Are Passerine Birds Reservoirs for Avian Influenza Viruses?

By

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(Under the Direction of Michael J. Yabsley)

ABSTRACT

Although peridomestic passerines have been involved in influenza A virus (IAV) outbreaks in poultry, there is little evidence indicating they serve as reservoirs for these viruses under natural conditions. Recent molecular-based detections in passerines have challenged this paradigm, suggesting additional research is warranted to define the role of these birds as IAV hosts. To address this, we reviewed the published literature reporting results from IAV surveillance of passerines and conducted prospective virologic and serologic surveillance of passerines for IAV's. Passerines were collected and tested from 102 terrestrial wild bird species from Georgia, New Jersey, Delaware, and Minnesota. IAV antibodies were detected in 4/3,868 serum samples (0.1%), all from Minnesota. No virus was detected in 900 swab samples by virus isolation or matrix real-time reverse transcriptase polymerase chain reaction. Data from this study demonstrates that passerines have a limited role in IAV natural history and should not be considered reservoirs.

INDEX WORDS: Avian influenza virus, bELISA, Passerines, RRT-PCR, Serology, Virus isolation

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CHAPTER 1

INTRODUCTION

Influenza A viruses (IAVs) have been isolated from over 100 avian species representing 26 different families (Olsen et al. 2006). Most detections of IAV have been from wild birds in the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds), which are widely considered the major reservoirs for this virus (Stallknecht and Shane 1988; Webster et al. 1992). However, other avian taxa can be infected and they have been suggested to play various roles in the transmission of IAV within the wild bird reservoir system and/or in outbreaks in aberrant hosts (i.e. domestic poultry). In particular, terrestrial wild birds in the order Passeriformes or other peridomestic species (i.e. pigeons, crows, etc.), are frequently considered a risk for IAV transmission because they are widespread, utilize a diversity of habitats, and often interface with wild birds, domestic animals, and humans. However, historically, IAV has rarely been isolated from passerines and other terrestrial birds despite extensive surveillance efforts (Boudreault et al. 1980; Diebel et al. 1985; Webster et al. 1992). Importantly, when IAV has been isolated from terrestrial wild birds, it often has been from peridomestic species associated with agricultural environments where IAV was likely circulating in domestic animals (i.e. poultry farms, live bird markets; Ellis et al. 2009; Siengsanan et al. 2009).

In recent years, multiple recent molecular-based surveillance studies have reported IAV detection in passerines at higher levels than traditional isolation-based surveillance efforts and some researchers have interpreted these novel data to suggest that passerines may be IAV reservoirs (Gronesova et al. 2008a; Gronesova et al. 2008b; Peterson et al. 2008; Fuller et al. 2010; Borovská et al. 2011; Cumming et al. 2011; Thinh et al. 2012). The benefits and disadvantages of virus isolation vs. molecular diagnostics (i.e. real-time reverse transcriptase polymerase chain reaction (RRT-PCR)) in wild bird IAV surveillance are well-established (Spackman et al. 2003). In regards to RRT-PCR, it is a sensitive screening tool to detect IAV infection; however, positive results can be difficult to interpret without additional confirmatory data, including virus isolation or sequencing. This is particularly true for RRT-PCR positive samples with high cycle threshold (Ct) values, from which virus is rarely isolated.

The recent molecular detections of IAV in passerines without confirmatory isolation have raised questions relating to the role of passerines in IAV epidemiology. Whether these diagnostic results reflect true positives (i.e. infected bird) and what the results mean in regards to IAV host reservoir status are unknown. To address these gaps in our knowledge, the objectives of this study were to: 1) Summarize existing literature on IAV surveillance in passerines and 2) Conduct a large-scale survey for IAV in passerines under a variety of settings (natural, peridomestic, and agriculture) using common diagnostic tools, including virus isolation in embryonated chicken eggs, RRT-PCR, and serology.

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CHAPTER 2

AVIAN INFLUENZA VIRUS – LITERATURE REVIEW

Description

Influenza A viruses belong to the *Orthomyxoviridae* family of segmented negative-sense RNA viruses divided into five genera, influenza types A, B, and C, Isavirus, and Thogotovirus (Suarez 2008). IAVs can be differentiated from type B and C influenza viruses based on the identity of the nucleoprotein (NP) and matrix (M1) proteins (Webster et al 1992). IAVs are enveloped viruses that contain single stranded RNA, ranging from 80 to 120nm spherical forms to filamentous forms that are several microns in length.

IAV contain eight gene segments encoding ten viral proteins including the surface proteins hemagglutinin (HA) and neuraminidase (NA). The IAV subtype is determined based on the combination of HA and NA glycoproteins. HA glycoproteins function to bind virus to host cells and is the major antigen for host immunity, while the NA glycoprotein releases virus from host cells following replication. To date, 16 HA and 9 NA antigenic subtypes have been detected and found in many different combinations (Olsen et al. 2006). The surface proteins of IAV can undergo antigenic drift, causing changes in HA and NA by mutation, and antigenic shift, which results in larger antigenic changes through reassortment of the genome (Murphy and Webster 1996; Webster 1999; Spackamn 2008). IAVs are classified as either high pathogenic (HPIAV) or low pathogenic (LPIAV). The World Organization for Animal Health distinguishes a virus as highly pathogenic when it has an intravenous pathogenicity index (IVPI) in 6-week-old chickens >1.2 or causes 75% mortality in 4, 8 week-old chickens infected intravenously. All viruses not meeting these criteria are considered low pathogenic. However, any H5 or H7 virus not meeting these criteria is sequenced and compared to other H5 and H7 viruses.

To facilitate classification of various IAVs, a specific nomenclature is used to describe each influenza virus isolate which includes: (1) antigenic type (A, B, or C); (2) host animal from which the virus was isolated; (3) geographic origin of the isolate; (4) laboratory or reference identification number of the isolate; (5) the year of isolation; and (6) the HA and NA subtypes (Suarez 2008). An example of this nomenclature for a IAV H7N7 virus isolated from a starling from Victoria, Australia in 1985 would be: A/Starling/Victoria/5156/85 (H7N7).

Host range and reservoirs

A wide variety of animals are known hosts for IAVs, including humans, pigs, horses, marine mammals, and birds (Webster 1992). Based on phylogenetic studies, there are numerous species-associated lineages of IAVs, which demonstrates that interspecies transmission depends on the species infected and possible host interactions. Wild birds are considered to be the source of influenza viruses in all other species.

The first isolate of IAV was during a mortality event of common terns (*Sterna hirundo*) in South Africa (Becker 1966). Although this report indicated that wild birds can become infected with HPIAV, the next detection of HPIAV in wild birds wasn't until 2002 when H5N1 HPIAV emerged in Asia and more recently spread throughout Eurasia and Africa. Subsequent investigations sought to determine the potential role of wild birds in the ecology of IAV (Hinshaw and Webster 1982). Serologic studies conducted from 1968-1972, detected IAV antibodies from numerous species of free-living wild birds (Esterday et al. 1968; Asplin 1970; Laver and Webster 1972; Stallknecht and Shane 1988). This demonstrated that IAVs not only naturally circulate in wild avian populations, but that this potential reservoir (i.e. wild birds) incorporates many different avian species (Stallknecht and Brown 2008). This was further confirmed through the isolation of IAVs from wedge –tailed shearwaters (*Puffinus pacificus*) in Australia and ducks in California (Downie and Laver 1973; Slemons et al. 1974).

To date, IAVs have been isolated from >100 species of wild birds in 12 orders and all known HA (H1-H16) and NA (N1-N9) subtypes have been isolated from wild birds (Stallknecht and Shane 1988; Olsen et al 2006). Wild birds associated with aquatic environments represent the group with the highest rate of IAV isolation. In particular, birds in the orders Anseriformes (ducks, geese and swans) and Charadriiformes (gulls, terns and waders), are considered the natural reservoirs for IAV; however, there is much variability in prevalence of IAV isolation between species and even families within these two orders (Stallknecht and Shane 1988). Among Anseriforms, the majority of virus isolates have come from dabbling ducks in the subfamily Anatinae with viruses reported from 47 of the 158 species of ducks and geese worldwide (Olsen et al. 2006). More IAV isolations have been reported from mallard (*Anas platyrhynchos*) than any other species (Stallknecht and Brown 2007); however, this species is one of the most studied host species. Most virus isolations from Charadriiformes are associated with birds in the families Laridae (gulls and terns) and Scolopacidae (sandpipers and turnstones). Although IAV has been isolated from a many gull and tern species, most isolations are from a single species, the ruddy turnstone (*Arenaria interpres*), and in particular, ruddy turnstones from a single site, Delaware Bay, USA (Stallknecht 1998; Stallknecht and Brown 2007; Hanson et al. 2008).

Although birds in the orders Anseriformes and Charradriiformes are considered the natural reservoirs for IAV, isolations have been reported from species in other orders utilizing aquatic habitats. A limited number of isolates have been acquired from birds in the orders

Ciconiiformes (ibis and herons), Gaviiformes (loons), Gruiformes (moorhen and coots), Pelecaniformes (pelicans and cormorants), Podicepdiformes (grebes), and Procellariformes (shearwaters and petrels) (Stallknecht and Shane 1988; Stallknecht and Brown 2008).

Transmission and maintenance

Transmission among wild birds is via the fecal-oral route (Hinshaw et al. 1979). Within ducks, IAV replicates in cells lining the lower gastrointestinal tract (Slemons and Easterday 1977; Webster et al. 1978) and large amounts of virus are shed in the feces (Webster et al. 1978; Hinshaw and Webster 1982). For example, experimentally-infected Muscovy ducks (*Cairina moschata*) excreted large amounts of fecal material with a viral titers as high as $1x10^{7.8}$ median embryo-infectious dose (EID_{50})/ml (Webster et al. 1978). Hinshaw et al. (1980) reported that duration of viral shedding among waterfowl hosts may be prolonged as experimentally-infected domestic Pekin ducks (*Anas platyrhychos*) excreted virus for over 28 days.

