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ABSTRACT: Wild bird surveillance for highly pathogenic avian influenza (HPAI) H5N1 virus from 2004 to 2007 in Thailand indicated that the prevalence of infection with avian influenza H5N1 virus in wild birds was low (1.0%, 95% confidence interval [CI]: 0.7–1.2, 60/6,263 pooled samples). However, the annual prevalence varied considerably over this period, with a peak of 2.7% (95% CI: 1.4, 4.1) in 2004. Prevalence dropped to 0.5% (95% CI: 0.3, 0.8]) and 0.6% (95% CI: 0.3, 1.0) in 2005 and 2006, respectively, and then increased to 1.8% (95% CI: 1.0, 2.6) in 2007. During this period, 16 species from 12 families of wild birds tested positive for H5N1 virus infection. All samples from juvenile birds were negative for H5N1 virus, whereas 0.6% (95% CI: 0.4, 0.9) of pooled samples from adult birds were positive. Most positive samples originated from peridomestic resident species. Infected wild bird samples were only found in provinces where poultry outbreaks had occurred. Detection of H5N1 virus infection in wild birds was reported up to 3 yr after eradication of the poultry outbreaks in those provinces. As observed with outbreaks in poultry, the frequencies of H5N1 outbreaks in wild birds were significantly higher in winter. Further understanding of the mechanisms of persistence and ongoing HPAI H5N1 transmission between wild birds and domestic poultry is needed.

Key words: H5N1, highly pathogenic avian influenza, surveillance, wild birds.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 virus causes severe disease and sudden death in avian species. In Thailand, an HPAI H5N1 outbreak was first reported during 2004 followed by five subsequent waves of HPAI H5N1 outbreaks in poultry as reported by the Department of Livestock Development (DLD), Government of Thailand (Thanapongtharm and Noimoh, 2006). These outbreaks affected more than 60 of 73 provinces, resulting in the culling of more than 62 million chickens (Tiensin et al., 2005). On 22 January 2008, a new outbreak in poultry was reported in a single province in Thailand (OIE, 2008).

Because outbreaks of HPAI H5N1 occurred in numerous countries across several continents within a short period, wild birds often were suggested as a source (Feare, 2007; FAO, 2008). Recently, wild bird surveillance programs for HPAI H5N1 have been established in many countries, including Thailand, with the objectives of early detection of HPAI H5N1 viruses in wild bird populations and determining the role of wild birds in transmission. National avian influenza surveillance of wild birds in Thailand has been conducted since 2004, under the authority of the Department of National Parks, Wildlife, and Plant Conservation (DNWPC), Government of Thailand (Photieng and Jamjomroon, 2006). In this study, we report changes in HPAI H5N1 virus prevalence in wild birds compared with patterns of H5N1 HPAI outbreaks in poultry over the collection period 2004–2007.

MATERIALS AND METHODS

Collection of field samples

Wild bird samples were collected through collaboration between the DNWPC and the Monitoring and Surveillance center for Zoonotic diseases in Wildlife and Exotic animals (MoZWE), Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand. Wild birds were caught using baited traps, hand nets, or mist nets, or they were shot by DNWPC staff. Between 2004 and 2005, various wild bird species were caught in different types of habitats in provinces where poultry were or were not affected. During 2006 and 2007, the survey program was targeted to particular areas where poultry outbreaks had occurred either recently or in the past. After live-capture, tracheal (or cloanal) and cloacal swabs were collected; for birds that were shot, tracheal and cloacal swabs were collected and in some cases carcasses also were submitted. Carcasses of birds found dead were submitted by the public via the government veterinary sectors. Individual or pooled (one to four birds from the same species and collected in same time and place) swabs were kept in viral transport media (VTM), which contained 0.5% (w/v) bovine plasma albumin, penicillin G (2×10^6 U/l), streptomycin (200 mg/l), gentamicin (250 mg/l), nystatin (0.5×10^6 U/l, polymyxin B (2×10^6 U/l), ofloxacin (60 mg/l), and sulfamethoxazole (0.2 g/l). All specimens were transported, chilled (at approximately 4 C) using ice boxes and/or mobile refrigerators, and delivered to the MoZWE laboratory within 48 hr.

