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Pathogenicity in domestic ducks and mice of highly pathogenic H5N1 clade 2.3.2.1 influenza viruses recently circulating in Eastern Asia

Hyun-Mi Kang [a], Jun-Gu Choi [a], Kwang-Il Kim [a], Bang-Sil Kim [a], Damdinjav Batchuluun [b], Tseren-Ochir Erdene-Ochir [b], Min-Chul Kim [a], Jun-Hun Kwon [a], Choi-Kyu Park [c,*], Youn-Jeong Lee [a,**]

[a] Avian Disease Division, Animal and Plant Quarantine Agency, 175 Anyangro, Anyangsi, Gyeonggido 430-757, Republic of Korea
[b] Avian Influenza Section, Transboundary Animal Disease Laboratory, State Central Veterinary Laboratory, Zaisan, Khan-Uul District, P.O. Box 53/03, Ulaanbaatar 210153, Mongolia
[c] College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea

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ABSTRACT

Influenza virus A (H5N1) clade 2.3.2.1 has recently caused widespread outbreaks of disease in domestic poultry and wild birds in Eastern Asia. In the current study, the antigenicity and pathogenicity of three clade 2.3.2.1 viruses (Ck/Kr/Gimje/08, Ws/Mongolia/1/09, and Ws/Mongolia/7/10) were investigated in domestic ducks and mice. The H5N1 influenza viruses in this study were antigenically similar to each other (*r*-values of 0.35–1.4). The three viruses replicated systemically in all tissues tested in domestic ducks, indicating high pathogenicity. However, the viruses produced different clinical signs and mortality rates: Ck/Kr/Gimje/08 and Ws/Mongolia/1/09 resulted in 100% mortality with severe neurological signs, whereas Ws/Mongolia/7/10 resulted in 50% mortality with relatively mild neurological signs. In mice, infection with Ck/Kr/Gimje/08 and Ws/Mongolia/7/10 resulted in weight loss that peaked at 4 days post-infection (22.3% and 20.8%, respectively), same MLD_{50} (2.2 Log_{10} EID_{50}) and systemic replication. The three viruses had K deletion at the −2 position of the HA1-connecting peptide (PQRERRRK-R), which is associated with increased virulence in domestic ducks and harbored NA stalk deletion, NS1 deletion and mutation of P42S in NS1, and full length (90aa) in PB1-F2, which confer increased virulence in mice. Our study shows that clade 2.3.2.1 viruses from Korea and Mongolia are antigenically similar and highly pathogenic in both domestic ducks and mice. Moreover, we provide molecular determinants of the clade 2.3.2.1 viruses associated with the pathogenicity in domestic ducks and mice, respectively.

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1. Introduction

Wild waterfowl are a natural reservoir for the 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes of the influenza A virus. Influenza viruses in wild aquatic birds have long been in a state of evolutionary equilibrium and infected hosts usually show no signs of disease (Webster et al., 1992; Fouchier et al., 2005). However, in the case of H5N1 highly pathogenic avian influenza (HPAI) viruses, some isolates have recently (since 2002) acquired the capacity for high pathogenicity in both domestic and wild ducks, causing disease and death in ducks in many countries (Chen et al., 2004; Sturm-Ramirez et al., 2004; Kim et al., 2011).

* Corresponding author. Tel.: +82 53 950 5973; fax: +82 53 950 5973.
** Corresponding author. Tel.: +82 31 467 1807; fax: +82 31 467 1814.
E-mail addresses: parkck@knu.ac.kr (C.-K. Park), leeyj700@korea.kr (Y.-J. Lee).

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In South Korea, there have been four outbreaks of H5N1 HPAI, in 2003–2004, 2006–2007, 2008 and 2010–2011. Mortality of domestic ducks intranasally inoculated with viruses isolated during the outbreaks was 0–25% and 0% for clade 2.5 (2003–2004 outbreak) and clade 2.2 (2006–2007 outbreak), respectively (Lee et al., 2005; Jeong et al., 2009). Pathogenicity in domestic ducks of viruses causing the 2003–2004 and 2006–2007 outbreaks in South Korea was therefore mild or low, which made it difficult to recognize viral infection at domestic duck farms. In contrast, the third (2008) and fourth (2010–2011) outbreaks caused by clade 2.3.2.1 viruses resulted in relatively high mortality (up to 50%) at duck farms, particularly in young ducklings (Kim et al., 2010, 2012). However, the exact pathogenicity of clade 2.3.2.1 Korean virus, which is the dominant genotype currently circulating in Eastern Asia, has not been analyzed under laboratory conditions. The silent nature of the infection of HPAI viruses in domestic ducks has caused the extensive spread of these viruses and it is therefore important to know their precise pathogenic phenotype in ducks.