Although the maintenance cycle of IAV is not fully understood, data suggest that continual bird-to-bird transmission and environmental persistence allows IAV to persist in nature. Although, knowledge of environmental persistence of IAVs is limited, IAVs have been isolated from surface water in Canada, Minnesota, and Alaska (Hinshaw et al. 1980; Halverson et al. 1983; Ito et al. 1985) and several laboratory trials have been conducted. Initially, Webster et al. (1978) showed that H3N2-inoculated fecal material and nonchlorinated river water at initial doses of $10^{6.8}$ EID_{50} and $10^{8.1}$ EID_{50}, respectively, remained infective for up to 32 days. More recently, it has also been demonstrated that IAVs can persist for extended periods in water at 4°C, 17°C, and 28°C and that temperature, pH, and salinity impact IAV persistence (Brown et al. 2006; Stallknecht et al. 1990a; Stallknecht et al. 1990b).

Among North American species of Anseriformes, peak IAV prevalence occurs in late summer and early fall as large numbers of waterfowl congregate on staging areas throughout Canada and the northern United States prior to fall migration (Hinshaw et al. 1985; Olsen et al. 2006). At these staging areas, high concentrations of susceptible juvenile birds become infected and IAV prevalence rates can exceed 30%. However, as birds arrive at wintering grounds, prevalence rates are often <1-2% (Stallknecht and Shane 1988). In contrast, the prevalence of AIVs among the Charadriiformes differ significantly spatially, temporally, and genetically (Kawaoka et al. 1988; Stallknecht and Shane 1988). For example, the only time and geographic site worldwide where consistent IAV isolations have been reported from shorebirds is at one migration stop-over site (Delaware Bay, USA) (Stallknecht and Shane 1988; Fouchier et al. 2003; Hanson et al. 2008). In other locations, the prevalence of IAVs in these shorebird species is generally very low or absent (Krauss et al 2004; Hanson et al. 2008).

The diversity of IAV subtypes varies among wild bird populations with variation occurring between hosts, geographic location, and year (Stallknecht and Brown 2008). All HA and NA subtypes, excluding H13 and H16, circulate in waterfowl in North America and Europe (Olsen et al. 2006). However, HA subtypes H3, H4, and H6 and NA subtypes N2, N6, and N8 are the most common IAVs isolated from North American waterfowl (Hinshaw et al. 1980; Stallknecht and Shane 1988; Sharp et al. 1993; Krauss et al. 2004). To date, surveillance studies in Charadriformes throughout the world have yielded isolates from 15 of the 16 HA subtypes (excluding H14) and all nine NA subtypes (Krauss et al. 2010). Common subtypes isolated from shorebirds include H5, H7, and H9 viruses (Krauss et al. 2004). While the predominant subtypes represented by gulls are H13 and H16.

IAVs in Passerines

Historically, passerines were considered to have little to no role in the epidemiology of IAV (Hinshaw and Webster 1982; Webster et al. 1992; Stallknecht and Shane 1988; Stallknecht 1997). Despite extensive surveillance efforts, IAVs have rarely been isolated from passerine birds (Boudreault et al. 1980; Diebel et al. 1985; Webster et al. 1992); however, when IAV has been reported, it most often is reported from peridomestic species associated with agricultural environments where IAV was present in domestic animals (i.e. poultry farms, live bird markets etc.; Ellis et al. 2009; Siengsanan et al. 2009). However, multiple recent molecular-based surveillance studies have reportedly detected IAV in passerines at higher levels than traditional isolation-based surveillance efforts and some researchers have interpreted these novel data to suggest that passerines may be IAV reservoirs (Gronesova et al. 2008a; Gronesova et al. 2008b; Peterson et al. 2008; Fuller et al. 2010; Borovská et al. 2011; Cumming et al. 2011; Thinh et al. 2012). One goal of the current research was to conduct a critical and comprehensive review of data regarding the role passerines play in the epidemiology of IAV, therefore, additional details on passerines and their potential role as IAV hosts can be found in Chapter 3.

Pet Trade of Passerines

IAVs have been isolated from passerines imported for the pet trade. In the United States, the first reports of IAVs from passerines were three virus isolates acquired in 1971 and 1972 from dying mynah birds in quarantines in California and Massachusetts (Butterfield et al. 1973). Three additional isolates were obtained from three species of finch housed in the same cage imported into California from Taiwan (Slemons et al. 1973a), while Slemons et al. (1973b) isolated 15 IAVs from several different passerine species imported into California from Thailand. A large surveillance study conducted from October 1973 to September 1981, testing of 2,882,111 birds resulted in IAV isolations from 170 birds of different passerines species (finches and canaries) (Senne et al. 1983). Isolation of IAVs of all species of exotic imported birds greatly declined over the period of the study with a prevalence of 64% in 1974 to 0.2% in 1981 (Senne et al. 1983). In another study conducted from 1982 and 1991, 146 of 274 (54%) IAV isolations made from imported birds were reported from passerines (Panigrahy et al. 1992). Several subtypes were isolated from passerines including H3N6, H3N8, H4N1, and H7N1 from pekin robins (*Leiothrix lutea*), H3N8 and H4N8 from canaries and finches, H4N8 from thrushes, H4N1 from silver-eared mesias (*Leiothrix argentauris*), H7N8 and H7N1 from magpie robins (*Copsychus saularis*), H7N1 from a blue and white flycatcher (*Cyanoptila cyanomelana*) and H4N8 from mynahs (Panigraphy et al. 1992). In addition, Panigraphy and Senne (1997) detected numerous subtypes from passerines intended for importation into the US between 1992 and 1996. Subtypes isolated from passerines varied by year with H3N8, H4N6, and H7N1 (1992); H3N6, H4N2, H4N6, H7N1 and H7N8 (1993); H4N6 and H7N1 (1994); none (1995); and H3N6, H3N8, H4N6, and H4N8 (1996). All IAVs isolated from 1992-1996 were determined to be nonpathogenic for chickens.

Other countries have conducted surveillance of passerines imported for the pet trade and IAVs have been isolated in the United Kingdom and Japan. The first report of an IAV isolated from an imported passerine in the United Kingdom was a H3N8 from a thrush (*Cactua sulphurea*; Alexander 1981). A follow-up study, Alexander and Allen (1977) isolated IAV from 42 of 170 different consignments of caged birds from India upon their arrival at Heathrow Airport, London. All isolates were H3N8 and were acquired from a diversity of species including tricolored nuns, finches, spice birds, and mynah birds. Two studies conducted in Japan detected 22 IAV isolates from birds being imported from Thailand and India (Fukumi et al.

1977; Nerome et al. 1978). Isolates were from 19 mynah birds from India (n=9) and Thailand (n=10). All isolates originating from India and two from Thailand were H3N8 and the remaining eight Thailand isolates were H4N8. Another study detected H4N6, H4N8, and H3N8 in mynahs imported into Japan (Ogawa et al. 1980).

Experimental Infection of Passerines

Experimental inoculation studies of passerines with both HPIAV and LPIAV indicate they are susceptible to infection, shed virus, and seroconvert, although few studies utilized LPIAVs. Nemeth et al. (2010) inoculated and subsequently challenged house sparrows (*Passer domesticus*) and European starlings (*Sturnus vulgaris*) with a LPIAV isolate (A/wild bird/California/08 (H3N8)), which was isolated from waterfowl in California. The project had two goals: 1) Inoculate sparrows and starlings to determine susceptibility and determine if transmission occurred to co-housed control birds and 2) Determine susceptibility of birds to a challenge inoculation. As determined with RT-PCR, more starlings (35/36; 97%) than sparrows (19/36; 53%) shed virus via oropharyngealy and only 3/36 (8%) birds of each species had detectable cloacal viral shedding. On average, starlings shed virus longer than sparrows (3.3 days and 1.5 days, respectively). All but one starling during the first goal seroconverted; however, when this bird was challenged in goal 2, it seroconverted. No transmission to co-housed controls was detected during the 28 day study. A similar study inoculated starlings with two LPIAV viruses (one from chickens [A/chicken/OH/494832/2007 (H2N3)] and one from a waterfowl species [A/Northern Shoveler/ 28926-3/2007 (H4N2)] from Ohio, US (Qin et al. 2010). Virus was detected from both tracheal and cloacal swabs of these inoculated starlings; however, birds infected with the H4N2 virus from the duck shed a higher average titer than birds infected with the H2N3 isolate from the chicken. Interestingly, only 40% and 60% of RRT-

PCR-positive tracheal and cloacal swabs from H2N3 and H4N2 infected birds, respectively, was positive by virus isolation. All birds had seroconverted by 14 days post inoculation (DPI; Qin et al 2010).

Prior to the outbreaks of Eurasian H5N1, only one study had inoculated passerines with HPIAVs. Nestorowicz et al. (1987) inoculated sparrows and starlings with two different viruses obtained from a chicken outbreak in Victoria, Australia (A/chicken/Victoria/1/85 (H7N7) and A/Starling/Victoria5156/85 (H7N7)). The mortality rate of starlings (100%) was higher than that of sparrows (30%) when inoculated with either H7N7 isolate.

