institute of Veterinary Research, 86 Truong Chinh, Dong Da, Hanoi, Vietnam

^cFood and Agriculture Organization, 3 Nguyen Gia Thieu, Hoan Kiem, Hanoi, Vietnam

^dMassachusetts Institute of Technology, Cambridge, MA 02139, USA

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 $10^{7.2}$ TCID₅₀ 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.

© 2013 Elsevier B.V. All rights reserved

*Corresponding author at: Research Center for Animal Hygiene and Food Safety, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan. Tel: +81 155 49 5893; fax: +81 155 49 5893. hogawa@obihiro.ac.jp (H. Ogawa).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

H5N1; influenza virus; pathogenicity; duck; systemic; conjunctiva

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, 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 the 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 and 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 from a moribund domestic duck in Quang Binh province belonging to the Central North of

Vietnam in late 2012. 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 $10^{7.2}$ TCID₅₀ 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 1,000 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/whocdscsrncs20025rev.pdf>). The sample to be tested was serially diluted (1:10) for the titration. Based on the cytopathic effect (CPE) observed 4 days post-inoculation (dpi), TCID₅₀ was calculated by the Behrens-Kärber method. The hemagglutination test using 0.5% chicken erythrocytes suspended in phosphate-buffered saline was performed on the cell culture supernatants to confirm that the observed CPE reflects the growth of the virus in the cells.

2.6. Detection of AIV nucleic acid in samples

Tubes containing cloacal or oropharyngeal or conjunctival swabs in media were vortexed well to ensure mixing, and then the swabs were removed from the media. The tissue samples were subjected to 20% homogenate preparations in VTM. Total RNA was extracted from the media or tissue samples using ISOGEN II. The extracted RNAs were tested for the presence of AIV by RRT-PCR using the M gene primers of influenza A virus (CDC protocol: http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf) and a one-step RT-PCR kit (Qiagen) in an IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Cycling conditions used were as follows: Stage 1 – 50°C for 30 min and 95°C for 15 min, and stage 2 – 40 cycles of 95°C for 10 sec, 58°C for 50 sec. The primers and probe used were as follows: forward primer 5'-gac cra tcc tgt cac ctc tga c-3', reverse primer 5'-agg gca tty tgg aca aak cgt cta-3', and probe FAM-tgc agt cct cgc tca ctg ggc acg-BHQ1. Any sample with a cycle threshold (Ct) value less than 40 was considered to be positive for the M gene.

3. Results

3.1. Genetic and phylogenetic analysis

In order to investigate the similarity between A/duck/Vietnam/QB1207/2012 (H5N1) and other H5N1 HPAIVs, a homology search for the HA gene sequence of the virus was performed. The results showed that the HA gene of A/duck/Vietnam/QB1207/2012 is similar to those of the H5N1 viruses (clade 2.3.2.1) isolated from domestic poultry in Vietnam during 2012 with the segment identities ranging from 99.64% to 99.82%. The HA cleavage site sequence of A/duck/Vietnam/QB1207/2012 (H5N1) showed the typical

sequence motif of a virulent-type, QRERRRKR/GLF. Phylogenetic analysis of the full-length HA gene of the HPAI H5N1 viruses clearly indicated that A/duck/Vietnam/QB1207/2012 belonged to the Eurasian lineage, and fell into clade 2.3.2.1. The strain was closely related to other HPAI H5N1 strains isolated from domestic poultry in Vietnam in 2012 as well as Indonesian strains in 2012 (Fig. 1). The HA nucleotide sequence obtained in this study is available from GenBank under accession number KF182741.

3.2. Pathogenicity of A/duck/Vietnam/QB1207/2012 (H5N1) in ducks

All the ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1) showed clinical signs of disease, which included depression, loss of appetite and respiratory distress, between 2 and 7 dpi. Three ducks started to recover from the disease after 7 dpi, and eventually survived the viral infection. Most ducks shed blue feces from 2 dpi to 10 dpi. Neurological signs such as tremor, paralysis, loss of balance and intermittent head shaking were observed in 4 ducks from 4 dpi, and the symptoms continued in those ducks until they died. Death of ducks was recorded from 3 dpi to 6 dpi with a peak number of deaths on 5 dpi (Fig. 2). The mortality of the ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1) was 67%. HI antibody was detected in the sera of 3 surviving ducks on 17 dpi with antibody titers between 1:256 (2 ducks) and 1:512 (1 duck). In the non-infected 2 ducks, no clinical signs were observed and neither duck seroconverted.

