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Isolation of avian influenza viruses from two different transhemispheric migratory shorebird species in Australia

Brief Report

A. C. Hurt[1,2], **P. M. Hansbro**[3], **P. Selleck**[4], **B. Olsen**[5,6], **C. Minton**[7], **A. W. Hampson**[1,2], and **I. G. Barr**[1,2]

[1]World Health Organisation Collaborating Centre for Reference and Research on Influenza, Parkville, Australia
[2]School of Applied Sciences, Monash University, Churchill, Australia
[3]Discipline of Immunology and Microbiology and the Hunter Medical Research Institute, School of Biomedical Sciences, University of Newcastle, Callaghan, Australia
[4]CSIRO Australian Animal Health Laboratory, Geelong, Australia
[5]Section for Zoonotic Ecology and Epidemiology, Department of Biology and Environmental Science, Kalmar University, Kalmar, Sweden
[6]Department of Infectious Diseases, Umeå University, Umeå, Sweden
[7]Australasian Wader Studies Group, Australia

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Summary. Shorebirds on their southerly migration from Siberia to Australia, may pass through Asian regions currently experiencing outbreaks of highly pathogenic H5N1 influenza. To test for the presence of avian influenza viruses in migratory shorebirds arriving in Australia during spring 2004, 173 cloacal swabs were collected from six species. Ten swabs were positive for influenza A, with H4N8 viruses detected in five red-necked stints and H11N9 viruses detected in five sharp-tailed sandpipers. No H5N1 viruses were detected. All isolated viruses were non-pathogenic in domestic chickens. These results further demonstrate the potential for migratory shorebirds to carry and potentially spread influenza viruses.

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Waterfowl are the natural reservoir of all influenza A viruses, which are generally carried as asymptomatic infections [24]. However, severe disease may occur when certain avian influenza viruses (AIV) are transmitted from this reservoir to domestic poultry and to other species including humans. Although there was previously little evidence of direct transmission from birds to humans, during

the last eight years there have been several instances of poultry-to-human transmissions resulting in a number of human fatalities. The first of these reports involved the fatal infection of a 3-year-old boy in Hong Kong in 1997 with a highly pathogenic avian influenza (HPAI) H5N1 virus [5]. In association with ongoing poultry outbreaks of H5N1 viruses over the following months, a further 17 human cases were recorded in Hong Kong, with 5 deaths [20]. Other influenza A subtypes have also been transmitted from poultry to humans with less severe consequences. Influenza H9N2 viruses were isolated from two young children in Hong Kong in 1999 [22], H7N7 strains from an outbreak in the Netherlands in 2003 infected 89 poultry workers, resulting in one death [11], and H7N3 infections were identified in two poultry workers from Canada in 2004 [21].

Since 2003, an HPAI H5N1 virus distantly related to the viruses in Hong Kong during 1997 [13] has emerged and now appears to be endemic in many countries in southeast Asia, resulting in the culling of millions of chickens and the death of over 70 humans. The currently circulating HPAI H5N1 strain causes significant disease in several species of waterfowl, which did not appear to have been seriously affected by HPAI H5 viruses from earlier outbreaks [8, 12]. One recent outbreak amongst migratory bar-headed geese at Qinghai Lake in Western China during May 2005 [4] resulted in the deaths of approximately 6000 birds. Qinghai Lake is located in a protected nature reserve with few poultry farms in the vicinity, which may indicate that the route of transmission was via contact with other migratory birds. Another outbreak in wild birds occurred three months later at Erkhel Lake in Mongolia, where H5N1 virus was identified in specimens collected from dead birds, suggesting that migratory birds may be involved with the carriage and spread of the virus [16]. H5N1 virus was not isolated from any live birds at Lake Erkhel, and so it remains unknown which species may be involved with the H5N1 transfer, however, given the limited studies of AIV in birds other than waterfowl, further surveillance of migratory birds including shorebirds remains a priority.

Over 20 species of shorebirds migrate from their breeding grounds in Siberia down the East Asian/Australian flyway to Australia and New Zealand each year. During this migration, birds stop over in many of the regions that are currently experiencing H5N1 outbreaks, and where the virus may now be endemic. Given that little is known regarding the ecology of influenza in shorebirds and that there are reports suggesting that migratory birds may play a role in the spread of H5N1, sampling of various species of transhemispheric migratory waders in Australia was conducted shortly after their arrival from the Northern Hemisphere during 2004, to look for evidence of AIV, including H5N1.

