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SUSCEPTIBILITY OF WOOD DUCKS TO H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS

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ABSTRACT: Since 2002, H5N1 highly pathogenic avian influenza (HPAI) viruses have caused mortality in numerous species of wild birds; this is atypical for avian influenza virus (AIV) infections in these avian species, especially for species within the order Anseriformes. Although these infections document the susceptibility of wild birds to H5N1 HPAI viruses and the spillover of these viruses from infected domestic birds to wild birds, it is unknown whether H5N1 HPAI viruses can persist in free-living avian populations. In a previous study, we established that wood ducks (*Aix sponsa*) are highly susceptible to infection with H5N1 HPAI viruses. To quantify this susceptibility and further evaluate the likelihood of H5N1 HPAI viral maintenance in a wild bird population, we determined the concentration of virus required to produce infection in wood ducks. To accomplish this, 25 wood ducks were inoculated intranasally at 12–16 wk of age with decreasing concentrations of a H5N1 HPAI virus (A/Whooper Swan/Mongolia/244/05 [H5N1]). The median infectious dose and the lethal dose of H5N1 HPAI virus in wood ducks were very low ($10^{0.95}$ and $10^{1.71}$ median embryo infectious dose [EID_{50}]/ml, respectively) and less than that of chickens ($10^{2.80}$ and $10^{2.80}$ EID_{50}/ml). These results confirm that wood ducks are highly susceptible to infection with H5N1 HPAI virus. The data from this study, combined with what is known experimentally about H5N1 HPAI virus infection in wood ducks and viral persistence in aquatic environments, suggest that the wood duck would represent a sensitive indicator species for H5N1 HPAI. Results also suggest that the potential for decreased transmission efficiency associated with reduced viral shedding (especially from the cloaca) and a loss of environmental fitness (in water), may be offset by the ability of this virus to be transmitted through a very low infectious dose.

Key words: *Aix sponsa*, avian influenza virus, BID_{50}, BLD_{50}, H5N1, highly pathogenic avian influenza, infectious dose, susceptibility, wood duck.

INTRODUCTION

Wild aquatic birds in the orders Anseriformes and Charadriiformes are the natural reservoirs for avian influenza virus (AIV) (Hinshaw and Webster, 1982; Stallknecht and Shane, 1988). Traditionally, viral infection in Anseriformes or Charadriiformes have not been associated with morbidity or mortality (Webster et al., 1992), and AIVs that are highly pathogenic to domestic poultry are not found in these wild aquatic bird populations (Suarez, 2000). However, in 2002, mortality associated with H5N1 highly pathogenic avian influenza (HPAI) virus infection was reported in wild and captive aquatic birds in two waterfowl parks in Hong Kong (Ellis et al., 2004). Since these outbreaks, H5N1 HPAI viruses have continued to cause mortality in wild birds in Asia (USGS, 2006). In 2005, H5N1 HPAI viruses spread into Europe and Africa, and epidemiologic observations and genetic studies suggest that this geographic dissemination of virus may have occurred through migratory waterfowl (Sabirovic et al., 2006). Although field data from the epidemics in Eurasia confirm that H5N1 HPAI viruses can cause mortality in wild birds and suggest that migratory waterfowl play a role in the epidemiology of these viruses, it is unknown whether H5N1 HPAI viruses can persist in wild avian populations.

Based on our current knowledge of AIV in wild birds, three factors are important for viral persistence in waterfowl popula-

