TEMPORAL, SPATIAL AND SPECIES PATTERNS OF AVIAN INFLUENZA VIRUSES AMONG WILD BIRDS

by

BRITTA ANN HANSON

(Under the direction of William R. Davidson)

ABSTRACT

Recent direct transmissions of avian influenza viruses (AIV) to humans and the continued threat of AIV outbreaks within domestic birds and livestock emphasize the need for current data of AIV within wild bird populations. However, in North America, the majority of the information on subtype prevalence and diversity is more than 10 years old and these were collected from relatively few species. This study examined the distribution of AIV among wild birds migrating through or wintering in the Atlantic, Mississippi and Central flyways of the U.S during 1998-2002.

During the fall seasons 1998-2000 in Minnesota, the predominant hemagglutinin (H) subtypes of AIV isolated from ducks were the H3, H4 and H6 subtypes. This is consistent with previous studies. However, unlike past surveys, more than 20% of the isolates were subtypes associated with human or poultry disease (H5, H7 and H9). In contrast, no H3, H4 or H6 viruses were detected in ducks sampled in Texas during February in 2001 and 2002. The 11-15% AIV prevalence in these wintering ducks was higher than expected and only the H2, H7 and H8 subtypes were isolated.

During April - September, 2000, AIVs were detected in shorebirds at only two of five locations sampled and the prevalence among individual shorebird species varied. Most AIVs (67.5%) were isolated from ruddy turnstone (*Arenaria interpres*) in the Delaware Bay. Unlike a previous survey of shorebirds in the northeastern U.S. in which the H9 and H13 subtypes predominated, the H10 and H12 subtypes comprised >60% of all isolates and no H9 or H13 subtypes were detected.

Given the unexpected predominance of the subtypes detected in this study, it appears that the subtypes most often associated with migrating waterfowl (H3, H4 and H6) and shorebirds (H9 and H13) are not necessarily predictable and may vary with time and location of sampling. Also, the 11-15% prevalence of AIV in wintering ducks in Texas suggests that assumptions regarding AIV as primarily a fall season event are not always accurate. It is likely that AIV ecology is not completely understood and the current results indicate that seasonal, species or site characteristics will greatly influence AIV subtype diversity and prevalence.

INDEX WORDS: Anseriformes, Avian influenza virus, Charadriiformes, Delaware Bay, Minnesota, Shorebird, Texas, Waterfowl

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BRITTA ANN HANSON

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by

BRITTA ANN HANSON

Approved:

Major Professor: William R. Davidson

Committee: David E. Stallknecht
Glenn O. Ware
Robert J. Warren

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2003

DEDICATION

To Mom, for everything.

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TABLE OF CONTENTS

Page

ACKNOWLEDGMENTS iv

CHAPTER 1: INTRODUCTION 1

CHAPTER 2: LITERATURE REVIEW 4

PUBLIC HEALTH 6

HOST RANGE 7

WILD MAMMALS 9

WILD BIRDS 10

MAINTENANCE AND TRANSMISSION BY WILD BIRDS 14

OBJECTIVES 16

THESIS FORMAT 17

LITERATURE CITED 18

CHAPTER 3: AVIAN INFLUENZA VIRUSES IN MINNESOTA DUCKS 1998 - 2000 27

SUMMARY 28

INTRODUCTION 29

MATERIALS AND METHODS 30

RESULTS 31

DISCUSSION 33

REFERENCES 37

ACKNOWLEDGMENTS 41

CHAPTER 4: AVIAN INFLUENZA VIRUSES AND PARAMYXOVIRUSES IN WINTERING AND RESIDENT DUCKS IN TEXAS 42
ABSTRACT 43
INTRODUCTION 43
MATERIALS AND METHODS 44
RESULTS 45
DISCUSSION 45
LITERATURE CITED 49
CHAPTER 5: IS AVIAN INFLUENZA VIRUS INFECTION IN SHOREBIRDS SPECIES OR LOCATION SPECIFIC? 53
ABSTRACT 54
INTRODUCTION 55
MATERIALS AND METHODS 56
RESULTS 59
DISCUSSION 59
LITERATURE CITED 66
CHAPTER 6: CONCLUSIONS 72
APPENDIX: AVIAN INFLUENZA VIRUSES IN WILD BIRDS 75
CITATIONS 87

CHAPTER 1

INTRODUCTION

Influenza A viruses naturally infect humans, wildlife and domestic animals. Influenza viruses cause human mortality and morbidity yearly and periodic epidemics have been responsible for more than 20 million deaths worldwide in the last century. Domestic livestock, particularly poultry and swine, also are at risk of infection and influenza outbreaks can cause severe economic losses to these industries.

Studies indicate that wild birds are the major reservoir for influenza viruses and represent the original source of all type A influenza viruses associated with domestic birds and mammals, wild mammals and humans. Unlike influenza viruses in humans, which are transmitted via a respiratory route, avian influenza viruses (AIV) are generally transmitted via a fecal-oral route, thus making fecal-contaminated water a potential source of infection for wild and domestic birds. In numerous surveys, birds of the order Anseriformes (the ducks, geese, and swans) typically have the highest AIV prevalence rate compared to other orders (Stallknecht et al., 1988). Ducks, particularly juvenile *Anas* spp., have the highest prevalence rate as they congregate prior to fall migration (Webster et al., 1992). Influenza A viruses also have been isolated from shorebirds (order Charadriiformes); however, compared to waterfowl surveys, AIV isolations from shorebirds are not frequently reported.

Of the 15 known hemagglutinin subtypes, the subtypes associated with human and poultry disease (H5, H7 and H9) are not frequently reported in wild birds. However, in North America few surveys of waterfowl have been reported since the early 1990's and our knowledge of shorebird AIV ecology, specifically the sandpipers (family Scolopacidae), is limited to two reports of isolations from birds migrating through the U.S more than ten years ago (Kawaoka et al., 1988; Saito et al., 1994). Given the implications of wild birds as reservoirs for potentially pathogenic AIV in humans and livestock, it is necessary to have current information on the ecology of AIVs circulating within the wild population (Alfonso et al., 1995; Webby et al., 2002).

CHAPTER 2

LITERATURE REVIEW

The influenza viruses belong to the genus *Influenzavirus*, the only genus of the *Orthomyxoviridae* family. These enveloped viruses contain single-stranded RNA and are approximately 80-120 nm in diameter. Influenza is subdivided into three types (A, B, and C) based on antigenicity. Influenza A viruses infect multiple species of birds and mammals but have evolved in humans, birds, swine and horses into genetically distinct types. This host restriction is believed to limit, but not eliminate, the probability and severity of interspecies transmission (Webster et al., 1992).

The envelope surrounding the influenza A virus contains two glycoproteins. The hemagglutinin (H) glycoprotein functions to bind the virus to host cells and serves as the major antigen associated with acquired host immunity. The neuraminidase (N) glycoprotein is responsible for the release of virus from host cells after replication (Webster et al., 1992). Influenza A viruses are classified based on their combination of one of 15 known H glycoproteins with one of nine known N glycoproteins. Due to its function of binding to the host cell to allow infection and its role in host immunity, the H glycoprotein is of primary concern when considering influenza transmission.

Genetically, the influenza viruses are highly variable (Fenner et al., 1987). Like other RNA viruses, the influenza viruses lack proofreading abilities and the genome is subject to point mutations. This is referred to as genetic drift. Influenza viruses also possess a segmented genome which can freely reassort during replication if two or more unique viruses infect the same cell. Such reassortment is referred to as genetic shift. These changes may produce a genetically distinct H gene to which the host population has no existing immunity. The introductions of new H variants have resulted in numerous deaths worldwide and are a constant concern for public and livestock health managers attempting to prevent disease due to influenza (Capua et al., 2002).

PUBLIC HEALTH

One of the first descriptions of an epidemic characterized by influenza-like symptoms in humans was recorded in 412 B.C. (Kaplan et al., 1977). From the 16th century until the late 19th century, additional accounts of probable influenza epidemics were noted by historians (Kaplan et al., 1977). In 1901, researchers found that influenza was caused by a filterable agent (Suarez et al., 2000) and following several experiments in the 1930's, the influenza virus was identified (Fenner et al., 1976). Retrospective research in the 20th century traced the first confirmed human influenza outbreak to 1889 (Alexander et al., 2000). An outbreak in 1900 was caused by an H3N8 virus. In 1918, the most deadly influenza pandemic to date killed an estimated 20-100 million people and was caused by an H1N1 virus. Three pandemics, all believed to have originated in Asia, have occurred since then: H2N2 (1957), H3N2 (1968) and H1N1 (1977) (Oxford, 2000). None of these pandemics had the high death toll associated with the 1918 outbreak, but there were significant impacts felt worldwide. For example, the combined death toll due to influenza-related deaths from the 1957 and 1968 outbreaks is estimated at 98,000 in the U.S. (Alexander et al., 2000). In 1996, an H7N7 virus was isolated from a woman in England, marking the first time humans were infected with a subtype other than H1, H2, or H3 (Alexander et al., 2000). An H5N1 outbreak killed six people in Hong Kong the next year. This outbreak caused significant concern due to its high virulence in poultry and apparent direct transmission from birds to humans. Prior to the

Hong Kong outbreak, it was believed that in order for an avian influenza virus to infect a human, the virus must first pass through domestic swine. In 1999, a second direct transmission from birds to humans was believed to have occurred when H9N2 viruses were isolated from two children in Hong Kong.

HOST RANGE

Domestic swine and horses

Swine are susceptible to avian, swine, and human influenza viruses. Mortality due to infection is low but the effects of influenza-associated morbidity causes an average loss of £7 per pig totaling approximately £65 million ($94 million U.S.) per year in the U.K. (Alexander et al., 2000).

Studies indicate that swine serve as a "mixing vessel" for avian and human influenza strains. When a pig is dually infected with an avian and human influenza strain, the easily mutable viruses can reassort, producing a novel subtype (Scholtissek et al., 1988; Webster et al., 1992). Transmission of influenza from swine to humans is well documented (Hinshaw et al., 1978; Rota et al., 1989; Wentworth et al., 1997) with isolated deaths reported (Webster et al., 1992). Only subtypes of H1 or H3 origin were isolated from swine prior to 1997. However, results from recent surveys suggest that little is known about the potential role swine play in the ecology of influenza transmission. For example, four H9 viruses were isolated from pigs in China (Peiris et al., 2001) during 1997-1998. An H4N6 virus, believed to be entirely of avian origin, was recovered from clinically affected swine in Canada (Karasin et al., 2000) in 1999. Recent retrospective surveys suggest that pigs in China had antibodies to H4 and H5 prior to 1983 and antibodies to H9 in 1998 (Ninomiya et al., 2002).

Only two subtypes, H3N8 and H7N7, have been identified in domestic and wild horses. Research on the genetic stability of equine influenza suggests that horses may be a dead-end host for influenza A viruses (Webster et al., 1992).

Domestic poultry

Highly pathogenic avian influenza (HPAI) can result in significant (up to 100%) mortality in domestic poultry but has been restricted to the H5 and H7 subtypes (Alexander et al., 2000). Decreased egg production, mild respiratory disease and other mild clinical symptoms are associated with low pathogenic avian influenza (LPAI) and are usually caused by one of the other H subtypes or a less virulent form of an H5 or H7 subtype (Alexander et al., 2000). Domestic ducks, chickens, turkeys and ratites are all susceptible to influenza infections.

