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## **INFLUENZA VIRUSES IN HEALTHY WILD BIRDS IN HONG KONG**

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Wild waterfowl are considered the natural reservoir of influenza A viruses. The recent outbreak of highly pathogenic avian influenza (HPAI) H5N1 in Asia which has now spread as far as Africa highlights the importance of defining the influenza virus gene pool in these birds and understanding the potential role played by migratory waterfowl in such HPAI outbreaks. Seventy-three influenza viruses were isolated from 16,724 samples collected from feral waterfowls or their fecal droppings during 2003-7 at Mai Po Marshes and Lok Ma Chau in Hong Kong. A diversity of influenza viruses representing hemagglutinin subtypes of H1, H2, H4, H5, H6, H7, H8, H9, H10 and H11; neuramidinase subtypes of N1, N2, N3, N4, N6, N7, N8 and N9, were isolated. Seventy-two of these 73 positive isolates were collected during the winter period coinciding with the southern migration of waterfowl along the East Asian flyway. No HPAI H5N1 viruses were isolated from healthy birds sampled in this study, though H5N1 viruses have been isolated from dead wild birds found in Hong Kong. Phylogenetic analyses of the HA gene of the H5 viruses isolated in the study showed that they clustered with other LP H5 viruses isolated from Hokkaido, Mongolia and Siberia but they seemed not to be very closely related to the HP H5N1. Six of 150 blood sample collected from wild ducks and one of 43 from shorebirds were tested to have antibody by neutralization tests for H5 subtype hemagglutinin.

### **INTRODUCTION**

Influenza viruses from wild aquatic birds contributed to the genesis of the pandemics of the last century which then led to significantly global mortality. For example the “Spanish flu” pandemic in 1918 is estimated to have killed over 25 millions of people [1]. It is suggested that the 1918 pandemic virus was a purely avian virus that adapted to efficient human-to-human transmission [2]. The pandemic viruses of 1957 and 1968 also acquired three or two gene segments from wild birds, respectively [3]. Reconstruction of the sequence of the hypothetical ancestral strain at the avian-human transition indicated that only six amino acids in the mature hemagglutinin molecule had been changed during the transition between the avian virus strain and the human pandemic strain [4]. More recently, a highly pathogenic avian influenza H5N1 virus has spread to affect poultry in a number of countries in Asia and beyond. This virus transmits zoonotically to humans, albeit inefficiently [5]. However, when it does, it often causes a fulminant viral pneumonia with high case-fatality. In some instances, wild birds and waterfowls are implicated in the spread of this infection to poultry. Studies in Siberia and Hokkaido (Japan)[6] have indicated that the NP gene of H11N9 viruses isolated from ducks in Kobayasky area in 1996 forms a cluster with that of the highly virulent H5N1 influenza viruses isolated from chickens and humans from 1997. This supports the contention that wild birds are the ultimate reservoir of the gene pool of influenza viruses. Influenza virus surveillance of wild birds, especially migratory birds, is therefore of vital importance for both animal and human health.

### **MATERIALS AND METHODS**

Routine sampling of the fecal droppings were carried out 2-3 times per week at designated areas in Mai Po Nature Reserve and Lok Ma Chau of Hong Kong during the waterfowl migratory months (October – March). The Mai Po Nature Reserve is one of the sites along the East Asia-Australian flyway where migratory waterfowls stop over and congregate during autumn – spring (Oct – Mar) each year. The largest group of wild waterfowl consist of ducks and grebes and a peak of around 30,000 of this species were counted each year in Hong Kong [7]. Trapping of birds was also done twice a month during the same period, depending on the number of birds present at the trapping site and the weather. Tracheal and cloacal swabs together with blood

samples were collected from the trapped birds. Samples were inoculated into 9-10 day old embryonated eggs and positive isolates were subtyped using standard antisera by the hemagglutination inhibition test and one-step RT-PCR (8). Sequencing was performed by BigDye Terminator V3.1 cycle sequencing kit on ABI PRISM 3700 DNA analyzer (Applied Biosystems). All sequence segments were assembled and aligned by BioEdit version 7. A phylogenetic tree was generated by neighbor-joining bootstrap analysis (1,000 replicates) using the Kimura 2-parameter model in MEGA, version 3.1. The sera collected from trapped birds were tested for H5 antibodies using the microneutralisation test.