As part of this surveillance, birds were caught from Fullerton Cove in the Hunter estuary, NSW, Australia, over a three-week period during November 2004 using funnel traps. After trapping, a paediatric swab was inserted into the cloaca of the bird and then placed in 4 ml of viral transport media. Samples were then either stored on ice or snap frozen in liquid nitrogen prior to being transported to the laboratory where they were stored at $-80\,^{\circ}\mathrm{C}$. The samples were thawed and total RNA extracted using the RNeasy kit (QIAGEN, Australia), with minor modifications to the manufacturer's protocol. Briefly, cloacal swabs from three

different birds (500 μl of each) were pooled and extracted with 1500 μl of RLT buffer, followed by 1500 μl of 70% ethanol, and debris was pelleted by centrifugation (5 min at 5000 *g*). Aliquots (750 μl) of the supernatant were then transferred to the RNeasy Column, and RNA was eluted with sterile water (50 μl). When an RNA 'pool' was found to be influenza positive by RT-PCR, each of the three cloacal swabs were re-extracted separately and tested individually.

To detect the presence of influenza A, RT-PCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Australia) according to the manufacturer's protocol. 5 μl of RNA was added to a total reaction of volume of 50 μl. Primers (10 μM) were based on those previously published [18] with modifications to the antisense primer (5′-CCCATGCAACTGGCAAGTGCACA-3′). The limit of detection of the RT-PCR assay for cell-culture isolates has been determined previously to be 0.6 $TCID_{50}$.

Initial haemagglutinin (HA) typing was by sequence analysis of the HA2 region using previously published primers [17]. RT-PCR was performed and the products purified using the QIAquick PCR Purification Kit (QIAGEN). Sequencing was performed using a Big Dye III kit (Perkin Elmer) and run on an ABI 310 genetic analyser at IMVS, Adelaide. Nucleotide sequences were analysed using DNASTAR v.5 (Lasergene, USA). Determination of the HA subtype was based on nucleotide similarity, where the homology of HA2 sequences was compared with other available HA2 sequences using BLAST on the Los Alomos National Laboratory (LANL) influenza sequence database (http://www.flu.lanl.gov). Full HA and neuraminidase (NA) sequence analysis was performed using primers previously published [9], with modification to the forward HA primer (Bm-HA-1) to improve specificity towards the subtype of virus as determined by HA2 sequencing (Primer Bm-H4-1 was used for viruses of H4 subtype (5′-TATTCGT CTCAGGGAGCAAAAGCAGGGGAAACAATGCTA-3′); Primer Bm-H11-1 was used for viruses of H11 subtype (5′-TATTCGTCTCAGGGAGCAAAAGC AGGGGATCAATGAAGA-3′).

RT-PCR positive swabs were inoculated into the allantoic cavity of 10–12-day-old embryonated hen's eggs and incubated at 35 °C for 3 days. Allantoic fluid was harvested and tested for haemagglutination activity with 1% turkey red blood cells. Viral culture allowed antigenic typing to be conducted to confirm the subtype of influenza-positive specimens, using a haemagglutination inhibition (HI) assay [2] and an NA inhibition assay [23].

To determine whether influenza viruses isolated in this study were susceptible to the neuraminidase inhibitor (NI) drugs oseltamivir carboxylate (Tamiflu) and zanamivir (Relenza), isolates were tested using a fluorescence-based NA enzyme inhibition assay [10]. To assess virus susceptibility to the adamantane anti-influenza drugs, amantadine or rimantadine, the matrix genes of influenza viruses were amplified and sequenced using methods described earlier and primers as previously published [9].

A total of 173 healthy transhemispheric migratory shorebirds, from 6 different species, were trapped and swabbed (Table 1). This represented the most prevalent shorebird species in the region, although two relatively common species, the Far

Table 1. Summary of the species and number of migratory shorebirds sampled, and the number and percentage of each species that were influenza A positive

Common name	Scientific name	No. of birds sampled	No. of influenza A positive swabs	% of influenza A positive
Sharp-tailed Sandpiper	*Calidris acuminata*	97	5	5
Curlew Sandpiper	*Calidris ferruginea*	14	0	0
Red-necked Stint	*Calidris ruficollis*	14	5	36
Red Knot	*Calidris canutus*	6	0	0
Bar-tailed Godwit	*Limosa lapponica*	29	0	0
Black-tailed Godwit	*Limosa limosa*	13	0	0

