Variation in the Responses of Wild Species of Duck, Gull, and Wader to Inoculation with a Wild-Bird-Origin H6N2 Low Pathogenicity Avian Influenza Virus

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Summary. There is poor understanding of host responses to avian influenza virus (AIV) infection in wild birds, with most experimental studies using captive-bred birds and highly pathogenic AIVs that have an early endpoint. The objective of this study was to experimentally assess antibody responses and patterns of viral excretion in wild birds challenged with a low pathogenicity AIV. Ruddy turnstomes (Arenaria interpre), silver gulls (Chroicocephalus novaehollandiae), and wandering whistling ducks (Dendrocygna arcuata) were challenged with a H6N2 virus, and blood, cloacal, and oropharyngeal (OP) swabs were analyzed from each bird over 28 days, with serology conducted on the ducks for a further 7 mo. Nineteen of 22 birds showed evidence of infection, with respiratory infection prevalent in the turnstomes and gulls as mostly low titer viral excretion to 4 days postinoculation (DPI) with gastrointestinal replication detected in only one turnstone. In AIV naive ducks, there was gastrointestinal tropism with moderately high titer viral excretion via the cloaca to 6 DPI and low-grade OP viral excretion to 4 DPI. The hemagglutination inhibition antibody response was poor in the ducks, declining from 19 to 56 DPI, with higher titer responses in the gulls and turnstomes. All infected birds responded with elevated nucleoprotein antibodies (in competitive enzyme-linked immunosorbent assay) by 7–10 DPI, and in the ducks these waned slowly after 42 DPI and were long-lived to at least 8 mo. The interspecies variability in response was consistent with a subtype that had adapted well in ducks, while the response of the turnstomes may have been influenced by preexisting immunity to AIV. These findings provide insight into AIV infection dynamics in wild birds and highlight the need for further research.

Key words: antibody, avian influenza virus, inoculation, viral excretion, wild birds

Abbreviations: AAHL = Australian Animal Health Laboratory; AD = average deviation; AIV = avian influenza virus; c-ELISA = competitive enzyme-linked immunosorbent assay; CG = cycle threshold; DAFWA = Department of Food and Agriculture WA; DPI = days postinoculation; ECE = embryonating chicken egg; EID50 = 50% egg infectious dose; GMT = geometric mean titer; H/HA = hemagglutinin; HI = hemagglutination inhibition; HPAI = highly pathogenic avian influenza; ID50 = 50% infectious dose; LP = low pathogenicity; N = neuraminidase; NP = nucleoprotein; OP = oropharyngeal; RRT-PCR = real-time reverse transcription polymerase chain reaction; VI = virus isolation; WA = Western Australia

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Since 2003, the epidemiology of highly pathogenic avian influenza (HPAI) H5N1 has changed significantly with transmission of virus from poultry to wild birds, respiratory tropism, and pathogenicity in wild bird hosts that are normally considered resistant to infection (22). The pathobiology and host responses of wild birds and poultry to challenge with HPAI H5N1 have therefore been the focus of a number of studies. However, most of these studies have an early end point with a narrow timeframe for assessment of the host responses to infection or are focused on...
assessing protective immunity following vaccination. Hence there is a poor understanding of the responses to avian influenza virus (AIV) infection by the wild bird humoral and cellular immune system and how this might be influenced by a multitude of interwoven factors such as virus subtype, virus adaptation, host life status, concurrent infections, and stressors. Responses by the immune system in wild birds are further complicated by the likelihood of multiple low pathogenicity AIV (LPAIV) challenges, and whether the acquired immunity from these exposures influences subsequent responses to further challenges. Furthermore in wild birds, mounting an immune response to AIV infection might come at a significant biological cost, potentially compromising functional activities such as migration and breeding (19, 21).

There are also many other unexplained immunological factors that could influence the epidemiology of AIV in wild birds that require further investigation. The influence of long-lasting nucleoprotein (NP) antibodies found in naturally infected mallards (Anas platyrhynchos) on immunity is unclear (1), and whether this is a factor in the phenomenon of cyclic periodicity of LPAIV infection, observed in some waterfowl populations, warrants further study. In their natural avian hosts, LPAIV primarily causes an innocuous, localized infection with intestinal tropism and fecal excretion of virus. In this study we sought to better understand host responses and infection dynamics following experimental challenge with an AIV in wild species of duck, gull, and wader that make up the three bird groups primarily sampled in AIV surveillance studies.