tions: 1) the ability to remain infective for long durations in aquatic habitats, 2) highly concentrated and prolonged viral shedding by birds in these populations, and 3) an ample supply of susceptible birds. The low pathogenic avian influenza (LPAI) viruses that naturally circulate in waterfowl populations, referred to herewith as "wild-type AIV," have evolved over time into the perfect host-parasite relationship (Webster et al., 1978), satisfying all three of these factors for viral maintenance. These LPAI viruses can persist for long durations in water (Stallknecht et al., 1990; Brown et al., 2007), and experimentally infected ducks shed high concentrations of virus for a prolonged duration via the fecal route (Webster et al., 1978). Susceptible birds and a high prevalence of AIV infections occur annually during the fall when juvenile ducks congregate at marshalling sites before fall migration (Halvorson et al., 1985). Compared with these wild-type AIV, H5N1 HPAI viruses do not appear to be as well-adapted to fulfill all of these prerequisites for maintenance in a wild bird population. Experimentally, H5N1 HPAI viruses remain infective in water for a shorter duration than wild-type viruses, suggesting the H5N1 HPAI viruses may not be as environmentally fit as the wild-type viruses (Brown et al., 2007). In addition, four out of five North American duck species experimentally infected with H5N1 HPAI viruses excreted low viral titers for short durations, and in all species, shedding was primarily associated with oropharyngeal (OP), rather than a cloacal, route (Brown et al., 2006). Potential losses in environmental fitness in aquatic habitats and the decreased viral shedding associated with H5N1 HPAI virus infections in most species of ducks may greatly reduce the viral burden in the aquatic environment. This implies that transmission must be very efficient in at least some aquatic bird species for these H5N1 HPAI viruses to be transmitted and potentially to persist in waterfowl populations. This increased transmission efficiency may be provided by the ability of these H5N1 HPAI viruses to infect a susceptible bird at a very low dose. Currently, there is no available information on infective dose for any H5N1 HPAI or wild-type AIV in any wild duck species.

Mortality reports from the ongoing H5N1 HPAI virus epidemics in Eurasia suggest that these viruses are especially virulent for some Anseriforme species (Sabirovic et al., 2006; USGS, 2006). In particular, field and experimental data indicate that wood ducks are highly susceptible to H5N1 HPAI virus infection (Ellis et al., 2004; Brown et al., 2006). However, all experimental infections to date that have evaluated H5N1 HPAI viruses in wood ducks and other duck species have been conducted with high viral inoculation doses, and the concentration of virus required to produce infection in this or any other wild Anseriforme species is currently unknown. Without this information, it is impossible to fully understand the sensitivity of wood ducks or other susceptible waterfowl species to infection with H5N1 HPAI viruses.

The objectives of this study were 1) to determine and evaluate the concentration of H5N1 HPAI virus required to produce infection or death in wood ducks, 2) to compare the median infectious and lethal dose in wood ducks to white-leghorn (WL) chickens, and 3) to evaluate the effect that H5N1 HPAI viral dose has on morbidity, mortality, and viral shedding.

MATERIALS AND METHODS

The virus

The H5N1 HPAI virus (A/Whooper Swan/Mongolia/244/05 [H5N1]; Mongolia/05) used in this study was obtained from the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service (ARS), US Department of Agriculture (USDA), Athens, Georgia, USA. This virus was originally isolated from a dead whooper swan (*Cygnus cygnus*) in Mongolia during a 2005 outbreak of H5N1 HPAI virus in waterfowl (OIE Disease In-

formation, 2005; Brown et al., 2006). The Mongolia/05 strain is in the Goose/Guandong/96 lineage and, phylogenetically, is included in clade 2 (World Health Organization Global Influenza Program Surveillance Network, 2005). Mongolia/05 was selected for use in this study because it is representative of the H5N1 HPAI viruses that have been reported from wild birds in Asia, Europe, and Africa (Brown et al., 2006).

Virus was propagated by second passage in 9- to 11-day-old specific pathogen free (SPF) embryonated chicken eggs. Allantoic fluid from the inoculated eggs was diluted in brain-heart infusion (BHI) medium to yield the final titers of $10^{1.5}$, $10^{3.0}$, $10^{4.5}$, and $10^{6.0}$ median embryo infectious doses (EID_{50}) per 0.1 ml (single bird inoculum) for the wood duck trial and $10^{1.0}$, $10^{3.0}$, and $10^{5.0}$ EID_{50}/0.1 ml for the WL chicken trial. Back-titers on these doses were determined in 9- to 11-day-old SPF chicken eggs and were $10^{1.9}$, $10^{3.1}$, $10^{4.9}$, $10^{6.1}$ EID_{50}/0.1 ml for the wood duck trial and $10^{0.9}$, $10^{3.1}$, $10^{5.1}$ EID_{50}/0.1 ml for the chicken trial. A sham-inoculum was prepared by diluting sterile allantoic fluid 1:30 in BHI.