In total, 6,263 pooled samples representing 15,660 individual wild birds were collected. In 2004, 552 (8.8% of total) samples were tested, representing a combination of individual and pooled samples from 692 birds. In 2005, 2,620 (41.8% of total) samples representing 7,562 birds were tested. In 2006, 2,070 (33.1% of total) samples representing 5,441 birds were tested; and in 2007, 1,021 (16.3% of total) samples representing 1,965 birds were tested. The survey included 50 provinces and more than 223 species of birds. Data for each sample collected were recorded on a field data sheet (either DNWPC or MoZWE forms) and included sampling date, species, age (juvenile, adult, or unknown), health status (no clinical signs, clinical signs, dead, or unknown), type of sample, and location.

Virus isolation and identification

Specimens were submitted to the virology laboratory at the MOZWE, Faculty of Veterinary Science, Mahidol University. If specimens were not processed within 24 hr, they were stored at −80 C. Submitted carcasses were necropsied and tissue samples, including trachea, lungs, brain, liver, spleen, and intestines, collected. Tissues were homogenized in a sterile chilled mortar and pestle with added VTM. The specimens were clarified by centrifugation at 2,500× G at 4 C for 15 min, and the supernatants were collected.

After filtration with a 0.22-μm filter, supernatants from swab and tissue samples were inoculated into Madin-Darby canine kidney (MDCK) cells or 11-day embryonated eggs. For MDCK cultures, 500 μl of sample was inoculated directly onto cells in 25-cm² tissue culture flasks and incubated at 37 C for 2 hr, at which time the supernatant was discarded and 5 ml of TPTK-trypsin medium was added (500 g/ml trypsin in minimal essential medium). Flasks were incubated at 37 C in a 5% CO_2 incubator and assessed for the presence of cytopathic effect daily for 4 days. The remainder of each specimen was stored at −80 C. For virus isolation using embryonic eggs, 200 μl of each sample was injected into the allantoic cavity of 11-day-old embryonated eggs in triplicate. Viability of embryos was monitored daily for 3 days. The infected eggs were chilled at 4 C overnight before allantoic fluids were collected.

Virus was initially identified by hemagglutination assay (HA) according to the World Health Organization (WHO) methodology (WHO, 2008). Briefly, serial twofold dilutions of tissue culture media or allantoic fluid were made in 50 μl of phosphate-buffered saline (PBS) on 96-well U-bottomed plates. To each well, 50 μl of 0.5% (v/v) chicken erythrocytes in PBS was then added. The plates were kept at 4 C for 1 hr, after which the HA titers were determined based on the last dilution showing complete hemagglutination.

Viral RNA was extracted from cell culture supernatants or allantoic fluid using a viral RNA extraction kit (QIAGEN, Valencia, California, USA). The multiplex reverse transcription-polymerase chain reaction (RT-PCR) was used to identify type and subtype of viruses. Using the nucleotide sequence available in the GenBank database, multiple sequence alignment of H5, N1, and M gene were performed using ClustalX, version 1.8 (ftp://ftp-igbmc. u-strasbg.fr/pub/ClustalX). The H5 and N1 primers were selected from conserved regions of 50 known sequences specific for H5N1 influenza A viruses. The M primers were also selected from conserved regions of at least 50 known sequences from influenza A viruses. Viruses were identified as type-A influenza viruses by using RT-PCR with the M gene

specific primer set (forward primer M-65F: 5′-CCGAGATCGCACAGAGACTTGAAGAT 3′, reverse primer M-400R: 5′-GGCAAGTGCAC-CAGCAGAATAACT-3′). Subtype was determined using the H5 specific primer set (forward primer H5-155F: 5′-ACACATGCYCARGACA-TACT-3′, reverse primer H5-699R: 5′-CTYTGRTTYAGTGTTGATGT-3′) and the N1 specific primer set (forward primer N1-1078F: 5′-ATGGTAATGGTGTTTGGATAG-GAAG 3′, reverse primer N1-1352R: 5′-AATGCTGCTCCCACTAGTCCAG-3′).

The RT-PCR was performed using a One-Step RT-PCR kit (QIAGEN) containing the appropriate primer mix. The 5 µl of reaction mixture contained denatured RNA, 10 µl of 5× OneStep RT-PCR buffer (QIAGEN), OneStep RT-PCR enzyme, 10 µl of 10 mM dNTP mix (QIAGEN), and 6 µl of primer mix (1.25 µmol each). RNase-free water was added to a total volume of 50 µl. Amplification of DNA was carried out at 50 C for 30 min and at 95 C for 15 min for reverse transcription followed by 35 cycles of denaturing at 94 C for 45 sec, annealing at 60 C for 45 sec, and extension at 72 C for 1 min. The PCR ended with a final extension step at 72 C for 10 min. The reference strain of influenza H5N1 virus (A/chicken/Thailand/vsmu-3-CBI/2005) was used as a positive control in the multiplex RT-PCR assays.