## RESULTS

During the 4-year surveillance period from Sep 03 to Feb 07, influenza viruses were isolated from 73 out of 16,724 (0.43%) fecal dropping samples collected from wild migratory waterfowls. These viruses were of hemagglutinin subtypes H1, H2, H4, H5, H6, H7, H8, H9, H10 and H11; neuraminidase subtypes N1, N2, N3, N4, N6, N7, N8 and N9. (Table 1). Seventytwo of these 73 positive isolates were collected during the winter period coinciding with the southern migration of waterfowl along the East Asian flyway. Nevertheless, none of the water samples collected from the ponds where these birds congregate was found to be positive for the virus. For nonwaterfowl, none of the 6499 samples collected was found to be positive for avian influenza during the same study period in 2003-06. The H5 and H7 subtype isolated from these fecal droppings of apparently healthy birds were low pathogenic influenza viruses. No HPAI H5 viruses were isolated in this study. Phylogenetic analyses of the HA gene of the LPAI H5 viruses isolated in the study revealed that these viruses clustered with other LPAI H5 viruses isolated from Hokkaido, Mongolia and Siberia. They were phylogenetically distinct from HPAI H5 viruses with amino acid homology of only 90%. Six out of 150 blood samples collected from wild ducks and one out of 43 blood samples from shorebirds, revealed neutralisation of LPAI H5N3 in microneutralisation assay providing evidence of circulation of these viruses in shore birds as well as in wild ducks.

**Table 1.** The number of different hemagglutinin and neuraminidase subtypes isolated from wild migratory birds in Hong Kong Sep 03-Feb 07.

## DISCUSSION

To date, 16 HA and 9 NA subtypes have been identified in wild birds [9-11] belonging to at least 90 species representing twelve different orders [12]. The aquatic birds of the orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, surfbirds, sandpipers) are believed to be the main reservoirs of all of these different subtypes of influenza A viruses [13]. Our findings have revealed that a diversity of avian influenza virus exist in aquatic birds in Hong Kong. On the other hand, the nonaquatic birds did not yield virus isolates. As only LPAI H5 can be isolated from these aquatic birds in our surveillance study and only a low percentage of birds have LPAI H5 antibody, we have no proof that HPAI H5 viruses are endemic in the migratory bird populations in this region. While this surveillance program in apparently healthy wild birds yielded negative results for HPAI H5N1, surveillance of dead wild birds carried out by the Agriculture, Fisheries and Conservation department of Hong Kong regularly identifies some birds with HPAI H5N1 infection. However, these positives are not all, or even predominantly obtained in migratory bird species. Although the poultry farms within Hong Kong have remained free of HPAI H5N1 since 2004, it remains possible that these wild birds picked up infection from infected poultry in the wider region. One limitation of this study is that most swabs tested were fecal droppings. Since recent studies suggest that viral titers of HPAI H5 viruses were higher in the tracheal rather than cloacal swabs (Osterhaus A – personal communication), we may have under-estimated the presence of HPAI H5N1 viruses. Phylogenetic analysis of the HA gene of the LPAI H5 viruses in this study has revealed that they clustered together with those viruses from

Hokkaido (Japan), Mongolia and Siberia. This demonstrated that there is a dynamic “mixing” of viruses longitudinally along the East Asian Pathway; as suggested previously [14]. The wide diversity of hemagglutinin and neuraminidase subtypes identified from wild waterfowl within a rather small geographic region re-emphasizes the diversity of influenza viruses in these species. Since the previous pandemics of 1957 and 1968 did not arise from highly pathogenic avian influenza viruses, it remains important to maintain surveillance of LPAI influenza subtypes as well.

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