3.3. Virus shedding from the ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1)

Virus titers in conjunctival, cloacal and oropharyngeal swabs taken from the infected group and non-infected ducks on 1-16 dpi were measured. Viruses were recovered from oropharyngeal swabs of all the infected ducks between 1 dpi and 7 dpi, but not on 8 dpi and later. The mean daily virus titer of the oropharyngeal samples ranged from $10^{1.9}$ to $10^{5.0}$ TCID₅₀/ml, and the highest mean titer of $10^{5.0}$ TCID₅₀/ml was obtained from the samples on 2 dpi. The mean virus titers of the oropharyngeal samples on 1 dpi and 3 dpi were $10^{4.5}$ and 10^4 TCID₅₀/ml, respectively, but the titers gradually decreased in the following days. Viruses were recovered from the conjunctival swabs between 1 dpi and 7 dpi similarly to the oropharyngeal swabs, although the mean viral titers of conjunctival swabs were lower than those of oropharyngeal swabs. The titers ranged from $10^{1.6}$ to $10^{3.8}$ TCID₅₀/ml, among which the highest titer was detected on 2 dpi. The mean titer on 3 dpi was $10^{3.1}$ TCID₅₀/ml, but those on other dates were lower than the titers on 2 dpi and 3 dpi. In the case of cloacal swabs, viruses were recovered from 1 dpi to 4 dpi, with the mean viral titers between $10^{1.6}$ and $10^{2.4}$ TCID₅₀/ml (Fig. 3).

RRT-PCR was performed on the RNAs extracted from the cloacal, oropharyngeal and conjunctival swab samples. Viral RNA was detected in the oropharyngeal swabs from 1 dpi to 16 dpi, at the end of the experiment (Fig. 4B). In the conjunctival swabs and cloacal swabs, viral RNA was detected from 1 dpi to 15 dpi and to 12 dpi, respectively (Figs. 4C and 4A). In most of the days, the mean Ct value in the oropharyngeal sample reflected the highest concentration of viral RNA, followed by the conjunctival sample (Fig. 4).

3.4. Viral replication in the ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1)

Table 1 shows the results of virus titration and viral RNA detection for the tissue samples, which were obtained from dead or sacrificed ducks. On 3 dpi, virus titers ranging from $10^{2.9}$ to $10^{7.6}$ TCID₅₀/ml were detected in the tissue samples of one duck that died. The highest virus titers were detected in the lung and heart samples, and the lowest titer was in the pancreas sample. In another duck sacrificed on 3 dpi, the titers ranged from $10^{2.2}$ to $10^{5.9}$ TCID₅₀/ml, all of which were lower in each tissue except pancreas than the titer of the duck that died from infection. Results of RRT-PCR confirming the presence of the viral M gene were similar to those in the virus titration in all the tissue samples of the two ducks. On 5 dpi

and 6 dpi, there were 5 ducks found dead, and viruses were recovered from the brains ($10^{4.6}$ to $10^{6.2}$ TCID₅₀/ml), lungs ($10^{4.2}$ to $10^{5.2}$ TCID₅₀/ml), hearts ($10^{2.2}$ to $10^{6.2}$ TCID₅₀/ml), intestines ($10^{2.2}$ to $10^{4.6}$ TCID₅₀/ml) and pancreas ($10^{2.2}$ to $10^{4.6}$ TCID₅₀/ml) of the 5 ducks. The results of RRT-PCR for those tissue samples were consistent with the results of viral titer determination. In the kidney samples, virus was recovered only from one duck while results of the RRT-PCR were positive for all samples of the 5 ducks with Ct values of 27•30. A similar result was obtained for the spleen samples except for one duck, which gave positive results both in the virus isolation and viral RNA detection. On 7 dpi, two ducks were scarified. Viruses were recovered from the intestine samples of the 2 ducks at the titer of $10^{2.2}$ TCID₅₀, and also in the lung and pancreas samples of 1 duck with the same titer obtained in the intestine samples. However, RRT-PCR for the viral M gene resulted in positives from more tissue samples from the 2 ducks, among which only one intestine sample was congruent to the results of virus titration (Table 1).

4. Discussion

The importance of characterizing HPAI H5N1 viruses is continuously being emphasized due to the fact that viruses have been mutating and changing their biological behaviors and directly threatening animal health and public health (Hulse-Post et al., 2005; Kwon et al., 2011; Sturm-Ramirez et al., 2004; Tumpey et al., 2002). In this study, we identified genetic characteristics of A/duck/Vietnam/QB1207/2012 (H5N1), which has been recently isolated from a domestic duck in Vietnam, and evaluated its pathogenic potential and viral shedding in experimental infection of domestic ducks. Genetic analyses showed that the virus is classified to clade 2.3.2.1 (Fig. 1). H5N1 viruses of clade 2.3.2 were first identified from ducks, geese and other mammals in China and Vietnam in 2005 (Chen et al., 2006; Robertson et al., 2006). H5N1 viruses of clade 2.3.2.1 were evolved from the H5N1 viruses of clade 2.3.2 and emerged in Vietnam in 2009 with their prevalence increasing towards the end of 2010 (Nguyen et al., 2012). In 2012, viruses of clade 2.3.2.1 were dominantly circulating in Vietnam. Several studies reported on the pathogenicity of H5N1 viruses of clade 2.3.2.1, which were isolated from wild birds until 2011 (Kwon et al., 2011; Sakoda et al., 2010; Soda et al., 2013). However, pathogenicity of the H5N1 HPAIV isolated from domestic ducks in Vietnam in 2012 has not been studied.

