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Experimental infection of mandarin duck with highly pathogenic avian influenza A (H5N8 and H5N1) viruses

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ABSTRACT

A highly pathogenic avian influenza (HPAI) H5N8 virus was first detected in poultry and wild birds in South Korea in January 2014. Here, we determined the pathogenicity and transmissibility of three different clades of H5 viruses in mandarin ducks to examine the potential for wild bird infection. H5N8 (clade 2.3.4.4) replicated more efficiently in the upper and lower respiratory tract of mandarin ducks than two previously identified H5N1 virus clades (clades 2.2 and 2.3.2.1). However, none of the mandarin ducks infected with H5N8 and H5N1 viruses showed severe clinical signs or mortality, and gross lesions were only observed in a few tissues. Viral replication and shedding were greater in H5N8-infected ducks than in H5N1-infected ducks. Recovery of all viruses from control duck in contact with infected ducks indicated that the highly pathogenic H5 viruses spread horizontally through contact. Taken together, these results suggest that H5N8 viruses spread efficiently in mandarin ducks. Further studies of pathogenicity in wild birds are required to examine possible long-distance dissemination via migration routes.

1. Introduction

Wild birds are considered a natural reservoir for avian influenza (AI) A viruses, which are classified according to the type of haemagglutinin (HA; H1–H16) and neuraminidase (NA; N1–N9) proteins expressed on the capsid surface (Webster et al., 1992; Olsen et al., 2006). In general, AI viruses do not cause clinical signs in poultry, whereas highly pathogenic avian influenza (HPAI) viruses belonging to certain H5 and H7 strains can cause high death rates and substantial economic losses.

In 2010, a reassortant H5N8 HPAI virus belonging to the Gs/GD lineage within clade 2.3.4.4 was isolated from swab samples taken from mallard ducks at a wholesale live bird market in eastern China; this virus was designated A/duck/Jiangsu/k1203/2010 (H5N8) (Zhao et al., 2013). Moreover, surveillance of live poultry markets in eastern China in 2013 identified an H5N8 virus in domestic ducks (Li et al., 2014). In early 2014, outbreaks of reassortant H5N8 viruses were reported in South Korea (Lee et al., 2014), and in September 2014 the H5N8 virus was detected in waterfowl in Russia, an intermediate location at which virus distribution occurs (Marchenko et al., 2015). Since then, H5N8 virus and reassortants have been detected in Europe, the western and central United States, and Canada (http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza).

Between 2013 and 2015, H5N8 virus has been isolated from wild birds of the orders *Accipitriformes*, *Anseriformes*, *Charadriiformes*, *Falconiformes*, and *Gruiformes* (Verhagen et al., 2015a). South Korea experienced many outbreaks of H5N8 HPAI in wild birds and domestic poultry from 2014 to 2015; indeed, H5N8 HPAI viruses were isolated from 58 wild birds, mainly members of the Anatidae Family (e.g., Baikal teals (ten cases), spot-billed ducks (five cases) and mallard ducks (four cases)). The virus was also isolated from captured, apparently healthy, and dead migratory wild birds. Similarly, wide geographic dissemination of H5N1 viruses (clade 2.2) belonging to the Gs/GD lineage was observed from 2005 to 2006; the virus spread from Qinghai Lake (China), to Siberia, and then to various countries in Asia, Europe, and Africa (Olsen et al., 2006; Salzberg et al., 2007). Moreover, clade 2.3.2.1 H5N1 virus was repeatedly detected in Southeast Asia in 2009–2010, from where it spread to poultry and wild birds in Europe (Li et al., 2011; Reid et al., 2011; Sharshov et al., 2010). It was presumed that these H5 viruses (including H5N8) were spread by migratory birds. This was confirmed by the isolation and analysis of HPAI H5 viruses

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from several wild bird species (Adlhoch et al., 2014; Ip et al., 2015; OIE, 2015; Saito et al., 2015; Verhagen et al., 2015b).

Although the role of wild birds in the spread of H5N8 appears clear, there is little information about the pathogenicity of H5N8 viruses in wild birds or the particular species involved. Therefore, the aim of the present study was to examine the pathogenesis and mode of transmission of a reassortant H5N8 virus and two H5N1 viruses in mandarin ducks (*Aix galericulata*), a representative migratory species belonging to the Family Anatidae.

2. Materials and methods

2.1. Animals

Mandarin ducks are a designated "natural monument" in South Korea; therefore, the present study used commercial captive-bred birds. Twenty nine adult ducks (14 males and 15 females) were examined. All birds were negative for H5 antibodies for a period of 1 week prior to the experiments and were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Animal and Plant Quarantine Agency (QIA) in South Korea. All experiments took place in a biosafety level 3-enhanced facility at QIA.

2.2. Viruses

The pathogenicity of a single H5N8 virus, A/broiler duck/Kr/Buan2/2014 (H5N8) (clade 2.3.4.4) [Buan2], was evaluated in mandarin ducks. Two representative H5N1 HPAI viruses, A/chicken/Kr/IS/2006 (H5N1) (clade 2.2) [IS] and A/mandarin duck/Kr/PSC24-24/2010 (H5N1) (clade 2.3.2.1) [PSC24-24], were also used for comparison. All viruses were propagated and titrated in specific pathogen-free eggs and stored at −70 °C until further use.