With the emergence of Eurasian H5N1, numerous studies have been conducted to investigate host range, pathogenicity, and reservoir potential of a wild range of bird species. Perkins and Swayne (2002) assessed the ability of A/chicken/Hong Kong/220/97 (H5N1) to infect and cause disease in zebra finches (*Taeniopygia guttata*), house finches (*Carpodacus mexicanus*), house sparrows, and European starlings. Zebra finches were most severely affected with 100% mortality, followed by house finches having significant morbidity and mortality (64% and 36%, respectively). In contrast, house sparrows exhibited only mild depression, no mortality, and lacked gross lesions and starlings exhibited neither clinical disease nor mortality and lacked any gross lesions. This study demonstrated that a single IAV isolate differed in pathogenicity among different bird species, even species within the same order. This same virus was then used in a subsequent inoculation study which included two mammalian species and seventeen avian species (including zebra finches, house finches, and starlings) (Perkins and Swayne 2003). Similar to Perkins and Swayne (2002), zebra finches had high rates of morbidity and mortality (100%), followed by house finches with delayed morbidity and lower mortality rates (78% and 57%, respectively). Again, house sparrows exhibited either no or mild clinical disease and no mortality, while starlings had no clinical disease or gross lesions.

A study by Boon et al. (2007) inoculated house sparrows and starlings with four different HPIAV H5N1 viruses that were acquired in the mid-2000s. Two viruses were from Thailand (A/duck/Thailand/144/2005 and A/quail/Thailand/551/2005) and two viruses were isolated in China (A/common magpie/Hong Kong/645/2006 and A/Japanese white-eye/Hong Kong/1038/2006). Variable mortality (66-100%) was noted in inoculated sparrows, depending on the virus strain; however, none of the starlings died. Reisolation of virus was obtained from oropharengeal and cloacal swabs from starlings and sparrows inoculated with any of the four viruses. There was no evidence of transmission to contact sparrows and only one contact starling became infected.

Subsequent studies were conducted to determine the effects of various median egg infectious doses (EID_{50}) on the susceptibility and pathogenicity of HPIAV to various bird species. Brown et al. (2009) inoculated house sparrows and rock pigeons (*Columbia livia*) with decreasing doses of A/whooper swan/Mongolia/244/05 (H5N1) and found that the sparrows in the medium and high dose groups died, whereas only 3 of 5 sparrows in the low dose group died. Both cloacal and oropharyngeal virus shedding was detected for all viral doses. Interestingly, only two of the five pigeons in the high dose group died pigeons and viral shedding was transient among all dose groups and viral titers were low. Similarly, Breithaupt et al. (2011) inoculated two species of passerines (blackcaps (*Sylvia atricapilla*) and red-billed queleas (*Quealea quelea*)) with A/*Cygbus cygnus*/Germany/R65/2006 (H5N1) using different doses (i.e. low and high). The blackcaps had 100% mortality among both the low and high dose groups, whereas the queleas had varying mortality rates (18% and 82% for low and high, respectively). IAV antibodies were detected among the two surviving queleas in the high dose group, where the nine remaining in the low dose group failed to seroconvert.

To investigate the potential effects of migration on susceptibility to IAVs, Kalthoff et al. (2009) inoculated three groups of stonechats (*Saxicola torquata*) from strongly migrating, weakly migrating, and non-migrating populations with A/*Cygbus cygnus*/Germany/R65/2006 (H5N1). The birds were inoculated in autumn, just before their natural migration period. Despite the presence of migratory restlessness in the strongly migrating group, there were no differences in susceptibility, survival time, clinical symptoms and viral shedding between the migratory status groups.

Collectively, through experimental trails we know that when exposed, passerines can become infected, shed virus and seroconvert to LPIAV and HPIAV. However, similar to other avian orders there are much variation among species and even families of passerines, but what is consistent is that viral shedding is typically short lived, shed at low viral titers, and there is little cage-mate transmission when exposed.

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CHAPTER 3

Are Passerine Birds Reservoirs for Avian Influenza Viruses?

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ABSTRACT

Although peridomestic passerine species have been involved in influenza A virus (IAV) outbreaks in poultry, there is little historic evidence to indicate they serve as reservoirs for these viruses under natural conditions. Recent molecular-based detections of IAV in terrestrial wild birds have challenged this paradigm and it has been suggested that additional research is warranted to better define the role of these birds as IAV hosts. To address this need, we reviewed the published literature reporting results from IAV surveillance of passerines. We also conducted prospective virologic and serologic surveillance of passerines for IAV's. The literature review included 59 publications from 1975-2013 that reported results from 829 species of passerines and other terrestrial birds. In our prospective study during 2010 and 2011, 3,868 serum samples and 900 swab samples were collected and tested from 102 terrestrial wild bird species from four states, including Georgia, New Jersey, Delaware, and Minnesota. Antibodies to the nucleoprotein of IAV were detected with a commercial blocking ELISA in 4/3,868 serum samples (0.1%), all from Minnesota. No virus was detected in 900 swab samples by virus isolation in embryonating chicken eggs or matrix real-time reverse transcriptase polymerase chain reaction. Results presented herein are consistent with historic literature; while passerines and terrestrial wild birds may have a limited role in the epidemiology of IAV when associated with infected domestic poultry or other aberrant hosts; there is no evidence supporting their involvement as natural reservoirs for IAV.

Key words: Avian influenza virus, bELISA, passerines, RRT-PCR, serology, virus isolation

INTRODUCTION

Influenza A viruses (IAVs) have been isolated from over 100 avian species representing 26 different families (Olsen et al. 2006). Most detections of IAV have been associated with birds in the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds), which are widely considered as the major reservoirs for these viruses (Stallknecht and Shane 1988; Webster et al. 1992). Infected birds in other avian taxa, including passerine species, have been reported (Olsen et al. 2006) but their potential to serve as reservoirs for these viruses has not been established. Passerine species can be infected with IAV experimentally (Perkins & Swayne 2002; Perkins & Swayne 2003; Boon et al. 2007; Brown et al. 2009; Nemeth et al. 2010; Breithaupt et al. 2011) and by contact with infected aberrant hosts (i.e. domestic poultry; Morgan & Kelly 1990). Historically, IAV has rarely been isolated from passerine birds despite extensive surveillance efforts (Boudreault et al. 1980; Diebel et al. 1985; Webster et al. 1992); however, when IAV has been reported, it most often is reported from peridomestic species associated with agricultural environments where IAV was present in domestic animals (i.e. poultry farms, live bird markets etc.; Ellis et al. 2009; Siengsanan et al. 2009).

Over time, testing methods for IAV have changed. Historically, virus detection relied on isolation and few isolates from passerine species were reported (Amin et al. 1980; Boudreault et al. 1980). More recently, molecular-based testing, especially conventional reverse transcriptase (RT-) and real-time reverse transcriptase (RRT-) polymerase chain reaction (PCR) assays, have become more common, and with increased detection of viral RNA, some authors have suggested that passerines may represent natural IAV reservoirs and potentially contribute to the maintenance and long-range movement of these viruses (Gronesova et al. 2008a; Gronesova et al. 2008b; Peterson et al. 2008; Fuller et al. 2010; Borovská et al. 2011; Cumming et al. 2011;

Thinh et al. 2012). There have also been recent improvements in the antibody testing related to IAV as demonstrated by the development and validation of both IAV and subtype specific ELISA tests (Brown et al. 2010; Lebarbenchon et al. 2012); however with few exceptions, serologic methods for IAV antibody detection have not been routinely applied to passerine species.

Recent reports of molecular detection of IAV in passerines, most often without confirmatory virus isolation, have raised questions relating to the role of terrestrial wild birds in IAV epidemiology; this is especially true under natural conditions where a domestic animal source of IAV is not available. To better evaluate the potential role of passerines in IAV epidemiology in North America, we conducted: 1) a critical literature review of all existing literature on IAV surveillance of passerines; and 2) a field study of passerine species at variety of settings (natural, peridomestic, and agriculture) using historic (virus isolation in embryonating chicken eggs) and more recent (RRT-PCR, and bELISA) diagnostic approaches.

MATERIALS AND METHODS

Literature Review

Thirty-seven years (1975-2012) of publications on IAV surveillance that included information on wild birds in the order Passeriformes were searched using Web of Knowledge (http://www.webofknowledge.com), Google Scholar (http://scholar.google.com), PubMed (www.ncbi.nlm.nih.gov/pubmed) and JSTOR (http://www.jstor.org). This was followed by hand searching the literature cited in all publications that included IAV data from passerine birds. Prevalence data and metadata, including sampling location (country), species, and testing method were extracted from each publication. In addition, studies were classified related to sampling under natural or unnatural conditions. Unnatural conditions were defined as situations where birds were sampled in association with known poultry outbreaks (LPIAV and HPIAV), live-bird markets, or where samples only included peridomestic species. Because many of these publications included other terrestrial birds in the orders Columbiformes, Cuculiformes, and Piciformes, results from these orders are also reported.

Prospective survey

Samples were collected during 2010 and 2011 in Georgia, New Jersey, Delaware, and Minnesota, USA. In order to maximize the likelihood of detecting IAV in terrestrial birds, sampling in New Jersey, Delaware and Minnesota was conducted at locations and times where peak levels of IAV activity are known to occur in recognized wild avian reservoirs (i.e. gulls, shorebirds, and ducks). In Georgia, birds were sampled throughout the year in Clarke, Madison, Oconee, and Tift counties. Surveillance in New Jersey and Delaware was conducted during May within Cape May and Kent counties, respectively, to correspond to the spring migratory stopover of shorebirds at Delaware Bay (Hanson et al. 2008). Surveillance in Minnesota was conducted during May, July, August and September at Agassiz National Wildlife Refuge (NWR) in Marshall Co. in close proximity to waterfowl habitat. Waterfowl and shorebirds also were being sampled at these sites as part of other ongoing studies.