Economic losses due to influenza outbreaks in poultry can be substantial. In 1983-84, an HPAI H5N2 outbreak in Pennsylvania and Virginia resulted in the loss of more than 17 million birds at a cost of more than $60 million for a government control program and $349 million to the consumer (Alexander et al., 2000). Similar losses were repeated in Mexico and Pakistan in the early 1990's (Perdue et al., 2000). The H5N1 Hong Kong outbreak in 1997 resulted in the culling of all of the island's 1.2 million chicken, duck, pigeon and quail in production or at market (Kolata, 1999). The chicken markets were closed for a month, resulting in undetermined loss of income for more than

1,000 farmers and sellers. In 2002, Virginia state officials depopulated 197 poultry farms (4.7 million birds) to prevent the spread of a LPAI H7 outbreak.

Transmission of virus from wild birds to domestic birds is believed to occur when infected wild birds utilize or contaminate water sources on poultry farms. Avian influenza viruses have been isolated from animal water sources used by ranged and housed turkeys in Minnesota (Sivanandan et al., 1991). Virus has also been isolated from bodies of water utilized by migrating waterfowl (Hinshaw et al., 1980; Markwell et al., 1982; Ito et al., 1995; Lai et al., 1999). Ranged birds may become infected after drinking virus-contaminated water or by direct transmission when mingling with wild birds (Alexander et al., 2000). This has led some researchers to advise limited contact between domestic and wild birds (Kawaoka et al., 1988; Capua et al., 1999).

WILD MAMMALS

Wild mammals may be involved in the epizootiology of influenza, but information on the role these animals play in the maintenance and transmission of AIV is limited. Influenza A viruses have been isolated from seals and whales, although the mortalities and morbidities associated with the isolations are uncertain (Webster et al., 1992). More recently, an influenza A virus was isolated from a muskrat (*Ondatra zibethicus*) in Siberia, marking the first report of an influenza isolation from a wild freshwater mammal (L'vov et al., 2001).

Mink (*Mustela vison*), raccoon (*Procyon lotor*), skunk (*Mephitis mephitis*) and thirteen-lined ground squirrels (*Citellus tridecemlineatus*) have been experimentally infected with AIV (Bailey, 1983; Okazaki et al., 1983a; Berg et al., 1990; Englund, 2000). Mortality and disease caused by influenza have been noted in farmed mink (Englund, 2000). In other studies, farmed mink were seropositive for influenza (Yagyu et al., 1982; Okazaki et al., 1983b). However, the role these mammals play in the ecology of influenza, in the wild or in captivity, is unknown.

WILD BIRDS

Ducks

Influenza A viruses representing all of the hemagglutinin and neuraminidase subtypes have been isolated from wild birds, specifically waterfowl and other aquatic species, leading researchers to suggest that birds are the primary source of all influenza A viruses (Hinshaw et al., 1980; Hinshaw et al., 1982; Sharp et al., 1993; Suss et al., 1994). An updated list of all AIV isolations from wild birds is provided in the Appendix Table 1. This reservoir was first identified during a 1961 epizootic in which more than 1300 common terns (*Sterna hirundo*) died (Becker, 1966). This represents the first and only confirmed outbreak among wild birds to date which has caused notable mortality. Experimental infections of mallards (*Anas platyrhynchos*) and Franklin's gulls (*Larus pipixan*) with AIV failed to produce clinical disease in these species (Homme et al., 1970; Bahl et al., 1977; Webster et al., 1978). In other studies, however, histologic changes in some tissues were detected upon necropsy of infected birds (Laudert et al., 1993a) and a decrease in egg production was noted for one week after experimental infection of mallards (Laudert et al., 1993b). Due to the low incidence of notable disease in proportion to the rate of isolations, it is speculated that wild birds are well-adapted to the avian influenza A virus and this is probably a reflection of a very long-term host-pathogen relationship (Webster et al., 1992).

Although influenza viruses have been isolated from more than 90 species within at least 12 bird orders, members of the Anseriformes (ducks, geese, and swans) have the overall highest isolation rate (15.2%) (Stallknecht et al., 1988). Isolation rates among all other orders are usually less than 3% (Stallknecht et al., 1988). Avian influenza virus is transmitted among waterfowl via the fecal-oral route (Webster et al., 1992) and an infected duck can excrete up to $10^{8.7}$ mean egg infectious doses of virus per gram of feces (Webster et al., 1978). Among waterfowl, isolation rates appear to be highest in the fall when large numbers of birds congregate on lakes and ponds prior to migration. Immunologically naïve juvenile ducks appear most susceptible to infection with prevalence rates greater than 50% possible in the fall (Hinshaw et al., 1985). Isolation rates from adults sampled at the same location as the juveniles are significantly lower (Hinshaw et al., 1982; Hinshaw et al., 1985; Hinshaw et al., 1986).

Considerable surveillance of waterfowl in the 1970's and 1980's forms the basis of our current knowledge of avian influenza ecology in North America (Kawaoka et al., 1988; Stallknecht et al., 1988; Stallknecht et al., 1990c; Slemons et al., 1991). Although all 15 hemagglutinin subtypes have been isolated from wild waterfowl (Sharp et al., 1997; L'vov et al., 2001), the H3, H4, or H6 subtypes have been isolated most frequently (Sharp et al., 1993). In contrast, the H5, H7, and H9 subtypes (those potentially pathogenic for humans or poultry) have been poorly represented in these reported isolations. For example, in surveys of AIV in North American waterfowl, the H5, H7, and H9 subtypes represent only 0.4%, 0.7%, and 0.4% of more than 3,100 isolates respectively (Webster et al., 1976; Boudreault et al., 1980; Hinshaw et al., 1980; Kocan et al., 1980; Smitka et al., 1981; Hinshaw et al., 1985; Nettles et al., 1985; Hinshaw et al., 1986; Kawaoka et al., 1988; Stallknecht et al., 1990c; Slemons et al., 1991; Alfonso et al., 1995). This has led some researchers to suggest that waterfowl may not represent reservoirs for all H subtypes (Sharp et al., 1993). However, reports of recent virus isolation attempts from waterfowl in North America are few (Ito et al., 1995; Lai et al., 1999), and surveillance of migrating waterfowl in the Atlantic and Mississippi flyways has not been reported since 1991.

Shorebirds

Since the 1961 AI epizootic in common terns, influenza viruses have been isolated from several species within the order Charadriiformes, the gulls, terns, and sandpipers. In a summary of AIV prevalence in birds the Charadriiformes had a 2.2% AIV prevalence rate overall (Stallknecht et al., 1988). However, most of the birds in that summary were members of the Laridae family (gulls and terns) and no sandpipers were sampled in North America. The most comprehensive survey of shorebirds published to date, since Stallknecht and Shane's review (1988), sampled 1,446 gulls, terns and sandpipers in the Delmarva Peninsula, U.S. over a two-year period beginning in May, 1985 (Kawaoka et al., 1988). Overall, AIV viruses were detected in 5.8% of shorebirds sampled (Kawaoka et al., 1988). Most isolates were obtained during sampling efforts in the months of May and September and less than 10 AIV viruses were obtained in June and October. No AIVs were detected in other months of sampling. The higher prevalence of AIV in those birds sampled in May and September coincides with the times of peak shorebird migration in the northeastern U.S. As in waterfowl, it is believed that AIV in shorebirds is also transmitted by a fecal-oral route and thus the high concentration of birds during migration may facilitate the spread of virus.

Kawaoka et al. (1988) reported that the H9 and H13 subtypes were most prevalent overall (26.1% and 17.9% respectively) and the H2, H4 and H11 subtypes each accounted for approximately 10-12% of the total isolates. The subtypes associated with highly pathogenic AIV in domestic poultry, H5 and H7, accounted for 3.6% of all isolates from shorebirds in 1985-1987 (Kawaoka et al., 1988). Based on reports of the predominance of the H3, H4, and H6 subtypes within North American ducks, the authors suggested that the predominant AIVs circulating within the shorebird population is different than those in the wild duck population.

In Kawaoka's report, the ruddy turnstone (*Arenaria interpres*) accounted for 11.2% of birds sampled but 40% of all AIV isolates. A similar association of prevalence variation among species can be observed in other aquatic bird families. Within the Anatinae subfamily, it appears that ducks within the tribe Anatini have a higher AIV prevalence rate compared to waterfowl of other tribes (Stallknecht, 1998). The ruddy turnstone was once classified in the Charadriidae family but is currently in the Scolopacidae family. Within the subfamily Scolopacinae, the turnstone is in the Arenariini tribe while the majority of the other sandpiper species are members of the Calidridini tribe. The reasons for the apparent higher prevalence among certain tribes of birds are unknown, but could relate to species susceptibility, habitat preferences or some other cause.

It is important to note that of the 1,446 samples in Kawaoka's study, only 497 samples were obtained from one of four species of sandpipers sampled. Only 37 of these samples were direct cloacal swabs from 2 species, the ruddy turnstone and red knot (*Calidris canutus*). The other 460 samples were collected as fecal samples off the ground in areas of shorebird concentrations and the species was assumed based on the birds seen in the area prior to collection. Only 2 AIV isolates were obtained from the cloacal swabs but at least 17 additional AIV viruses were identified from the fecal samples and attributed to either ruddy turnstones or sanderlings (*C. alba*). Both Laridae and Scolopacidae species forage in close proximity on the beaches and birds of both families will arrive and depart frequently. The reliability of identifying samples through indirect sampling techniques such as feces collection is unknown and any results from such methods could be misinterpreted.

A survey of ring-billed gulls (*L. delawarensis*) conducted during 1977-1979 in Baltimore, MD detected AIV in 2% of birds sampled (Graves, 1992). Prevalence ranged 0-10% during the year with most isolates detected during April and August – October. The H11 and H13 represented more than 50% of the subtypes isolated. The H2, H5, H6 and H9 were also detected. No AIVs were detected from young (6 weeks or younger) royal terns (*Thalasseus maximus*), herring gulls (*L. argentatus*) or laughing gulls (*L. atricilla*).

MAINTENANCE AND TRANSMISSION BY WILD BIRDS

Both waterfowl and shorebirds are implicated in the maintenance cycle of influenza among wild birds and the potential transmission to domestic animals

(Alexander, 2000). For example, phylogenetic analysis of a H5N2 AIV in domestic birds suggests that it is closely related to a ruddy turnstone AIV (Saito et al., 1994). In Minnesota, wild waterfowl are commonly suggested as the likely source of influenza infections in domestic turkeys (Karunakaran et al., 1983; Halvorson et al., 1985). Other studies have suggested that avian influenza viruses were transmitted to swine (Karasin et al., 2000; Peiris et al., 2001; Ninomiya et al., 2002) although no specific modes of transmission were reported.

As mentioned previously, the prevalence of AIV in ducks is usually highest during the fall. Sampling efforts on duck wintering grounds and during spring migration usually result in recovery of virus from < 2% of birds sampled (Webster et al., 1976; Stallknecht et al., 1991; Webster et al., 1992). Isolation rates from ducks, swans, and gulls ranged from 1-7% in the summer (Graves, 1992; Ito et al., 1995; Okazaki et al., 2000).

Given that the highest prevalence and subtype variation is detected in ducks during fall migration with few isolations during other seasons, it is unclear how the relatively large variety of subtypes of AIV are maintained within bird orders from year to year. Sharp et. al (1993) speculated that shorebirds were partly responsible for the maintenance of influenza in ducks due to the recovery of one H11 virus from one duck during the nesting season in Canada. However, extensive surveys of shorebirds for influenza during summer and winter seasons have not been reported and this does not explain how shorebirds, which primarily are infected with H9-H13 subtypes, could be responsible for the large variety of H3, H4, and H6 subtypes detected in waterfowl each fall.

