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Age at infection affects the pathogenicity of Asian highly pathogenic avian influenza H5N1 viruses in ducks

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Abstract

The Asian highly pathogenic avian influenza (HPAI) H5N1 viruses have changed from producing no disease or mild respiratory infections in ducks to some strains causing systemic disease and death. Differences in pathogenicity between four of these viruses as well as the effect of host age on the outcome of infection were studied in ducks. Three of the viruses were highly lethal in 2-week-old ducks and induced severe neurological dysfunction. Neurological signs were also observed in 5-week-old ducks inoculated with one of these viruses; however mortality was low. The fourth virus studied did not induce neurological signs in 2-week-old ducks, but did produce moderate mortality. This virus caused no clinical signs or death in 5-week-old ducks. All viruses studied were isolated from oropharyngeal and cloacal swabs, and also from brain, heart, lung and muscle tissues, demonstrating systemic infection. All viruses evaluated transmitted efficiently to contact ducks. Phylogenetic analysis of the viruses studied and other Asian H5N1 HPAI viruses with diverse pathogenicity in ducks, showed changes in several genes, but none clearly associated with pathogenicity. In conclusion, the pathogenicity of circulating H5N1 HPAI viruses in ducks varies depending on the virus strain and the age of the duck and correlates with the level of viral replication in tissues. High titers of virus in organs, high viral shedding, and variable mortality enable ducks to circulate H5N1 HPAI viruses.
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Keywords: Highly pathogenic avian influenza; H5N1; Ducks; Pathogenicity; Age

1. Introduction

The natural host species and reservoir for avian influenza (AI) viruses are wild aquatic birds, especially of the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (shorebirds, gulls, terns, and auks) (Stallknecht, 1998; Swayne and Halvorson, 2003). Most AI viruses replicate preferentially in the gastrointestinal tract of ducks producing an asymptomatic enteric infection, and are excreted at high levels in feces and transmitted through the fecal–oral route (Hinshaw et al., 1980; Webster et al., 1978). Normally, ducks naturally or experimentally infected with H5 and H7 highly pathogenic AI (HPAI) viruses for chickens and turkeys develop only subclinical to mild disease (Alexander et al., 1986; Cooley et al., 1989; Perkins and Swayne, 2002; Shortridge et al., 1998). However, the pathobiology of AI in ducks has changed, with some Asian HPAI H5N1 viruses replicating systemically and producing neurological dysfunction and death (Ellis et al., 2004; Hulse-Post et al., 2005; Kishida et al., 2005; Lee et al., 2005; Nguyen et al., 2005; Sturm-Ramirez et al., 2004, 2005; Zhou et al., 2006). The only previous cases of reported deaths in ducks due to HPAI occurred in an experimental infection with an H7N1 virus (Alexander et al., 1978) and during the H7N1 outbreak in Italy in 1999–2000, where Muscovy ducks were found dead and presented lesions and viral antigen staining in the brain (Capua and Mutinelli, 2001).

In experimentally infected ducks, a zoonotic avian influenza H5N1 virus from the Hong Kong outbreak in 1997 produced an innocuous infection characterized by transient shedding and no clinical disease (Perkins and Swayne, 2001). Beginning in 2001, some isolates of HPAI H5N1, also of Asian origin related to the Hong Kong 1997 viruses, were found to replicate in internal organs of ducks, however did not produce clinical dis-

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ease or mortality (Tumpey et al., 2002). In late 2002, HPAI H5N1 outbreaks in two Hong Kong parks caused the death of many resident avian species, including waterfowl (Ellis et al., 2004). Experimental infection studies using a goose and an egret isolate from this outbreak confirmed the high lethality, with central nervous system involvement and broad tissue infectivity, of these H5N1 viruses in ducks (Lee et al., 2005; Nguyen et al., 2005; Sturm-Ramirez et al., 2004). Subsequent research in which 4–6-week-old ducks were inoculated with 23 different H5N1 influenza viruses isolated in Asia between 2003 and 2004, showed that circulating viruses caused different pathology in ducks, these viruses varying from non-pathogenic to highly lethal (Hulse-Post et al., 2005; Sturm-Ramirez et al., 2005).

A difference in age susceptibility was observed during a natural outbreak of H5N1 influenza virus in commercial ducks in South Korea. In 14-day-old meat ducks the virus produced increased morbidity and up to 12% of mortality in the affected flock, with microscopic lesions observed in the pancreas, liver, brain, and heart. However, adult ducks at eight breeder duck farms also affected during this outbreak showed decreased egg production and feed consumption, but no mortality (Kwon et al., 2005). Experimental infection of 5-week-old ducks with a H5N1 duck isolate from an outbreak in Japan did not produce mortality, however viruses were recovered from multiple organs including the brain, and neurological signs were associated with high titers of the virus in brain (Kishida et al., 2005).