During 2011, additional IAV surveillance of passerines and other peridomestic birds was conducted on two domestic turkey farms in Wright county, Minnesota that had recently (~2-4 weeks) experienced an outbreak of H7N9 low pathogenic avian influenza virus.

Birds were captured with mist nets and as by-catch during waterfowl capture using rocket nets; on turkey farms some additional birds were collected with shotguns. Birds were banded, aged and gender was determined based on plumage characteristics (Pyle 1997, Sibley 2000). A blood sample was collected via jugular venipuncture from all birds weighing >8 grams; in no cases did the total blood sample exceed 1% of the birds total body weight. Blood was centrifuged and serum was placed into 2.0 ml Sarstedt tubes (Sarstedt, Inc. Newton, NC). Cloacal and oropharyngeal (OP) swabs were collected using sterile tipped applicators (Puritan Medical Products Company LLC, Guilford, ME) and placed in separate tubes containing 1.0 ml Brain Heart Infusion media (Becton Dickinson and Co., Sparks, MD) supplemented with penicillin G (1,000 units/ml), streptomycin (1 mg/ml), kanamycin (0.5 mg/ml), gentamicin (0.25 mg/ml), and amphotericin B (0.025 mg/ml; Sigma Chemical Company, St. Louis, MO). Serum and swab samples were stored at -20C and -80C, respectively, until processed.

All serum samples were tested for antibodies to IAV. Virus isolation and RRT-PCR testing were used on a limited basis to test specific species that were reported positive in the IAV literature and birds testing positive by bELISA. All swab samples collected around poultry farms previously infected with H7N9 were tested by both RRT-PCR and virus isolation.

Serum samples were tested for AI antibodies using commercially available bELISA test kits (FlockCheck AI MultiS-Screen anti-body test kit, Idexx Laboratories, Westbrook, ME) following manufacturer's instructions. Cloacal and OP swabs were thawed, vortexed for 15 s, and centrifuged at 1500x *g* for 15 minutes to allow antibiotics to reduce bacterial contamination. For virus isolation, the supernatant was inoculated (0.33 ml/egg) into 9-11 day old specific-pathogen free (SPF) embryonated chicken eggs via the allantoic route (Stallknecht et al. 1990). Eggs were incubated at 37C for 120hr and evaluated daily for viability. Amnio-allantoic fluid (AAF) was collected for testing by hemagglutination (HA) assay (Swayne et al. 2008). For RRT-RCR, RNA was extracted from cloacal and OP swab material using a modified protocol with the MagMAX™-96 AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX) and Thermo Electron KingFisher magnetic particle processor (Thermo Electron Corporation, Waltham, MA)

(Das et al.2009) . Real-time RT-PCR (RRT-PCR) was carried out to screen for IAV by amplification of the matrix (M) genes described (Spackman et al. 2002). Samples with a cycle threshold (Ct) value equal or less than 40.00 were considered positive.

RESULTS

Literature Review

We found 60 publications from 1975-2012, that contained surveillance data on IAV in passerines and associated terrestrial birds (Table 1); combined these publications included 829 species from 4 orders (Columbiformes, Cuculiformes, Passeriformes, and Piciformes). The majority of birds, 745/829 (89.9%), were from the order Passeriformes. Reported testing methods varied between studies (Figure 1), but most utilized virus isolation and/or PCR to detect IAV. Several publications are listed multiple times throughout the literature review, utilizing several different types of testing methods. Only nine publications tested for antibodies to IAV; serologic assays included bELISA, Indirect ELISA (iELISA), Hemagglutination Inhibition (HI), and Agar Gel Immunodiffusion (AGID). Only three of the nine publications provided both serologic and virus detection data.

Based on 24 publications that utilized virus isolation as the primary testing method, 119 isolations were reported from 25,438 (0.47%) tested birds. Most positive results 92/119 (77.30%) were associated with sampling of birds in an unnatural event or setting. A minority of the IAV isolations (27/119; 22.69%) was from terrestrial birds under natural settings, with all 27 isolations associated with two publications (Boudreault et al. 1980; Amin et al. 1980). These publications each reported one IAV subtype from all positive passerines sampled (H1N1, Boudreault et al.1980; H4N6, Amin et al. 1980).

Molecular based surveillance was used in 19 publications reporting 248 PCR positives from a total of 29,258 (0.85%) birds. All of these positive results were from birds sampled in a natural setting. However, in 4 of these 19 publications, sampling was conducted in areas and times when HPAI H5N1 was present. Only nine publications attempted to confirm positive PCR results by virus isolation. Combined these nine publications reported 74 PCR positives from 10,477 samples (0.71%); only nine viruses were isolated, all from one publication associated with Eurasian H5N1 (Kou et al. 2009).

Birds were serologically tested for antibodies to IAV in 10 publications. Combined, antibodies were reported from 216/3,553 (6.08%) birds. Most reported seropositive birds were associated with unnatural settings 210/216 (97.22%), while only 6/216 (2.78%) were reported from birds sampled from natural settings. Only four publications reported virus isolation results for seropostive birds. Of the 393 birds tested, only one was virus isolation positive (from unnatural settings).

Overall, based on virus isolation, PCR, or serologic results, 128 species representing 34 families were reported positive for IAV (Table 2). These included a whole lot of variation related to differences in feeding habits, habitat utilization etc. No predominant IAV subtypes were reported from passerines (Table 3) and results varied greatly between publications. As previously stated, limited diversity was reported from birds tested by virus isolation (Boudreault et al. 1980; Amin et al. 1980). In contrast, numerous subtypes were reported from a few PCR-based studies (Gronesova et al. 2008a; Gronesova et al. 2008b; Borovská et al. 2011).

Prospective survey

During 2010 and 2011, serum samples were collected from 3558 terrestrial wild birds in four states (GA [*n*=1200], NJ/DE [*n*=280], and MN [*n*=2078]), representing 102 species and 22 families (Table 4). Three of the serum samples (0.08%) were positive for antibodies to the NP of IAV, including a Baltimore oriole (*Icterus galbula*), American robin (*Turdus migratorius*) and

American redstart (*Setophaga ruticilla*) (Table 5). All three positive birds were sampled at Agassiz NWR in northwestern MN. We screened 236 samples using both RRT-PCR and virus isolation, and an additional 353 samples using virus isolation and all were IAV negative. These negative results included the three seropositive birds.

One of 310 (0.32%) wild bird serum samples, from an European starling (*Sturnus vulgaris*), collected around IAV positive turkey farms in MN during 2011, tested positive for antibodies to the NP of IAV (Table 6). All 311 swabs collected from birds on the poultry farm tested negative for IAV by virus isolation and RRT-PCR.

DISCUSSION

Virus isolation has long been accepted as the reference standard for IAV virus detection. Virus isolation was the most commonly used diagnostic used throughout the literature review. Overall, there was an extremely low prevalence (0.45%) reported, with over 75% of the reported IAV's associated with Eurasian H5N1. Only two publications (Boudreault et al. 1980 & Amin et al. 1980) reported IAV's from birds associated with a natural setting, and in each study, one subtype was reported for all passerines sampled, H1N1 and H4N6 respectively. Although both of these subtypes are common among sampled waterfowl (Olsen et al. 2006; Wilcox et al. 2011), such a restricted subtype diversity is suspect. In relation to the Boudreault et al. (1980) study, isolates were reported from a broad geographic area and to date such isolations have not been repeated. Likewise, the precise sampling techniques used in the Amin et al. (1980) study are not well described and could have involved the sampling of captive birds. It also is not feasible to eliminate the possibility of laboratory contamination, especially in the case of the Bouldreault et al. (1980) study which also included waterfowl. Overall, if the results of these two studies are not included in the combined results for the 24 publications reporting virus isolation results, overall prevalence decreases from 0.47% to 0.37%.

Several recent molecular surveillance studies have reportedly detected IAV in passerines at higher levels than traditional isolation-based surveillance efforts; these results have been interpreted as an indication that passerines may be IAV reservoirs (Gronesova et al. 2008a; Gronesova et al. 2008b; Peterson et al. 2008; Fuller et al. 2010; Borovská et al. 2011; Cumming et al. 2011; Thinh et al. 2012). The benefits and disadvantages of virus isolation vs. molecular diagnostics (i.e. real-time reverse transcriptase polymerase chain reaction (RRT-PCR) in wild bird IAV surveillance are well-established (Spackman et al. 2003). Although molecular techniques provide sensitive screening tools to detect IAV infection; positive results can be difficult to interpret without additional confirmatory data, such as virus isolation or sequencing. This is particularly true for RRT-PCR positive results with high cycle threshold (Ct) values, from which virus is rarely isolated. Even under ideal transport, storage and processing conditions positive virus isolation results from RRT-PCR positive samples may be less than 33% (Munster et al. 2007).

Several problems were noted among those publications that reported results based solely on molecular diagnostics. First, every study that concluded that passerines represented an IAV reservoirs provided no confirmatory isolation data from PCR positive birds (Gronesova et al. 2008a; Gronesova et al. 2008b; Peterson et al. 2008; Fuller et al. 2010; Borovská et al. 2011; Cumming et al. 2011; Thinh et al. 2012). In particular, Fuller et al. (2010) implicated golden-crowned kinglets (*Regulus satrapa*) as IAV reservoirs; this was based on a sample size of two birds with one testing positive. Of the nine publications that reported virus isolation results as a confirmatory test of PCR positives, isolates were reported from one publication and this was associated with Eurasian H5N1 outbreaks (Kou et al. 2009). A second problem was encountered related to variation in PCR protocols represented in these studies. Several publications (Gronesová et al 2007; Gronesová et al. 2008a; Gronesová et al. 2008b; Borovská et al. 2011) used nested PCR to screen birds for IAV's instead of RRT-PCR which has been validated for wild birds (Spackman et al. 2003). Considering the potential for cross contamination while using nested PCR protocols, it may be understandable why the highest IAV prevalence estimates reported for passerines in the literature were reported by these authors. None of these results were confirmed through virus isolation.