Influenza virus has been isolated from freshwater sources associated with wild aquatic birds (Hinshaw et al., 1980; Halvorson et al., 1983; Sivanandan et al., 1991; Ito et al., 1995; Lai et al., 1999) and it is suggested that water may play a role in the maintenance of AIVs. The temperature, pH, and salinity of water affect influenza A virus survival rates (Stallknecht et al., 1990a) with infectivity noted after 91 days and linear regression models predicting persistence of infectivity for more than 200 days (Stallknecht et al., 1990b) under experimental conditions. Given that influenza virus can survive freeze-thaw cycles (Grieff et al., 1966; Smith et al., 1974) some researchers suggest that influenza viruses may freeze in lake or pond waters over winter and, upon thawing, could potentially contribute to the maintenance of the virus as birds arrive for the nesting season (Shoham, 1993).

Although our knowledge of influenza in birds and humans has improved greatly since the virus was first identified, it is clear that many aspects of influenza virus ecology are uncertain. To what extent AIV in ducks and shorebirds influences human and poultry disease is unknown and should be augmented with current data. Only with recent data can the potential and perceived threats wild birds pose to human and domestic animal health be lessened.

OBJECTIVES

This study was designed to examine the distribution of AIV among wild birds migrating through or wintering in the Atlantic, Mississippi and Central flyways of the U.S. during 1998-2002.

The objectives of this study were to:

1. provide current data on the AIV subtype diversity present in migrating North American wild ducks from sites previously sampled during 1973-1976;
2. examine the prevalence and serotype diversity of AIV in waterfowl at a wintering site in Texas;
3. examine the current prevalence, serotype diversity and temporal and spatial variations of AIVs circulating in multiple shorebird species in the Atlantic, Mississippi and Central flyways.

THESIS FORMAT

This thesis is written in manuscript format with chapters 2, 3 and 4 as separate manuscripts. Chapter 1 is a review of the ecology of AIV in humans, domestic animals and wild birds. Chapter 2, entitled "Avian influenza viruses in Minnesota ducks 1998-2000", examines the prevalence and subtype diversity of AIV in migrating mallards and pintails in a region sampled during the 1970's. Chapter 3, entitled, "Avian influenza viruses and paramyxoviruses in wintering and resident ducks in Texas", details the unexpectedly high prevalence of AIV in waterfowl wintering in a coastal region of Texas. In Chapter 4, entitled, "Is avian influenza virus infection in shorebirds species and location specific?" the results of AIV surveillance in shorebirds is discussed along with possible reasons for our findings. Chapter 5 serves to summarize all our findings and emphasize the need for continued surveillance of wild birds for AIV.

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CHAPTER 3

AVIAN INFLUENZA VIRUSES IN MINNESOTA DUCKS 1998 - 2000[1]

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[1] Hanson, B.A., D. E. Stallknecht, D. E. Swayne, L. A. Lewis, and D. A. Senne. Accepted for publication in the Proceedings of the Fifth International Avian Influenza Symposium.

SUMMARY: Although wild ducks are known to be a major reservoir for avian influenza viruses (AIV), there are few recent published reports of surveillance directed at this group. Predominant AIV hemagglutinin (HA) subtypes reported in previous studies of ducks in North America include H3, H4, and H6, with the H5, H7, and H9 subtypes not well-represented in these host populations. The objective of this study was to determine if these subtype patterns have persisted. Each September from 1998 to 2000, cloacal swabs were collected from wild ducks banded in Roseau and Marshall counties, Minnesota. Mallards (*Anas platyrhynchos*) were sampled all years and northern pintails (*A. acuta*) were sampled only in 1999. Influenza viruses were isolated from 11%, 14%, and 8% of birds during 1998, 1999, and 2000, respectively. Prevalence, as expected, was highest in juveniles, ranging 11-23% in mallards. Viruses representative of the HA subtypes 2, 3, 4, 5, 6, 7, 9, 10, 11, and 12 were isolated. Viruses in the H5, H7, and H9 subtypes, which are associated with highly pathogenic influenza in poultry or recent infections in humans, were not uncommon and each of these subtypes was isolated in 2 out of the 3 years of surveillance.

Keywords: avian influenza, mallard, pintail, Minnesota, poultry

Abbreviations: AIV = avian influenza virus; BHI = Brain Heart Infusion; HA = hemagglutinin; MEM = Minimal Essential Media; NA = neuraminidase; NVSL = National Veterinary Services Laboratory; SPF = specific-pathogen-free; WMA = wildlife management area.

INTRODUCTION

Ducks, geese, and swans of the order Anseriformes are the primary reservoir for avian influenza viruses (AIV) and have been implicated in the spread of influenza to domestic poultry (8,13). In the 1990's, at least 21 countries reported isolating AIV from wild or domestic birds (20) and highly pathogenic strains affected more than 14 million domestic poultry (28). All of these highly pathogenic influenza outbreaks have been associated with AIVs of the hemagglutinin (HA) subtypes H5 and H7, which represent the only subtypes linked to highly pathogenic influenza outbreaks in poultry since the 1950's (28). In 1997, an H5 AIV (H5N1) also represented the first suspected direct transmission of AIV from poultry to humans and was responsible for six deaths in Hong Kong (1). More recently in 1999, an H9N2 virus was isolated from two patients in Hong Kong and in this case direct avian-to-human transmission also was suspected (17). The threat of continued introductions of these viruses to humans or to poultry populations and the recognition of wild birds as the reservoir for these viruses reaffirms the need for surveillance of these reservoir populations to understand the potential for the emergence of pathogenic human and avian strains.

Considerable surveillance of waterfowl in the 1970's and 1980's forms the base of our current knowledge of avian influenza ecology in North America (14,23,26,27). Although all 15 HA subtypes have been isolated from wild waterfowl (18,21), the H3, H4, or H6 subtypes have been isolated most frequently (22). In contrast, the H5, H7, and H9 subtypes have been poorly represented in these reported isolations. For example, in surveys of AIV in North American waterfowl, the H5, H7, and H9 subtypes represent only 0.4%, 0.7%, and 0.4% of more than 3,100 isolates respectively (2,6,9-11,14,15,19, 23,24,27,30). This has led some researchers to suggest that waterfowl may not represent reservoirs for all HA subtypes (22). Reports of recent virus isolation attempts from waterfowl in North America are few (12,16), and surveillance of migrating waterfowl in the Atlantic and Mississippi flyways has not been reported since 1991. The objective of this study is to provide current information on the AIV subtype diversity present in migrating North American wild ducks from sites previously sampled during 1973-1976 (4,5).

MATERIALS AND METHODS

In mid-September 1998-2000, cloacal swabs were collected from ducks captured on Thief Lake Wildlife Management Area (WMA), Roseau River WMA, or Agassiz National Wildlife Refuge in Roseau and Marshall counties, Minnesota during annual waterfowl banding programs conducted by the Minnesota Department of Natural Resources and the U.S. Fish and Wildlife Service. Adult and juvenile mallards (*Anas platyrhynchos*) were sampled all years and northern pintail (*A. acuta*) in 1999 only. An emphasis on juvenile mallards was based on previous reports of high isolation rates from this species and age class (11,29) and the abundance of this species at the sites. Ducks were sexed, aged, and banded by wildlife agency personnel. Cloacal swabs were collected using sterile cotton-tipped applicators (Puritan®, Hardwood Products Company, Guilford, MN) and placed in sterile polypropylene tubes (Corning Inc., Corning, NY) containing 4 ml of transport media supplemented with penicillin G (10,000 u/ml), streptomycin (2mg/ml), kanamycin (0.6 mg/ml), gentamicin (1 mg/ml), and amphotericin B (0.02 mg/ml) (Sigma Chemical Company, St. Louis, MO). Either Minimal Essential Media (MEM) (Sigma Chemical Company) or Brain Heart Infusion media (BHI) (Becton

Dickinson, Sparks, MD) were used in 1998-99 whereas only BHI was used in 2000. Samples were stored on ice in the field and were shipped overnight (approximately 24-48 hr on ice total) to the laboratory where they were frozen at –70° C until processed.

Samples were thawed, vortexed and centrifuged at 1,500 *g* for 15 minutes. The supernatant was inoculated (0.25 ml/egg) via the allantoic route into four 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs. Hemagglutination testing was completed as previously described (27) except only two eggs were used during the second egg passage of passage one negative samples. All isolates were serotyped using hemagglutinating inhibition and neuraminidase inhibition tests at the National Veterinary Services Laboratory (NVSL), Veterinary Services, Animal and Plant Health Inspection Service, U.S. Department of Agriculture.

Differences in prevalence estimates among species, age class, and transport media were tested using Chi Square analysis (25).

RESULTS

Avian influenza viruses were detected during all three years with 154 AIVs isolated from 1423 (10.8%) sampled ducks (Table 1.1). Prevalence of infection in mallards (18%) sampled during 1999 was significantly higher than observed in northern pintails (2.9%) ($P < 0.0001$). A significant difference in prevalence between juveniles (16.6%) versus adults (2%) was detected ($P < 0.0001$). Prevalence of AIV also was higher in mallard juveniles (17%) than in mallard adults (2.1%) ($P < 0.0001$). No significant difference in isolation rates between pintail adults and juveniles was detected ($P = 0.11$), probably because of the low sample size of juveniles.

Table 3.1. Prevalence of avian influenza virus in migratory ducks, Roseau and Marshall counties, Minnesota, September 1998-2000.

Year	Species	Age	Total (no. infected/no. sampled) %
1998	Mallard	Juvenile	41/375 (10.9)
1999	Mallard	Adult	2/87 (2.3)
		Juvenile	58/247 (23.4)
	Pintail	Adult	1/96 (1.0)
		Juvenile	3/37 (8.1)
2000	Mallard	Adult	8/380 (2.1)
		Juvenile	41/201 (20.3)
Total			154/1423 (10.8)

As expected, the H3, H4, and H6 subtypes predominated representing 18.5%. 28.5%, and 16% respectively of all AIV isolations (Figure 1.1). These subtypes and the H11 subtype were detected each year. Thirty-two viruses of the H5, H7, and H9 subtypes were isolated, representing 7.4%, 3.4%, and 10.7% of all isolates respectively. Each was identified in two of the three years in mallards and one H7 AIV was isolated from a juvenile pintail. All nine neuraminidase (NA) subtypes were represented, and 32 HA NA subtype combinations were detected (Table 1.2). More than one HA and/or NA were identified in 15 isolates (1.2% of total). These were interpreted as dual infections, which are not uncommon in waterfowl (21).

To avoid species and age-related variation, comparison of AIV isolation rates between samples stored in BHI (22.0%) versus MEM (11.2%) were restricted to juvenile mallards. Virus isolation attempts were more successful from samples stored in BHI ($P = 0.0005$).

DISCUSSION

Variation in prevalence by age and year of sampling is consistent with previous AIV surveys. The higher isolation rate observed in juveniles versus adults agrees with other studies (2,10,11). Yearly prevalence ranged from 10.9- 23.4% in juvenile mallards. However, very high prevalence estimates, exceeding 60%, have been reported in other years and locations (11).