MATERIALS AND METHODS

Wild birds in captivity. To minimize the welfare concerns of captivity and transport, suitable species were selected that were likely to readily adapt to captivity and could be caught locally. Of the Charadriiformes, the ruddy turnstone ( Arenaria interpres) has been successfully kept in captivity (12), and the silver gull ( Chroicocephalus novaehollandiae) has adapted to human activity in a commensal relationship. Ruddy turnstones (n = 6), aged as 1 yr old by an ornithologist, and silver gulls (n = 9), aged as immature except for one adult, were caught at Broome, Western Australia (WA: 18° S, 122° E). Seven wandering whistling ducks ( Dendrocygna arcuata) raised as orphans locally and well adapted to captivity were aged as 5 mo old at the start of the trial. At a Broome wildlife care facility, each species was caged separately, provided water and food ad libitum, and allowed to adapt to captivity. The trial was approved by the WA Department of Conservation and Environment, Murdoch University Animal Ethics Committee, and the Chief Veterinary Officer of the Department of Food and Agriculture WA (DAFWA).

Experimental design. Prior to inoculation, blood, oropharyngeal (OP), and cloacal swabs were collected from each bird to test for any preexisting exposure to an AIV subtype and to provide experimental negative control data. The inoculum, a low passage LPAIV propagated in embryonating chicken eggs (ECEs) from the original stock of A/ Eurasian coot/WA/2727/79/H6N2, had an infectivity titer of 10^6.95 50% egg infectious dose (EID50)/0.1 ml, as calculated using the Spearman-Kärber method (20) in 9–11-day-old ECEs. Each treated wild bird was administered a 1 ml dose of inoculum equivalent to an infectivity titer of 10^6.75 EID50, divided into 0.5 ml by the OP route, 0.15 ml in each nares, and 0.1 ml in each conjunctiva. One bird of each species was left as an untreated in-contact control. Cloacal and OP swabs were collected from each bird every second day postinoculation (DPI) until 16 DPI and thereafter every 3 days in the ducks, with a final sample taken from all birds at 28 DPI. Sterile wooden and aluminum cotton-tipped swabs (Eurotubo, Deltalab, Rubi, Spain) were used for the ducks and gulls and turnstones, respectively. All swabs were collected and stored at −170°C as individual samples in 1 ml vials of viral transport media that consisted of Hanks’ balanced salts, amino acids supplement solution, and 0.035% NaHCO3, 0.1% yeast extract, and 0.5% lactalbumin hydrolysate (Difco Labs, Becton, Dickinson and Company, Baltimore, MD), 2000 IU penicillin G, 5000 μg streptomycin, and 112 μg amphotericin B (Sigma-Aldrich. St. Louis, MO). A blood sample was collected from each bird every 3 days from 7 to 28 DPI to determine the persistence of NP c-ELISA antibodies in the ducks, testing continued at variable intervals (range 14–35 days) until their release at 8 mo postinoculation. Samples taken from the right jugular vein in the turnstomes and gulls (0.4 and 1 ml, respectively) and from the medial metatarsal vein in the ducks (1 ml) were transferred into 1.1 ml gel microtubes (Sarstedt AG and Co., Nümbrecht, Germany). Following clot retraction at ambient temperature, tubes were centrifuged (Tony Seiko Capsule HF-120 microcentrifuge, Tokyo, Japan) for 5 min at 2000 x g at 20°C until analysis. The body weight and welfare of the birds were closely monitored during the trial.

Serology. All sera were tested at DAFWA with a NP competitive enzyme-linked immunosorbent assay (NP c-ELISA) targeting influenza virus A group–specific NP antibodies using reagents supplied by the Australian Animal Health Laboratory (AAHL) and based on standard methods (18). At DAFWA, optical densities were read at 450 nm (Multiskan EX Microplate Photometer, Thermo Labsystems, China), with test serum results calculated as the percentage inhibition of binding of the monoclonal antibody in the absence of any serum. Sera with >60% inhibition were interpreted as positive, 40%–60% as equivocal, and <40% as negative.