2.3. Experimental design

To assess the pathogenicity of the viruses, mandarin ducks were inoculated with either the Buan2 H5N8 virus, the main genotype in South Korea, or one of two H5N1 HPAI viruses (IS or PSC24-24). To test pathogenicity, 0.1 ml of each viral isolate containing a 50% Egg Infective Dose (EID_{50}; $10^{6.5}$ virions) was inoculated intranasally into seven mandarin ducks. After 8 h, two non-infected mandarin ducks (contact group) were co-housed with the seven birds infected with each of the viruses. The control group (two non-infected mandarin ducks) was inoculated with 0.1 ml of phosphate buffered saline via the same route. Two of the seven infected mandarin ducks from each inoculated group were then sacrificed at 3 days post-infection (p.i.) and brain, trachea, lung, kidney, spleen, heart, cecal tonsil, liver, muscle (leg), intestine (including pancreas), and proventriculus tissue were collected aseptically for virus recovery. Swab samples from the oropharynx (OP) and cloaca (CL) were collected daily (on Days 1–7), and then on Days 10 and 14 p.i. The remaining inoculated birds (five per group) were observed clinically for 14 days.

For virus isolation, each OP and CL sample was suspended in 1 ml of maintenance medium containing antibiotics (Antibiotic-Antimycotic, Invitrogen, USA) and then homogenized to yield a solution with a tissue wt/vol of 10%. Samples were then centrifuged at 3500 rpm for 5 min and 0.1 ml of supernatant was titrated on chicken embryo fibroblast cells to determine the median tissue culture infective dose ($TCID_{50}$). Virus growth was determined by observing the cytopathic effect and HA activity. Virus titers were calculated as described previously (Reed and Muench, 1938). The limit of virus detection was <0.5 $TCID_{50}$/0.1 ml. Statistical analysis was performed using the Student's *t*-test and a *p* value of <0.05 was considered statistically significant.

2.4. Histological analysis of lesions

Two mandarin ducks from each inoculated group were sacrificed at 3 days p.i. to look for evidence of histopathologic lesions in the same tissues used for virus recovery (pancreas, spleen, and cecal tonsil tissue was not tested). Briefly, tissues were fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin, sectioned (approximately 5 μm thick), mounted on glass slides, and stained with hematoxylin and eosin.

2.5. Serological assays

Pre-inoculation serum was collected from each bird and confirmed as serologically negative for H5 antibodies in a hemagglutinin inhibition assay according to standard procedures (OIE, 2015). Sera was collected from surviving mandarin ducks on Day 14 p.i. and the antibody response was measured. All sera were treated with a receptor-destroying enzyme to remove non-specific inhibitors (WHO, 2002).

3. Results

3.1. Clinical signs, mortality, and sero-conversion in mandarin ducks

None of the mandarin ducks infected with the H5N8 or H5N1 viruses by inoculation or after contact with infected birds died or exhibited clinical symptoms. However, all mandarin ducks inoculated with H5N8 or in contact with H5N8-infected ducks sero-converted, with relatively high antibody titers. Likewise, all mandarin ducks inoculated with H5N1 or in contact with H5N1 virus-inoculated birds sero-converted, although the titers were lower than those observed in birds infected with H5N8 (Table 1).

3.2. Viral replication and transmission in mandarin ducks

The replication and transmissibility of a virus in the host animal have a major influence on the magnitude of an outbreak. Therefore, we next compared the pathogenicity of the H5N8 virus (Buan2) with that of the two H5N1 viruses (IS and PSC24-24). The H5N8 virus was re-isolated from swab samples (OP, $10^{0.5-1.8}$ $TCID_{50}$/0.1 ml; CL, $10^{0.5-2.9}$ $TCID_{50}$/0.1 ml) taken on Days 1–6 p.i. The titer of the H5N8 virus in the CL at 5 days p.i. was significantly higher than that of the IS and PSC24-24 viruses ($p < 0.05$). H5N1 was also recovered from the OP ($10^{0.5-1.4}$ $TCID_{50}$/0.1 ml) and CL ($10^{0.5-1.1}$ $TCID_{50}$/0.1 ml) swabs from infected ducks (Fig. 1A and C).

To determine whether HPAI viruses are efficiently transmitted between mandarin ducks, we attempted to isolate virus from OP and CL swabs obtained from the birds in the contact groups. All three H5 viruses were recovered successfully (Fig. 1B and D).

The H5N8 virus was isolated from tissues collected from mandarin ducks euthanized on Day 3 p.i. The virus replicated in the trachea, proventriculus, intestine (pancreas), cecal tonsil, lung, and heart, although the titers were relatively low ($10^{0.5-2.3}$ $TCID_{50}$/0.1 ml). The H5N1 IS and PSC24-24 viruses replicated only in some tissues (trachea and cecal tonsil, and lung) (Table 2).

3.3. Histopathologic lesions

All three challenge groups showed evidence of histopathologic lesions at 3 days p.i. Lesions included congestion or hemorrhage, multifocal hepatic necrosis accompanied by infiltration of lymphocytes and macrophages, chronic cholangitis, and diffuse congestion in the lungs (Fig. 2).