In 1999, the difference between isolation rates of pintails and mallards is more extreme than reported in other studies when AIVs were isolated from both species at similar rates (6,10,23). Although our sample size of juvenile pintails was small, only one AIV was detected among 96 adults. Hinshaw et al. detected AIV in adult pintails 5-29%

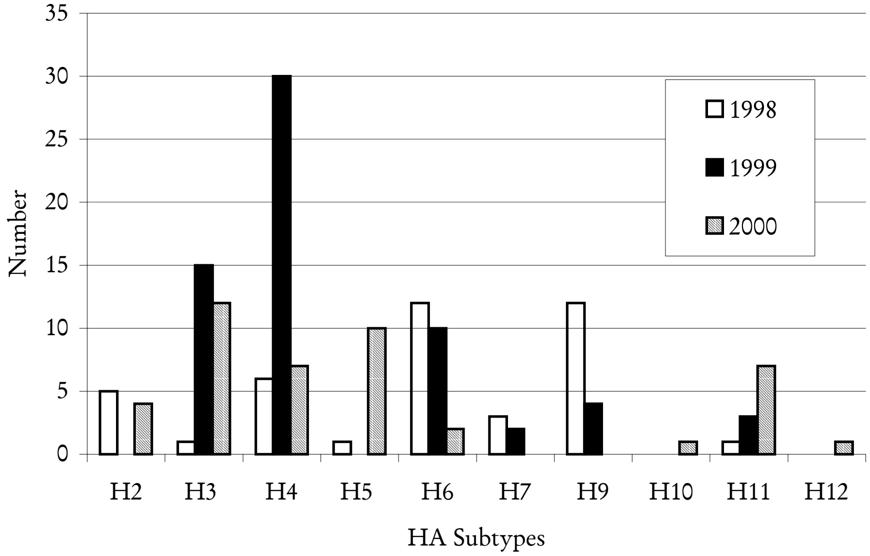

Figure 3.1. Number of HA subtypes in 149 avian influenza viruses isolated from waterfowl from Roseau and Marshall counties, Minnesota, September 1998-2000.

Table 3.2. Antigenic classification of avian influenza subtypes isolated from ducks (N = 154) in Roseau and Marshall counties, Minnesota, September 1998-2000.

Antigenic subtype	No.
H2N2	3
H2N3	3
H2N4	1
H2N9	2
H3N1	1
H3N2	4
H3N4	2
H3N5	1
H3N6	4
H3N8	7
H3N9	2
H4N2	2
H4N3	1
H4N6	21
H4N8	17
H4N9	1
H5N2	7
H5N3	2
H5N5	1
H6N2	1
H6N3	1
H6N4	1
H6N5	5
H6N6	1
H6N8	12
H7N3	4
H7N7	1
H9N2	16
H10N7	1
H11N2	2
H11N9	9
H12N5	1
H3N4,8	7
H6N1,4	2
H4N4,8	1
H5N1,4	1
H6N4,8	1
H5,6N6	1
H2,5N2,6	1
H?N2	2
H?N4,8	1

of the time (10). One possible explanation for this study's lower detection rate is the pintail's population fluctuations over the last two decades (7). The resulting age structures of the current population could be radically different than birds sampled 20 years ago; and thus, it may be possible that the majority of adult pintails sampled have already acquired immunity to AIV.

The predominance of the H3, H4, and H6 virus subtypes, which represented 63.8% of all isolates, coincides with more than 30 years of surveillance data (2,6,9-11,14,15,19,23,24,27,30) suggesting that this serotype predominance may be a stable characteristic of AIVs in North American waterfowl. Likewise, all NA subtypes were detected and the high prevalence of the N2, N6, and N8 subtypes (24%, 17.5%, and 23.4% respectively) was consistent with other studies (22). Unlike other studies, however, the isolation rates of the H5, H7, and H9 subtypes (21.5% combined) were higher, and in no other study were so many of these particular subtypes isolated within a three-year span. In fact, the isolation of all three subtypes has been reported only in Alberta during a 15-year study (11). Furthermore, these subtypes were not detected at all in several studies (2,24,27,30). From individual survey results, AIVs of the H5, H7, and H9 subtypes have never exceeded 8% (23), 1.6% (14), and 2.6% (14) of the total isolates, respectively. It is interesting that in a previous survey within the same region of Minnesota during 1973-1976, the H5 virus subtype, as in this study, was isolated during two years, but the H7 and H9 subtypes were not detected in ducks (3). At least two H5 and one H9 subtypes were detected in domesticated turkeys in the state during 1972-1979 (3).

Although previous studies suggest the H5, H7, and H9 virus subtypes are rare in North American waterfowl (14,22,29), the present study isolated these subtypes more than 20% of the time. It is interesting to note that in more recent work in Brazoria County, Texas, AIVs were isolated from 11 of 96 ducks sampled in February and that the H7 subtype represented five of these isolates (unpublished data). Whether this is because of to the location of sampling, a real change over time, or some other factor is unknown, but these results suggest that spatial and temporal variation related to AIV in waterfowl populations may deserve additional attention. In North America, AIV isolations with serotyping results from waterfowl has been reported only from approximately 30 locations over the past 30 years. Surveillance has not been reported in British Columbia, Saskatchewan, or Manitoba in Canada, and has been completed in fewer than 20 states in the U.S. Surveillance of ducks migrating along the Atlantic and Mississippi flyways has not been reported for more than 10 years. Perhaps our knowledge of AIV ecology needs to be supplemented with new surveillance efforts from a broad range of locations, species, and seasons.

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CHAPTER 4

AVIAN INFLUENZA VIRUSES AND PARAMYXOVIRUSES IN WINTERING AND RESIDENT DUCKS IN TEXAS[1]

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[1] Hanson, Britta A., David E. Swayne, Dennis A. Senne, David Lopbries, Justin Hurst, David E. Stallknecht. To be submitted to the Journal of Wildlife Diseases.

ABSTRACT: Cloacal swabs were collected from teal (*Anas crecca*., *A. cyanoptera, A. discors*), mottled duck (*A. fulvigula*) and northern pintail (*A. acuta*) in Brazoria County, Texas during February and August, 2001 and February, 2002. Prevalence of avian influenza virus (AIV) was 11%, 0% and 15% respectively. The AIV hemagglutinin (H) subtypes H2 and H7 were detected both years and the H8 subtype was detected in 2001. Avian paramyxovirus type 1 (APMV-1) was isolated from 13% of mottled ducks sampled in August and 30.7% of teal in February, 2002. The seasonal prevalence of both viruses and the AIV subtypes detected in this study are not typical based on previous reports of these viruses from North American ducks.

Key words: avian influenza virus, avian paramyxovirus, mottled duck, pintail, teal, Texas

INTRODUCTION

Wild birds represent the natural reservoir for avian influenza viruses (AIV) and avian paramyxoviruses (APMV), both of which are potential health threats to domestic poultry worldwide (Alexander, 2000). Although there has been significant progress in understanding the epidemiology of AIV and APMV in wild bird populations, questions remain relating to the maintenance of these viruses in nature. In previous surveys of North American ducks, the prevalence of AIV has been consistently highest during the fall season. This has been attributed to the concentration of immunologically naïve juvenile ducks during premigration staging, and in general, few AIV viruses are isolated from ducks on their wintering grounds or during other seasons (Webster et al., 1992). Although all 15 known hemagglutinin (H) subtypes have been represented in AIVs isolated from wild waterfowl (Sharp et al., 1997; L'vov et al., 2001), the predominate subtypes reported from North American ducks are the H3, H4 and H6 subtypes (Sharp et al., 1993).

Wild ducks also are considered the natural hosts of the APMV-1 (*Newcastle disease virus*), APMV-4, APMV-6, APMV-8 and APMV-9 serotypes (Alexander, 2000). Prevalence of APMV in wild ducks may vary due to multiple factors including location, species, sex, age, and season of sampling (Stallknecht et al., 1991). However, the epidemiology of APMV is poorly understood and unlike AIV, clear seasonal peaks in the transmission of APMV have not been detected.

Most North American studies investigating the prevalence of AIVs and APMVs in wild ducks have focus on mallards (*Anas platyrhynchos*) captured during the fall season in Canada and the northern half of the U.S. However, since transmission of both AIVs and APMVs has been documented in ducks wintering on the gulf coast of the U.S. (Stallknecht et al., 1990b; Stallknecht et al., 1991), additional information on the epidemiology of these viruses in wintering waterfowl is warranted. The objective of this study was to estimate the prevalence of AIV and APMV in both migratory and resident duck species in the gulf coast region of Texas, with an emphasis on winter-time sampling.

MATERIALS AND METHODS

Ducks were captured at pre-baited sites using rocket nets over freshwater marsh that is artificially and seasonally inundated (October - March) at Peach Point Wildlife Management Area in Brazoria County, Texas. Cloacal swabs were collected by Texas Parks and Wildlife Department personnel using sterile cotton-tipped applicators

(Puritan®, Hardwood Products Company, Guilford, MN) and placed in 3 ml of Brain Heart Infusion media (Becton Dickinson and Co., Sparks, MD) supplemented with penicillin G (10,000 u/ml), streptomycin (2mg/ml), kanamycin (0.6 mg/ml), gentamicin (1 mg/ml), and amphotericin B (0.02 mg/ml) (Sigma Chemical Company, St. Louis, MO). Samples were stored on ice in the field, shipped overnight (approximately 24-72 hr on ice total) and frozen at –70° C until processed.

For virus isolation, samples were thawed, vortexed and centrifuged at 3,000 rpm for 15 minutes. The supernatant was inoculated (0.25 ml/egg) via the allantoic route into four 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs. Eggs were incubated at 37º C for 72 hr and hemagglutination testing was completed as previously described (Stallknecht et al., 1990b). For negative samples, amnio-allantoic fluid was pooled by sample, diluted 1:10 in sterile phosphate-buffered saline, and repassaged into 2 additional eggs. All isolates were serotyped using hemagglutinin inhibition and neuraminidase inhibition tests at the National Veterinary Services Laboratory, Veterinary Services, Animal and Plant Health Inspection Service, U.S. Department of Agriculture.

RESULTS

During 2001 and 2002, 258 ducks were sampled and 57 hemagglutinating agents were detected. Prevalence by year, month and species is presented in Table 2.1. The sex and age of the birds were not consistently recorded and were not included in the analysis.

DISCUSSION

Based on previous reports of the low prevalence of AIV in Gulf coast wintering ducks (Stallknecht et al., 1990b), the >10% isolation rate during both winters was unexpected. In addition, the AIV subtypes isolated in this study have not been frequently detected in

Table 4.1. Occurrence of avian influenza virus (AIV) and avian paramyxovirus (APMV) isolated from ducks, Brazoria County, Texas.

Year	Month	Species	N	AIV	APMV	No. serotype isolates
2001	February	Blue-winged teal (*Anas discors*)	32	7 (0.22 ± 0.14)[A]	0	H2N4 (n = 3), H7N3 (n = 3), H7N4 (n = 1)
2001	February	Cinnamon teal (*A. cyanoptera*)	2	1 (0.5 ± 0.7)	0	H8N4 (n = 1)
2001	February	Green-winged teal (*A. crecca*)	29	2 (0.07 ± 0.09)	0	H7N3 (n = 1), H8N4 (n = 1)
2001	February	Mottled duck (*A. fulvigula*)	3	0	0	
2001	February	Pintail (*A. acuta*)	30	1 (0.03 ± 0.06)	0	H8N4 (n = 1)
2001	August	Mottled duck	87	0	11 (0.13 ± 0.07)	APMV-1 (n = 11)
2002	February	Blue-winged teal	75	11 (0.15 ± 0.08)	24 (0.32 ± 0.11)	H2N4 (n = 1), H2N9 (n = 1), H7N3 (n = 2), H7N4 (n = 2), H?N3 (n = 3), H?N4 (n = 2), APMV-1 (n = 23), APMV-7 (n = 1)

[A] No. (Prevalence ± C.I.)

ducks. For example, of more than 3,100 isolates from North American ducks during 1974-1990, the H2, H7 and H8 each represented <1% of the total isolates respectively (Webster et al., 1976; Boudreault et al., 1980; Hinshaw et al., 1980; Kocan et al., 1980; Smitka et al., 1981; Hinshaw et al., 1985; Nettles et al., 1985; Hinshaw et al., 1986; Kawaoka et al., 1988; Stallknecht et al., 1990b; Slemons et al., 1991; Alfonso et al., 1995). The more commonly reported AIV H subtypes from ducks, including the H3, H4 and H6, were not detected in this study.