The hemagglutination inhibition assay (HI) was performed with 4 HAU/25 μl of the live homologous H6N2 antigen after pretreatment of sera with 10% chicken red blood cells, consistent with standard methods (15). Any sera with NP c-ELISA results suggestive of prior exposure to AIV were tested by HI against other HA subtypes available at AAHL including H1, 2, 3, 4, 5, 7, 8, 9, 10, 12, 13, and 15. The HI titer was expressed as the reciprocal of the highest dilution that caused complete inhibition of agglutination. A HI titer of ≥16 is considered a positive result in chickens under OIE guidelines (15).

Virology. The swabs were tested individually for the presence of influenza A RNA by Taqman Type A real-time reverse transcription polymerase chain reaction (RRT-PCR) according to the AAHL method of Heine and Trinidad (4). At DAFWA, minor variations included extraction of RNA from 100 μl samples using a Magmax 96 viral isolation kit (Ambion Inc., Austin, TX) on a Magmax Express magnetic particle processor (Biosystems, Life Technologies, Corp., Mulgrave, Australia) according to the manufacturer’s instructions. A cycle threshold (Ct) value < 37 was considered positive with Cc ≥ 37–40 indeterminate and Cc ≥ 40 negative. All swabs from the ducks were tested, with the number of gull and turnstone swabs analyzed determined by the progressive results from RRT-PCR testing. The results of RRT-PCR testing logged the titrations of extracted RNA from 100 μl of the inoculum were used to produce a standard curve. Near virus stock (10^6.95 EID50/0.1 ml) gave a Ct value of 20, and the limit of detection by RRT-PCR was a 10^−6 dilution with a Ct value of 39.2, equivalent to an estimated 10^6.92 EID50/0.1 ml. Individual swab viral titers for each positive Ct value were then extrapolated from the standard curve, providing an estimate of excretion based on the detection of amplicons that can include live and inactivated viral material.

The presence of viable virus was confirmed by standard ECE virus isolation (VI) methods (15) with minor variations, including the use of eggs from a commercial hatchery in a region free of AIV infection and where NDV and AIV vaccination is not used, and at each passage, inoculation of a 0.2 ml sample dose into each of two eggs. The prechallenge negative controls were tested in pools of three by species, and postchallenge, all samples with Ct values < 40 and 140 Ct negative samples were individually tested. A subsample of HA positive samples were confirmed as H6 virus by HI testing with 4 HAU of virus against anti-H6 reference serum.

Statistical methods. Descriptive analysis of continuous data with mean, median, average deviation (AD), SD, statistical differences between means, and assessment of data by Spearman’s correlation coefficient (r) were all analyzed in either Microsoft Excel (2007) or
RESULTS

Prechallenge. All birds appeared to be clinically healthy and were negative for AIV by RRT-PCR and VI. When the sera were tested by NP c-ELISA, only the turnstones (6/6; median of 57% inhibition) had evidence of past exposure to AIV. All sera tested negative for H6 antibodies by HI, and the six turnstone sera also tested negative for HI antibodies to other available HA subtypes at AAHL.

Postchallenge. Birds were considered to be infected if they had evidence of excreted viral RNA, or seroconversion with increasing levels of NP or HI antibodies in sequential serum samples. Using these criteria, 19 of 22 birds were considered to be infected postchallenge including one in-contact duck. The data for the three uninfected birds (two gulls and one turnstone) were excluded from statistical analysis. All birds appeared clinically healthy during the month after inoculation with most birds gaining weight.

By 7 DPI, all infected birds were positive by NP c-ELISA with median values significantly elevated ($P < 0.001$) and at their highest level (>80% inhibition) in all three species (Fig. 1). In the ducks, the high median c-ELISA value was sustained to 28 DPI and thereafter waned slowly (Fig. 1) with all ducks still positive by 56 DPI. At 8 mo postinoculation the ducks had a median NP c-ELISA of 57% inhibition, just below the positive cutoff threshold. In the gulls after 10 DPI, the median NP c-ELISA antibody level tapered off more rapidly than the ducks and turnstones and was at 51% inhibition by 28 DPI (Fig. 1). At this point, two gulls were at prechallenge values (<40%), and two gulls had high values (77%, 78%). In the turnstones, the median NP c-ELISA antibody value declined more slowly compared to the gulls and by 28 DPI was at 76% inhibition.