Traditionally, serologic detection methods have been underutilized in wild bird surveillance due to the poor sensitivity of testing methods (AGID; Brown et al. 2010). Serologic based diagnostics were under represented in our literature review and in total there were results from approximately 3,553 birds. More recently, Brown et al. (2010) validated the use of a species-independent commercially available bELISA, which can be used as an additional application for wild bird IAV surveillance. Overall, results from passerines included in this literature review had an IAV antibody prevalence of 6.08%; Brown et al. (2010) reported antibody prevalence in known reservoirs, such as, Anseriformes and Charadriiformes, of 41.7% and 31.4% respectively. If passerines played a significant role in IAV epidemiology it would be expected that they have antibody prevalence somewhat similar to that of known reservoirs. Furthermore, nearly 98% of the seropositive birds included in this literature review were associated with an unnatural setting. If passerine birds sampled from natural areas are considered, a total of 400 birds are represented from two studies with an overall antibody prevalence of 0.0%.

Overall, there is little or no conclusive evidence supporting a role for passerines in the natural history of IAV. The possible exception to this relates to peridomestic birds associated with infected domestic poultry as there is clear evidence that some poultry IAVs can replicate in passerine species (Morgan and Kelly 1990). However, even in this case there are no data to indicate that poultry adapted IAVs could be maintained in populations of passerine birds.

The results of our prospective study support these conclusions. Of the 900 and 547 birds tested by virus isolation and RRTPCR, respectively, none tested positive. Likewise, even though birds were sampled from areas where IAVs were present in the environment and were being detected in shorebirds and ducks, only three of 3558 (0.08%) tested positive for NP antibodies to IAV. Passerines can be infected with wild bird IAVs (Nemeth et al. 2010) and they do seroconvert (Brown et al. 2009; Nemeth et al. 2010). Although it may be reasonable to assume that these seropositive birds resulted from previous infections with IAV, the extremely low antibody prevalence suggests a rare event most likely associated with spillover. Results from the H7N9 infected farm samples also are consistent with the literature and the failure to detect virus in these birds provides no indication of virus maintenance in these peridomestic birds. It is interesting that NP antibodies were detected in one starling at this site. Although this still represents a very low antibody prevalence (1/310, 0.32%) it is higher than the background prevalence observed in birds sampled from natural settings.

Currently, there are many well validated techniques to detect IAV or antibodies to IAV in wild birds, and as in our prospective study, these can easily be incorporated into a study design to provide validation of field results. This failure to confirm results was the major short-coming of the published studies that reported IAVs from passerine birds. An additional problem encountered when reviewing these manuscripts relates to incomplete information as location, birds associated with outbreaks and species sampled.

As a cautionary note, the order Passeriformes makes up over half of all known bird species (Dickenson 2003) and there may be isolated cases where a passerine species may play a role in local IAV epidemiology. To date, this has not been demonstrated but if such investigations are warranted they should be approached with a diagnostic strategy that provides results that can and are confirmed.

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Table 1. Avian influenza virus sampling from land birds separated based on testing method.

Testing Method	Study	Location	Results #pos./#tested	Positive Species
PCR				
	Baumer et al. 2010	Switzerland	0/98	N/A
	Borovská et al. 2011	Slovakia	71/529	Great Reed Warbler (*Acrocephalus arundinaceus*) n=7, Moustached Warbler (*Acrocephalus melanopogon)* n=2, Eurasian Reed Warbler (*Acrocephalus scirpaceus*) n=24, Sedge Warbler (*Acrocephalus schoenbaenus*) n=28, European Greenfinch (*Carduelis chloris*) n=1, Savi's Warbler (*Locustella luscinioides*) n=4, Lesser Whitethroat (*Sylvia curruca*) n=1, Bearded Parrotbill (*Panurus biarmicus*) n=4
	Cumming et al. 2011	Africa	15/441	Chestnut-backed Sparrowlark (*Eremopterix leucotis*) n=3, Barn Swallow (*Hirundo rustica*) n=1, African Pipit (*Anthus cinnamomeusI*) n=1, Yellow-throated Longclaw (*Macronyx croceus*) n=1, Southern Grey-headed Sparrow (*Passer diffusus*) n=1, Red-billed Quelea (*Quelea quelea*) n=1, Southern Masked-weaver (*Ploceus velatus*) n=1, Village Weaver (*Ploceus cucullatus*) n=2, Yellow Bishop (*Euplectes capensis*) n=1, African Red-eyed Bulbul (*Pycnonotus nigricans*) n=1, Little Rush-warbler (*Bradypterus baboecala*)n=1, Willow Warbler (*Phylloscopus trochilus*) n=1
	Dusek et al. 2009	United States	0/-[A]	N/A
	Fouchier et al. 2003	Northern Europe	0/-	N/A
	Fuller et al. 2010	United States	39/4341	American Goldfinch (*Spinus tristis*), Cedar Waxwing (*Bombycilla cedrorum*), Veery (*Catharus fuscescens*), Hermit Thrush (*Catharus guttatus*), Swainson's Thrush (*Hylocichla ustulata*), Yellow-rumped Warbler (*Setophaga coronata*), Alder Flycatcher (*Empidonax alnorum*), Yellow-breasted Chat (*Icteria virens*), Dark-eyed Junco (*Junco hyemalis*), Song

			Sparrow (*Melospiza melodia*), Fox Sparrow (*Passerella iliaca*), MacGillivray's Warbler (*Geothlypis tolmiei*), Black-headed Grosbeak (*Pheucticus melanocephalus*), Western Tanager (*Piranga ludoviciana*), Golden-crowned Kinglet (*Regulus satrapa*), Northern Waterthrush (*Parkesia noveboracensis*), Bewick's Wren (*Thryomanes bewickii*), American Robin (*Turdus migratorius*), Pacific-slope Flycatcher (*Empidonax difficilis*), Dusky Flycatcher (*Empidonax oberholseri*), Western Wood-Peewee (*Contopus sordidulus*), Cassin's Finch (*Carpodacus cassinii*)
Globig et al. 2009	Germany	0/640	N/A
Grosnova et al. 2008a	Western Slovakia	32/105	Great Reed Warbler (*Acrocephalus arundinaceus*) n=1, Moustached Warbler (*A. melanopogon*) n=1, Reed Warbler (*A. scirpaceus*) n=10, Sedge Warbler (*A. schoenobaenus*) n=8, Reed Bunting (*Emberiza schoeniclus*) n=3, Swallow (*Hirundo rustica*) n=3, Savi's Warbler (*Locustella luscinioides*) n=4,White Wagtail (*Motacilla alba*) n=1, Blackcap (*Sylvia atricapilla*) n=1
Grosnova et al. 2007	Slovakia	11/42	Moustached Warbler (*Acrocephalus melanopogon*) n=1, Reed Warbler (*A. scirpaceus*) n=3, Great Reed Warbler (*A. arundinaceus*) n=1, Bearded Tit (*Panurus biarmicus*) n=1, Reed Bunting (*Emberizia schoeniclus*) n=3, Blue Tit (*Parus caeruleus*) n=1, Swallow (*Hirundo rustica*) n=1
Grosnova et al. 2008b	Slovakia	27/79	Common Cuckoo (*Cuculus canorus*) n=1, Moustached Warbler (*Acrocephalus melanopogon*) n=1, Reed Warbler (*A. scirpaceus*) n=3, Great Reed Warbler (*A. arundinaceus*) n=1, Bearded Tit (*Panurus biarmicus*) n=1, Reed Bunting (*Emberizia schoeniclus*) n=3, Blue Tit (*Parus caeruleus*) n=3, Swallow (*Hirundo rustica*) n=1, Blackcap (*Sylvia atricapilla*) n=5, Robin (*Erithacus rubecula*) n=2, Chiffchaff (*Phylloscopus collybita*) n=1, Dunnock (*Prunella modularis*) n=1, European Tree Sparrow (*Passer montanus*) n=3, Black Redstart (*Phoenicurus ochruros) n=1*

Hesterberg et al. 2009	Europe	15/13,088	Passeriformes n=9, Columbiformes n=6
Ip et al. 2008	Alaska	0/1,927	N/A
Lebarbenchon et al. 2007	France	0/627	N/A
Lebarbenchon et al. 2010	France	0/950	N/A
Lefrancois et al. 2010	Caribbean	0/42	N/A
Munster et al. 2007	Africa, Asia, & Europe	0/4,649	N/A
Peterson et al. 2008	China	24/928	Yellow-throated Bunting (*Emberiza elegans*) n=2, Little Bunting (*Emberiza pusilla*) n=1, Grey Wagtail (*Motacilla cinerea*) n=1, White-browed Shortwing (*Brachypteryx montana*) n=1, Oriental Magpie-robin (*Copeychus saularis*) n=1, Hill Blue-flycatcher (*Cyornis hanyumas*) n=1, Fujian Niltava (*Niltava davidi*) n=1, Small Niltava (*Niltava macgrigoriae*) n=1, Plumbeous Water-redstart (*Rhyacornis fuliginosus*) n=1, Yellow-browed Tit (*Sylviparus modestus*) n=1, Cinnamon Sparrow (*Passer rutilans*) n=2, Brownish-flanked Bush-warbler (*Cettia fortipes*) n=1, *Phylloscopus sp.* n=1, Southern Blyth's Leaf-warbler (*Phylloscopus reguloides*) n=2, Radde's Warbler (*Phylloscopus schwarzi*) n=1, Grey-cheeked Fulvetta (*Alcippe morrisonia*) n=2, Moustached Laughingthrush (*Garrulax cineraceus*) n=1, Streak-breasted Scimatar-babbler (*Pomatorhinus ruficollis*) n=1, Grey-throated Babbler (*Stchyris nigriceps*) n=1, Japanese White-eye (*Zosterops japonicus*) n=1
Thinh et al. 2012[D]	Vietnam	14/185	Japanese White-eye (*Zosterops Japonicus*) n=10, Puff-throated Bulbul (*Alophoixus pallidus*) n=2, White-tailed Robin *(Cinclidium leucurum)* n=1, Striped Tit-Babbler *(Macronous gularis*) n=1
Snoeck et al. 2011	Africa	0/587	N/A