The distinctly different AIV subtype assemblage in ducks tested in this study suggest that ducks in wintering areas, such as the coastal region of Texas, may play a unique role in the maintenance of many AIVs. This may be especially true for the less common AIV H subtypes. Although our sample size was small given the considerable population of ducks wintering in the management area (approx. 20,000, T. Merindino, personal comm.), it is interesting that none of the AIV subtypes typically associated with ducks was detected and AIV prevalence greater than 10% was detected during February in both years. This suggests that previously uncommon AIV subtypes may be transmitted among these wintering ducks. This is further supported by the isolation of H10N7 AIVs from a blue-winged teal (*A. discors*) and a northern shoveler (*A. clypeata*) sampled during April, 2000 approximately 100 miles south of Brazoria county (unpublished data).

Our results may represent the combined effects of multiple factors including: prior exposure and acquired immunity; host migratory patterns; or environmental influences on the virus.

Based on numerous published reports of the prevalence of H3, H4 and H6 AIV subtypes from ducks sampled during late-summer and fall, population immunity to these specific subtypes should be high. Prior exposure and resulting immunity has been suggested as an explanation of the low prevalence of AIV in adult ducks versus juvenile ducks (Hinshaw et al., 1985; Webster et al., 1992) and is consistent with our failure to isolate AIVs representing the H3, H4 or H6 subtypes in this study. As evidence suggests that natural infections of AIV do not afford cross-protection between AIV subtypes (Suss et al., 1994), it is possible that wintering ducks would remain susceptible to those subtypes that do not predominate during the premigration and migration periods.

The species composition of ducks sampled in this study also may have influenced our results, because, it is plausible that the infected birds detected in Texas represent birds with little or no previous exposure to AIVs. Blue-winged teal, from which most of the AIVs were isolated, generally depart the breeding grounds earlier than most species, often arriving on the wintering grounds by September (Bellrose, 1980). This suggests that these early migrants may have minimal or no previous AIV exposure and thus represent a susceptible population on wintering areas. Avian influenza viruses have been reported from this species cohabiting with other ducks species during migration (Hinshaw et al., 1980;) as well as upon arrival to wintering areas in Louisiana (Stallknecht et al., 1990b). Although the prevalence of AIV was low in Louisiana, in both cases the more common H3, H4 and H6 subtypes predominated during the period regardless of location.

Although information is limited, persistence of AIV in water is influenced by the temperature, salinity and pH (Stallknecht et al., 1990a) and variation in environmental persistence between individual AIV isolates has been detected under experimental conditions. Although speculative, it may be possible that environmental conditions as well as variation in waterfowl populations influence local subtype diversity.

Both APMV-1 and APMV-7 have been previously reported from wild ducks (Stallknecht et al., 1991), although APMV-7 is generally associated with pigeons and doves (Alexander, 2000). The 30.7% APMV-1 prevalence detected in this study is the highest prevalence reported from wintering ducks. However, based on negative results from the first year of sampling, APMV-1 infections in these ducks may be a sporadic event.

Given the strong bias toward sampling ducks, especially mallards, during the fall season, it is not surprising that the ecology of both AIVs or APMVs are not fully understood. From these results, it appears that some of the assumptions regarding the seasonality of transmission and the virus subtype host associations of AIVs are not as clear as previously thought and that our understanding of APMV in wild ducks is incomplete. The transmission and maintenance of both of these viruses in free-living duck populations involves multiple interactions between many host species, many different subtypes of viruses, and occurs within many different environments. For these reasons, care must be taken in generalizations regarding the epidemiology of both AIVs and APMVs.

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CHAPTER 5

IS AVIAN INFLUENZA VIRUS INFECTION IN SHOREBIRDS SPECIES OR LOCATION SPECIFIC?[1]

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[1] Hanson, Britta A, David E. Swayne, Dennis A. Senne, David E. Stallknecht. To be submitted to the Journal of Wildlife Diseases upon analysis of additional data from years 2001 and 2002.

ABSTRACT

Since the initial isolation from common terns (*Sterna hirundo*) in 1961, avian influenza viruses (AIV) have been isolated from more than 90 species of wild birds. Most isolates have been reported from species in the order Anseriformes but AIVs also have been reported from at least 11 additional avian orders. Extensive surveys of AIVs in shorebirds, specifically those in the family Scolopacidae, are few with only one report of a survey of multiple species in the northeastern U.S. Results of that survey suggested that the predominant AIV hemagglutinin (H) subtypes were H9 and H13 (44% of all isolates) while the 6 neuraminidase (N) subtypes isolated were distributed fairly evenly. In this study, shorebirds migrating along the Atlantic and Gulf coasts of North America were sampled during spring and fall migration (April - September 2000). More than 2,300 shorebirds were sampled and 40 AIVs were isolated. Most AIVs (67.5%) were isolated from ruddy turnstones (*Arenaria interpres*) during spring migration in the Delaware Bay region. In contrast to the previous study, the H10 and H12 subtypes comprised >60% of all isolates and no H9 or H13 subtypes were detected. These results demonstate that the predominance of AIV subtypes in North American shorebirds varies over time and suggests that AIV infection among shorebirds may be localized and species specific.

Key words: avian influenza virus, Delaware Bay, least sandpiper, ruddy turnstone, shorebird.

INTRODUCTION

After the 1961 avian influenza virus (AIV) epizootic which killed approximately 1,300 common terns (*Sterna hirundo*) in South Africa (Becker, 1966), more than 21,000 samples from wild birds have yielded more than 2,300 AIV isolates (Stallknecht et al., 1988). To date, most isolates have been reported from birds of the order Anseriformes. Based on all available published data prior to 1988, birds of the order Charadriiformes, which include the gulls, terns, and sandpipers, had an overall AIV prevalence rate of 2.2% (Stallknecht et al., 1988). In a subsequent study, however, AIV viruses were isolated from 5.8% of shorebirds sampled in the Delmarva Peninsula in the northeastern U.S. (Kawaoka et al., 1988). In that report, the ruddy turnstone accounted for 11.2% of birds sampled but 40% of all AIV isolates. The most prevalent AIV hemagglutinin (H) subtypes reported from this study were the H9 and H13 (26.1% and 17.9% respectively) while the H2, H4 and H11 subtypes each accounted for approximately 10-12% of the total isolates. Based on reports of the predominance of the H3, H4, and H6 subtypes within North American ducks, the authors suggested that the predominant AIVs circulating within shorebird populations differed from those predominating in the wild duck populations.

Although no known morbidity or mortality of wild birds has been attributed to AIV since the 1961 outbreak, wild birds have been implicated in the transmission of AIV to domestic poultry (Karunakaran et al., 1983; Halvorson et al., 1985). In addition, AIV has been isolated from ranged poultry water sources (Sivanandan et al., 1991), leading some to advise limited contact between domestic and wild birds (Kawaoka et al., 1988). Viruses representing the H5 and H7 subtypes, which are the H subtypes associated with highly pathogenic AIV in domestic poultry, have been isolated from shorebirds in the Delaware Bay region (Kawaoka et al., 1988; Saito et al., 1994). Potential for transmission of AIV from shorebirds to domestic poultry is not well understood, but phylogenetic analysis of two H5 AIVs isolated from chickens in live-bird markets in Florida and Pennsylvania during 1993 suggests that they were closely related to an H5 AIV isolated from a turnstone in the Delaware Bay during May, 1991 (Saito et al., 1994). In addition, analysis of the H gene of another H5 AIV isolated from a ruddy turnstone in the Delaware Bay suggests that it shares a common ancestor with viruses isolated from live poultry market chickens in Florida, an emu from Texas and a domestic turkey in Minnesota (Garcia et al., 1997). Recent direct transmissions of H5 and H9 AIVs from domestic birds to humans has increased the concern of potential pandemics following the introduction of novel or recombinant viruses to the human population (Perdue et al., 2000). Both the H5 and H9 subtypes have been isolated from shorebirds in the Delmarva Peninsula (Kawaoka et al., 1988; Saito et al., 1994).

Subsequent surveys of shorebirds have not been reported from the Delmarva peninsula or other North American locations. The objectives of this study were to examine the current prevalence and serotype diversity of AIVs in shorebirds migrating through the Delaware Bay region and to determine if AIV infections in shorebirds are dependent upon species or location.

MATERIALS AND METHODS

During April - September 2000, shorebirds were captured via cannon net, mist net or walk-in traps by various state, federal and private agencies at five locations (Figure 3.1).

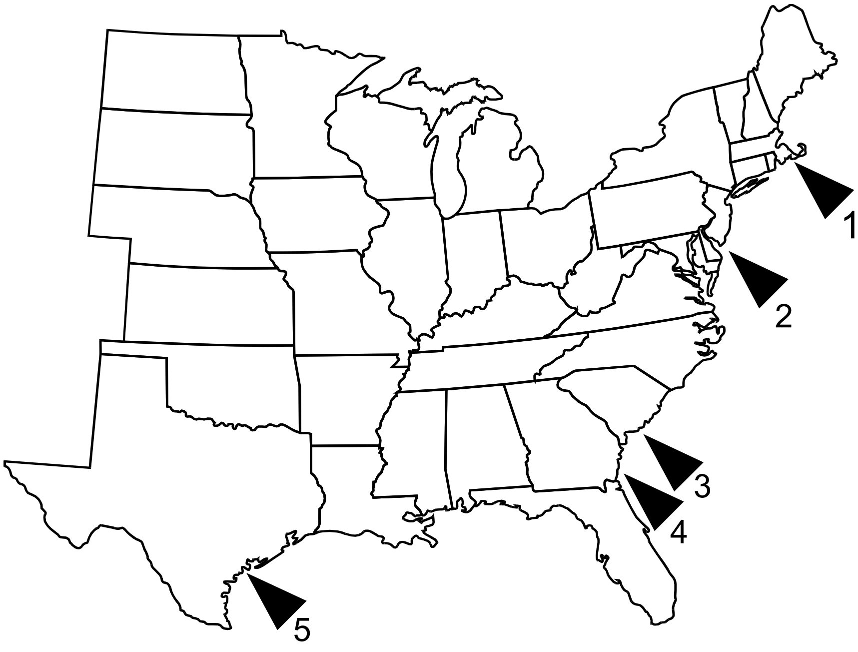

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Figure 5.1. Locations of avian influenza sampling, April - September, 2000. 1. Bird Island, MA 2. Delaware Bay, NJ and DE 3. Harbor Island, SC 4. Little Egg Island, GA 5. Aransas National Wildlife Refuge (Myrtle Foester-Whitmire Division), TX

Numbers of species sampled are listed by location (Table 3.1). Cloacal swabs were collected using sterile cotton-tipped applicators (Puritan®, Hardwood Products Company, Guilford, MN) and placed in sterile polypropylene tubes (Corning Inc., Corning, NY) containing 3 ml of Brain Heart Infusion media (BHI) (Becton Dickinson, Sparks, MD) supplemented with penicillin G (10,000 u/ml), streptomycin (2mg/ml), kanamycin (0.6 mg/ml), gentamicin (1 mg/ml), and amphotericin B (0.02 mg/ml) (Sigma Chemical Company, St. Louis, MO). Most samples were stored on ice in the field (< 8 hr) and then placed in liquid nitrogen or on dry ice. All samples were then stored at –70° C until processed.