For all species, mean HI titers peaked later (around 9–12 days later in the ducks and 3 days later in the other species) than the NP c-ELISA values, which peaked between 7 and 10 DPI (Fig. 1). Moreover, differences in HI antibody responses (Fig. 1) and the proportion test positive by DPI (Fig. 2) were found between the three species. In the ducks, six of seven birds had positive HI titers ($\geq$16) with the highest reciprocal titer of 32 detected in only one bird, which persisted for approximately 6 days. By 28 DPI, four ducks still had positive HI titers, and by 42 DPI, no ducks had positive HI titers. From 42 DPI to 6 mo postinoculation three ducks had HI titers of eight with the other ducks having lower or no detectable titers. After this, HI titers of eight were detected in two other ducks that may have been related to an unexplained spike in NP c-ELISA values at 6 mo.

In the gulls, all NP c-ELISA-positive gulls had a positive HI antibody test result at least once postchallenge. Mean HI titers were elevated earlier (7 DPI), peaking markedly higher than the ducks and turnstones at 10–13 DPI (Fig. 1), which was related to higher HI values (titers of 64–128) detected in three gulls. The gulls with higher titers also showed longer persistence of HI antibody with consistently positive HI titers in two gulls; however, by 28 DPI, the mean HI titer was lower in the gulls compared to the ducks (Fig. 1). In the turnstones, the overall low mean HI titer relates to the variable individual responses with little or no HI antibody response in 3 of 5 birds, even though these birds had high positive NP c-ELISA antibody responses. In the two HI positive turnstones, a moderate HI titer of 64 was detected at 10–13 DPI; however, this level was short-lived, and by 28 DPI, only one of these birds had a HI titer of eight.

To determine the degree of Spearman’s correlation ($r_s$) between NP and HI antibody responses, individual birds with HI reciprocal titers $\geq$8 were analyzed, with statistical correlation found only in two ducks and in two gulls.

Respiratory and intestinal viral shedding. The intestinal tract was the primary site for H6N2 replication in the ducks, with virus
detected from one or more of the cloacal swabs from six of seven (85.7%) ducks. Cloacal viral excretion was prevalent in the ducks from 2 to 6 DPI, peaking at 4 DPI with an estimated median viral titer of $10^{4.58}$ EID$_{50}$/0.1 ml swab (Fig. 3) and highest individual viral titer of $10^{4.86}$ EID$_{50}$/0.1 ml swab. There were no confirmed virus isolations from the cloaca after 8 DPI, and the few RRT-PCR tests with high $C_t$ values probably represent residual RNA from neutralized virus.

The oropharynx was also a site for viral replication in the ducks, but compared to the intestinal tract, this was transient with low viral titers. Virus was isolated from one or more of the OP swabs from 5 of 7 ducks at 2 DPI and 2 of 7 ducks at 4 DPI; however, no virus was isolated after 4 DPI (Fig. 3). The titers were low, with the highest median viral titer of $10^{1.69}$ EID$_{50}$/0.1 ml swab at 4 DPI. The recovery of virus at 2 and 4 DPI from the oropharynx of the untreated duck shows that AIV infection was readily acquired either directly or indirectly from the challenged birds. Indeterminate RRT-PCR results were also detected at 28 DPI in two ducks; however, these samples were negative when retested after one freeze-thaw cycle. Virus could not be isolated from cloacal samples by 10 DPI and from OP samples by 6 DPI, with significant differences in estimated median duration of viral shedding from the cloaca (6 days) compared to the oropharynx (2 days). The viral load, estimated as median viral titer in the swab, was at all times higher from the cloaca than from the oropharynx (Fig. 3).

In the gulls there was no evidence of viral shedding based on RRT-PCR testing of cloacal swabs ($n = 52$) to 12 DPI and from virus isolation ($n = 27$) to 6 DPI. Conversely, respiratory replication was prevalent but of short duration, with no evidence of viral shedding after 4 DPI (Fig. 4). At 2–4 DPI, estimated median viral titers were higher from the oropharynx of the gulls compared with those from the oropharynx of the ducks and the turnstones (Figs. 3 and 4). At 2 DPI this was attributable to the high viral titer from one gull of $10^{3.26}$ EID$_{50}$/0.1 ml swab. The isolation of virus and high viral titers show that the H6N2 virus replicated in the oropharynx of some gulls, and that the results were not from any residual virus following inoculation.