Eastern curlew and the Pacific golden plover, did elude capture. No dead birds were observed or swabbed. In addition, it was noted that many of the different species were found to be feeding on the same mudflats during falling tide, and also roosting in the same areas. Following analysis of the 173 samples, five of 97 swabs taken from sharp-tailed sandpipers and five of 14 swabs from red-necked stints were found to be positive for influenza A virus by RT-PCR (Table 1). Swabs taken from the remaining 4 species did not contain any detectable influenza A viruses. Each of the five influenza A-positive swabs from red-necked stints were taken on a different day over a 7-day period, while three of the five positive swabs from sharp-tailed sandpipers were taken on different days. At the time of swabbing, the tip of a single tail feather was cut off to ensure that none of the birds were re-sampled. All sampling was conducted in the same area.

Sequence analysis of the full HA and NA genes of the RT-PCR influenza A-positive swabs, and subsequent comparison with public sequence databases, identified that the five samples from sharp-tailed sandpipers were all A(H11N9), while the five samples from red-necked stints were all A(H4N8). No H5N1 viruses were detected. Variability within the five H11N9 and the five H4N8 sequences was found to be extremely low, with only 1–2 synonymous nucleotide changes found between the same subtype sequences.

Phylogenetic comparison of the full HA nucleotide sequence of the A/Red-necked Stint/Australia/1/2004 virus and all H4 sequences (longer than 1000 bp) available from public databases revealed that the sequences grouped into two major clusters. These clusters clearly separated strains from birds of Eurasian/Australasian origin from strains from birds of American origin (Fig. 1). The HA gene of the red-necked stint virus was found to be most closely related to the A/Budgerigar/Hokkaido/1/77 virus, however the level of similarity was only 87%, suggesting that none of the H4 sequences in the public databases have a particularly close genetic relationship with the strain identified in this study. A similar phylogenetic analysis of the full HA sequence of A/Sharp-tailed Sandpiper/Australia/6/2004 was also conducted, however there were only 4 other H11 sequences (longer than 1000 bp) available on the sequence databases with

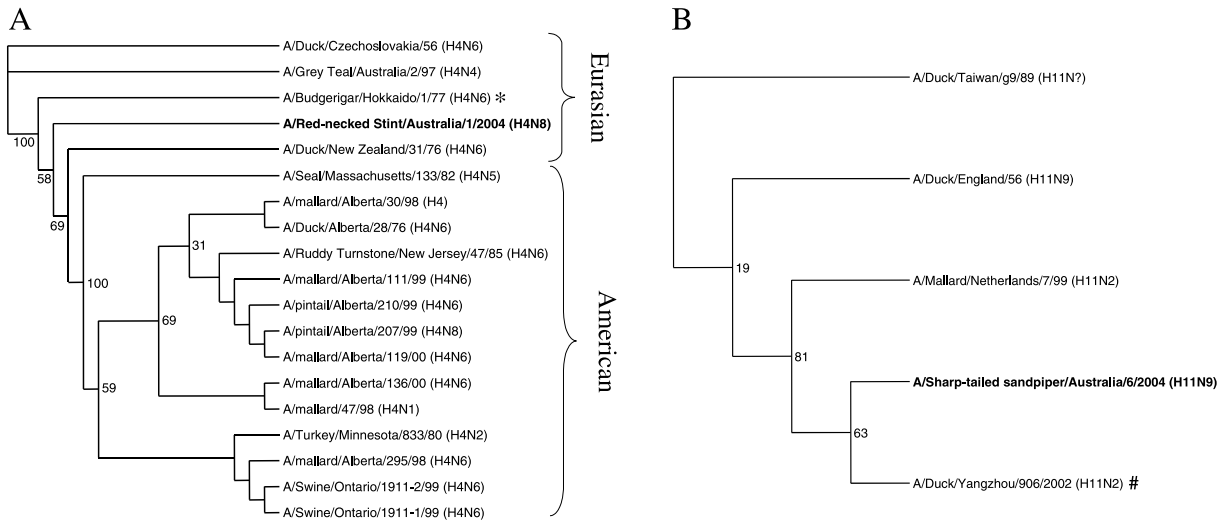

Fig. 1. Phylogenetic analysis of the full HA genes from influenza viruses isolated in this study in comparison with sequences available on public sequence databases. **A** represents H4 strains; *bold text* virus isolated in this study, * virus with highest nucleotide similarity, 87%. **B** represents H11 strains; *bold text* virus isolated in this study, # virus with highest nucleotide similarity, 94%. The numbers in the tree represent bootstrap values (100 replicates). The HA sequences for A/Red-necked Stint/Australia/1/2004 and A/Sharp-tailed sandpiper/Australia/6/2004 are available from GenBank (Accession numbers are DQ327834 and DQ327835, respectively)

which to compare. The HA gene of the sharp-tailed sandpiper virus was most closely related to A/Duck/Yangzhou/906/2002, the most recent H11 sequence, with 94% nucleotide similarity.