It has been reported that avian H5N1 viruses showed differences in ability to cause disease in experimentally infected ducks, and the resultant disease ranged from the complete absence of clinical signs to severe neurological dysfunction and death (Brown et al., 2006; Chen et al., 2004; Hulse-Post et al., 2005; Isoda et al., 2006; Lee et al., 2005; Sturm-Ramirez et al., 2004 and 2005; Zhou et al., 2006). Data from these studies showed that the presence of viral RNA and infective viruses in a variety of tissues correlated with the severity of clinical signs and the mortality observed in the ducks inoculated with the H5N1 viruses. The ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1) showed clinical signs including depression, neurological signs, and a high mortality of 67% (Fig. 2). Results of the virus titrations for the tissue samples of the ducks that died on 3 dpi showed systemic infection with high titers ($>10^6$ TCID₅₀/ml) of virus in all the tissues sampled including brain, lung and heart and lower titers for the pancreas. Similar results were obtained for other ducks, which died by 6 dpi, albeit the titers were lower, especially in the tissues other than brain and lung (Table 1). Most of the ducks dying from infection showed neurological signs, suggesting that the death of these ducks was likely to be associated with damage in the central nervous system. The results obtained in this study seem to be consistent with the previous studies performed for the H5N1 HPAIV of clade 2.3.2.1 isolated from wild birds until 2011 (Kwon et al., 2011; Sakoda et al., 2010; Soda et al., 2013), confirming that the H5N1 isolate of clade 2.3.2.1 could cause illness and death in a large proportion of domestic ducks.

It is understood that most low pathogenic AIVs replicate preferentially in the gastrointestinal tract, and apparently have little or perhaps no impact on the host bird's health. Ducks excrete a high concentration of AIV in their feces, and can transmit these viruses via the fecal-oral route to other birds including ducks in their population and to poultry (Webster et al., 1978). However, in late 2002, the biology of the H5N1 influenza viruses was found to be different from other AIVs when the H5N1 viruses were isolated from the upper respiratory tract of dead wild ducks at high titers (Brown et al., 2006; Hulse-Post et al., 2005; Pantin-Jackwood and Swayne, 2007; Sturn-Ramirez et al., 2005). In the current study, viruses were recovered from lung tissue at higher titers than those in intestine, pancreas, spleen and kidney in most ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1) (Table 1). In addition, virus titers in the oropharyngeal swabs were much higher than those in the cloacal swabs. Virus was detected up to 7 dpi in oropharyngeal swabs but only up to 4 dpi in cloacal swabs (Fig. 3). These results may suggest that the prolonged viral shedding would create favorable circumstances for environmental contamination and transmission of the recent HPAI H5N1 viruses via cross-contact and/or other routes. The importance of viral shedding from the respiratory tracts, as well as the conjunctiva, in transmission between wild and domestic birds should be examined further. In the current study, a high dose of virus was intranasally inoculated into ducks, and this could result in the higher virus titers in the oropharyngeal and conjunctival samples compared to the cloacal swabs. Additional studies should explore different routes of infection such as intraocular, intracloacal and oral routes, and different doses of viral inoculation in order to simulate natural infection and assess data that may be more relevant to fully understanding the current H5N1 situation in the field.