Size-specific PCR products (335 base pairs [bp] for M, 544 bp for H5, and 274 bp for N1) that were obtained from the multiplex PCR in several field experiments were sequenced to evaluate the specificity of the assay. The known concentration RNA received from previously identified virus (A/chicken/Thailand/vsmu-3-BKK/2004) was prepared for a sensitivity test. Copy number of virus RNAs was calculated by using median tissue culture infective dose values and measured by using *TaqMan* real-time RT-PCR according previously published methods (Ng et al., 2005; WHO, 2008). To perform sensitivity tests, the RNAs were serially diluted 10-fold, ranging from 10^6 to 10 copies/µl. All HA-positive samples were identified and subtyped by using multiplex RT-PCR. For samples that were HA positive but RT-PCR negative for all three targets (M, H5, and N1), attempts were made to detect the Newcastle viruses by using specific primer to RT-PCR (data not shown). For samples that were positive for M gene only, their amplicons were sequenced and nucleotide blasted by using the basic alignment sequence tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These M-positive samples also were subtyped by using

H1-H15 specific primers for RT-PCR reaction (Lee et al., 2001).

Statistical analysis

Both field data records and laboratory results were entered into an Excel, version 2003 (Microsoft, Redmond, Washington) worksheet and kept at MoZWE as the avian influenza wild bird surveillance database. The database was analyzed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The prevalence of avian influenza H5N1 virus isolated from wild bird samples with 95% confidence intervals (CI) was determined, and Pearson's chi-square analysis was used to determine significantly different prevalence results in each field category. However, results from the different capture techniques were amalgamated to determine the final result.

RESULTS

Overall, 60 of 6,263 pooled samples (1.0%, 95% CI: 0.7, 1.2) tested positive for H5N1 virus. The peak annual prevalence was found in the first year of the outbreak and the annual prevalence significantly decreased in the following years ($P<0.0001$). Between 2005 and 2006, the annual prevalence of the virus remained stable but rose significantly in 2007 (chi-square, $P<0.005$). However, these overall annual prevalence contained variation in species. The positive pooled samples collected throughout this period were taken from 16 different wild bird species in 12 families (Table 1), including Rock Pigeon (*Columba livia*), Tree Sparrow (*Passer montanus*), Common Myna (*Acridotheres tristis*), Asian Pied Starling (*Sturnus contra*), Common Koel (*Eudynamys scolopacea*), Black Drongo (*Dicrurus macrocercus*), White-vented Myna (*Acridotheres grandis*), Scaly-breasted Munia (*Lonchura punctulata*), Plain Backed Sparrow (*Passer flaveolus*), unidentified pond heron species, unidentified heron species, unidentified dove species (all residential species), the Kentish Plover (*Charadrius alexandrinus*), Brown-headed Gull (*Larus brunnicephalus*), Asian Open-billed Stork (*Anastomus oscitans*) (all winter visitors), and duck species (both

TABLE 1. Positive wild bird families and their health status in the wild bird surveillance during 2004–2007.

