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Pathogenicity and transmissibility of three avian influenza A (H5N6) viruses isolated from wild birds

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Abstract Since 2013, highly pathogenic H5N6 avian influenza viruses (AIVs) have emerged in poultry and caused sporadic human infections in Asia. The recent discovery of three new avian H5N6 viruses – A/oriental magpie-robin/Guangdong/SW8/2014 (H5N6), A/common moorhen/Guangdong/GZ174/2014 (H5N6) and A/Pallas's sandgrouse/Guangdong/ZH283/2015 (H5N6) – isolated from apparently healthy wild birds in Southern China in 2014–2015 raises great concern for the spread of these highly pathogenic AIVs (HPAIVs) and their potential threat to human and animal health. In our study, we conducted animal experiments and tested their pathogenicity in ducks, chickens and mice. Ducks can carry and shed the H5N6 HPAIVs, but show no ill effects. On the other hand, these H5N6 HPAIVs can efficiently infect, transmit and cause death in chickens. Due to the overlap of habitats, domestic ducks play an important role in circulating AIVs between poultry and wild birds. Our results raise the possibility that wild birds disseminate these H5N6 HPAIVs to poultry along their flyways and thus pose a great threat to the poultry industry. These viruses are also highly pathogenic to mice, suggesting they pose a potential

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threat to mammals and, thus, public health. One virus isolated in 2015 replicates much more efficiently and is more lethal in these animals than the two other viruses isolated in 2014. It seems that the H5N6 viruses tend to be more lethal as time passes. Therefore, it is necessary to vigilantly monitor H5N6 HPAIVs in wild birds and poultry.

Introduction

Wild birds are always considered to be the natural reservoirs for low pathogenic avian influenza viruses (LPAIVs), as well as being the source of infection of poultry in nature.[1] During their introduction into poultry, LPAIVs have the potential to mutate into highly pathogenic avian influenza viruses (HPAIVs). Recent studies showed that wild birds can also carry the HPAIVs, such as H5N1[2] and H5N8.[3,4] Migratory birds can rapidly disseminate them along their flyways, thus posing a great threat to both global poultry production and public health.

The highly pathogenic H5 subtypes, panzootic in poultry, continue to evolve and pose a major challenge to animal and human health.[5] The H5N1 HPAIVs were first detected in China in 1996 (A/goose/Guangdong/1/1996). Since then, it has become endemic in poultry in Asia, Europe and Africa, causing great economic loss. It has also repeatedly infected humans. Since the first diagnosed case of human infection in 1997 in Hong Kong,[6–8] the virus has accumulated an exceedingly high fatality rate of more than 60%. Phylogenetic analysis of H5 protein sequences document its rapid evolution and the formation of distinct clades. In particular, the viruses have reassorted with different NA subtypes, including N1, N2, N3, N5, N6 and N8.[9–14] Novel viral subtype H5N6, which belongs to clade 2.3.4.4, has circulated within poultry in Southeast and East Asia[13,15,16] and caused human infections since 2013.[17] Human infections of H5N6 reappeared in December 2015 in Guangdong Province, and poultry was considered to be the source of these infections.[18] Previously, we isolated three H5N6 HPAIVs from apparently healthy wild birds in 2014–2015: A/oriental magpie-robin/Guangdong/SW8/2014 (H5N6) (Abbreviation: SW8), A/common moorhen/Guangdong/GZ174/2014 (H5N6) (Abbreviation: GZ174) and A/Pallas's sandgrouse/Guangdong/ZH283/2015 (H5N6) (Abbreviation: ZH283).[19] Introductions of AIVs from wild birds to domestic poultry occasionally cause outbreaks and great losses of the domestic birds.[20] Previously, isolates of H5N6 HPAIVs in Asia were mainly recovered from dead wild birds or environmental fecal samples.[21] The occurrence of non-lethal H5N6 HPAIVs in wild birds raises the possibility that they can spread. To better understand their potential threat to poultry, we investigated their transmission, and pathogenicity in ducks and chickens. Previous studies showed that avian H5N6 viruses of clade 2.3.4.4 were less pathogenic in mice and ferrets than their parental clade 2.3.4 H5N1 virus, but they acquired the ability of contact transmission in ferrets.[22] Therefore, we also investigated the pathogenicity of these three H5N6 HPAIVs in mice.

Materials and methods

Ethics statement

We conducted all animal experiments under the guidance of both the Guangdong Provincial Center for Disease Control and Prevention's Institutional Animal Care and Use Committee and the Association for Assessment and Accreditation of Laboratory Animal Care International. South China Agricultural University's Committee on the Ethics of Animal Experiments of Animal Biosafety Level 3 (ABSL-3) reviewed and approved the study protocols (permit no. 2015-10). All methods were carried out in accordance with these approved guidelines.

Replication, pathogenicity and transmissibility of three viruses in ducks and chickens

In vivo pathogenicity studies of the wild birds' H5N6 influenza viruses were conducted in accordance with the World Organization for Animal Health (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf). In brief, one-week-old, clinically healthy Muscovy ducks and six-week-old specific-pathogen-free (SPF) white Leghorn chickens were used in this study. For each virus, the inoculated groups (six birds for each group, n = 6) were inoculated intranasally with 10^6 EID_{50} (egg infective dose at which 50% of inoculated eggs are infected) of the indicated virus in a 0.2 mL volume in an ABSL-3 lab. The contact groups (n = 3) were inoculated intranasally with 0.2 mL phosphate-buffered saline (PBS) and then placed in physical contact (in the same cage, sharing food and water) with birds that had been inoculated with the virus 24 h previously. A control group of six chickens were inoculated intranasally with 0.2 mL of PBS. Two days later, three inoculated birds from each group were euthanized humanely to test for viral replication in lung, kidney, spleen, cecal tonsils, bursa of Fabricius, trachea, pancreas, liver, heart and brain. The remaining birds were observed for clinical symptoms, morbidity and mortality for up to 14 days. Oropharynx and cloacal swabs were collected and suspended in 1 mL PBS for viral detection of viral shedding at 2, 3, 5, 7 and 9 days post-infection (DPI). All tissues and swabs were collected and titrated for virus infectivity in SPF chick embryos by using the EID_{50} method, and calculated by using the Reed–Muench method.[23] Seroconversion of all surviving birds on 14 DPI was confirmed by the hemagglutination inhibition (HI) test.

Replication and pathogenicity of three viruses in mice

Six- to seven-week old female SPF BALB/c mice weighing 12–14 g were purchased from the Medical Laboratory Animal Center of Guangdong Province, China, and housed in ABSL-3 isolators. The mice were divided randomly into three infection groups (n = 11 for each group) and a mock-infected control group (n = 5). Each of the three infection groups was lightly anesthetized with CO_2 before intranasal inoculation with 50 μL of 10^6 EID_{50} of one of the three H5N6 viruses,