In the turnstones, virus was detected from the cloaca of one bird at 10 DPI, with an estimated titer of $10^{3.14}$ EID$_{50}$/0.1 ml swab. There was no evidence of viral shedding before or after this sample point, and this bird had a concurrently raised HI value (titer of 64 at 13 DPI). As found in the gulls, shedding of viral material in the turnstones was more prevalent from the oropharynx than from the cloaca, with all birds positive by RRT-PCR at 2 DPI, although no viable virus was isolated (Fig. 4). As found in the other species, excretion of viral material from the oropharynx was transient and low-titer, indicating low-grade infection.

**DISCUSSION**

In this study, the host responses to LPAIV challenge in three wild bird species were measured and compared by serological and virological testing. This was the first time this had been undertaken in these three species at the one time. Given the logistics and limitations of this study, a number of host-virus factors may have influenced the response to the virus challenge in the different species. For instance, 1) the birds were at different stages of adjustment to captivity, namely, the gulls and turnstones were wild caught with less time to adapt, whereas the ducks were raised as orphans in captivity; 2) the birds had different AIV immune status, since the turnstones had serological evidence of previous exposure to an AIV and their immune system may have limited viral replication, whereas the ducks were AIV naive and at a different stage of maturity; 3) a high infectivity dose of inoculum was used to provide a sufficient challenge to the gulls and turnstones since the H6N2 virus was likely to be better adapted in ducks given the prevalence of H6 subtypes found in waterfowl (14). Furthermore, there was a lack of data on
the infectious dose (ID<sub>50</sub>) required in shorebirds at the time of the trial, though a recent study in ruddy turnstones suggests an ID<sub>50</sub> for LPAIV of at least 10<sup>3.6</sup> EID<sub>50</sub>/0.1 ml (2).

Following inoculation with H6N2 virus, infected birds had responses consistent with an LPAIV including localized viral replication and asymptomatic infection. Marked elevation in NP c-ELISA antibody levels within 14 DPI was consistent with other studies in domestic ducks (6,17) and ruddy turnstones (2). In this study, median NP c-ELISA values were maintained at high levels (>80% inhibition) to 42 DPI in the ducks, and this finding may be a useful diagnostic guide for determining the time since primary AIV infection in naive ducks. A transient spike in NP c-ELISA values in most ducks at 6 mo postinoculation could not be explained, but may have been associated with infection by another non-H6 subtype, or an unusual immune response as the ducks reached sexual maturity. By 8 mo postinoculation the median NP c-ELISA value was just below the positive cutoff for this test, suggesting that NP antibodies are relatively long-lived in ducks, consistent with other studies (1).

Antibodies to H6 HA were not detected in all infected birds, however; where detected, significant HI titers followed elevated NP c-ELISA levels by approximately 3 days in the gulls and turnstones and 9–12 days in the ducks. Diagnostic HI titers (≥16) were detected in 6 of 7 ducks from 10 to 19 DPI; however, by 42 DPI no ducks were positive by HI. The HI titers were consistently low, and only one duck had a higher value (titer of 32) of short duration. These results concur with other reports that conclude ducks have weak and short-lived HI antibody responses (9) and would explain why there was poor Spearman’s correlation between NP and HI antibody responses in infected ducks. It has been suggested that the weak HI antibody response of ducks may be associated with higher survival rates of AIV in waterfowl that favor the perpetuation and ecology of AIV in these hosts (11).

The results of the duck serology in the present study indicate a sharp decline in HI antibodies between 19 and 56 DPI and a gradual decline in NP antibodies after 42 DPI. The decline in antibodies around this time has been previously shown to have a bearing on the immune response in domestic ducks with resistance to secondary infection at 28 DPI, and infection with higher (anamnestic) HI titers and no viral shedding at 46 DPI, suggesting that a secondary immune mechanism rapidly shut down infection (9). Other studies have assessed the influence of cross-protective immunity in mallards (1,8); however, few, if any studies have included wild birds. Further research is required to determine whether protective immunity to AIV in wild birds wanes around 28–46 days after primary infection. The findings from extensive longitudinal wild bird surveillance studies of ducks and shorebirds in North America show that the prevalence of infection with several subtypes is cyclical, with a 2–3–year periodicity (10). Whether this cyclical phenomenon is due to long-lived NP antibodies as reported in this and other studies (1), and primed HI immunity that result from waves of infection in these populations, conferring immunity against reinfection for 12 mo or more, requires further research.