Family	2004				2005				2006				2007				Total samples
	H/UI[a]	Sick/dead	Total sample	% Positive (95% CI)	H/UI	Sick/dead	Total sample	% Positive (95% CI)	H/UI	Sick/dead	Total sample	% Positive (95% CI)	H/UI	Sick/dead	Total sample	% Positive (95% CI)	
Cuculidae	0	0	0	0 (0.0, 0.0)	0	0	0	0 (0.0, 0.0)	0	1	3	33.33 (0.0, 86.7)	0	0	4	0 (0.0, 0.0)	8
Columbidae	1[b]	1	148	1.4 (0.0, 3.2)	2	2	839	0.61 (0.0, 1.2)	3	5	250	3.2 (1.0, 5.4)	2	4	337	2.46 (0.5, 4.4)	1,594
Dicruridae	0	0	1	0 (0.0, 0.0)	0	0	0	0 (0.0, 0.0)	1	0	9	11.11 (0.0, 31.6)	0	0	0	0 (0.0, 0.0)	11
Anatidae	1[b]	0	14	7.14 (0.0, 20.6)	0	0	28	0 (0.0, 0.0)	0	0	22	0 (0.0, 0.0)	0	0	20	0 (0.0, 0.0)	85
Laridae	0	0	3	0 (0.0, 0.0)	1[b]	0	23	4.35 (0.0, 12.7)	0	0	28	0 (0.0, 0.0)	0	0	55	0 (0.0, 0.0)	110
Ardeidae	0	0	98	0 (0.0, 0.0)	0	0	236	0 (0.0, 0.0)	0	1	298	0.34 (0.0, 1.0)	2	0	109	1.83 (0.0, 4.4)	744
Estrildidae	0	0	0	0 (0.0, 0.0)	0	0	31	0 (0.0, 0.0)	0	0	40	0 (0.0, 0.0)	1	0	17	5.88 (0.0, 17.1)	89
Charadriidae	0	0	5	0 (0.0, 0.0)	0	0	1	0 (0.0, 0.0)	1	0	41	2.44 (0.0, 7.2)	0	0	35	0 (0.0, 0.0)	83
Emberizidae	0	0	107	0 (0.0, 0.0)	3	0	566	0.53 (0.0, 1.1)	0	0	37	0 (0.0, 0.0)	3	0	97	3.09 (0.0, 6.5)	813
Sturnidae	0	0	88	0 (0.0, 0.0)	1[b]	0	220	0.45 (0.0, 1.3)	1	0	118	0.85 (0.0, 2.5)	5	0	135	3.7 (0.5, 6.9)	568
Ciconiidae	7[b]	5	45	26.67 (13.7, 39.6)	5	0	547	0.91 (0.1, 1.7)	0	0	345	0 (0.0, 0.0)	0	0	82	0 (0.0, 0.0)	1,036
Unidentified	0	0	0	0 (0.0, 0.0)	0	0	2	0 (0.0, 0.0)	0	0	17	0 (0.0, 0.0)	1	0	2	50 (0.0, 119.3)	22
Others	0	0	43	0 (0.0, 0.0)	0	0	127	0 (0.0, 0.0)	0	0	862	0 (0.0, 0.0)	0	0	128	0 (0.0, 0.0)	1,160
Total	9	6	552	2.72 (1.4, 4.1)	12	2	2620	0.53 (0.3, 0.8)	6	7	2,070	0.63 (0.3, 1.0)	14	4	1,021	1.76 (1.0, 2.6)	

[a] H/UI = healthy appearance/unidentified health status.

[b] Number of pooled wild bird samples with unidentified health status.

residential and winter visitors; Lekagul and Round, 1991). Even though many studies stated that wild waterfowl play a role as natural reservoirs of avian influenza viruses (Stallknecht and Shane, 1988; Munster et al., 2007; Webster et al., 2007), there was no significant difference between H5N1 detection in waterfowl and nonwaterfowl in this study.

Interestingly, there was no significant difference between prevalence of H5N1 detection in waterfowl and nonwaterfowl groups in this survey. All 178 pooled samples from juvenile birds were negative for H5N1 virus, whereas 31 of 4,899 (0.6%) samples from adults were positive (95% CI: 0.4, 0.9). However, there were 1,186 samples with no record of age. Overall, 0.6% (95% CI: 0.4, 0.8) of apparently healthy birds (30/4,897 pooled samples) tested positive, compared with 4.1% for birds sampled that were found dead (19 of 462 pooled samples, 95%CI: 2.3, 5.9). Families of wild birds that tested positive with their recorded health status are shown in Table 1. However, there were 833 samples with unknown health status.

Analysis of the data revealed that samples collected from birds opportunistically found dead were significantly more likely to test positive to H5N1 than samples from apparently healthy birds (chi-square, $P<0.0001$). Tissue samples from carcasses were significantly more likely to be positive for H5N1 (9.9%, 95% CI: 5.9, 13.9) than swabs (0.6%, 95%CI 0.4–0.8, $P<0.0001$). Positive samples were detected from specimens collected from wild birds in 12 of 50 (24%) provinces sampled, including Bangkok, Nakhon Sawan, Phra Nakhon Si Ayutthaya, Kanchanaburi, Nakhon Pathom, Suphan Buri, Chanthaburi, Nakhon Phanom, Ratchaburi, Ang Thong, Samut Prakan, and Buri Ram. Analysis of data in comparison with data on poultry outbreaks showed that, as with the poultry, H5N1 virus was first detected in wild birds in 2004 and that the peak prevalence of both poultry and wild bird outbreaks occurred during this year. Similarly with poultry outbreaks, the frequency of infected wild bird samples increased significantly during winter ($P<0.005$). However, positive wild bird cases were only found in the provinces where domestic poultry outbreaks were reported, and wild bird outbreaks apparently did not spread throughout the country at the rate found with outbreaks in poultry (Fig. 1).