Because of the lack of H4 and H11 data available on public sequence databases, it is difficult to speculate about the origin of these viruses and whether they were contracted from waterfowl within Australia or were carried into Australia from Asia by migratory species. H4 viruses are one of the most common subtypes found in ducks throughout the world [6], however when compared with viruses in the sequence databases, the H4 virus from the red-necked stint still remained distinct, with only 87% nucleotide identity to the closest match. H11 viruses are somewhat less common, with this study representing the first identification of this subtype in Australia since 1975 [1, 7]. However, this is probably the result of a lack of surveillance rather than the absence of the virus. To better understand the spread of influenza viruses by migratory birds, it is essential that increased surveillance is conducted, that results are widely reported and that sequences are deposited into public databases for future reference.

Of the ten RT-PCR-positive swabs inoculated into embryonated hen's eggs, all five of the H4N8 viruses were successfully cultured, however only two of the H11N9 viruses could be propagated. Subsequent antigenic analysis of both the HA and NA from cultured viruses confirmed the subtyping result determined by gene sequence analysis. Viruses cultured in eggs were also used to determine the

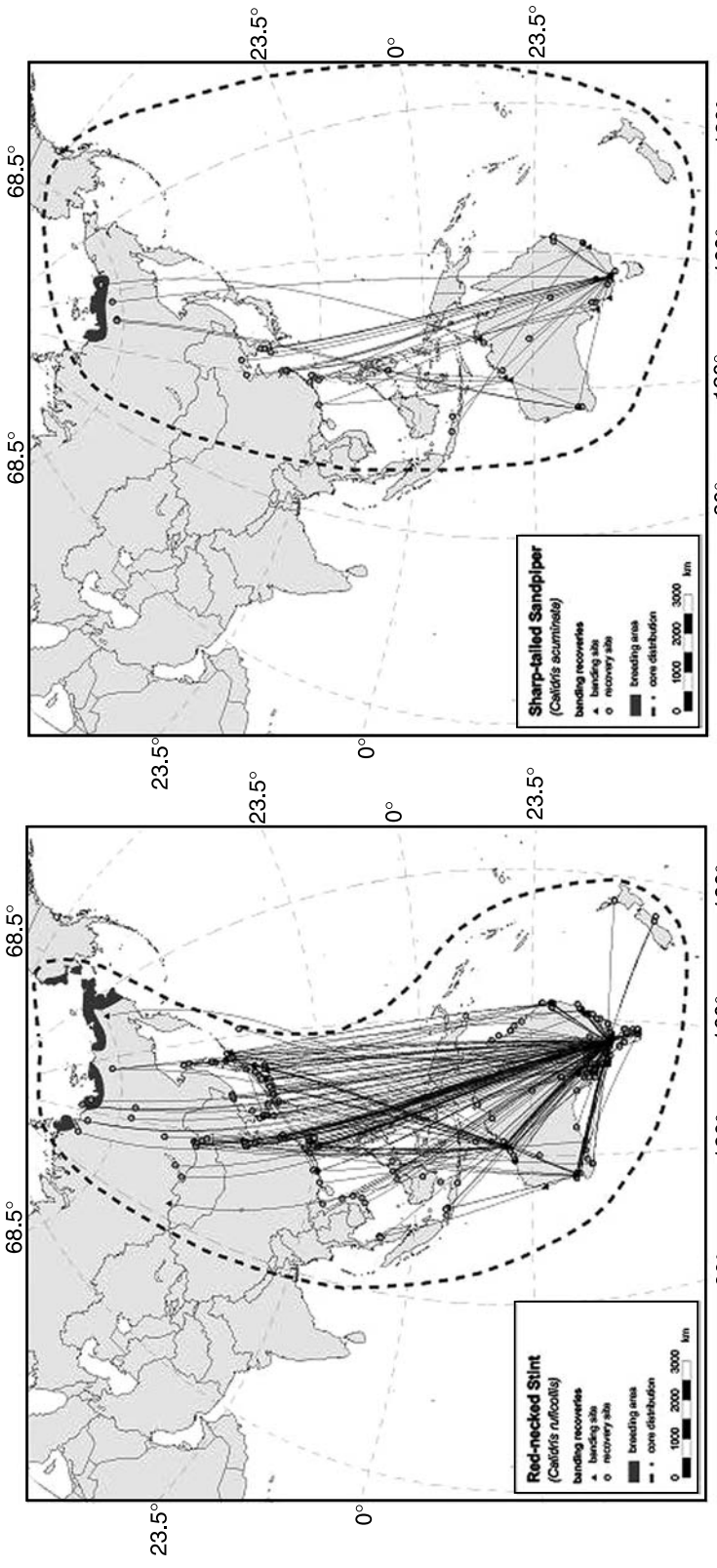

Fig. 2. Banding recovery and flag sighting data displaying migratory paths for red-necked stints and sharp-tailed sandpiper. Figures kindly provided by Wetlands International [15]

potential to cause disease in domestic chickens and an intravenous pathogenicity index (IVPI) calculated [19]. Both H4N8 and H11N9 viruses were found to be non-pathogenic in chickens, with an IVPI value of 0 for both.

As anti-influenza drugs will be essential components of the response to a future pandemic, it is important to determine the sensitivity of any newly identified influenza viruses to these drugs. A NA enzyme-inhibition assay demonstrated that both the N8 and N9 from the red-necked stint and sharp-tailed sandpiper viruses were highly sensitive to zanamivir (Relenza) (IC_{50} values of 1.63 nM and 1.54 nM, respectively) and oseltamivir carboxylate (Tamiflu) (IC_{50} values of 1.07 nM and 0.35 nM, respectively). These IC_{50} values were similar to currently circulating human N1- and N2-sensitive viruses [10]. Sequence analysis of the matrix gene (data not shown) suggested that these avian viruses would also be sensitive to the adamantane anti-influenza drugs, amantadine and rimantadine.