Animals

Twenty-five captive-bred wood ducks were acquired from a private breeder at 12–16 wk of age (Chenoa Farms, Martin, Tennessee, USA). This age was selected because it corresponds to the time in nature when North American ducks would most likely be infected; the peak prevalence of AIV in wild waterfowl is associated with premigration staging in the late summer/early fall (Halvorson et al., 1985). Both male and female ducks were included in approximately equal numbers. Ducks were housed in groups of five in self-contained isolation units that were ventilated under negative pressure with high-efficiency particulate air (HEPA)–filtered air. The birds were maintained under continuous lighting, and food and water were provided ad libitum.

Fifteen WL chickens (*Gallus gallus domesticus*) were acquired at 3 wk of age from an SPF flock maintained at SEPRL. The chickens were housed in groups of five in negative-pressure, HEPA-ventilated, stainless-steel isolation cabinets units. The birds were maintained under continuous lighting, and food and water were provided ad libitum.

General care was provided in accordance with the guidelines of the Institutional Animal Care and Use Committee, as outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Craig et al., 1999) and under an animal use protocol approved by the Institutional Animal Care and Use Committee at both SEPRL and the University of Georgia (UGA). All experiments were performed in the USDA-certified Biosafety Level 3 (BSL 3)-Ag facility at SEPRL (Barbeito et al., 1999).

Virus isolation and serologic testing

Cloacal and OP swabs were stored at −70 C until virus isolations and titrations were performed. Standard procedures were used for virus isolation from cloacal and OP swabs using SPF embryonated chicken eggs (Swayne et al., 1998). Viral titers for positive samples were determined by microtiter endpoint titration (Reed and Muench, 1938) using primary cultures of chicken embryo fibroblasts (CEFs). In this protocol, sequential 10-fold dilutions (10^{-1} to 10^{-8}) were made of the clarified swab suspension in serum-free Eagle's minimum essential medium (MEM) supplemented with antibiotics (100 U penicillin G with 100 μg streptomycin sulfate/ml). Each well of a 96-well microtiter plate received 100 μl of cell suspension and 50 μl of the appropriate virus dilution. Primary cultures of CEFs from 9- to 11-day-old SPF chicken embryos were used for these cell suspensions. Final cell suspensions consisted of 3×10^6 CEF/ml suspended in serum-free MEM supplemented with antibiotics. Supplemental trypsin was not added to these assays. The covered plates were incubated at 37 C under 5% carbon dioxide (CO_2) for 96 hr. Examination for cytopathic effects was performed with light microscopy and plates were then stained with 1% crystal violet in 10% neutral buffered formalin for further confirmatory examination. Endpoints were recorded as 100% monolayer destruction. Viral titers were expressed as median tissue culture infectious dose ($TCID_{50}$)/ml. The minimal detectable limit of this assay is $10^{1.96}$ $TCID_{50}$/ml. Serologic testing was performed via the agar gel precipitin (AGP) test using standard procedures (Swayne et al., 1998).