This is the first study in which conjunctival swabs were evaluated for their reliability as samples to detect the H5N1 virus in an experimental infection using ducks. No other studies have demonstrated the duration and amount of the virus shedding from the conjunctiva following H5N1 infection. The current study was conducted to confirm the virus detection in conjunctival swabs from ducks following H5N1 infection, and to further clarify the time course of this detection. Under the experimental conditions used in the current study, it was found that the H5N1 virus was efficiently recovered from the conjunctival samples of infected ducks. Virus was recovered from the conjunctival swabs from 1 dpi to 7 dpi as for oropharyngeal swabs. Virus titers in the conjunctival swabs were higher than those in cloacal swabs, though not as high as those in oropharyngeal swabs (Fig 3). Similar results were obtained in RRT-PCR in which viral RNA was detected up to 12 dpi, 15 dpi and 16 dpi in cloacal, conjunctival and oropharyngeal swabs, respectively (Fig. 4). Detection of the viral M gene in the conjunctival and oropharyngeal swabs for periods longer than that for the cloacal swabs could be associated with the efficient viral replication in respiratory and conjunctival tissues rather than in gastrointestinal tissues. In nature, it may happen that a prolonged period of viral shedding from the respiratory tissues of infected ducks creates a favorable circumstance for the spread of viruses over a large area, important in a country such as Vietnam where the free-grazing duck is still a common way to raise domestic ducks. On the other hand, we should be cautious in assessing the relationship between viral detection and the presence of infectious virions, important to assessing disease risk and for effective disease control measures. In the current study, the viral gene was detected in the oropharyngeal and conjunctival swabs until 15 dpi or later, though the infectious virus was only detected until 7 dpi. The results would suggest that viral gene detection after 8 dpi does not necessarily indicate the risk of virus transmission from an infected duck. Brown et al. (2013) cautioned against a possible overestimation of the risk of environmental transmission of virus by using viral gene detection, based on their results obtained from an infection study using wild ducks and low pathogenic avian influenza virus showing a discrepancy between the molecular detection of virus and an ability to amplify samples in culture. Such

information should be effectively utilized to improve the diagnostic methods in avian influenza surveillance and ultimately to predict and control disease.

Results in the current and our previous study (Bui et al., 2013) would suggest that the conjunctival swab may be a good additional or alternative sample taken from ducks, although cloacal and oropharyngeal swabs have long been used for the detection of AIVs in surveillance and diagnosis. The amount of H5N1 RNA and virus titers were high in conjunctival swabs and also indicated a longer period of shedding than a cloacal swab did. In addition, the conjunctival swab contained a larger amount of the virus in comparison with the tracheal swab of a whooper swan infected with H5N1 virus in our previous study (Bui et al., 2013), but the viral titer was higher in the oropharyngeal swab than in the conjunctival swab of the domestic ducks infected with the H5N1 virus in this study until 4 dpi (Fig. 3). As suggested above, this discrepancy might be related to the difference in the route of infection, i.e., intranasal route of infection used in this study and a natural infection in the previous study. Further studies using other H5N1 strains and also wild birds would clarify how and when the viruses are shed from the infected birds and the reliability of the conjunctival samples for diagnosis and surveillance.

5. Conclusion

This study revealed that A/duck/Vietnam/QB1207/2012 (H5N1) of clade 2.3.2.1, an H5N1 HPAIV recently circulating in Vietnam, is lethal to domestic ducks and replicates efficiently in tissues including the lung, brain and heart. The virus was shed from the upper respiratory track and conjunctiva, which poses a concern of the high risk of virus spread through cross-contact and/or environmental transmission. Shedding of the H5N1 HPAIV from the conjunctiva of the ducks implies that ocular tissues could be involved in an infection route for the virus and/or the target of virus replication. The high titer of virus and prolonged detection of viral RNA in the conjunctival swabs suggests that the conjunctival swab may be a good additional or alternative sample for surveillance and diagnosis of AIV. This addition may help maximize efficient AIV surveillance in wild birds and domestic poultry, especially for HPAIV.

Acknowledgments

We would like to thank Nguyen Thi Bo, Nguyen Thi Hanh and Vu Viet Hung in National Institute of Veterinary Research (NIVR), Vietnam, for their technical assistance. This work was partially supported by a Grant-in-Aid for the Bilateral Joint Projects of the Japan Society for the Promotion of Science, Japan, the Heiwa Nakajima Foundation, Japan, the US National Institute of Allergy and Infectious Diseases (NIAID contracts HHSN2662007000010C), and with the contribution from the NIVR project funded by MARD of Vietnam on the Study to generate poultry influenza inactivated H5N1 vaccine derived from the local isolates of Vietnam.

References

- Alexander DJ. A review of avian influenza in different bird species. *Vet Microbiol.* 2000; 74(1–2):3–13. [PubMed: 10799774]
- Brown JD, Berghaus RD, Costa TP, Poulson R, Carter DL, Lebarbenchon C, Stallknecht DE. Intestinal excretion of a wild bird-origin H3N8 low pathogenic avian influenza virus in mallards (*Anas platyrhynchos*). *J Wildl Dis.* 2012; 48(4):991–998. [PubMed: 23060500]
- Brown JD, Stallknecht DE, Beck JR, Suarez DL, Swayne DE. Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses. *Emerg Infect Dis.* 2006; 12(11):1663–1670. [PubMed: 17283615]
- Bui VN, Ogawa H, Ngo LH, Baatartsogt T, Abao LN, Tamaki S, Saito K, Watanabe Y, Runstadler J, Imai K. H5N1 highly pathogenic avian influenza virus isolated from conjunctiva of a whooper swan with neurological signs. *Arch Virol.* 2013; 158(2):451–455. [PubMed: 23053526]