The multiplex RT-PCR products consisted of 335 bp for the M gene, 544 bp for the H5 gene, and 274 bp for the N1 gene and were visualized by gel electrophoresis. Some positive specimens were subjected to nucleotide sequencing (GenBank accession EF178520 and EU716171 [M gene], EF178517 and EF178528 [H5 gene], EF178519 and EF178529 [N1 gene] and BLAST [http://blast.ncbi.nlm.nih.gov/Blast.cgi] search to confirm the M, H5, and N1 gene detection). The sensitivity of the multiplex RT-PCR was determined using 10-fold serial dilutions of known concentrations of RNA of H5N1 virus. The DNA bands were visible at RNA standard dilution as low as 10^3 copies/μl.

DISCUSSION

From the surveillance of wild birds in Thailand from 2004 to 2007, it is apparent that avian influenza H5N1 virus has been detected at a low level in wild bird populations since the first wild bird positive sample was found in February 2004. In this study, the annual prevalence in 2005 and 2006 significantly decreased compared with 2004 and then rose significantly in 2007.

The surveillance program operating during 2004–2006 was a more general survey, with random surveillance over a wider area of the country. In 2007, the surveillance was targeted toward areas that had poultry outbreaks; this targeted approach may explain the increase in prevalence observed in 2007, but prevalence still was lower than that observed in
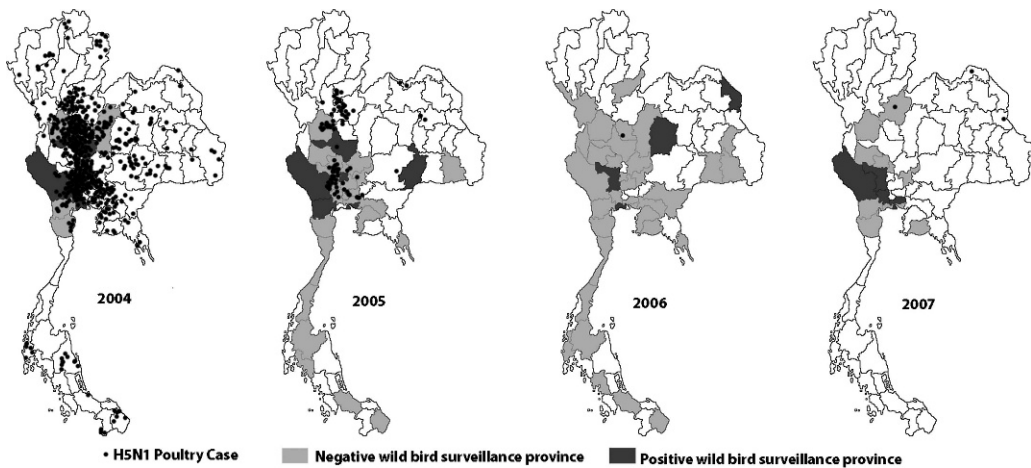
FIGURE 1. Thai provinces where domestic poultry outbreak cases were reported and wild bird surveillance conducted (modified map; Thanapongtharm and Noimoh, 2006).

2004. It should be noted that true prevalence estimates are based on the assumption that only one sample in the pool was positive; however, in this study prevalence (based on number of infected pools) may be overestimated because pooled samples contain between one and four individual bird samples. Our results suggest that spillover of HPAI H5N1 viruses from poultry to wild birds is an important factor. However, it is still not clear whether the virus persists in wild birds in the absence of detectable HPAI H5N1 in domestic birds. Other possible HPAI H5N1 virus sources would include contaminated environments from previous outbreaks and/or subclinical infected domestic poultry; surveillance for HPAI H5N1 in poultry in Thailand is mostly based on detection of clinical signs.