Experimental design

Wood ducks were evenly divided into five treatment groups with five birds in each group. Each group was intranasally (IN) inoculated with either a sham-inoculum or one of four different viral doses of the Mongolia/05; doses consisted of $10^{1.5}$, $10^{3.0}$, $10^{4.5}$, or $10^{6.0}$ EID_{50} in a volume of 0.1 ml per bird. Before inoculation, blood was collected from all ducks for serologic testing via the AGP test to ensure that birds did not possess antibodies to AIV. In addition, cloacal and OP swabs were collected

from each bird for virus isolation before inoculation to verify that the ducks were not shedding AIV at the start of the study. After inoculation, birds were observed daily for morbidity and mortality. Morbidity was defined as any clinical abnormality observed after inoculation with virus, including weakness, cloudy eyes, respiratory difficulty, shivering, crowding, ruffled feathers, hemorrhage on the unfeathered skin, or neurologic signs. Cloacal and OP swabs were collected from all ducks on 0, 2, 4, 6, and 14 days postinoculation (DPI). In addition, cloacal and OP swabs were collected from all ducks that were found dead or were euthanized because of severe neurologic clinical signs. At 14 DPI, blood was collected from the surviving birds for serologic testing, and all remaining ducks were euthanized with intravenous (IV) administration of sodium pentobarbital (100 mg/kg). The mean death time (MDT) was determined for each group and expressed as DPI. The median bird infectious dose (BID_{50}) and median bird lethal dose (BLD_{50}) were calculated for each of the four viral-inoculated groups using the Spearman and Karber method (Finney, 1964) and expressed as EID_{50}. This method was performed with the assumption that no birds became infected or died with a viral dose of 10^0 EID_{50}. Infection for BID_{50} calculation in wood ducks was determined based on virus isolation (in birds that died) and serologic testing (in surviving birds). Lethality for the BLD_{50} calculation in wood ducks was determined based on mortality.

Fifteen chickens were evenly divided into three groups and birds in each group were IN inoculated with one of three different doses of the Mongolia/05 virus, including $10^{1.0}$, $10^{3.0}$, and $10^{5.0}$ EID_{50} in a 0.1 ml volume per bird. Before inoculation, serum was collected from each bird to ensure that they were serologically naïve to AIV. After inoculation, the birds were monitored daily for morbidity and mortality. Morbidity was defined using the same standards as described for the wood ducks. Cloacal and OP swabs were not collected from chickens in this study. At 10 DPI, blood was collected from the surviving chickens for serologic testing via the AGP test. The surviving chickens were then euthanized by IV administration of sodium pentobarbital (100 mg/kg). The MDT, BID_{50}, and BLD_{50} were determined as described for the wood duck experiment. Infection for BID_{50} calculation in chickens was determined based on mortality and serologic testing. Lethality for the BLD_{50} calculation in chickens was determined based on mortality.

RESULTS

Morbidity, mortality, and serologic testing

Morbidity and mortality data are summarized in Table 1. None of the sham-inoculated wood ducks exhibited morbidity or mortality. All five wood ducks inoculated with $10^{4.5}$ and $10^{6.0}$ EID_{50} of the Mongolia/05 virus died, and the MDT in these groups was 6.8 and 5.2 DPI, respectively. One of the wood ducks in the $10^{4.5}$ EID_{50} group died on 14 DPI, which was 8–10 days after the other four ducks in this group. All cloacal and OP swabs collected from this bird after 4 DPI were negative for AIV on virus isolation, including swabs collected at the time of death. These results suggest that this wood duck did not die as a direct result of H5N1 HPAI viral infection, but rather, most likely succumbed to secondary infections or lesions associated with the viral infection. Discounting this wood duck, the corrected MDT for wood ducks that died from H5N1 HPAI infection in this group was 5.0 DPI. Four of the five wood ducks in each group inoculated with the $10^{1.5}$ and $10^{3.0}$ EID_{50} viral doses died, and the MDT were 6.3 and 5.5 DPI, respectively. The two wood ducks that survived did not exhibit clinical signs of disease at any time during the trial, but both ducks developed antibodies to AIV as detected via the AGP test. We were unable to determine a low viral dose that did not cause any mortality or seroconversion in wood ducks, which prohibited calculating a specific median dose for infectivity or lethality. Alternatively, we calculated the BID_{50} and BLD_{50} based on the data at the lowest inoculation dose, with the assumption that the 10^0 EID_{50} dose caused no morbidity or seroconversion, and reported these values. The results of this study indicate that wood ducks have a BID_{50} titer of $10^{0.95}$ EID_{50} and a BLD_{50} titer of $10^{1.71}$ EID_{50}.