Samples were thawed, vortexed and centrifuged at 3,000 rpm for 15 minutes. The supernatant was inoculated (0.25 ml/egg) via the allantoic route into four 9-day-old specific-pathogen-free embryonated chicken eggs. Eggs were incubated at 37º C for 72 hr and hemagglutination testing was completed as previously described (Stallknecht et al., 1990b). For negative samples, amnio-allantoic fluid was pooled by sample, diluted 1:10 in sterile phosphate-buffered saline, and repassaged into two additional eggs. All isolates were serotyped using hemagglutinating inhibition and neuraminidase inhibition tests at the National Veterinary Services Laboratory, Veterinary Services, Animal and Plant Health Inspection Service, U.S. Department of Agriculture.

Differences in prevalence estimates among species, location and season were tested using Chi Square analysis (Sokal et al., 1981).

RESULTS

Avian influenza viruses were isolated from 39 shorebirds in the Delaware Bay and one shorebird in Texas (Table 3.1). Shorebirds sampled in the spring had a significantly higher prevalence of AIV compared to birds sampled during the fall ($P < 0.0001$). Prevalence of AIV also was higher in birds migrating through Delaware Bay (2.6%) than all other sites (0.1%) ($P < 0.0001$). Prevalence of AIV infection in turnstones sampled was significantly higher than observed in all other species ($P < 0.0001$). Ruddy turnstones accounted for 12.4% of all birds sampled but 67.5% of all AIV isolates from shorebirds.

The H10 and H12 subtypes represented 60.4% of all AIV isolations (Figure 3.2). Viruses of subtypes H2, H3, H4, H5, H6, H7 and H11 also were detected. Of the nine known neuraminidase (N) subtypes, only N2 was not represented in the AIV isolates.

DISCUSSION

Overall, shorebirds migrating through the Delaware Bay during May had the highest prevalence of AIV compared to shorebirds at other locations and times sampled in this study. The low prevalence or failure to detect AIV in shorebirds at sites other than the Delaware Bay is consistent with several other studies (Slepuskin et al., 1972; Zakstelskaya et al., 1974; Bahl et al., 1977; Boudreault et al., 1980; Thorsen et al., 1980; Honda et al., 1981; Tsubokura et al., 1981; Abenes et al., 1982; Sinnecker et al., 1983; Mackenzie et al., 1984; Nettles et al., 1985; Slemons et al., 1991; Astorga et al., 1994; Okazaki et al., 2000). Although the reasons for this spatiotemporal cluster are not clear, one possibility is the high concentration of shorebirds at that site in May. Up to 1.5

Table 5.1. Occurrence of avian influenza virus in birds sampled at five locations in the eastern and central U.S. during 2000.

Location	Date	Species	N	AIV (%)	Subtype
Bird Island, MA	May 23 - 26				
		Common tern (*Sterna hirundo*)	105		
		Tern feces (off ground)	102		
Delaware Bay, DE and NJ	May 15 - 25				
		Dunlin (*Calidris alpina*)	85	1 (1.2)	H11N6
		Least sandpiper (*C. minutilla*)	127		
		Red knot (*C. canutus*)	587	7 (1.2)	H6N1 (2) H10N7 (4) H12N5 (1)
		Ruddy turnstone (*Arenaria interpres*)	296	27 (9.1)	H5N3 (1) H6N4 (1) H7N9 (2) H10N7 (8) H11N6 (1) H12N4 (4) H12N5 (10)
		Sanderling (*C. alba*)	134		
		Semipalmated sandpiper (*C. pusilla*)	226	3 (1.3)	H11N6
		Short-billed dowitcher (*Limnodromus griseus*)	12		

Table 5.1. Continued.

Location	Date	Species	N	AIV (%)	Subtype
Delaware Bay (continued)		Feces (off ground near juvenile gulls)	14	1	H4N6
Harbor Island, SC	April 16 - 19				
		Red knot	202		
		Short-billed dowitcher	24		
Little Egg Island, GA	September 10 - 16				
		Red knot	140		
		Short-billed dowitcher	16		
Aransas National Wildlife Refuge (Myrtle Foester-Whitmire Division), TX	April 14 - 17				
		Dunlin	10		
		Least sandpiper	38	1 (2.6)	H3N8
		Long-billed dowitcher (*L. scolopaceus*)	42		
		Semipalmated sandpiper	122		
		Stilt sandpiper (*C. himantopus*)	67		

Table 5.1. Continued.

Location	Date	Species	N	AIV (%)	Subtype
Aransas NWR (continued)		Western sandpiper (*C. mauri*)	13		
		Miscellaneous species*	22		

* Black-necked stilt (*Himantopus mexicanus*) (4), Black skimmer (*Rynchops niger*) (1), Killdeer (*Charadrius vociferous*) (2), Lesser yellowlegs (*Tringa flavipes*) (9), Pectoral sandpiper (*Calidris melanotos*) (3), Semipalmated plover (*Charadrius alexandrius*) (3).

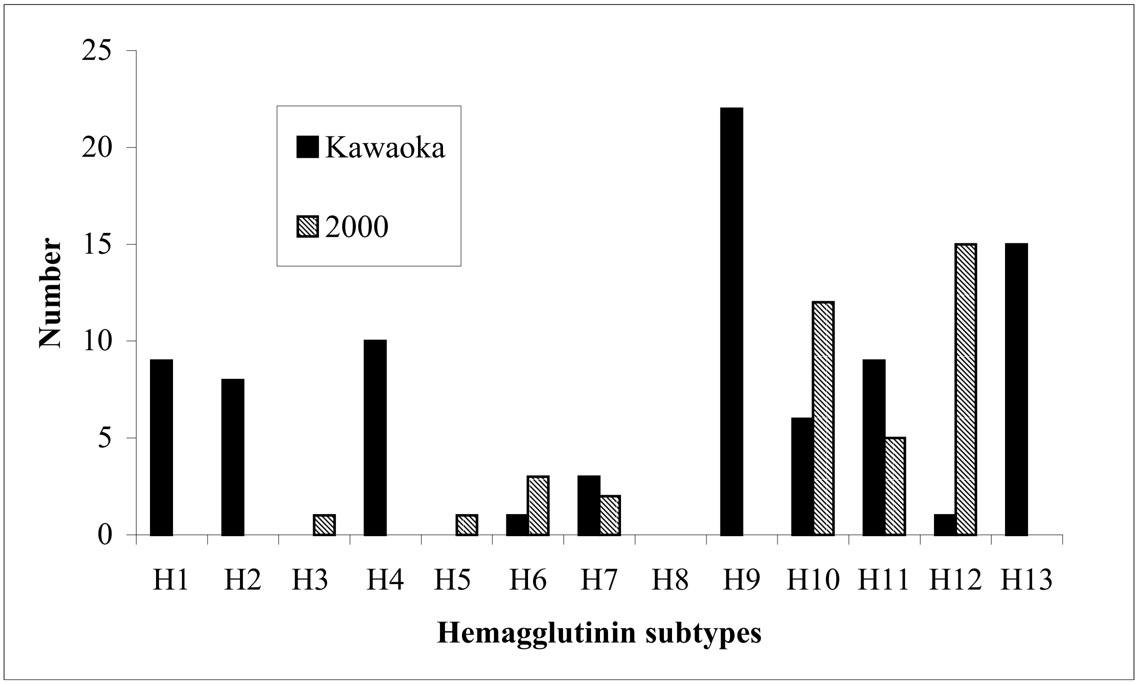

Figure 5.2. Hemagglutinin subtypes isolated from shorebirds sampled in the northeastern U.S. during May 1985 to May 1987 (Kawaoka et. al., 1988) and during May 2000.

million shorebirds utilize the shores of the Delaware Bay during spring migration and will concentrate in densities as high as 40 birds/m^2 at high-quality feeding sites (P. Atkinson, personal comm.). Given that AIV is primarily transmitted via a fecal-oral route (Webster et al., 1992), it is likely that these crowded conditions facilitate virus transmission. Total shorebird numbers and densities are usually lower and species diversity is typically not as high at other sites sampled compared to the Delaware Bay.

The preponderance of the H10 and H12 AIV subtypes in this study differed from the predominance of the H9 and H13 subtypes in the previous study of shorebirds migrating through the Northeastern U.S (Figure 3.2). This difference is not surprising given that temporal differences in H subtype prevalence are observed in waterfowl as well (Sharp et al., 1993). It should be noted that most of the H13 subtypes isolated in Kawaoka's study were from gulls, which were not sampled in this study. However, the lack of H9 subtypes and the predominance of the H10 and H12 in this study shows that subtype diversity varies temporally. It is unknown what influences the subtype prevalence from year-to-year or why the subtypes commonly found in shorebirds are not frequently isolated from waterfowl. Only three AIVs of domestic poultry or human concern (H5, H7 and H9 subtypes) were isolated, suggesting that only a small percentage of shorebirds migrating through the Delaware Bay were infected with these subtypes during May 2000.

Like the previous study involving multiple shorebird species (Kawaoka et al., 1988), the ruddy turnstone had the highest AIV prevalence of birds sampled in the family Scolopacidae. The reasons for this are unknown. A similar phenomena is observed within the family Anatindae: ducks of the tribe Anatini appear to have a higher prevalence of AIV than other duck tribes (Stallknecht, 1998). The ruddy turnstone is the only member of the tribe Arenarini while most of the other shorebirds sampled belong to the tribe Calidrini. Experimental infections of shorebirds could help determine whether there are unique physiological characteristics which make certain species more susceptible to infection with influenza.

Further study is also needed to fully understand the ecology of AIV in shorebird populations of the Delaware Bay and to what extent the behavior of the shorebirds influence transmission. There is circumstantial evidence that many of the shorebirds separate by species while roosting at night. For example, red knots appear to prefer sandy coastal habitats whereas the turnstone will utilize any area, including wetlands and exposed mudflats (H. Sitters, personal comm.). Given that AIV is transmitted primarily by a fecal-oral route in birds and that the virus can persist for weeks in water under favorable conditions (Stallknecht et al., 1990a), it seems plausible that some habitats could be more conducive to virus transmission. For example, wetlands have characteristics, such as shallow pools of water, that may increase the transmission of AIV versus habitats such as sandbars that usually lack stagnant water and are washed over by high tides twice a day.

Another possible explanation for the higher prevalence relates to the ruddy turnstone's feeding behavior. Unlike most other sandpipers, ruddy turnstones will consume carrion and human food wastes (Nettleship, 2000) and they are adept at digging and moving objects to reach their prey. They also search for food in unnatural areas (e.g., parking lots). Perhaps their feeding strategy exposes them to pathogens not normally encountered on the beach.

The only other isolate from a shorebird outside the Delaware Bay, an H3N8 from a least sandpiper (*Calidris minutilla*) in Texas, is the first known report of an AIV isolated from this species. Negative results or low prevalence of AIV infections at other sites are difficult to interpret. This may relate to the low sample size of birds at other sites or the absence of ruddy turnstones in association with other shorebird species outside the Delaware Bay. Regardless, the isolation of AIV from multiple species in the Delaware Bay and the low prevalence of AIV in birds at other sites suggests that unique population or location characteristics may play a role in the increased detection of AIV in the Delaware Bay. Since the bay is the last major stopover for many species prior to reaching the breeding grounds, it is critical that shorebirds maximize their time feeding and resting. It is unknown what effect, if any, an infection with AIV has on their ability to prepare for the flight to the breeding grounds. With many shorebird populations believed to be in decline (Morrison et al., 1994), it is important to understand all the factors contributing to this decline, including the possible role of disease.

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CHAPTER 6

CONCLUSIONS

Although influenza has been recognized as an important disease of humans for at least 100 years, our understanding of avian influenza in the natural reservoir (wild birds) is limited to approximately 30 years of data. Many of the assumptions regarding avian influenza in wild bird populations are based on research conducted in the 1970's. With the relatively recent recognition of avian influenza viruses (AIV) contributing to human influenza outbreaks and the potential for economic losses in the domestic poultry industry, it is important that our historical knowledge of influenza viruses is supplemented with current data in order to better understand and predict influenza outbreaks in both humans and birds.

