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Avian Influenza Viruses in Wild Land Birds in Northern Vietnam

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ABSTRACT: Given a paucity of data on the occurrence of avian influenza viruses (AIVs) in wild passerines and other small terrestrial species in Southeast Asia and the importance of highly pathogenic Asian-strain H5N1 outbreaks in humans and domestic poultry in these areas, we focused on surveillance for influenza A viral nucleic acids and antibodies for AIVs in wild-caught birds in northern Vietnam. Four of 197 serum samples collected in 2007 from Black-crested Bulbul (*Pycnonotus melanicterus*), Crow-billed Drongo (*Dicrurus annectans*), Buff-breasted Babbler (*Pellorneum tickelli*), and Black-browed Fulvetta (*Alcippe grotei*) were antibody positive for the H5 subtype. Fourteen of 193 samples collected in 2008 were positive for the influenza A viral M gene by real-time reverse transcriptase-polymerase chain reaction. These included samples from 10 Japanese White-eyes (*Zosterops japonicus*), two Puff-throated Bulbuls (*Alophoixus pallidus*), one White-tailed Robin (*Cinclidium leucurum*), and one Striped Tit-babbler (*Macronous gularis*). Almost all positive samples were from bird species that forage in flocks, including Japanese White-eyes with an unusually high prevalence of 14.9%. We collected samples from birds from three habitat types but detected no strong pattern in prevalence. Our results suggest that attention should be given to terrestrial species, particularly flocking passerines, in AIV surveillance and monitoring programs.

Key words: avian influenza virus (AIV), Japanese White-eye, Passeriformes, Vietnam.

Recent outbreaks of disease with highly pathogenic avian influenza (HPAI) viruses of the H5N1 suptype in domestic poultry and humans have drawn attention to the potential role of wild birds in transmission and maintenance of these pathogens and have motivated new surveillance efforts for avian influenza viruses (AIVs) in wild bird populations (e.g., Gaidet et al., 2007). While most affected wild bird species inhabit wetlands or aquatic habitats (Olsen et al., 2006; Stallknecht and Brown, 2007), HPAI H5N1 has been isolated from wild-caught passerines including live Eurasian Tree Sparrows in China (Kou et al., 2005) and a dead one in Hong Kong (Ellis et al., 2004). A recent HPAI H5N1 isolate from a tree sparrow linked to limited transmission among humans in China (Liu et al., 2010) and a relatively high prevalence of AIVs in passerines in the USA (Fuller et al., 2010) also motivate a closer look at the occurrence of AIVs in wild-caught passerines. Little information is available about the occurrence of AIVs in wild-caught birds in Southeast Asia, including Vietnam, an area experiencing a relatively high incidence of outbreaks in humans and domestic poultry (Alexander, 2007; Hien et al., 2009; Brown, 2010). Our study focused on surveillance for the presence of influenza A virus nucleic acids and antibodies for AIVs in wild-caught forest-dwelling birds in northern Vietnam.

The research was conducted in and near Cuc Phuong National Park (CPNP; 20°14′–20°24′N; 105°29′–105°44′E) and Tam Dao National Park (TDNP; 21°21′–21°42′N; 105°23′–105°44′E) in northern Vietnam. Birds were captured using mist nets in plots at each of three habitat types at the two National Parks: forest interior, forest edge, and human-dominated landscapes. Details of the dominant tree species and spatial relationships of the plots are described elsewhere (Vu, 2009). Sample sizes were determined by time available in the field, laboratory processing capacity, and access permission. Up to 10 μl of blood/g of body mass was collected via jugular venipuncture from all birds weighing >12 g.

In 2007 blood was collected from 197 birds of 45 species representing 15 families

TABLE 1. Avian species, number of positive birds, total sample size, and hemagglutinin subtypes for avian influenza (AI) virus antibody-positive birds sampled in Cuc Phuong National Park, northern Vietnam, June and July 2007.[a]

Species sampled by family	No. positive	Sample size	Subtype(s)
Corvidae			
Dicrurus annectans	1	8	H5
Pycnonotidae			
Pycnonotus jocosus	1	16	H9
Pycnonotus melanicterus	1	8	H5
Sylviidae			
Alcippe grotei	1	16	H5
Pellorneum tickelli	2	14	H5, H6
Total	6	62[b]	H5, H6, H9