This inconsistent pathogenicity with HPAI H5N1 virus infections in ducks appears to be related in part to virus strain differences and in part to the ducks age at infection. In order to better understand the varying severity of disease caused by Asian HPAI H5N1 viruses in ducks, we studied the differences in pathogenicity, including clinical response, viral titers in tissues and extent and duration of viral shedding, associated with HPAI H5N1 virus infection by inoculating Pekin ducks at two different ages with different strains of HPAI H5N1 viruses. These viruses were isolated from a human case of H5N1 in Vietnam (A/Vietnam/1203/04), a human case from Thailand (A/Thailand PB/6231/04), and a crow isolate also from Thailand (A/Crow/Thailand/04). For comparison, the original egret isolate from the Hong Kong parks outbreak (A/Egret/HK/757.2/02) was also included in the study. The pathobiological changes associated with infection with these viruses, including gross and microscopic lesions and viral antigen distribution in tissues, has been reported elsewhere (Pantin-Jackwood and Swayne, 2007).

2. Materials and methods

2.1. Viruses

The following HPAI H5N1 viruses were used in this study: A/Vietnam/1203/04 (received courtesy of Kanta Subbarao, U.S. National Institutes of Health), A/Thailand PB/6231/04 and A/Crow/Thailand/04 (received courtesy of Chantanee Buranathai, Department of Livestock Development, Thailand) and A/Egret/HK/757.2/02 (kindly provided by Trevor Ellis, Agriculture Fisheries and Conservation Department, Hong Kong). Virus stocks were propagated in embryonating chicken eggs as previously described (Pantin-Jackwood and Swayne, 2007). A sham inoculum was made using sterile allantoic fluid diluted 1:300 in brain heart infusion (BHI) medium. Serial titrations were performed in embryonating eggs and 50% egg infectious dose (EID_{50}) titers were determined by testing hemagglutination activity (Swayne et al., 1998). Titration endpoints were calculated by the method of Reed and Muench (Reed and Muench, 1938). All experiments using HPAI H5N1 viruses, including work with animals, were performed in biosecurity level-3 Ag (BSL-3-Ag) facilities at Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service, United States Department of Agriculture (USDA) (Barbeito et al., 1995), and all personnel were required to wear a powered air protection respirator with high efficiency particulate air (HEPA)-filtered air supply (3M^{TM}, St. Paul, MN).

2.2. Pathogenicity studies in 2-week-old ducks

Two-week-old Pekin ducks were intranasally (IN) inoculated to determine the pathogenicity of the four H5N1 viruses previously mentioned. Serum samples were collected from a representative number of ducks prior to inoculation to ensure that the birds were serologically negative for AIV as determined with the agar gel precipitin (AGP) test (Beard, 1970). Ducks were housed in self-contained isolation units (Mark 4, Controlled Isolation Systems, San Diego, CA) that were ventilated under negative pressure with HEPA-filtered air and maintained under continuous lighting. Feed and water were provided with ad libitum access. General care was provided as required by the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Craig et al., 1999). The experimental design has been previously described (Pantin-Jackwood and Swayne, 2007). Briefly, ducks were separated into a control group and four virus-inoculated groups. The control group contained five ducks and these were IN inoculated with 0.1 ml of a sham inoculum. Two control birds were euthanized at 2 and 14 days post-inoculation (dpi), and their tissues collected in 10% neutral buffered formalin solution for histopathologic evaluation. At 14 dpi, serum was collected from the three remaining control birds for AGP testing to ensure that controls remained serologically negative to AI virus. The four virus-inoculated groups, each containing 10 birds, were inoculated IN with inoculum containing 10^5 EID_{50}/ml of the viruses. To study transmission of the viruses, two uninfected ducks (contact birds) were placed in each isolator with the inoculated birds at 2 dpi. Two birds from each group were euthanized at 2 dpi and their tissues collected in 10% neutral buffered formalin solution to determine microscopic lesions and the extent of virus replication in tissues as previously reported (Pantin-Jackwood and Swayne, 2007). Portions of the brain, lung, skeletal muscle, heart and kidney were collected in BHI with antibiotics for virus isolation. The remaining eight birds were observed for signs of illness over a 14-day period during which time the clinical signs and weights were recorded. Oropharyngeal and cloacal swabs were collected from all ducks each day from 1 to 7 days and from remaining ducks on 10 and 14 days dpi. Tis-