In this study, the features of experimental H6N2 infection were consistent with that expected of LPAIV infection in ducks with prominent intestinal tropism and replication of short duration (9). Cloacal shedding of virus at moderate levels was detected to 6 DPI in 6 of 7 ducks; however, infectious virus was not detected after 8 DPI. Viral replication was also detected at low levels to 4 DPI in the oropharynx of 6 of 7 ducks, including the one duck negative by cloacal sampling.

In contrast to the ducks, there were significant early HI and NP antibody responses detected in infected gulls around 7–10 DPI, and HI titers were the highest (titer of 128) detected in this study. However, the HI antibody responses were short lived, and by 28 DPI most gulls had low HI titers. Viral shedding in the gulls was detected only from respiratory samples and, with the exception of one gull that had a high viral titer of 10<sup>6.28</sup> EID<sub>50</sub>/0.1 ml swab at 2 DPI, was mostly at low levels of excretion for a short duration (2–4 days). These findings suggest that the gulls were susceptible to infection from a subtype not adapted to persist in this host, and that humoral or cell-mediated responses rapidly cleared the infection.

The life history of the turnstones used in this study was not known, though prechallenge NP c-ELISA results indicate these birds had been previously exposed to AIV. After virus challenge, these birds had rapid elevation of NP c-ELISA levels that persisted at moderately high levels to 28 DPI. Only two turnstones had moderate anti-H6 antibody responses (HI titers of 64), which would have been expected to effectively shut down virus infection. The turnstones showed RRT-PCR evidence of low titer OP virus replication for 2–4 days, though viable virus was not detected. Interestingly, one bird had moderate viral excretion from the cloaca (estimated 10<sup>5.17</sup> EID<sub>50</sub>/0.1 ml swab at 10 DPI; previous C<sub>r</sub> result of 38 at 2 DPI); however, this did not persist to 13 DPI when this bird had a H6 HI antibody titer of 64. The lack of HI antibody response in most of the turnstones and limited OP replication suggests that this virus did not initiate a
significant infection. Possibly their immune systems had been primed, as evident from the preexisting NP c-ELISA antibodies, and if that was the case, existing cell-mediated and humoral immune responses may have limited viral replication postchallenge. This was not able to be investigated further in this study.

Although cloacal shedding featured prominently in the ducks and would constitute an important mode of virus transmission in the aquatic environment, overall 17 of 19 infected birds shed viral particles from the oropharynx at least once postinoculation. Other authors have found prominent respiratory shedding of LPAIV in wild birds, suggesting an important role for respiratory replication and transmission in wild birds (2,7). In this study H6N2-challenged ducks had low viral titers and short duration of shedding (10^{3.36–1.69} EID_{50}/0.1 ml swab from 2–4 DPI) via the oropharynx, suggestive of a lesser role for respiratory shedding than cloacal shedding. Further study is needed to determine the conditions under which respiratory replication and shedding could be important in LPAIV transmission.

The immune system of wild birds has been poorly studied, and the ruddy turnstone is suggested as a suitable, interesting candidate for further AIV infection studies of the avian immune system. This species has all the attributes that correlate with a strong immune system such as longevity and low reproductive rates (12) and has scavenging behaviors that expose it to more pathogens or immunogens (3). Whether this immunocompetence compromises other physiological processes important to this species, such as transequatorial migration and breeding, is poorly understood (21).

The findings from this study show the interspecies variability in host responses to infection with a HA subtype, which suggests this virus was well adapted to ducks. In the gull and turnstone hosts, virus replication was more prominent from the respiratory tract, and this may have elicited a stronger anti-HA immune response in an attempt to shut down infection. Furthermore, in the turnstones, preexisting immunity to AIV may have also influenced the immune response. Whether gastrointestinal viral replication is an indicator of adaptation in the host, and conversely, whether respiratory replication stimulates a greater host immune response, should be investigated further. Overall, these findings are consistent with other studies, where subtypes well adapted in one host behave differently in incidental hosts (6,13,16). This study provides further understanding of AIV infection dynamics in wild birds and suggests the need for further research in the aspects referred to above.

REFERENCES


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