[a] The following taxa (n) were tested and found antibody negative for all AI subtypes tested: Alcedinidae, *Alcedo atthis* (11); Chloropseidae, *Chloropsis cochinensis* (2); Columbidae, *Chalcophaps indica* (1); Corvidae, *Aegithina tiphia* (8), *Cissa hypoleuca* (1), *Crypsirina temia* (1), *Hypothymis azurea* (11), *Hemipus picatus* (1) *Tephrodornis gularis* (6), *Terpsiphone paradise* (2); Laniidae, *Lanius schach* (2); Meropidae, *Nyctyornis athertoni* (1); Muscicapidae, *Cinclidium leucurum* (1), *Copsychus saularis* (6), *Cyornis hainanus* (1), *Ficedula hyperythra* (1), *Niltava macgrigoriae* (1), *Tarsiger cyanurus* (6); Nectrainiidae, *Arachnothera longirostra* (1); Paridae, *Parus major* (2); Passeridae, *Lonchura punctulata* (2), *Passer rutilans* (1); Picidae, *Dendrocopus hyperythrus* (1); Pittidae, *Pitta elliotii* (1); Pycnonotidae, *Alophoixus pallidus* (6), *Iole propinqua* (5), *Pycnonotus aurigaster* (6), *Pycnonotus finlaysoni* (4), *Pycnonotus jocosus* (16), *Pycnonotus sinensis* (1); Sylviidae, *Alcippe rufogularis* (6), *Macronous gularis* (4), *Malacopteron cinereum* (2), *Napothera crispifrons* (1), *Pellornuem ruficeps* (10), *Pomatorhinus hypoleucos* (1), *Pomatorhinus ruficollis* (1), *Stachyris nigriceps* (11), *Stachyris striolata* (2), *Timalia pileata* (3); Trogonidae, *Harpactes erythrocephalus* (1).

[b] The total number of all birds sampled in Cuc Phuong National Park in 2007 was 197.

from five orders (Columbiformes [$n=1$], Coraciiformes [$n=12$], Passeriformes [$n=182$], Piciformes [$n=1$], and Trogoniformes [$n=1$]) in CPNP. The blood was placed in serum separator tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA), and AIV subtype-specific antibodies were detected using the hemagglutination inhibition (HI) test (Pedersen, 2008). Seven HI tests, specific for antibodies against H3, H4, H5, H6, H7, H9, and H11 hemagglutinin subtypes, were run for each sample. The determination of antibodies against other hemagglutinin subtypes was not performed given a limited amount of serum collected from small birds. An HI titer of 8 (corresponding to a 1:8 dilution) or higher was considered positive.

The results of the HI test (Table 1) indicated that serum samples from four passerines including Black-crested Bulbul (*Pycnonotus melanicterus*), Crow-billed Drongo (*Dicrurus annectans*), Buff-breasted Babbler (*Pellorneum tickelli*), and Black-browed Fulvetta (*Alcippe grotei*) were antibody positive for the H5 subtype. Of these, the Buff-breasted Babbler was also H6 antibody positive. Additionally, a Red-whiskered Bulbul (*Pycnonotus jocosus*) was antibody positive for the H9 subtype. None of the samples were positive for H3, H4, H7, or H11 subtypes, and no birds from the other orders were antibody positive.

In 2008 cloacal and oropharyngeal (OP) swabs were collected from 193 wild birds of 24 species, representing 11 families and three orders (Coraciiformes [$n=8$], Passeriformes [$n=181$], and Piciformes [$n=4$]) captured in TDNP. Cloacal swabs were collected from 192 birds; OP swabs were collected from 191. The cloacal and OP swabs from each bird were stored separately in cryogenic vials containing viral transport medium (World Health Organization, 2006) and were processed at the Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Aliquots from samples of the same type and species were pooled (up to five samples together), and the remainder of each original sample was preserved in RNA Later (Ambion, Applied Biosystems, Austin, Texas, USA) and stored at −80 C for future analysis as needed. Aliquots of the pooled samples were assayed by rRT-PCR directed at the conserved viral matrix gene (M gene; Spackman et al., 2002). Samples with Ct values <40 were considered positive. All

TABLE 2. Species, numbers of positive samples, and sample sizes for birds assayed for the influenza A viral matrix gene (M gene) using real-time reverse transcriptase-polymerase chain reaction using cloacal or oropharyngeal (OP) swab samples. Listed are those species for which either a cloacal or oropharyngeal swab sample was positive. Birds were captured and sampled at Tam Dao National Park, Vietnam, in July 2008.[a]

Species sampled by family	Cloacal		OP	
	No. positive	Sample size	No. positive	Sample size
Muscicapidae				
Cinclidium leucurum	0	2	1	2
Pycnonotidae				
Alophoixus pallidus	0	10	2	10
Macronous gularis	0	3	1	3
Zosteropidae				
Zosterops japonicus	1	66	9	67
Total	1	81[b]	13	82[b]

[a] The following taxa (*n* cloacal swabs, *n* OP swabs) were assayed for the viral M gene and found negative: Alcedinidae, *Alcedo atthis* (8, 8); Dicaeidae, *Dicaeum concolor* (3, 3); Muscicapidae, *Copsychus saularis* (1, 1), *Enicurus schistaceus* (2, 2); Nectariniidae, *Aethopyga christinae* (2, 2), *Aethopyga siparaja* (2, 1), *Arachnothera jugularis* (2, 2); Paridae, *Parus major* (15, 15); Passeridae, *Lonchura punctulata* (1, 1); Picidae, *Sasia ochracea* (4, 4); Priniidae, *Prinia rufescens* (2, 1), *Orthotomus sutorius* (10, 10), *Pycnonotus aurigaster* (5, 5), *Pycnonotus finlaysoni* (2, 2), *Pycnonotus jocosus* (24, 24); Sylviidae, *Alcippe morrisonia* (7, 7), *Pellorneum tickelli* (10, 10), *Stachyris nigriceps* (5, 5), *Stachyris striolata* (4, 4), *Yuhina zantholeuca* (2, 2).