All five of the WL chickens inoculated with the $10^{5.0}$ EID_{50} dose died and the MDT was 2 DPI. Three of the five chickens died at the $10^{3.0}$ EID_{50} dose

TABLE 1. Morbidity, mortality, and virus isolation data from wood ducks and chickens inoculated intranasally with different doses of the A/Whooper Swan/Mongolia/244/05 (H5N1) highly pathogenic avian influenza virus.

Group ID (EID_{50})[a]	Morbidity (sick/total)	Mortality (dead/total)	MDT[b] (days)	AMT[c] (oropharyngeal/cloacal) (log_{10} $TCID_{50}$/ml)	BID_{50}/BLD_{50}[d] (log_{10} EID_{50}/ml)
Wood Duck					0.95/1.71[e]
$10^{1.5}$	4/5	4/5	6.3	5.01/2.74	
$10^{3.0}$	4/5	4/5	5.5	4.38/1.96	
$10^{4.5}$	5/5	5/5	6.8 (5.0)[f]	5.14/2.91	
$10^{6.0}$	5/5	5/5	5.2	4.43/3.74	
White Leghorn Chicken					2.80/2.80
$10^{1.0}$	0/5	0/5	–	NP[g]	
$10^{3.0}$	3/5	3/5	4.3	NP	
$10^{5.0}$	5/5	5/5	2.0	NP	

[a] Median Embryo infectious dose (EID_{50}) of virus inoculated intranasally (IN) to each of the groups of wood ducks and chickens.

[b] MDT = mean death time.

[c] AMT = average mean titer in oropharyngeal and cloacal swabs collected from birds that died; $TCID_{50}$ = tissue culture infectious dose.

[d] BID_{50} = median bird infectious dose; BLD_{50} = median bird lethal dose.

[e] These values were calculated with the assumption that an inoculation dose of 10^0 EID_{50} resulted in no morbidity, mortality, or seroconversion.

[f] MDT for this group after disregarding a single outlying value of one wood duck that died on 14 days postinfection (DPI).

[g] NP = not performed.

and the MDT in this group was 4.3 DPI. None of the chickens died that were inoculated with $10^{1.0}$ EID_{50} of the Mongolia/05 virus. None of the surviving chickens at any viral dose produced antibodies to AIV so the BID_{50} and BLD_{50} titers in this species were equal and calculated to be $10^{2.80}$ EID_{50} (Table 1).

Virus isolation

Virus isolation results from the wood ducks are summarized in Table 1. None of the sham-inoculated ducks excreted virus or developed antibodies to AIV. Viral shedding was detected in all wood ducks in each of the four viral-inoculated groups on 2 DPI. In ducks that died, viral shedding was detected at all other sampling points in OP swabs and generally viral titers increased from 2 DPI until death. There were exceptions in which viral titers in some ducks remained constant or slightly decreased just before death. The average OP titer in the wood ducks that died with evidence of active viral infection (discounting the one wood duck in the $10^{4.5}$ EID_{50} group mentioned above) was $10^{4.7}$ $TCID_{50}$/ml (range= $10^{3.3}$–$10^{5.8}$ $TCID_{50}$/ml). Viral titers in all antemortem and postmortem swabs were higher in OP swabs than cloacal swabs in each of the 20 wood ducks infected in this study, and cloacal shedding did not occur at all time points in every duck.

Viral shedding in the two wood ducks that survived was either at a very low titer or for a brief duration. The surviving wood duck in the $10^{1.5}$ group excreted moderately high titers (average OP titer=$10^{4.3}$ $TCID_{50}$/ml), but virus was not detected after 4 DPI in OP swabs or 2 DPI in cloacal swabs. The surviving wood duck in the $10^{3.0}$ group shed virus for 6 DPI in OP swabs and 4 DPI in cloacal swabs, but titers were very low (average OP and cloacal titer was less than $10^{1.96}$ $TCID_{50}$/ml).