Previous studies have reported that avian influenza viruses are most often isolated from juvenile birds (Stallknecht and Shane, 1988). Stallknecht and Brown (2007) reported that prevalence of avian influenza virus infection in juvenile ducks can exceed 30% in premigrating season. In contrast, in this study all 178 samples from juvenile birds were negative for H5N1, whereas prevalence of samples from adults was 0.6%; however, age data

were not available for 29 positive samples. Some factors that could have contributed to this result include inaccurate age classification, insufficient samples of juveniles for specific species, insufficient age distribution at the point of sampling and location, and variations in age and species susceptibility (specifically related to population immunity). In addition, there may have been some bias in the current study, because sampling of wild species was mainly done at feeding areas where immature birds are less common. However, if species interaction is a factor in the transmission pathway for wild species, immature animals may have less time and spatial chance (in terms of movement from their nesting sites) to be infected. In addition, immature birds are more susceptible to HPAI H5N1 (Pantin-Jackwood et al., 2007) and may have been more likely to die after infection.

In our surveillance, 4.1% of 462 found dead birds were infected with H5N1 virus. However, wild bird carcasses are difficult to detect in the wild; Wobeser and Wobeser (1992) found that 70% of bird carcasses were removed by natural causes within 24 hr. In addition, Brown et al. (2008) stated that HPAI-infected wild birds can shed the virus before and after

symptomatic onset. It is likely that different bird species have varying susceptibility to HPAI H5N1 infection and therefore some wild bird species could be expected to be more resistant to this disease (Boon et al., 2007). Some apparently healthy wild birds were also positive for H5N1 virus in this study. Overall, 50% (30/60) of the positive samples were collected from apparently healthy birds, 32% (19/60) from dead birds, and the health status of the remainder (11/60) was not reported.

One of main transmission pathways for waterfowl is the fecal-oral route via contaminated water (Brown et al., 2007). It has been demonstrated that avian influenza viruses can persist in water and remain infective for extended durations at temperatures that are compatible with field conditions (28 C and 17 C; Stallknecht et al., 1990). Thus, contamination and persistency of the viruses in environment may play important roles in the disease transmission. Additional studies on species susceptibility, virus persistence, and duration and level of virus shedding are required to understand the pattern of H5N1 virus circulation in wild bird populations.

Existing surveillance data for avian influenza outbreaks in poultry in Thailand provided via a collaboration between MoZWE and DLD, the DLD website (http://www.dld.go.th/home/bird_flu/birdflu.html), and the OIE website (http://www.oie.int/downld/AVIAN%20INFLUENZA/A_AI-Asia.htm) were reviewed, and results were compared with our wild bird surveillance data. Outbreaks of HPAI H5N1 in wild birds were first detected in 2004 as well as in domesticated poultry (Tiensin et al., 2007). In this survey, only 12 provinces of 50 had positive wild birds found, whereas poultry outbreaks were found in 60 of the 73 provinces throughout Thailand (Tiensin et al., 2005). Thus, the outbreaks in the wild birds do not seem to have spread widely through out the country. Unlike the general pattern of outbreaks in poultry where the disease occurred with higher frequency in the central provinces due to the high density of rice fields and paddling ducks (Gilbert et al., 2006), outbreaks in wild birds were only found in those provinces where domestic poultry outbreaks were reported.

Poultry outbreaks increased significantly during winter (from November to February) compared with summer (from March to May) and the rainy (from June to October) season (Thanapongtharm and Noimoh, 2006); this temporal pattern also was similar to the seasonal frequency of positive wild bird samples detected in this study. Many factors may be involved in this spread, not only through the movement of wild bird species but also through the movement of humans, domestic poultry, poultry products, farm waste and poultry feed. Understanding the interaction of all of these transmission pathways in the epidemiology of H5N1 avian influenza will contribute substantially to the long-term control of H5N1virus.

In summary, outbreaks of HPAI in wild bird populations in Thailand occurred subsequent to outbreaks in domestic poultry. There was a decrease in the number of infected wild birds between 2004 and 2006; however, the prevalence increased in 2007, which may be associated with targeted surveillance. The infected wild bird species shared habitat and feeding areas with humans and/or domesticated poultry. Based on detection of virus in healthy birds it is possible that some wild bird species may be less susceptible to HPAI H5N1 viruses. In Thailand, the movement of wild bird species is considered to be of lower risk than movements of poultry in the spread of HPAI, but wild birds may play a role in the local persistence and transmission of the virus. Therefore, it is important to conduct additional studies to more fully understand the pattern of viral transmission in wild bird populations, contamination and persistence of the virus in environment, and the relationships between species and factors involved in the spread of HPAI H5N1.

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