- Chan PK. Outbreak of avian influenza A(H5N1) virus infection in Hong Kong in 1997. *Clin Infect Dis.* 2002; 34(Suppl 2):S58–64. [PubMed: 11938498]
- Chen H, Deng G, Li Z, Tian G, Li Y, Jiao P, Zhang L, Liu Z, Webster RG, Yu K. The evolution of H5N1 influenza viruses in ducks in southern China. *Proc Natl Acad Sci U S A.* 2004; 101(28):10452–10457. [PubMed: 15235128]
- Chen H, Smith GJ, Li KS, Wang J, Fan XH, Rayner JM, Vijaykrishna D, Zhang JX, Zhang LJ, Guo CT, Cheung CL, Xu KM, Duan L, Huang K, Qin K, Leung YH, Wu WL, Lu HR, Chen Y, Xia NS, Naipospos TS, Yuen KY, Hassan SS, Bahri S, Nguyen TD, Webster RG, Peiris JS, Guan Y. Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. *Proc Natl Acad Sci U S A.* 2006; 103(8):2845–2850. [PubMed: 16473931]
- Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. *Arch Virol.* 2001; 146(12):2275–2289. [PubMed: 11811679]
- Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, Krauss S, Scholtissek C, Puthavathana P, Buranathai C, Nguyen TD, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proc Natl Acad Sci U S A.* 2005; 102(30):10682–10687. [PubMed: 16030144]
- Isoda N, Sakoda Y, Kishida N, Bai GR, Matsuda K, Umemura T, Kida H. Pathogenicity of a highly pathogenic avian influenza virus, A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. *Arch Virol.* 2006; 151(7):1267–1279. [PubMed: 16502281]
- Kida H, Yanagawa R, Matsuoka Y. Duck influenza lacking evidence of disease signs and immune response. *Infect Immun.* 1980; 30(2):547–553. [PubMed: 7439994]
- Kishida N, Sakoda Y, Isoda N, Matsuda K, Eto M, Sunaga Y, Umemura T, Kida H. Pathogenicity of H5 influenza viruses for ducks. *Arch Virol.* 2005; 150(7):1383–1392. [PubMed: 15747052]
- Kuiken T. Is low pathogenic avian influenza virus virulent for wild waterbirds? *Proc Biol Sci.* 2013; 280(1763):20130990. [PubMed: 23740783]
- Kwon HI, Song MS, Pascua PN, Baek YH, Lee JH, Hong SP, Rho JB, Kim JK, Poo H, Kim CJ, Choi YK. Genetic characterization and pathogenicity assessment of highly pathogenic H5N1 avian influenza viruses isolated from migratory wild birds in 2011, South Korea. *Virus Res.* 2011; 160(1–2):305–315. [PubMed: 21782862]
- Lee CW, Suarez DL, Tumpey TM, Sung HW, Kwon YK, Lee YJ, Choi JG, Joh SJ, Kim MC, Lee EK, Park JM, Lu X, Katz JM, Spackman E, Swayne DE, Kim JH. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. *J Virol.* 2005; 79(6):3692–3702. [PubMed: 15731263]
- Monne I, Fusaro A, Al-Blawi MH, Ismail MM, Khan OA, Dauphin G, Tripodi A, Salviato A, Marangon S, Capua I, Cattoli G. Co-circulation of two sublineages of HPAI H5N1 virus in the Kingdom of Saudi Arabia with unique molecular signatures suggesting separate introductions into the commercial poultry and falconry sectors. *J Gen Virol.* 2008; 89(Pt 11):2691–2697. [PubMed: 18931064]
- Nguyen DC, Uyeki TM, Jadhao S, Maines T, Shaw M, Matsuoka Y, Smith C, Rowe T, Lu X, Hall H, Xu X, Balish A, Klimov A, Tumpey TM, Swayne DE, Huynh LP, Nghiem HK, Nguyen HH, Hoang LT, Cox NJ, Katz JM. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. *J Virol.* 2005; 79(7):4201–4212. [PubMed: 15767421]
- Nguyen T, Rivailler P, Davis CT, Hoa do T, Balish A, Dang NH, Jones J, Vui DT, Simpson N, Huong NT, Shu B, Loughlin R, Ferdinand K, Lindstrom SE, York IA, Klimov A, Donis RO. Evolution of highly pathogenic avian influenza (H5N1) virus populations in Vietnam between 2007 and 2010. *Virology.* 2012; 432(2):405–416. [PubMed: 22818871]
- Pantin-Jackwood MJ, Swayne DE. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. *Avian Dis.* 2007; 51(1 Suppl):250–259. [PubMed: 17494561]
- Perkins LE, Swayne DE. Pathogenicity of a Hong Kong-origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. *Avian Dis.* 2002; 46(1):53–63. [PubMed: 11924603]