PCR & VI				
	Caron et al. 2011	Africa	13/310^{B} 0/13	Passeriformes n=13
	Foti et al. 2011	Italy	0/-	N/A
	González-Reiche et al. 2012	Guatemala	2/709 0/2	Brown-crested Flycatcher (*Mionectes tyrannulus*) n=1, Golden-fronted Woodpecker (*Melanerpes aurifrons*) n=1
	Qin et al. 2010	United States	18/328^{C} 3/156 0/-	European Starling (*Sturnus vulgaris*)
	Račnik et al. 2008	Slovenia	1/670 0/670	Common Starling (*Sturnus vulgaris*) n=1
	Kou et al. 2009	China	32/7,891 9/-	Rock Dove (*Columbia livia*) n=6, Madagascar Bulbul (*Hypsipetes leucocephalus*) n=2, Plain Mountain Finch (*Leucosticte nemoricola*) n=1, Grey-cheeked Fulvetta (*Alcippe morrisonia*) n=2, Eurasian Sky lark (*Alauda arvensis*) n=1, Scaly Thrush (*Zoothera dauma*) n=2, Green-backed Tit (*Parus monticolus*) n=1, Rock Petronia (*Petronia petronia*) n=1 Tree Sparrow (*Passer montanus*) n=15, White-collared Yuhina (*Yuhina diademata*) n=1 N/A
	Gilbert et al. 2012	Mongolia	2/10 0/74	Asian Short-toed Lark (*Calandrella cheleensis*) n=1, Bluethroat (*Luscinia svecica*) n=1
	Cappelle et al. 2011^{D}	West Africa	1/380 0/1	Red-billed Quelea (Quelea quelea) n=1
	Williams et al. 2012	Peru	5/489 0/5	White-bellied Woodstar(*Chaetocercus mulsant*) n=1, Lacrimose Mountain Tanager (*Anisognathus lacrymosus*) n=1, Russet-crowned Warbler (*Basileuterus coronatus*) n=1, Greater Scythebill (*Campylorhamphus pucherani*) n=1, Peruvian Wren (*Cinnycerthia peruana*) n=1

Virus Isolation				
	Amin et al. 1980	Egypt	8/-	Garden Warbler (*Sylvia borin*) n=1, Common Redstart (*Phoenicurus phoenicurus*) n=1, Common Whitethroat (*Sylvia communis*) n=1, Tree Warbler (*Hippolais icterina*) n=1, Red-backed Shrike (*Lanis collurio*) n=1, Barn Swallow (*Hirundo rustica*) n=1, Willow Warbler (*Phylloscopus trochilus*) n=1, Yellow Wagtail (*Motacilla flava*) n=1
	Boudreault et al. 1980	Quebec and Ontario	19/436	Alder Flycatcher (*Empidonax alnorum*) n=3, Hermit Thrush (*Catharus guttatus*) n=1, Swainson's Thrush (*Hylocichla ustulata*) n=1, Tennessee Warbler (Oreothlypis peregrina) n=3, Yellow Warbler (*Setophaga petechia*) n=1, Magnolia Warbler (*dendroica magnolia*) n=1, Yellow-rumped Warbler (*Setophaga coronata*) n=2, Yellow-throated Warbler (*Setophaga dominica*) n=2, American Redstart (*Setophaga ruticilla*) n=3, Purple Finch (*Carpodacus purpureus*) n=1, Song Sparrow (*Melospiza melodia*) n=1
	Capua et al. 2000	Italy	2/47	House Sparrow (*Passer domesticus*) n=1, Collared Dove (*Streptopelia decaocto*) n=1
	Deibel et al. 1985	United States	0/1,296	N/A
	De Marco et al. 2003	Italy	0/83	N/A
	Douglas et al. 2007	Barbados	0/3	N/A
	Ellis et al. 2009	China	28/15,568	Magpie Robin (*Copsychus saularis*) n=2, Crested Myna (*Acridotheres cristatellus*) n=1, Common Magpie (*Pica pica*) n=5, Japanese White-eye (*Zosterops japonicus*) n=2, Munia (*Lonchura sp.*) n=1, White-backed Munia (*Lonchura striata*) n=2, House Crow (*Corvus splendens*) n=5, Scaly-breasted Munia (*Lonchura punctulata*) n=3, Lsrge-billed Crow (*Corvus validus*) n=1, Blue Magpie (*Urocissa sp.*) n=1, Silver-eared Mesia (*Leiothrix argentauris*) n=2, Chestnut Munia (*Lonchura atricapilla*) n=1, Long-tailed Shrike (*Lanius schach*) n=1, Red-billed Starling (*Sturnus sericeus*)n=1

	Graves 1989	United States	0/282	N/A
	Grešíková et al. 1978	Slovakia	0/192	N/A
	Honda et al. 1980	Japan, Hokkaido	0/533	N/A
	Kou et al. 2005	China	4/38	Tree Sparrow (*Passer montanus*) n=4
	Kulak et al. 2010	Ukraine	0/45	N/A
	Kwon et al. 2005	South Korea	3/3	Korean Magpie (*Pica pica sericea*) n=3
	Lipkind et al. 1979	Israel	1/42	Starling (*Sturnus vulgaris*) n=1
	Lipkind et al. 1981	Israel	1/439	Starling (*Sturnus vulgaris*) n=1
	Liu et al. 2010	China	1/68	Tree Sparrow (*Passer montanus*) n=1
	Mase et al. 2004	Japan	9/9	Crow n=9
	Nettles et al. 1985	United States	0/2,107	N/A
	Poetranto et al. 2011	Indonesia	1/1	Tree Sparrow (Passer montanus) n=1
	Schnebel et al. 2007	Germany	0/543	N/A
	Siengsanan et al. 2009	Thailand	39/3,182	Cuculidae n=1, Columbidae n=23, Dicruridae n=1, Estrilidae n=1, Emberizidae n=6, Sturnidae n=7
	Muzyka et al. 2012	Ukraine	0/243	N/A
	Ellis et al. 2004	China	2/70	Tree Sparrow (*Passer montanus*) n=1, Wild Pigeon (*Columbia livia*) n=1
	Morgan and Kelly 1990	Australia	1/208	Starling (Sturnus vulgaris) n=1
Serology				
	Al-Attar et al. 2008[E]	Iraq	49/120 30/120	Pigeon (*Columbia livia*) n=49,30
	Brown et al. 2010	United States	0/242	N/A
	Hadipor et al. 2011	Iran	152/200	House Sparrow (*Passer domesticus*) n=152
	Morishita et al. 1999	United States	0/1,709	N/A
	Cappelle et al. 2011	West Africa	0/158	N/A

	Thinh et al. 2012	Vietnam	6/184	Crow-billed Drongo (*Dicrurus annectans*) n=1, Red-whiskered Bulbul (*Pycnonotus jocosus*) n=1, Black-crested Bulbul (*Pycnonotus melanicterus*) n=1, Black-browed Fulvetta (*Alcippe grotei*) n=1, Buff-breasted Babbler (*Pellorneum tickelli*) n=2
Serology & VI				
	Johnson et al. 1977	United States	1/387[F] 0/301	American Crow (*Corvus brachyrhynchos*) n=1
	Kida and Yanagawa 1980	Japan, Hokkaido	2/47 0/54	Rock Pigeon (*Columbia livia*) n=1
	Kohls et al. 2011	Germany	2/487 0/-	Wood Pigeon (*Columba palumbus*) n=2
	Romváry and Tanyi 1975	Hungary	4/19 1/19	Collared Dove n=1

[A] Total sample size not indicated.

[B] PCR results listed above virus isolation results.

[C] Qin et al. 2010 screened European Starling (*Sturnus vulgaris*) digestive tracts and tracheal samples.

[D] Cappelle et al. 2011 is listed twice in the table, first in the PCR/VI section and second in the serology section.

[E] Al-Attar et al. 2008 compared indirect ELISA results over Haemagglutination Inhibition results.

[F] Serology results provided above virus isolation results.

Table 2. Avian families representing associated with publications reporting IAV positive passerines and other terrestrial birds.