In this study, some prevalence and seasonal results were similar to previous surveys of wild ducks; however, not all were consistent with previous work. For example, given the predominance of the H3, H4 and H6 subtypes in numerous surveys of wild waterfowl, it is not surprising that these subtypes represented the majority of subtypes isolated from ducks in Minnesota in this study. However, unlike past surveys, more than 20% of the isolates from ducks were subtypes associated with human or poultry disease (H5, H7 and H9). In previous studies, viruses of these subtypes have not been reported frequently from wild waterfowl. In contrast, no H3, H4 or H6 viruses were detected in ducks sampled in Texas. As the Texas work was unique with regards to period of sampling and location, the results suggest that the detection of the differing subtypes may be related to season and location. However, species susceptibility and behavior may also play a role. For example, the blue-winged teal, by departing for the breeding grounds earlier than most other duck species, may contribute to the maintenance of AIV on the wintering grounds.

These same seasonal, site and species characteristics also may play a role in the transmission of AIV among shorebirds. The low detection of AIV at sites other than the Delaware Bay suggests that there are some unique site or species characteristics involved in the maintenance of the virus. Since ruddy turnstones were not present at the other sites sampled in this study, it is impossible to distinguish the factors that contribute to the differing detection levels. However, given the higher incidence of AIV in ruddy turnstones compared to other species both in this study and a previous study, it is likely that the ruddy turnstone plays an important role in the transmission of AIV within the Delaware Bay shorebird population. Perhaps there are some innate physiological characteristics which make turnstones more susceptible to AIV infection. It is also possible that their feeding or roosting behaviors contribute to the transmission of AIV in the Delaware Bay. Whatever the reason, it appears that the Delaware Bay has a unique combination of factors that increase the detection of AIV.

It is likely that the ecology of AIV today is changing and will continue to change reflecting the natural cycles of host/disease relationships. Yet even though the ecology has changed, our assumptions are primarily based on data collected in the 1970's. These current surveys suggest that previous studies and assumptions regarding avian influenza epidemiology should not be solely relied on for predicting future outbreaks or making generalizations about the ecology of AIV. Without current data, our knowledge of AIV is limited and possibly outdated and inaccurate for use today. By understanding the current ecology of AIV, our ability to take actions to decrease transmission of AIV to domestic birds and to make informed statements regarding the perceived threat wild birds pose to both humans and domestic poultry will improve.

APPENDIX

AVIAN INFLUENZA VIRUSES IN WILD BIRDS

Appendix Table 1. Reports of avian influenza viruses from wild birds and corresponding citations.

Order/Species	**No. sampled/ no. positive**	**Country**	**Citation**
Anseriformes			
American black duck			
(Anas rubripes)	23/2	U.S.	(Rosenberger et al., 1974)
(Anas rubripes)	517/47	Canada	(Boudreault et al., 1980)
(Anas rubripes)	40/12	U.S.	(Deibel et al., 1985)
(Anas rubripes)	20/-	U.S.	(Hinshaw et al., 1986)
(Anas rubripes)	74/7	U.S.	(Slemons et al., 1991)
American wigeon			
(Anas americana)	62/11	Canada	(Hinshaw et al., 1980)
(Anas americana)	33/1	U.S.	(Slemons et al., 1991)
(Anas americana)	-/3	U.S.	(Slemons et al., 1975)
(Anas americana)	19/2	U.S.	(Alfonso et al., 1995)
(Anas americana)	32/3	Canada	(Boudreault et al., 1980)
(Anas americana)	22/1	U.S.	(Kocan et al., 1980)
Australian shelduck			
(Tadorna tadornoides)	74/3	Australia	(Mackenzie et al., 1984)
Blue-winged teal			
(Anas discors)	12/1	U.S.	(Slemons et al., 1991)
(Anas discors)	9/1	Canada	(Hinshaw et al., 1978)
(Anas discors)	120/33	Canada	(Boudreault et al., 1980)
(Anas discors)	838/132	Canada	(Hinshaw et al., 1980)
(Anas discors)	196/21	U.S.	(Deibel et al., 1985)
(Anas discors)	17/1	U.S.	(Hinshaw et al., 1986)
(Anas discors)	142/6	U.S.	(Stallknecht et al., 1990)
(Anas discors)	-	U.S.	(Slemons et al., 1974)
(Anas discors)	-/3	U.S.	(Slemons et al., 1975)
Bufflehead			
(Bucephala albeola)	3/1	Canada	(Hinshaw et al., 1980)
(Bucephala albeola)	7/1	U.S.	(Slemons et al., 1991)
Canada goose			
(Branta canadensis)	663/4	U.S.	(Ito et al., 1995)
(Branta canadensis)	7/3	Canada	(Boudreault et al., 1980)

Order/Species	No. sampled/ no. positive	Country	Citation
(Branta canadensis)	52/1	U.S.	(Rosenberger et al., 1974)
(Branta canadensis)	562/-	U.S.	(Hinshaw et al., 1986)
Canvasback			
(Aytha valisineria)	47/2	Canada	(Hinshaw et al., 1980)
(Aytha valisineria)	14/1	Canada	(Hinshaw et al., 1978)
Cinnamon teal			
(Anas cyanoptera)	-	U.S.	(Slemons et al., 1974)
Common shelduck			
(Tadorna tadorna)	582/-	France	(Hannoun et al., 1980)
(Tadorna tadorna)	263/3	France	(Hannoun, 1977)
Egyptian goose			
(Alopochen aegyptiacus)	102/4	South Africa	(Pfitzer et al., 2000)
Eurasian coot			
(Fulica atra)	1312/13	Germany	(Suss et al., 1994)
Eurasian wigeon			
(Anas penelope)	14/-	France	(Hannoun et al., 1980)
Falcated teal			
(Anas falcata)	-/1	former Soviet Union	(Isachenko et al., 1974)
Gadwall			
(Anas strepera)	-	U.S.	(Slemons et al., 1974)
(Anas strepera)	-/1	various	(Ottis et al., 1983)
(Anas strepera)	266/2	U.S.	(Stallknecht et al., 1990)
(Anas strepera)	65/1	U.S.	(Nettles et al., 1985)
(Anas strepera)	43/1	Canada	(Thorsen et al., 1980)
(Anas strepera)	52/6	Canada	(Hinshaw et al., 1980)
Garganey			
(Anas querquedula)	-	Romania	(Iftimovici et al., 1980)
Graylag goose			
(Anser anser)	95/1	former GDR	(Sinnecker et al., 1983)
(Anser anser)	611/4	Germany	(Suss et al., 1994)
Greater white-fronted goose			
(Anser albifrons)	-/1	U.S.	(Slemons et al., 1975)

Order/Species	**No. sampled/ no. positive**	**Country**	**Citation**
Green-winged teal			
(Anas crecca)	10/4	former GDR	(Sinnecker et al., 1983)
(Anas crecca)	126/1	U.S.	(Kocan et al., 1980)
(Anas crecca)	-	U.S.	(Slemons et al., 1974)
(Anas crecca)	-/4	U.S.	(Slemons et al., 1975)
(Anas crecca)	154/9	U.S.	(Slemons et al., 1991)
(Anas crecca)	-/1	Iceland	(Webster et al., 1981)
(Anas crecca)	37/3	Canada	(Hinshaw et al., 1980)
(Anas crecca)	59/-	France	(Hannoun et al., 1980)
(Anas crecca)	222/4	U.S.	(Stallknecht et al., 1990)
(Anas crecca)	29/2	Japan	(Kida et al., 1979)
(Anas crecca)	-/2	Japan	(Mikami et al., 1987)
(Anas crecca)	40/7	Japan	(Abenes et al., 1982)
(Anas crecca)	50/7	Canada	(Boudreault et al., 1980)
Grey teal			
(Anas gracilis)	115/3	Australia	(Mackenzie et al., 1984)
Long-tailed duck			
(Clangula hyemalis)	157/2	former GDR	(Sinnecker et al., 1983)
Mallard			
(Anas platyrhynchos)	91/7	U.S.	(Kocan et al., 1980)
(Anas platyrhynchos)	-	U.S.	(Slemons et al., 1974)
(Anas platyrhynchos)	242/1	U.S.	(Nettles et al., 1985)
(Anas platyrhynchos)	1322/-	U.S.	(Hinshaw et al., 1986)
(Anas platyrhynchos)	43/1	U.S.	(Rosenberger et al., 1974)
(Anas platyrhynchos)	-/13	U.S.	(Slemons et al., 1975)
(Anas platyrhynchos)	416/30	U.S.	(Slemons et al., 1991)
(Anas platyrhynchos)	60/1	U.S.	(Smitka et al., 1981)
(Anas platyrhynchos)	-/47	various	(Ottis et al., 1983)
(Anas platyrhynchos)	3/2	former GDR	(Ottis et al., 1980)
(Anas platyrhynchos)	-	U.S.	(Hinshaw et al., 1985)
(Anas platyrhynchos)	669/6	U.S.	(Webster et al., 1976)
(Anas platyrhynchos)	396/30	Canada	(Thorsen et al., 1980)
(Anas platyrhynchos)	46/9	Canada	(Boudreault et al., 1980)

Appendix Table 1 continued.

Order/Species	No. sampled/ no. positive	Country	Citation
(Anas platyrhynchos)	673/97	Canada	(Hinshaw et al., 1978)
(Anas platyrhynchos)	9/1	former Soviet Union	(Gresikova et al., 1978)
(Anas platyrhynchos)	-	Canada	(Hinshaw et al., 1985)
(Anas platyrhynchos)	270/113	U.S.	(Deibel et al., 1985)
(Anas platyrhynchos)	269/8	former Czechoslovakia	(Turek et al., 1983)
(Anas platyrhynchos)	330/53	former GDR	(Sinnecker et al., 1983)
(Anas platyrhynchos)	-/3	former Soviet Union	(Isachenko et al., 1974)
(Anas platyrhynchos)	-	former Soviet Union	(Roslaya et al., 1974)
(Anas platyrhynchos)	38/1	France	(Hannoun, 1977)
(Anas platyrhynchos)	60/4	U.S.	(Bahl et al., 1975)
(Anas platyrhynchos)	2899/849	Canada	(Hinshaw et al., 1980)
(Anas platyrhynchos)	184/24	U.S.	(Bahl et al., 1977)
(Anas platyrhynchos)	240/25	U.S.	(Alfonso et al., 1995)
(Anas platyrhynchos)	321/8	New Zealand	(Stanislawek et al., 2002)
(Anas platyrhynchos)	122/5	Japan	(Yamane et al., 1979)
(Anas platyrhynchos)	-/2	Japan	(Mikami et al., 1987)
(Anas platyrhynchos)	12/1	Hungary	(Romvary et al., 1976a)
(Anas platyrhynchos)	47/16	Isreal	(Lipkind et al., 1981)
(Anas platyrhynchos)	111/1	Hungary	(Stunzner et al., 1980)
(Anas platyrhynchos)	76/-	France	(Hannoun et al., 1980)
Mottled duck			
(Anas fulvigula)	68/2	U.S.	(Stallknecht et al., 1990)
Mute swan			
(Cygnus olor)	812/13	Germany	(Suss et al., 1994)
(Cygnus olor)	282/1	U.S.	(Graves, 1992)
(Cygnus olor)	503/6	former GDR	(Sinnecker et al., 1983)
Northern pintail			
(Anas acuta)	4/1	Isreal	(Lipkind et al., 1981)
(Anas acuta)	1/1	Japan	(Kida et al., 1979)
(Anas acuta)	233/12	Japan	(Yamane et al., 1978)
(Anas acuta)	-	U.S.	(Deibel et al., 1985)
(Anas acuta)	-	U.S.	(Slemons et al., 1974)