- Robertson SI, Bell DJ, Smith GJ, Nicholls JM, Chan KH, Nguyen DT, Tran PQ, Streicher U, Poon LL, Chen H, Horby P, Guardo M, Guan Y, Peiris JS. Avian influenza H5N1 in viverrids: implications for wildlife health and conservation. *Proc Biol Sci*. 2006; 273(1595):1729–1732. [PubMed: 16790404]
- Sakoda Y, Sugar S, Batchluun D, Erdene-Ochir TO, Okamatsu M, Isoda N, Soda K, Takakuwa H, Tsuda Y, Yamamoto N, Kishida N, Matsuno K, Nakayama E, Kajihara M, Yokoyama A, Takada A, Sodnomdarjaa R, Kida H. Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory. *Virology*. 2010; 406(1):88–94. [PubMed: 20673942]
- Salzberg SL, Kingsford C, Cattoli G, Spiro DJ, Janies DA, Aly MM, Brown IH, Couacy-Hymann E, De Mia GM, Dung do H, Guercio A, Joannis T, Maken Ali AS, Osmani A, Padalino I, Saad MD, Savic V, Sengamalay NA, Yingst S, Zaborsky J, Zorman-Rojs O, Ghedin E, Capua I. Genome analysis linking recent European and African influenza (H5N1) viruses. *Emerg Infect Dis*. 2007; 13(5):713–718. [PubMed: 17553249]
- Shortridge KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, Kodihalli S, Krauss S, Markwell D, Murti KG, Norwood M, Senne D, Sims L, Takada A, Webster RG. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology*. 1998; 252(2):331–342. [PubMed: 9878612]
- Smith GJ, Fan XH, Wang J, Li KS, Qin K, Zhang JX, Vijaykrishna D, Cheung CL, Huang K, Rayner JM, Peiris JS, Chen H, Webster RG, Guan Y. Emergence and predominance of an H5N1 influenza variant in China. *Proc Natl Acad Sci U S A*. 2006; 103(45):16936–16941. [PubMed: 17075062]
- Soda K, Usui T, Uno Y, Yoneda K, Yamaguchi T, Ito T. Pathogenicity of an H5N1 Highly Pathogenic Avian Influenza Virus Isolated in the 2010–2011 Winter in Japan to Mandarin Ducks. *J Vet Med Sci*. 2013; 75(5):619–624. [PubMed: 23318574]
- Starick E, Beer M, Hoffmann B, Staubach C, Werner O, Globig A, Strebelow G, Grund C, Durban M, Conraths FJ, Mettenleiter T, Harder T. Phylogenetic analyses of highly pathogenic avian influenza virus isolates from Germany in 2006 and 2007 suggest at least three separate introductions of H5N1 virus. *Vet Microbiol*. 2008; 128(3–4):243–252. [PubMed: 18031958]
- Sturm-Ramirez KM, Ellis T, Bousfield B, Bissett L, Dyrting K, Rehg JE, Poon L, Guan Y, Peiris M, Webster RG. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *J Virol*. 2004; 78(9):4892–4901. [PubMed: 15078970]
- Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humbert J, Seiler P, Puthavathana P, Buranathai C, Nguyen TD, Chaisingh A, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? *J Virol*. 2005; 79(17):11269–11279. [PubMed: 16103179]
- Tam JS. Influenza A (H5N1) in Hong Kong: an overview. *Vaccine*. 2002; 20(Suppl 2):S77–81. [PubMed: 12110265]
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 2011; 28(10):2731–2739. [PubMed: 21546353]
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994; 22(22):4673–4680. [PubMed: 7984417]
- Tumpey TM, Suarez DL, Perkins LE, Senne DA, Lee JG, Lee YJ, Mo IP, Sung HW, Swayne DE. Characterization of a highly pathogenic H5N1 avian influenza A virus isolated from duck meat. *J Virol*. 2002; 76(12):6344–6355. [PubMed: 12021367]
- Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology*. 1978; 84(2):268–278. [PubMed: 23604]
- WHO. [accessed on 15.05.13] Cumulative number of confirmed human cases of avian influenza A (H5N1) reported to WHO. 2013. http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/
- Xu X, Subbarao, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology*. 1999; 261(1):15–19. [PubMed: 10484749]

Zhou JY, Shen HG, Chen HX, Tong GZ, Liao M, Yang HC, Liu JX. Characterization of a highly pathogenic H5N1 influenza virus derived from bar-headed geese in China. *J Gen Virol.* 2006; 87(Pt 7):1823–1833. [PubMed: 16760384]

We performed an infection study of ducks using the H5N1 HPAIV isolated in Vietnam. The H5N1 virus caused severe disease with neurological symptoms in domestic ducks. The H5N1 virus replicated efficiently in the lung, brain and heart of domestic ducks. The virus was shed from the oropharynx and conjunctiva rather than from the cloaca. Conjunctival swab should be additional or alternative sample for HPAI diagnosis.