Positive Families	Publications
Alaudidae	Cumming et al. 2011; Gilbert et al. 2012; Kou et al. 2009
Bombycillidae	Fuller et al. 2010
Cardinalidae	Fuller et al. 2010
Columbidae	Al-Attar et al. 2008; Ellis et al. 2004; Kida & Yanawanga 1979; Kohls et al. 2011; Kou et al. 2009; Romvary & Tanyi 1975; Siengsanan et al. 2009
Corvidae	Ellis et al. 2009; Johnson et al. 1977; Kwon et al. 2005; Mase et al. 2004
Cuculidae	Gronesova et al. 2008; Siengsanan et al. 2009
Dendrocolaptidae	Williams et al. 2012
Dicruridae	Siengsanan et al. 2009; Thinh et al. 2012
Emberizidae	Boureault et al. 1980; Fuller et al. 2010; Gronesova et al. 2007; Gronesova et al. 2008a, Gronesova et al. 2008b; Peterson et al. 2008; Siengsanan et al. 2009
Estrillidae	Ellis et al. 2009; Siengsanan et al. 2009
Fringillidae	Borovská et al., 2011; Boudreault et al. 1980; Gronesova et al. 2008a; Fuller et al. 2010; Kou et al. 2009;
Hirundinidae	Amin et al. 1980; Cumming et al. 2011; Gronesova et al. 2007; Gronesova 2008a; Gronesova 2008b
Laniidae	Amin et al. 1980; Ellis et al. 2009
Locustellidae	Cumming et al. 2011
Motacillidae	Amin et al. 1980; Cumming et al. 2011; Gronesova et al. 2008a; Peterson et al. 2008
Muscicapidae	Amin et al. 1980; Ellis et al 2009; Gilbert et al. 2012; Gronesova et al. 2008b; Peterson et al. 2008
Paridae	Gronesova et al. 2007; Gronesova et al. 2008b; Kou et al. 2009; Peterson et al. 2008
Parulidae	Boudreault et al. 1980; Fuller et al. 2010; Williams et al. 2012
Passeridae	Cumming et al. 2011; Ellis et al. 2004; Gronesova et al. 2008b; Hadipor et al. 2011; Kou et al. 2005;Kou et al. 2009; Liu et al. 2011; Peterson et al. 2008; Poetranto et al. 2011
Phylloscopidae	Peterson et al. 2008
Picidae	González-Reiche et al. 2012
Ploceidae	Capelle et al. 2011; Cumming et al. 2011
Prunellidae	Grosnova et al., 2008b
Pycnonotidae	Cumming et al. 2011; Kou et al. 2009; Thinh et al. 2012
Regulidae	Fuller et al. 2010
Remizidae	Gronesova et al. 2008a
Strunidae	Ellis et al. 2009; Lipkind et al. 1978a; Lipkind et al. 1981; Morgan & Kelly 1990; Qin et al. 2010; Račnik et al. 2008; Siengsanan et al. 2009
Sylviidae	Amin et al. 1980; Borosková et al. 2011; Cumming et al. 2011; Gronesova et al. 2007;Gronesova et al 2008a; Gronesova et al. 2008b; Peterson et al 2008
Thraupidae	Williams et al. 2012
Timaliidae	Borovská et al., 2011; Ellis et al. 2009; Gronesova et al. 2008b; Gronesova et al.

	2007; Kou et al. 2009; Peterson et al. 2008; Thinh et al. 2012
Troglodytidae	Fuller et al. 2010; Williams et al. 2012
Turdidae	Boudreault et al. 1980; Gronesova et al. 2008b; Fuller et al. 2010; Kou et al. 2009; Peterson et al. 2008;
Tyrannidae	Boudreault et al. 1980; Fuller et al. 2010; González-Reiche et al. 2012
Zosteropidae	Ellis et al. 2009; Peterson et al. 2008

Table 3. Subtypes reported from passerines and other terrestrial birds testing positive from various diagnostics.

Publication	Subtype
Al-Attar et al. 2008	H9N3
Borovská et al. 2011	H6N4; H8N3; H11N3; H11N8; H12N6; H2N3; H11N6; H12N2; H1N3; H9N3; H3N1; H3N3; H6N3; H7N3; H8N6; H10N3; H11N1; H12N3; H12N4; H13N1; H2N1; H14N3; H8N1; H1N1; H14N3; H2N8; H6N8; H12N1; H12N3; H13N2; H15N4; H5N5; H7N1; H14N4; H11N5; H8N7
Boudreault et al. 1980	H1N1
Caron et al. 2011	H5
Cumming et al. 2011	H5
Ellis et al. 2004	H5N1
Ellis et al. 2009	H5N1
Gronesova et al. 2008a	H10N2; H9N2; H7N5; H13N1; H2N5; H6N5; H12N2; H12N5; H13N1; H9N5; H11N3; H12N3; H12N?; H10N2; H11N5; H13N?; H11N2; H4N?; H12N5; H3N?; H13N3
Gronesova et al. 2008b	H3N2; H12N1; H3N5; H9N5; H7N2; H9N2; H13N2; H10N3; H12N5; H?N6; H7N?; H?N2 ;H6N5; H10N6; H7N6; H1N6
Hadipour et al. 2011	H9N2
Hesterberg et al. 2009	H5N1
Kwon et al. 2005	H5N1
Lipkind et al. 1979	H1N1
Lipkind et al. 1981	H7N7
Liu et al. 2010	H5N1
Mase et al. 2004	H5N1
Morgan and Kelly 1990	H7N7
Poetranto et al. 2011	H5N1
Siengsanan et al. 2009	H5N1
Thinh et al. 2012	H5; H6; H9

Table 4. Order, family and total of each species sampled for prospective study not associated with H7N9 poultry outbreak.

Order/Family	Species
Columbiformes	
Columbidae	Common Ground-Dove (*Columbina passerina*) n=11, Mourning Dove (*Zenaida macroura*) n=31, Rock Pigeon (*Columba livia*) n=1
Cuculiformes	
Cuculidae	Black-billed Cuckoo (*Coccyzus erythropthalmus*) n=6
Piciformes	
Picidae	Downy Woodpecker (*Picoides pubescens*) n=16, Hairy Woodpecker (*Picoides villosus*) n=4, Northern Flicker (*Colaptes auratus*) n=9, Red-bellied Woodpecker(*Melanerpes carolinus*) n=12, Yellow-bellied Sapsucker(*Sphyrapicus varius*) n=18
Passeriformes	
Bombycillidae	Cedar Waxwing (*Bombycilla cedrorum*) n=17
Cardinalidae	Indigo Bunting (*Passerina cyanea*) n=39, Northern Cardinal (*Cardinalis cardinalis*) n=292, Rose-breasted Grosbeak (*Pheucticus ludovicianus*) n=16, Summer Tanager (*Piranga rubra*) n=2
Corvidae	American Crow(*Corvus brachyrhynchos*) n=1, Black-billed Magpie (*Pica hudsonia*) n=1, Blue Jay (*Cyanocitta cristata*) n=40
Emberizidae	Clay-colored Sparrow (*Spizella pallida*) n=5, Chipping Sparrow (*Spizella passerina*) n=18, Eastern Towhee (*Pipilo erythrophthalmus*) n=28, Field Sparrow (*Spizella pusilla*) n=8, Fox Sparrow (*Passerella iliaca*) n=3, Harris's Sparrow (*Zonotrichia querula*) n=10, LeCounte's Sparrow (*Amphispiza belli*) n=1, Licoln's Sparrow (*Melospiza lincolnii*) n=24, Savannah Sparrow (*Passerculus sandwichensis*) n=13, Dark-eyed Junco (*Junco hyemalis*) n=165, Seaside Sparrow (*Ammodramus maritimus*) n=7, Song Sparrow (*Melospiza melodia*) n=253, Swamp Sparrow (*Melospiza georgiana*) n=150, White-crowned Sparrow (*Zonotrichia leucophrys*) n=17, White-throated Sparrow (*Zonotrichia albicollis*) n=120
Fringillidae	American Goldfinch (*Spinus tristis*) n=165, House Finch (*Carpodacus mexicanus*) n=65, Purple Finch (*Carpodacus purpureus*) n=59
Hirundinidae	Barn Swallow (*Hirundo rustica*) n=6, Cliff Swallow (*Petrochelidon pyrrhonota*) n=16, Purple Martin (Progne subis) n=4, Tree Swallow (Tachycineta bicolor) n=6
Icteridae	Baltimore Oriole (*Icterus galbula*) n=38, Brown-headed Cowbird (*Molothrus ater*)n=97, Bobolink (*Dolichonyx oryzivorus*) n=1, Boat-tailed Grackle (*Quiscalus major*) n=3, Common Grackle (*Quiscalus quiscula*) n=49, Orchard Oriole (Icterus spurius) n=16, Red-winged Blackbird (*Agelaius phoeniceus*) n=353, Yellow-headed Blackbird (*Xanthocephalus xanthocephalus*) n=9
Mimidae	Brown Thrasher (Toxostoma rufum) n=56, Gray Catbird (*Dumetella carolinensis*) n=95, Northern Mockingbird (*Mimus polyglottos*) n=43