Order/Species	No. sampled/ no. positive	Country	Citation
(Anas acuta)	111/5	U.S.	(Slemons et al., 1991)
(Anas acuta)	-	Canada	(Hinshaw et al., 1985)
(Anas acuta)	112/8	Canada	(Hinshaw et al., 1978)
(Anas acuta)	62/-	France	(Hannoun et al., 1980)
(Anas acuta)	39/8	Canada	(Boudreault et al., 1980)
(Anas acuta)	745/256	Canada	(Hinshaw et al., 1980)
Northern shoveler			
(Anas clypeata)	33/10	Canada	(Boudreault et al., 1980)
(Anas clypeata)	-/2	Japan	(Mikami et al., 1987)
(Anas clypeata)	25/1	U.S.	(Slemons et al., 1991)
(Anas clypeata)	-	U.S.	(Slemons et al., 1974)
Pacific black duck			
(Anas superciliosa)	233/12	Australia	(Mackenzie et al., 1984)
Redhead			
(Aythya americana)	46/1	Canada	(Hinshaw et al., 1980)
(Aythya americana)	-/3	U.S.	(Slemons et al., 1975)
Ring-necked duck			
(Athya collaris)	18/2	Canada	(Boudreault et al., 1980)
(Aythya collaris)	-/1	U.S.	(Slemons et al., 1975)
Ruddy duck			
(Oxyura jamaicensis)	34/1	Canada	(Hinshaw et al., 1980)
(Oxyura jamaicensis)	-	U.S.	(Slemons et al., 1974)
Spot-billed duck			
(Anas poecilorhyncha)	40/12	Japan	(Yamane et al., 1979)
(Anas poecilorhyncha)	334/8	Japan	(Yamane et al., 1978)
(Anas poecilorhyncha)	5/1	Japan	(Abenes et al., 1982)
Tufted duck			
(Aythya fuligula)	-/6	various	(Ottis et al., 1983)
(Aythya fuligula)	40/2	Japan	(Tsubokura et al., 1981a)
(Aythya fuligula)	197/1	Japan	(Tsubokura et al., 1981b)
Tundra swan			
(Cygnus columbianus)	-/3	Japan	(Otsuki et al., 1984)
(Cygnus columbianus)	290/8	Japan	(Otsuki et al., 1987)

Appendix Table 1 continued.

Order/Species	**No. sampled/ no. positive**	**Country**	**Citation**
(Cygnus columbianus)	90/27	Japan	(Tsubokura et al., 1981a)
White-winged scoter			
(Melanitta fusca)	38/3	former GDR	(Sinnecker et al., 1983)
Wood duck			
(Aix sponsa)	12/2	Canada	(Boudreault et al., 1980)
(Aix sponsa)	639/15	U.S.	(Deibel et al., 1985)
Charadriiformes			
Arctic tern			
(Sterna paradisaea)	20/3	former Soviet Union	(Zakstelskaya et al., 1975)
(Sterna paradisaea)	28/2	former GDR	(Sinnecker et al., 1983)
Black-headed gull			
(Larus ridibundus)	316/7	former GDR	(Sinnecker et al., 1983)
Black-tailed gull			
(Larus crassirostris)	175/13	Japan	(Tsubokura et al., 1981a)
Common murre			
(Uria aalge)	100/1	former Soviet Union	(Sazonov et al., 1977)
Common tern			
(Sterna hirundo)	3/3	South Africa	(Becker, 1966)
(Sterna hirundo)	49/3	former Soviet Union	(Zakstelskaya et al., 1974)
(Sterna hirundo)	-/1	former Soviet Union	(L'vov, 1978)
Dunlin			
(Calidris alpina)	38/1	Japan	(Honda et al., 1981)
Eurasian woodcock			
(Scolopax rusticola)	-	former Soviet Union	(Roslaya et al., 1974)
(Scolopax rusticola)	-	former Soviet Union	(Isachenko et al., 1974)
Franklin's gull			
(Larus pipixcan)	30/1	U.S.	(Hinshaw et al., 1982)
Great black-backed gull			
(Larus marinus)	-/1	U.S.	(Hinshaw et al., 1982)
Herring gull			
(Larus argentatus)	23/2	former Soviet Union	(Zakstelskaya et al., 1975)
(Larus argentatus)	466/9	U.S.	(Kawaoka et al., 1988)

Appendix Table 1 continued.

Order/Species	**No. sampled/ no. positive**	**Country**	**Citation**
(Larus argentatus)	-/1	U.S.	(Hinshaw et al., 1982)
Laughing gull			
(Larus atricilla)	118/4	U.S.	(Kawaoka et al., 1988)
Lesser noddy			
(Anous tenuirostris)	254/1	Canada	(Mackenzie et al., 1984)
Ring-billed gull			
(Larus delawarensis)	189/2	U.S.	(Nettles et al., 1985)
(Larus delawarensis)	-/1	Canada	(Campbell, 1999)
(Larus delawarensis)	3403/70	U.S.	(Graves, 1992)
(Larus delawarensis)	3024/64	U.S.	(Hinshaw et al., 1982)
Ruddy turnstone			
(Arenaria interpres)	-	U.S.	(Saito et al., 1994)
(Arenaria interpres)	162/15	U.S.	(Kawaoka et al., 1988)
Sanderling			
(Calidris alba)	133/4	U.S.	(Kawaoka et al., 1988)
Sandwich tern			
(Sterna sandvicensis)	351/1	former GDR	(Sinnecker et al., 1983)
Slender-billed gull			
(Larus genei)	-/3	former Soviet Union	(L'vov, 1978)
Sooty tern			
(Sterna fuscata)	294/1	Australia	(Mackenzie et al., 1984)
Spur-winged lapwing			
(Vanellus spinosus)	2/1	India	(Manjunath et al., 1981)
Temmick's stint			
(Calidris temminckii)	-	former Soviet Union	(Zakstelskaya et al., 1975)
White-winged tern			
(Chlidonias leucoptera)	-	former Soviet Union	(Roslaya et al., 1974)
Ciconiiformes			
Glossy ibis			
(Plegadis falcinellus)	-	Romania	(Iftimovici et al., 1980)

Order/Species	**No. sampled/ no. positive**	**Country**	**Citation**
Gray heron			
(Ardea cinerea)	-/11	former Soviet Union	(Roslaya et al., 1975)
(Ardea cinera)	84/27	former Soviet Union	(Roslaya et al., 1974)
(Ardea cinerea)	-	Romania	(Iftimovici et al., 1980)
Squacco heron			
(Ardeola ralloides)	-	Romania	(Iftimovici et al., 1980)
Columbiformes			
Collard dove			
(Streptopelia decaocto)	19/1	Hungary	(Romvary et al., 1975)
Galliformes			
Ring-necked pheasant			
(Phasianus colchicus)	20/2	Hungary	(Romvary et al., 1976a)
Rock partridge			
(Alectoris graeca)	126/2	Isreal	(Lipkind et al., 1981)
Gaviiformes			
Arctic loon			
(Gavia arctica)	-	Romania	(Iftimovici et al., 1980)
Red-throated loon			
(Gavia stellata)	4/1	former Soviet Union	(Zakstelskaya et al., 1975)
Gruiformes			
American coot			
(Fulica americana)	14/1	Canada	(Boudreault et al., 1980)
(Fulica americana)	58/3	U.S.	(Slemons et al., 1975)
Eurasian coot			
(Fulica atra)	14/1	Australia	(Mackenzie et al., 1984)
(Fulica atra)	4/1	Hungary	(Romvary et al., 1976a)
(Fulica atra)	-/15	various	(Ottis et al., 1983)
(Fulica atra)	64/4	Isreal	(Lipkind et al., 1981)

Appendix Table 1 continued.

Order/Species	**No. sampled/ no. positive**	**Country**	**Citation**
Passeriformes			
American redstart			
(Setophaga ruticilla)	31/3	Canada	(Boudreault et al., 1980)
Barn swallow			
(Hirundo rustica)	-/1	Egypt	(Amin et al., 1980)
Black-faced bunting			
(Emberiza spodocephala)	-	former Soviet Union	(Roslaya et al., 1974)
Carrion crow			
(Corvus corone)	-	former Soviet Union	(Isachenko et al., 1974)
Common jackdaw			
(Corvus monedula)	-	former Soviet Union	(Isachenko et al., 1974)
Common redstart			
(Phoenicurus phoenicurus)	-/1	Egypt	(Amin et al., 1980)
Common whitethroat			
(Sylvia communis)	-/1	Egypt	(Amin et al., 1980)
Dark-eyed junco			
(Junco hyemalis)	15/1	Canada	(Boudreault et al., 1980)
European starling			
(Sturnus vulgaris)	42/1	Isreal	(Lipkind et al., 1979)
Garden warbler			
(Sylvia borin)	-/1	Egypt	(Amin et al., 1980)
Hermit thrush			
(Catharus guttatus)	10/1	Canada	(Boudreault et al., 1980)
House sparrow			
(Passer domesticus)	-/1	Hungary	(Romvary et al., 1976b)
Icterine warbler			
(Hippolais icterina)	-/1	Egypt	(Amin et al., 1980)
Purple finch			
(Carpodacus purpureus)	20/1	Canada	(Boudreault et al., 1980)

Order/Species	**No. sampled/ no. positive**	**Country**	**Citation**
Red-backed shrike			
(Lanius collurio)	-/1	Egypt	(Amin et al., 1980)
Song sparrow			
(Melospiza melodia)	41/1	Canada	(Boudreault et al., 1980)
Spotted flycatcher			
(Musicapa striata)	-/1	former Soviet Union	(Isachenko et al., 1974)
Swainson's thrush			
(Catharus ustulatus)	45/1	Canada	(Boudreault et al., 1980)
Tennessee warbler			
(Vermivora peregrina)	44/3	Canada	(Boudreault et al., 1980)
Willow flycatcher			
(Empidonax traillii)	34/3	Canada	(Boudreault et al., 1980)
Willow warbler			
(Phylloscopus trochilus)	-/1	Egypt	(Amin et al., 1980)
Yellow vented bulbul			
(Pycnonotus goiaver personatus)	1/1	Malaysia	(Ibrahim et al., 1990)
Yellow wagtail			
(Motacilla flava)	-/1	Egypt	(Amin et al., 1980)
Yellow warbler			
(Dendroica petechia)	38/1	Canada	(Boudreault et al., 1980)
Yellow-breasted bunting			
(Emberiza aureola)	-	former Soviet Union	(Roslaya et al., 1974)
Yellow-rumped warbler			
(Dendroica coronata)	22/2	Canada	(Boudreault et al., 1980)
Yellow-throated warbler			
(Dendroica dominica)	8/2	Canada	(Boudreault et al., 1980)
Pelecaniformes			
Cormorant			
(Phalacrocorax carbo)	4500/18	Germany	(Suss et al., 1994)
(Phalacrocorax carbo)	-	Romania	(Iftimovici et al., 1980)

Order/Species	**No. sampled/ no. positive**	**Country**	**Citation**
Piciformes			
Great-spotted woodpecker			
(Dendrocopos major)	-	former Soviet Union	(Roslaya et al., 1974)
Podicipediformes			
Pied-billed grebe			
(Podilymbus podiceps)	1/1	Canada	(Boudreault et al., 1980)
Procellariiformes			
Wedge-tailed shearwater			
(Puffinus pacificus)	201/1	Australia	(Downie et al., 1973)
(Puffinus pacificus)	289/2	Australia	(Downie et al., 1977)
(Puffinus pacificus)	531/3	Australia	(Mackenzie et al., 1984)

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