This is one of the few recent reports of detection, isolation and characterisation of AIV from migratory shorebirds within the East Asian/Australasian flyway. The significance of this flyway, and the birds that migrate along it, has increased substantially since 2003 in light of the outbreaks of highly pathogenic H5N1 viruses throughout Eastern Asia. Outbreaks of HPAI H5N1 in wild birds in isolated regions have suggested that migratory birds may be involved with the spread of this virus. We identified influenza subtypes H4N8 and H11N9 in two different species of migratory shorebirds that have been shown through banding recovery and flag sighting data to pass through countries heavy affected by H5N1, including Vietnam, Indonesia and China, on their southerly migration to Australia (Fig. 2). Most migratory shorebird species using this flyway have similar stopover locations throughout the Asian region currently affected by H5N1.

The role of migratory shorebirds in the spread of highly pathogenic H5N1 and the pathogenicity of these viruses in the different species that migrate throughout the East Asian/Australasian flyway are currently unknown. There has, however, been a recent preliminary report of H5N1 being detected by RT-PCR in a migratory shorebird (green sandpiper) in Russia [3]. Apparent differences in susceptibility to various influenza subtypes were observed in our study, with red-necked stints having exclusively H4N8 and sharp-tailed sandpipers only H11N9, even though these species regularly occur in the same habitats when feeding and roosting. The only other report of influenza detection in red-necked stints in Australia was in 1985, where interestingly, the majority of subtypes isolated were also H4N8, the same subtype found in this species in the current study [14]. This may indicate that there is an association of certain subtypes with certain species of birds. The H11N9 from this study represents the first known report of AIV detection in sharp-tailed sandpipers.

These findings contribute to the little knowledge that is currently available regarding AIV infections and their spread in migratory shorebirds. Given the results from this study and the large distances that these birds can travel in a short time, it is important that AIV surveillance is increased in Australia and throughout the region to better understand the potential risk of migratory shorebirds transferring highly pathogenic H5N1 into countries currently uninfected by the virus.

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Author's address: Dr. Aeron C. Hurt, WHO Influenza Centre, WHO Collaborating Centre for Reference and Research on Influenza, 45 Poplar Rd., Parkville, Vic. 3052, Australia; e-mail: aeron.hurt@influenzacentre.org