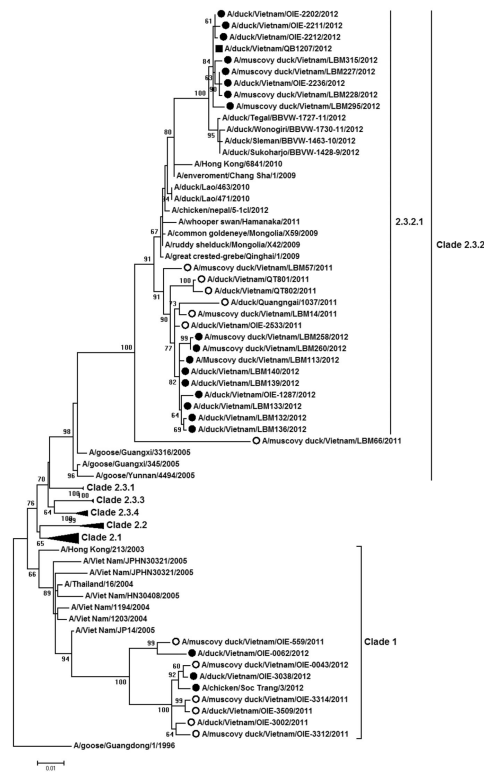


Fig. 1. Phylogenetic tree of the full-length HA genes of HPAI H5N1 viruses. A/duck/Vietnam/QB1207/2012 (H5N1) (*), H5N1 strains isolated in 2011 from Vietnam (○), H5N1 strains isolated in 2012 from Vietnam (●) and other representative strains of H5N1 are shown in the tree. The evolutionary history was inferred using the maximum likelihood method. The evolutionary distances were calculated using the Tamura-Nei model. Numbers at each branch point indicate bootstrap values > 60% in the bootstrap interior branch test. All positions containing gap and missing data were eliminated. Phylogenetic analysis was conducted in MEGA5. The scale bar indicates 0.01 nucleotide substitutions per site.

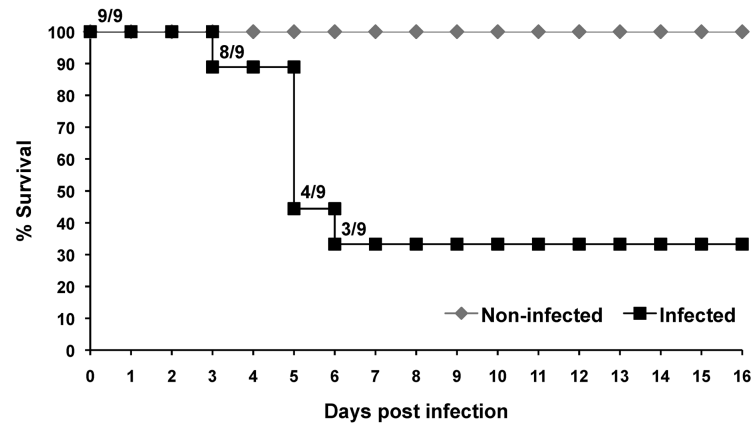


Fig. 2.

Survival rates of the ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1) and mock infection. Ducks were infected with $10^{7.2}$ TCID₅₀ of the virus in 200 μ l. The graph does not include the results of ducks that were scarified for tissue collection on 3 (1 duck) and 7 dpi (2 ducks). The numbers of surviving per total ducks in the infected group are indicated above the survival curve.

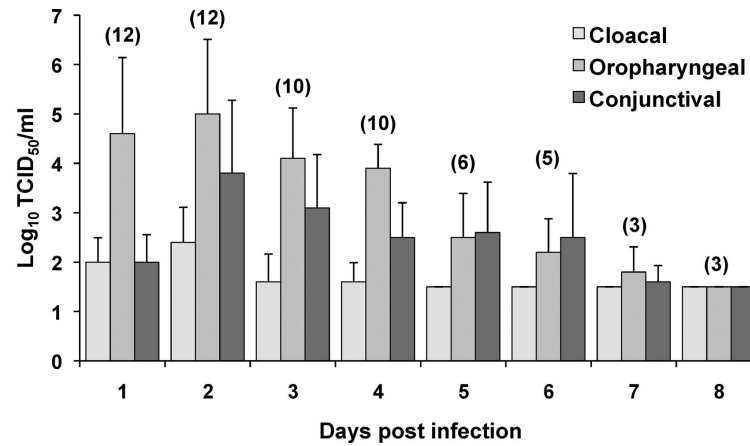


Fig. 3. Mean virus titer in the cloacal, oropharyngeal, and conjunctival swabs of ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1). Ducks were infected with $10^{7.2}$ TCID₅₀ of the virus, and swab samples were collected daily from the ducks to determine the virus titers using MDCK cells. The dashed line indicates the limit of detection ($10^{1.5}$ TCID₅₀/ml). Mean virus titers and standard deviations were expressed as Log₁₀ TCID₅₀/ml. Numbers in parentheses represent the sample numbers.

