Detection, isolation, and characterization of avian influenza viruses from waterfowl in wildlife refuges in the southeastern United States (2006-2011)

by

Teresa Villan Dormitorio

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Approved by

Joseph J. Giambrone, Chair, Professor of Poultry Science
Gary R. Hepp, Professor Emeritus of Forestry and Wildlife Sciences
Kenneth S. Macklin, Professor and Extension Specialist of Poultry Science
Hongzhuan Wu, Associate Professor at Alabama State University

ABSTRACT

Avian influenza (AI) is an infectious disease of birds caused by type A influenza viruses. These AI viruses (AIV) commonly infect poultry and wild birds; however, some AIV strains have infected and caused mortality in a variety of mammals including humans. Direct transmission of AIV from poultry to man have been demonstrated, and human influenza pandemic viruses have been reported to contain two or more novel genes that were very similar to those found in wild birds.

Wild aquatic birds represent major natural reservoirs of influenza A viruses and have been implicated as a continuous source of virus for domestic birds and other animal species including humans. All of the known hemagglutinin (H1-H16) and neuraminidase (N1-N9) influenza subtypes have been detected in wild waterfowl. While the virus does not usually cause clinical disease in these birds, severe illness may occur when the virus crosses the species border to poultry.

In this study, cloacal swabs were collected from hunter-killed or nesting waterfowl from wildlife refuges in Alabama, Georgia, and Florida. Out of 1260 swab samples inoculated into embryonated eggs, 64 allantoic fluids (AF) agglutinated red blood cells and 29 were RRT-PCR positive for the matrix gene of AIV. Nineteen AIV and 3 avian paramyxovirus subtypes were identified. No H5 or H7 isolates were found. RRT-PCR was found to be more sensitive and specific than AC-ELISA, since it detected AIV from AF with a hemagglutination titer as low as 4. Phylogenetic analysis of the H gene sequence of an Alabama H10N7 isolate showed close similarity (98%) to more recent

isolates, but only 90% related to an H10N7 isolated 38 years ago. Sequencing analysis of the H gene of four H1N1 isolates revealed that they were 92-97% similar to previously published H1N1 isolates including one, which came from swine. Experimental studies showed that an H1N1 isolate in embryonated eggs was eliminated within 24 hours of litter composting when the temperature reached 66^0C. Continuous surveillance and characterization of AIVs in wild birds will help in the understanding of the origin, evolution, transmission and control of present and future influenza outbreaks.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIV	Avian influenza virus
APMV	Avian paramyxovirus
CRBC	Chicken red blood cells
NDV	Newcastle Disease Virus
NVSL	National Veterinary Services Laboratories
AC-ELISA	Antigen Capture-Enzyme Linked Immunosorbent Assay
RRT-PCR	Real-time Reverse Transcription-Polymerase Chain Reaction
HPAI	High Pathogenic Avian Influenza
LPAI	Low Pathogenic Avian Influenza
USDA	United States Department of Agriculture
USAID	United States Agency for International Development

CHAPTER I: GENERAL INTRODUCTION

Avian influenza viruses (AIVs) can affect domestic poultry, as well as exotic pet birds, and wild migratory birds. AIVs have also been isolated, although less frequently, from mammalian species including rats, mice, weasels, ferrets, pigs, cats, tigers, dogs, horses, as well as humans.

Wild aquatic birds are the natural reservoir of AIVs. Ecological studies have established them as a primary source of virus for domestic poultry as well as other animal species including humans (Slemons et al., 1974; Webster et al., 1992). From this reservoir, viruses are sometimes transmitted to other host species and can continue to spread and sporadically cause mortality. Avian influenza viruses (AIVs) from wild birds usually cause no disease; however, some subtypes (H5 and H7) may undergo mutation and become highly pathogenic, especially in more susceptible domestic species (Capua and Alexander, 2009). All of the known 16 hemagglutinin (H) and 9 neuraminidase (N) AIV subtypes, and most H-N combinations have been detected in wild birds, predominantly ducks, geese, and shorebirds (Fouchier et al., 2005). AIVs are known to replicate in the intestinal tracts of ducks and contaminate water habitat through shedding of virus in feces. This process plays an important role in spreading AIVs. Occasionally, AIVs are transmitted directly to man and one recent example is the H5N1 virus isolated in 1997 from patients in Hong Kong. The same virus caused an outbreak on Hong Kong's chicken farms and resulted in a high mortality of birds. In an attempt to eradicate the disease, all of Hong Kong's poultry (approx.1.5 million birds) were slaughtered, which

may well have prevented adaptation of the avian H5N1 virus to man and, cause a subsequent human pandemic.