[b] The total number of all cloacal swabs tested was 192, and the total number of OP swabs tested was 191.

samples from pools positive for the M gene were inoculated individually into embryonated chicken eggs (Woolcock, 2008). Also, separate aliquots of the pools were passaged through eggs twice. Isolates were subjected to hemagglutination assay to determine ability to hemagglutinate. The presence of influenza A viruses in the isolates at this stage was confirmed by rRT-PCR for the M gene. We attempted to determine the subtypes of these isolates by HI test but were unsuccessful because of low virus concentrations.

Nine OP and one cloacal swab samples collected from 10 Japanese White-eyes (*Zosterops japonicus*) were M gene positive (Table 2); all of these birds were captured in the human-dominated landscape. Additionally, OP swab samples collected from two Puff-throated Bulbuls (*Alophoixus pallidus*) were positive. Following passage through eggs twice, one oropharyngeal swab sample from a White-tailed Robin (*Cinclidium leucurum*) in the forest interior and one oropharyngeal swab sample from a Striped Tit-Babbler (*Macronous gularis*) at the forest edge were positive for the viral M gene by rRT-PCR. In short, 14 swab samples were positive for the viral M gene, for a prevalence of 7.3% where 93% (13 of 14) of the positive samples were OP swabs and the remaining sample was a cloacal swab (Table 2). All positive samples were from passerines.

Serum samples from four birds captured at CPNP had antibodies specific to H5 avian influenza virus subtypes. Whether these antibodies were elicited in response to an HPAI or to an LPAI H5 subtype virus is unknown, but outbreaks of HPAI H5N1 were recorded in domestic poultry in the human-dominated areas surrounding CPNP in the spring of 2007 and in civets kept in CPNP in 2005 (Roberton et al., 2006) and 2008 (Vietnam Department of Animal Health, 2009). While reports of LPAI H5 viruses in passerines are infrequent relative to waterfowl, this is an important order from which isolates of AIVs from wild-caught animals have been made (reviewed by Stallknecht et al., 2007). In either case our results suggest that wild passerines could play a role in circulating AIVs in the environment in northern Vietnam, as the birds move locally or as they share habitat with domestic birds (Fuller et al., 2010).

The proportion of influenza A virus M gene-positive swab samples (7.3%) we observed was higher than the prevalence reported in a recent study in southeastern China (24 of 939 samples or 2.3%),

geographically close to northern Vietnam (Peterson et al., 2008). The use of only intestinal material as samples by Peterson et al. (2008) could explain the difference. Comparing our swab sample types, only 0.5% of cloacal samples were positive for the M gene, much lower than the 6.8% for OP swab samples. Using only cloacal swab samples for processing may lower the chances of detecting a current infection or exposure and may lead to underestimates of the prevalence of either HPAI or LPAI viruses (Parmley et al., 2011). We suggest collecting and processing both types of swab samples for detection of AIVs and, if possible, more comprehensive evaluation of positive samples for specific avian influenza virus strains.

Sociality is thought to enhance pathogen transmission among animals (Côté and Poulin, 1995), in part because transmission is often density-dependent (Anderson and May, 1979). The five birds that were AIV antibody positive were species that, at least occasionally, forage in flocks. Similarly, three of the four species that were M gene-positive forage in flocks. Flocks can include dozens of individuals of single or mixed species during the nonbreeding season (Lee et al., 2005) when birds are likely to be roosting or moving locally together. As with waterfowl (Munster and Fouchier, 2009), flocking behavior in passerines might enhance transmission of pathogens among birds due to frequent social interactions, such as food or water sharing or allogrooming, leading to relatively higher prevalence in more social species.

Among the flocking species in our study, Japanese White-eyes had the highest prevalence of influenza A virus (15%, $n=67$). Given a relatively high AIV prevalence, that they are abundant and easy to capture, and can live in close contact with humans (65 of the 67 in this study were caught in human-dominated landscapes), the Japanese White-eye could be a useful focal species for AIV surveillance or monitoring programs. This species has a broad geographic range and is distributed in most parts of East and Southeast Asia, where many of the recent outbreaks of the HPAI H5N1 virus have been recorded. Using the same focal species could enhance data comparisons about AIVs among regions of Asia as well as serve as a sentinel species for detection of outbreaks.

In conclusion, more attention should be given to wild-caught passerine birds in AIV surveillance and monitoring due to the role these species may play in the circulation of AIVs and the paucity of AIV surveillance data on them. Among passerines, social, flocking species in general, and Japanese White-eyes, in particular, may be effective focal or sentinel species in AIV surveillance or monitoring programs in Southeast Asia. Last, both OP and cloacal swabs should be collected and processed if both HPAI and LPAI virus detection is of interest.

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