Worldwide, HPAI outbreaks occur in migrating wild birds as well as in domestic poultry. In Mongolia, H5N1 HPAI viruses were isolated from dead wild waterfowl in 2005, 2006 and 2009–2010. The infections in 2005 and 2006, caused by clade 2.2 viruses, occurred in species of the Anatidae family such as the whooper swan, bar-headed goose, and common goldeneye. The infections in 2009–2010, caused by clade 2.3.2.1 viruses, affected a wider variety of waterfowl, including gulls and great crested grebes as well as species of the Anatidae family (OIE, 2005, 2006, 2009a, 2010).

Clade 2.3.2 virus belongs to a type of H5N1 virus ongoing significantly viral evolution (Uchida et al., 2008; Usui et al., 2009; Kang et al., 2011). Phylogenetic analysis of clade 2.3.2.1 isolates from Mongolia in 2009–2010 revealed that polymerase acidic gene of these isolates was similar to that of clade 2.5 HPAI viruses, but different from viruses isolated in Russia, Japan and South Korea in 2008 (Kang et al., 2011). This novel variant of clade 2.3.2.1 in Eastern Asia appears to have become established in poultry populations, fueling a new wave of cross-continent spread of HPAI viruses from Asia to Europe, similar to the spread of the clade 2.2 virus in 2005 and 2006 (Jiang et al., 2010; Defra, 2011; Li et al., 2011). Indeed, there have been subsequent and widespread outbreaks induced by this clade in domestic poultry and wild birds in Vietnam, Nepal, Myanmar, Bulgaria, Romania, Hong Kong, China, Russia, Mongolia, Japan and South Korea in 2010–2011, which highlights the potential for cross-species transmission between migrating wild birds and poultry (OIE, 2010, 2011; Kim et al., 2011).

Although several reports have compared the antigenicity and pathogenicity between clade 2.3.2.1 viruses and other virus clades (Sakoda et al., 2010; Sun et al., 2011), there has hitherto rarely been a comparison of the pathogenicity of different Eastern Asian viruses amongst clade 2.3.2.1. Therefore, we compared the antigenic relationships and pathogenic differences in domestic ducks and mice of the three highly pathogenic H5N1 clade 2.3.2.1 viruses from Korea (2008) and Mongolia (2009 and 2010) that are currently circulating in Eastern Asia.

2. Materials and methods

2.1. Viruses

The antigenic relationships and pathogenicity of three H5N1 viruses were evaluated. Ck/Kr/Gimje/08 was isolated from the index case farm of the 2008 Korean outbreak. Ws/Mongolia/1/09 and Ws/Mongolia/7/10 were donated by the State Central Veterinary Laboratory in Mongolia (Fig. 1). The viruses were propagated and titrated in specific pathogen-free (SPF) eggs and stored at −70 °C until further use.

2.2. Antigenic analyses

Antigenic analyses were performed by a hemagglutination inhibition (HI) test using chicken antisera against the tested viruses generated as described previously (OIE, 2009b). To generate the antisera, 4-week-old SPF chickens were injected with 0.5 ml of oil emulsion-inactivated virus, and sera were collected at 3 weeks after injection. The HI tests used 8 HAU (hemagglutination units) of antigen and 1% chicken erythrocytes, and the *r*-values were subsequently calculated as described previously (Archetti and Horsfall, 1950).

2.3. Experimental infection of domestic ducks and mice with clade 2.3.2.1 influenza viruses

To assess the pathogenicity of the H5N1 isolates, Ck/Kr/Gimje/08, Ws/Mongolia/1/09 and Ws/Mongolia/7/10 were inoculated into domestic ducks (Pekin ducks, Korea). For the pathogenicity test in domestic ducks, 0.1 ml of each H5N1 isolate containing $10^{6.5}$ 50% egg infective dose (EID_{50}) was inoculated intranasally into eight 2-week-old domestic ducks per group. For the control group, 0.1 ml of PBS was inoculated by the same route into five domestic ducks.

Oropharyngeal (OP) and cloacal (CL) swab samples were collected at 1, 3, 5, 7, and 10 days post-infection (d.p.i.). On the death of the ducks, brain, trachea, lung, kidney, spleen, heart, cecal tonsil and liver tissues were collected aseptically for virus recovery. The remaining ducks were observed clinically for 14 days. For virus isolation, each swab sample was suspended in 1 ml of maintenance medium (MM) cells with antibiotics (Antibiotic–Antimycotic, Invitrogen, USA) and each tissue sample was homogenized in MM with antibiotics to a final concentration of 10% wt/vol. Samples were then centrifuged at 3000 rpm for 10 min and 0.1 ml of supernatant was titrated in chicken embryonated fibroblast (CEF) cells to determine the median tissue culture infective dose ($TCID_{50}$). The sera were collected from surviving ducks to measure the antibody response.

Female 6–8-week-old BALB/c mice (eight per group) were infected intranasally with 10-fold serial dilutions from 10^0–10^7 EID_{50}/50 μl of Ck/Kr/Gimje/08 and Ws/

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