Because most wild bird species considered to be AIV reservoir are migratory, they are suspected of having a role in the spread of AIV. With the recognition of the human health implications due to AI, especially as a result of the spread of Asian H5N1 virus, AI infections have attracted the attention of veterinary and human scientists. Moreover, influenza A viruses of the H1N1 subtype have caused substantial morbidity and mortality in humans such as the pandemics of 1918 and 2009. Thus, there has been increased virus surveillance and establishment of AIV databases around the world. There is a global effort to collect and characterize AIV to identify genetic mutations and antigenic properties, which are useful in developing vaccines and controlling influenza.

Influenza viruses are inherently unstable. As these RNA viruses lack a genetic proof-reading mechanism, small errors that occur when the virus replicates, go undetected and uncorrected. Specific mutations and evolution in influenza viruses cannot be predicted, making it difficult if not impossible to know if or when a virus might acquire the properties needed to spread easily and infect humans. This difficulty is increased by the present lack of understanding concerning specific mutations, which may lead to increased transmissibility of the virus among humans. If and when, such a mutation occurs, there will be little warning when AI becomes a major threat to animals and humanity. Therefore, it is essential to prepare before a disaster occurs, as the time scale for the start of a pandemic disease cannot be predicted.

Since 1977, when the first human infections with the H5N1 (also known as bird flu) were documented, the virus has undergone changes. These changes have affected patterns of virus transmission and spread among domestic and wild birds. Studies have shown that H5N1 viruses from current outbreaks, when compared with viruses from 1997 and 2003, have become more lethal in experimentally infected chickens and mice, and are also hardier, surviving several days longer in the environment (WHO, 2006; Stallknecht et al., 2010). Other studies have shown that the virus is not yet fully adapted to poultry and continues to evolve (Webster and Govorkova, 2006; WHO, 2006).

The H5N1 outbreak has since spread to 16 countries in Asia, Africa, the Pacific Rim, Europe, Canada, and the Middle East and had resulted in the death and slaughtering of billions of poultry. People from these areas have also become ill with these avian viruses, in which more than half of those infected died. As of July 17, 2015, the cumulative number of confirmed human cases of H5N1infection reported to WHO is 844 with 449 deaths (53%) (WHO, 2015). The majority of these infections and deaths have occurred in rural areas of developing countries. It could well be argued that, given a lack of resources for diagnosis and reporting, an accurate figure would be somewhat higher. Although WHO reports only laboratory confirmed cases, it is evident from these numbers that H5N1 is lethal to humans, but difficult to spread. Residing deep in airways, the virus is not easily expelled by coughing and sneezing, the usual route of spread. Two research groups reported results that help explain the difficulty of virus spread in humans. The group of Yoshihiro Kawaoka of University of Wisconsin, Madison, tested various tissues of the human respiratory tract for receptors to which the virus can bind. Human flu preferentially bind to α2,6 galactose receptors that populate the human respiratory tract

from nose to lungs (Shinya et al., 2006). Avian viruses prefer α 2,3 galactose receptors that are common in birds but thought to be absent in humans. Using marker molecules that bind to one receptor or the other, the team found that humans also have α 2,3 galactose receptors, but only in and around the alveoli, structures deep in the lungs. Another group from Rotterdam, Netherlands, led by Thijs Kuiken, used more direct techniques to show that H5N1 readily binds to alveoli but not to tissues higher up in respiratory tract (Kuiken, 2006). This pattern is consistent with autopsies that have shown heavy damage to lungs but little involvement of upper respiratory tract. Findings explain, at least in part, the localization and severity of H5N1 viral pneumonia in humans.