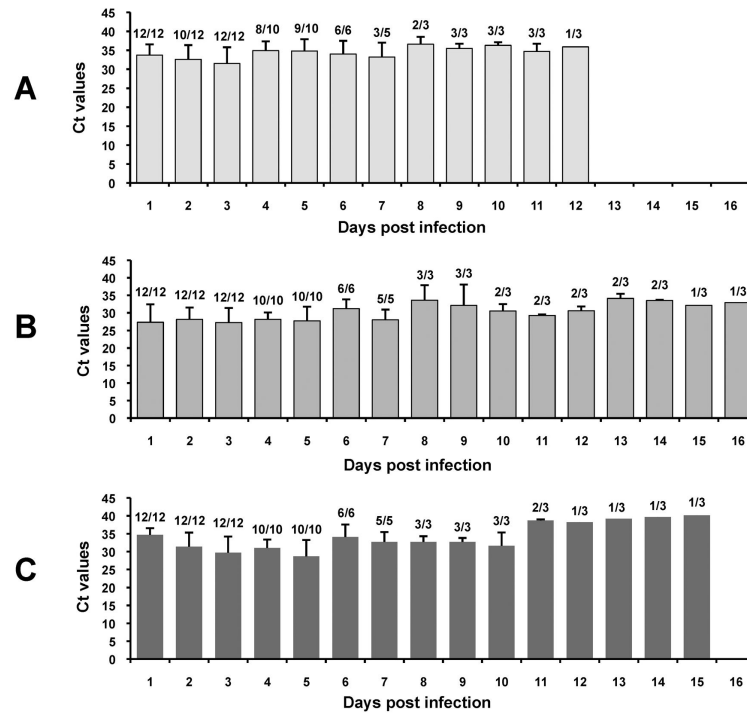


Fig. 4. Viral M gene detection by RRT-PCR for the swab samples from cloaca (A), oropharynx (B) and conjunctiva (C) of the ducks infected with $10^{7.2}$ TCID₅₀ of A/duck/Vietnam/QB1207/2012 (H5N1). The results are shown as mean and standard deviation of Ct values. The graphs (A, B, C) do not include the results of samples of which the Ct values were “undetermined” in the assay. The rates above the bars indicate the ratio between positive ducks and total number of ducks examined.

Table 1

Virus titers and Ct values in RRT-PCR analysis of the viral M gene obtained for samples of the ducks infected with A/duck/Vietnam/QB1207/2012 (H5N1)

DPI	Duck status	Virus titer in log ₁₀ TCID ₅₀ /ml (Ct values)						
		Brain	Lung	Heart	Intestine	Pancreas	Spleen	Kidney
3	Dead	6.9 (12.35)	7.6 (16.61)	7.6 (13.95)	6.9 (18.08)	2.9 (29.98)	6.6 (20.56)	6.2 (23.38)
	Sacrificed	2.9 (23.59)	5.9 (22.43)	3.9 (19.24)	5.2 (23.64)	4.6 (25.74)	2.2 (23.81)	3.2 (25.10)
5	Dead	5.6 (16.77)	4.9 (22.08)	2.6 (21.00)	3.9 (22.42)	4.6 (16.97)	4.6 (32.02)	— (30.92)
		4.6 (13.78)	4.2 (25.20)	2.2 (23.37)	2.2 (28.05)	2.2 (27.83)	— (N)	2.2 (28.83)
		5.9 (15.39)	5.2 (22.07)	6.2 (16.98)	4.6 (23.83)	3.2 (19.93)	— (29.27)	— (27.72)
		6.2 (21.13)	5.2 (24.69)	6.2 (16.19)	3.9 (23.16)	2.2 (23.48)	— (30.93)	— (28.75)
6	Dead	5.9 (15.44)	4.2 (26.37)	3.9 (21.45)	4.2 (21.45)	2.2 (18.42)	— (28.36)	— (27.33)
7	Sacrificed	— (N)	— (30.7)	— (28.24)	2.2 (31.32)	— (N)	— (36.92)	— (34.81)
		— (33.84)	2.2 (N)	— (N)	2.2 (N)	2.2 (N)	— (N)	— (N)

DPI: day post infection, —: undermined viral titer, N: undetermined in the RRT-PCR