DISCUSSION

The results of this study indicate that wood ducks are highly susceptible to

infection with H5N1 HPAI viruses and relatively small concentrations of these viruses are required to produce infection and death in this species. As a comparative measure of wood duck susceptibility, lower concentrations of H5N1 HPAI virus are required to produce infection and death in wood ducks than in domestic chickens, which are one of the most susceptible avian species to H5N1 HPAI virus (Perkins and Swayne, 2003).

Because only two ducks survived in this study, accurate conclusions cannot be made on the risk of surviving birds transmitting or geographically disseminating H5N1 HPAI virus during an outbreak in waterfowl. However, as opposed to chickens, in which there was no serologic evidence of infection in surviving birds, wood ducks that survived did seroconvert and shed virus suggesting that asymptomatically infected wood ducks could contribute to the transmission and spread of virus during an outbreak, though viral shedding is at a lower titer (average maximum OP titer for both survivors=$10^{3.13}$ $TCID_{50}$/ml; average OP titer for all nonsurvivors=$10^{5.11}$ $TCID_{50}$/ml) and for a shorter duration than birds that eventually succumbed to infection.

Ducks and chickens in this experiment were housed in groups making it impossible to determine whether viral infection in each bird occurred from the initial viral inoculum or transmission from another infected bird within the same housing unit. Consequently, the MDT may be overestimated in this study. However, housing chickens individually did not affect the BID_{50} (Swayne, pers. comm.) and the comparable susceptibility of wood ducks would suggest that similar results would be expected. In addition, all inoculated birds were shedding virus at 2 DPI, suggesting that these infections resulted from the original inoculum. After correcting for the one outlying duck in the $10^{4.5}$ group that died late in the study without viral shedding, the MDT appears to be negatively associated with viral dose. However, differences between dose groups were minor and not statistically significant, suggesting that this dose-related variation would have little if any impact on the extent of environmental viral contamination or potential H5N1 HPAI viral spread via infected birds.

Experimental studies evaluating the transmission of H5N1 HPAI virus in wood ducks indicate the following: 1) H5N1 HPAI viruses are virulent in wood ducks, causing high morbidity and mortality; 2) infected wood ducks shed virus for 4–7 days, which is primarily in respiratory secretions and less in the feces (Brown et al., 2006); and 3) very small concentrations of virus produce infection and death in wood ducks. Thus, the low BID_{50} titer of wood ducks may compensate for the more rapid viral degradation of H5N1 HPAI viruses in aquatic habitats. Taken together, these data suggest that the wood duck would represent a very effective indicator species for H5N1 HPAI virus. Although the information provides some insight related to the potential for H5N1 HPAI viruses to be maintained in waterfowl populations, these wood duck data are not sufficient to indicate or suggest that wood ducks or other wild birds could maintain these viruses in nature. Under natural conditions, the biology of wood ducks or other duck species may be much more important in understanding reservoir potential than susceptibility. In a previous study, five other species of North American ducks shed low viral titers for short durations, even after inoculation with high concentrations of H5N1 HPAI virus (Brown et al., 2006). It is possible that H5N1 HPAI virus epidemics in wild waterfowl are dependent on the presence of highly susceptible species like wood ducks, which may represent a small component of the avian community; such epidemics may represent short-term spill-over events that are driven by species composition and the specific ecological, climatic, or environmental conditions that may influence avian distribution and

behavior. This theory is consistent with field data from the H5N1 HPAI outbreaks in Europe during 2005–2006, in which the majority of mortality involved limited Anseriforme species, such as mute swans (*Cygnus olor*) and tufted ducks (*Aythya fuligula*) (Sabirovic et al., 2006). It is believed that severe weather in Eastern Europe during the winter of 2005–2006 disrupted the migration patterns of waterfowl and resulted in the congregation of high concentrations of these susceptible species, in which the H5N1 HPAI virus outbreaks could occur (Sabirovic et al., 2006).

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