Although more than half of H5N1 infected human beings have died, so far, human infection is still considered rare. Close contact with infected poultry and other domestic birds remains the most important source of human infections and to date, no human cases have been linked to exposure to wild birds. The virus does not spread easily from birds to humans or readily from person to person. Unfortunately, there are some indications that such a pattern of sustained human-to-human transmission may be eminent. Recent findings suggest that the 1918 "Spanish flu" pandemic may have resulted from a similar interspecies transmission event in which a purely avian virus adapted directly to human-to-human transmission without prior reassortment (Taubenberger, J., 2005).

Few scientists are prepared to say that H5N1 will not cause a human pandemic, because ingredients for it to happen are evident. We are seeing in real time an unprecedented evolution of a virus that has a pandemic potential. It is the first time in history that such an epizootic occurred. Nevertheless, it is heartening to note that

scientists, government agencies and regulators, biologics and poultry industries all over the world get together to attempt to deal with the problem. There is currently a "World Health Organization Global Influenza Program Surveillance Network" with nearly 100 members and collaborating laboratories, comprised of AI experts worldwide. Daily information and updates on research findings, reviews and commentaries indicate a round-the-clock effort of everyone concerned. In the USA, millions of dollars have been released for AI pandemic preparation. United States Agency for International Development (USAID) and other private agencies have recruited AI experts to be included in the database of volunteers, who may be called to help other countries if needed. The USDA and other departments and agencies of the Federal Government have taken steps to safeguard the country and its livestock populations from HPAI.

The spread of H5N1 across the globe highlights our vulnerability for the emergence of novel subtypes of influenza virus. Yet despite our fears of pandemic human disease, H5N1 is primarily a disease of birds. The establishment of regional sublineages suggests that H5N1 virus is perpetuated in poultry largely through a lapse in biosecurity rather than by continued reintroduction of viruses by migrating birds. A study has shown that H5N1 virus has persisted in its birthplace, southern China, for almost 10 years and has been repeatedly introduced into neighboring (e.g., Vietnam) and distant (e.g., Indonesia) regions, establishing "colonies" of H5N1 virus throughout Asia that exacerbate the pandemic threat. The best approach to avert the threat is to control H5N1 virus infection at its source, domestic poultry. Sustained and aggressive efforts to control H5N1 circulation in commercial and backyard poultry are mandatory to avoid catastrophic public health consequences.

New outbreaks of AI reported to the World Organization for Animal Health (OIE) have decreased, as might be expected from the seasonal pattern of the disease, but new cases of H5, H7, and H9 were reported during August 2015 in Africa, Asia, the Americas and Europe. Since December 2014, the United States Department of Agriculture (USDA) has confirmed 223 cases of highly pathogenic avian influenza (HPAI) in the Pacific, Central, and Mississippi flyways (USDA-APHIS, 2015a). The HPAI virus causing the US outbreaks is believed to have originated from a wild bird that was infected with an HPAI H5N8 virus from Asia and a low pathogenic North American strain of avian influenza at the same time. The viruses then recombined creating a new mixed origin viruses containing the Asian-origin H5 part of the virus, which is highly pathogenic to poultry (USDA, 2015). USDA has identified two mixed-origin viruses in the Pacific flyway: the H5N2 virus and new H5N1 virus. The new H5N1 virus is not the same virus as the H5N1 virus found in Asia that has caused human illness and spread globally (USDA-APHIS, 2015b).

The current widespread disease in the USA has been found in wild birds, as well as in a backyard and commercial poultry flocks, mostly in the Midwest. Since April, 2015, the outbreak has not moved south, or spread to the Atlantic flyway, however, experts predict the disease maybe in all four flyways this fall (USDA-APHIS, 2015a). As of June 17, 2015, 223 cases infected chickens, turkeys or mixed poultry from 10 Midwestern states have been reported and confirmed with a total of 48,091,293 affected birds (USDA-APHIS, 2015c). No new outbreaks have been reported since June. So far, the cost of depopulation, clean up, and indemnification by USDA to farmers, and loss in profits is about $3.3 billion (USDA-APHIS, 2015c). As a result of culling and mortality

due to infection, 10% of egg layers and 3.5% of turkeys in the Midwest were lost. In addition, sixteen percent of US poultry exports were embargoed by other countries (USDA-APHIS, 2015d).