Paridae	Black-capped Chickadee (*Poecile atricapillus*) n=34, Carolina Chickadee (*Poecile carolinensis*)n=14, Tufted Titmouse (*Baeolophus bicolor*) n=85
Parulidae	American Redstart (*Setophaga ruticilla*) n=27, Black-and-white Warbler (*Mniotilta varia*) n=5, Black-thraoted Blue Warbler (*Setophaga caerulescens*) n=1, Canada Warbler (*Cardellina canadensis*) n=1, Cape May Warbler (*Setophaga tigrina*) n=4, Common Yellowthroat (*Geothlypis trichas*) n=74, Chestnut-sided Warbler (*Setophaga pensylvanica*) n=1, Kentucky Warbler (*Geothlypis formosa*) n=1, Magnolia Warbler (*Setophaga magnolia*) n=3, Nashville Warbler (*Oreothlypis ruficapilla*) n=6, Northern Waterthrush (*Parkesia noveboracensis*) n=5, Ovenbird (*Seiurus aurocapillus*) n=3, Palm Warbler (Setophaga palmarum) n=1, Prairie Warbler (*Setophaga discolor*) n=5, Tennessee Warbler (*Oreothlypis peregrina*) n=13, Wilson's Warbler (*Cardellina pusilla*) n=1, Yellow-breated Chat (*Icteria virens*) n=2, Yellow Warbler (*Setophaga petechia*) n=181, Yellow-rumped Warbler (Setophaga coronata) n=30
Passeridae	House Sparrow (*Passer domesticus*) n=82
Polioptilidae	Blue-gray Gnatcatcher (*Polioptila caerulea*) n=7
Regulidae	Golden-crowned Kinglet (*Regulus satrapa*) n=1, Ruby-crowned Kinglet (*Regulus calendula*) n=4
Sittidae	Brown-headed Nuthatch (*Sitta pusilla*) n=1, White-breasted Nuthatch (*Sitta carolinensis*) n=3
Sturnidae	European Starling (*Sturnus vulgaris*) n=35
Troglodytidae	Carolina Wren (*Thryothorus ludovicianus*) n=50, House Wren (*Troglodytes aedon*) n=4, Marsh Wren (*Cistothorus palustris*) n=3
Turdidae	American Robin (*Turdus migratorius*) n=116, Eastern Bluebird (*Sialia sialis*) n=12, Hermit Thrush (*Catharus guttatus*) n=18, Swainsons Thrush (*Catharus ustulatus*) n=10, Thrush Sp. n=1, Veery (*Catharus fuscescens*) n=57
Tyranidae	Acadian Flycatcher (*Empidonax virescens*) n=1, Eastern Kingbird (*Tyrannus tyrannu*) n=6, Eastern Phoebe (*Sayornis phoeb*) n=29, Eastern Wood-Pewee (*Contopus virens*) n=7, Great Crested Flycatcher (*Myiarchus crinitus*) n=8, Least Flycatcher (*Empidonax minimus*) n=100, Trails Flycatcher (*Empidonax sp.*) n=25, Willow Flycatcher (*Empidonax traillii*) n=1, Yellow-bellied Flycatcher (Empidonax flaviventris) n=8
Vireonidae	Philadelphia Vireo (*Vireo philadelphicus*) n=6, Red-eyed Vireo (*Vireo olivaceus*) n=23, Warbling Vireo (*Vireo gilvus*) n=17, White-eyed Vireo (Vireo griseus) n=3, Yellow-throated Vireo (*Vireo flavifrons*) n=4

Table 5. Total samples tested by serology and virus isolation.

	Serology			Virus Isolation		
	GA	MN	NJ/DE	GA	MN	NJ/DE
Columbiformes						
Columbidae	0/37	0/3	0/3	NT	NT	NT
Cuculiformes						
Cuculidae	NT[A]	0/6	NT	NT	NT	NT
Piciformes						
Picidae	0/19	0/37	0/3	NT	NT	NT
Passeriformes						
Bombycillidae	0/1	0/16	NT	NT	0/8	NT
Cardinalidae	0/308	0/16	0/25	NT	NT	NT
Corvidae	0/35	0/4	0/3	NT	NT	NT
Emberizidae	0/206	0/606	0/10	0/2	0/112	NT
Fringillidae	0/81	0/208	NT	0/2	0/67	NT
Hirundinidae	NT	0/19	0/13	NT	0/17	NT
Icteridae	0/64	1/410[B]	0/92	NT	0/13	NT
Mimidae	0/61	0/51	0/26	0/10	NT	0/1
Paridae	0/96	0/34	0/3	0/1	NT	NT
Parulidae	0/45	1/306[C]	0/13	0/25	0/118	NT
Passeridae	0/67	NT	0/15	NT	NT	NT
Polioptilidae	0/4	NT	0/3	NT	NT	NT
Regulidae	0/5	NT	NT	0/6	NT	NT
Sittidae	0/1	0/3	NT	NT	NT	NT
Sturnidae	0/5	NT	0/30	NT	NT	NT
Troglodytidae	0/42	0/6	0/9	0/13	NT	NT
Turdidae	0/100	1/144[D]	0/26	0/15	0/73	0/18
Tyranidae	0/20	0/160	0/5	NT	0/83	NT
Vireonidae	0/3	0/49	0/1	0/5	NT	NT
Total	0/1200	3/2078	0/280	0/79	0/491	0/19

[A] None tested

[B] Positive sample from Baltimore oriole (*Icterus galbula*)

[C] Positive sample from American redstart (*Setophaga ruticilla*)

[D] Postive sample from American robin (*Turdus migratorius*)

Table 6. Total of each species sampled and tested on and within close proximity of H7N9 infected turkey farms.

Species	Serology	VI/PCR
American Goldfinch (*Spinus tristis*)	0/7	0/7
American Robin (*Turdus migratorius*)	0/13	0/13
Baltimore Oriole (*Icterus galbula*)	0/1	0/1
Barn Swallow (*Hirundo rustica*)	0/24	0/24
Brown-headed Cowbird (*Molothrus ater*)	0/4	0/4
Chipping Sparrow (*Spizella passerina*)	0/8	0/8
Common Yellowthroat (*Geothlypis trichas*)	0/1	0/1
Downy Woodpecker (*Picoides pubescens*)	0/2	0/2
Eastern Phoebe (*Sayornis phoebe*)	0/1	0/1
European Starling (*Sturnus vulgaris*)	1/110	0/110
Gray Catbird (*Dumetella carolinensis*)	0/6	0/6
House Sparrow (*Passer domesticus*)	0/94	0/94
House Wren (*Troglodytes aedon*)	NT[A]	0/1
Red-winged Blackbird (*Agelaius phoeniceus*)	0/15	0/15
Rock Pigeon (*Columba livia*)	0/5	0/5
Rose-breasted Grossbeak (*Pheuticus ludovicianus*)	0/1	0/1
Song Sparrow (*Melospiza melodia*)	0/9	0/9
Swamp Sparrow (*Melospiza georgiana*)	0/5	0/5
Yellow Warbler (*Setophaga petechia*)	0/4	0/4
Total	1/310	0/311

[A] None tested

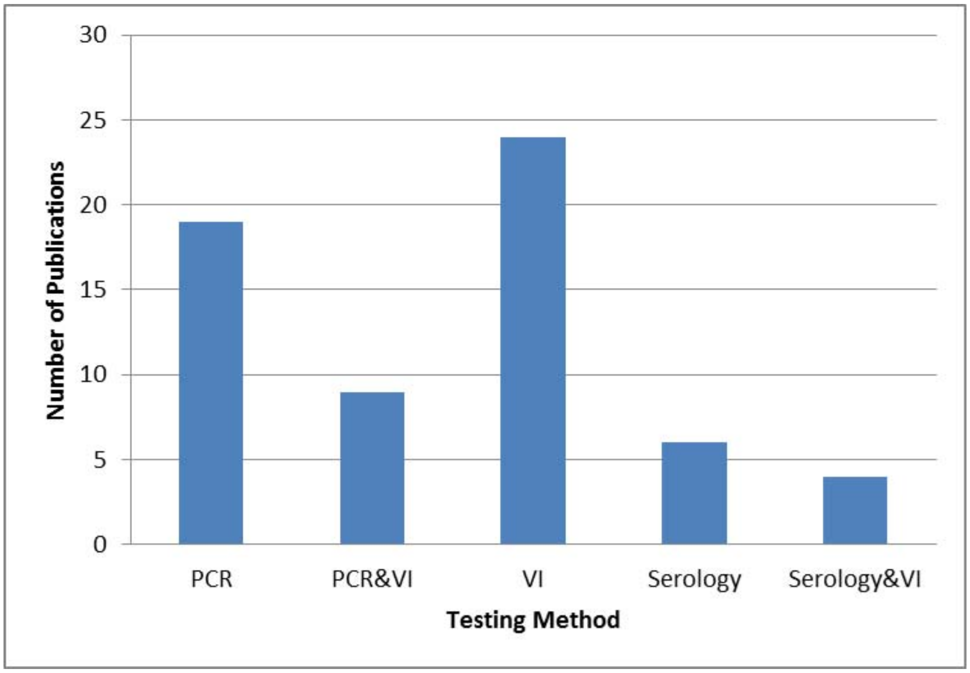

Figure 1.Breakdown of primary testing methods used for publications listed in the literature review.

CHAPTER 4

CONCLUSIONS

The purpose of this study was to gain a better understanding of the role passerines play in IAV natural history. To address this, the objectives were to: 1.) Summarize existing literature on IAV surveillance in passerines; and 2.) Conduct a large-scale survey for IAV in passerines under a variety of settings (natural, peridomestic, and agriculture) using common diagnostic tools, including virus isolation in embryonating chicken eggs, RRT-PCR, and serology.

Overall, there is little or no conclusive evidence supporting a role for passerines in the natural history of IAV. Recent molecular detections of IAV in passerines without confirmatory isolation have raised questions relating to the actual role of passerines in IAV epidemiology. This failure to confirm results was the major short-coming of the published studies reporting IAVs from passerines. With many well validated diagnostic techniques for IAV detection, similar to our prospective study, further investigations should be approached with sound diagnostic strategies providing results that can and are confirmed.

The possible exception relates to peridomestic birds associated with infected domestic poultry. It was noted throughout the literature review that spillover from domestic poultry to passerines (i.e. H5N1 outbreaks) can and does occur. However, even in this case there is no data to indicate that poultry adapted IAVs can be maintained in populations of passerine birds.

The results of our prospective study also support these conclusions. None of the birds tested by virus isolation or RRT-PCR were IAV positive and only a small percentage (0.08%) tested positive for NP antibodies to IAV. Likewise, results from the H7N9 infected farm samples also are consistent with the literature and the failure to detect virus in these birds provides no indication of virus maintenance in peridomestic birds.