The objectives of this research work were to detect, isolate, and characterize AIVs from wild waterfowl in wildlife refuges of the southeastern USA (Alabama, Georgia, and Florida). Virus isolation in embryonating eggs, AC-ELISA, and real-time PCR were used then compared to determine the most rapid, sensitive and cost-effective method for detection of AIV in wild birds. Subtypes of the AIV isolates were determined by the National Veterinary Services Laboratories (Ames, Iowa). Gene segments of some H1N1 isolates were cloned or amplified and DNA sequences determined. Phylogenetic analyses were conducted to determine genetic differences or similarity with other published H1N1s, including those that caused the 2009 pandemic. In addition, experimental studies were conducted to determine virus survival during composting.

AIVs undergo rapid and continuous mutation events as a natural consequence of adaptation in poultry. The rate is slower in the natural hosts, namely wild waterfowl, but even in these hosts evolution generates novel strains. The information generated here is useful for scientists and will contribute to the understanding of the influenza virus. Knowledge gained from these findings will be used for developing a more proactive and effective intervention program for AI. These data will be integrated into existing poultry health practices as well as state management systems, and will help poultry producers prevent AIV's entrance into their flocks. In addition, this project provided baseline data in support of the USA Homeland Security efforts to assist in distinguishing between

naturally emerging and purposely introduced type A influenza viruses in poultry in the southeastern USA.

CHAPTER II: REVIEW OF LITERATURE

2.1 The history of influenza A viruses

Avian influenza (AI) was first described when the deadly form of the disease caused massive outbreaks in poultry in northern Italy in 1878 (Lupiani and Reddy, 2009). It was originally termed “fowl plague”, and was confused with the acute septicemic form of fowl cholera. In 1901, the causative agent of this disease was shown to be ultra-filterable (i.e. virus) and, it was demonstrated in 1955 that there was a close relationship between this agent and mammalian influenza viruses (Alexander and Brown, 2000).

Infections in susceptible avian species can be divided into two forms based on the severity of the clinical disease produced. The very virulent viruses (formerly termed fowl plague) now called highly pathogenic avian influenza (HPAI) may cause poultry mortality as high as 100% (Alexander and Brown, 2000). These viruses have been restricted to subtypes H5 and H7, although not all viruses of these subtypes cause HPAI. For over 100 years, HPAI rarely occurred, which was often self-limiting or rapidly controlled by slaughter (Alexander and Brown, 2000). However, on some occasions, infection has become widespread in poultry and had severe implications for animal health and economy of the country. For example, in the Pennsylvania, USA epidemic (1983-84), more than 17,000,000 birds were slaughtered or died with an estimated cost to the US government of $60,000,000, excluding cost borne by the farmers (Lasley, 1986).

The first HPAI outbreak affecting poultry in the USA occurred in 1924-25. In 1975, outbreaks in chicken flocks caused by low pathogenic (LP) H4N8 were reported in

Alabama, with 69% mortality, including 31% mortality in a single day in one flock (Johnson et al., 1977). In 1995, 178 turkey farms in Minnesota were infected with H9N2, resulting in the worst economic loss due to influenza recorded in a single year (approximately $6,000,000) (Halvorson et al., 1998).

Outbreaks of HPAI in the last 50 years have been relatively uncommon around the world and have had limited spread within a country or region with one major exception, Asian lineage H5N1 that was first identified in 1996 (Suarez, 2010). This lineage of virus has spread to over 60 countries and has become endemic in poultry in at least four countries. The highly pathogenic strain of the H5N1 was first isolated and characterized in a domestic goose in the southern Guangdong province of China in 1996 (Whitworth et al., 2007). In the following year, the first H5N1 outbreak occurred in domestic poultry in Hong Kong, resulting in the culling of over 1.5 million chickens in an effort to contain and eliminate the disease. This outbreak also led to the infection of 18 people (with 6 fatalities) in what would be the first documented human death from the H5N1 virus. In the majority of cases, people obtained HPAI Asian H5N1 virus infection after direct or close contact with infected or dead poultry. Currently, HP Asian H5N1 virus does not transmit efficiently from person to person. Some cases of limited, non-sustained human-to-human transmission have likely occurred.

The ongoing outbreak of H5N1 among birds with occasional transmission to human beings is of major concern because of intriguing parallels between the H5N1 virus and the 1918 H1N1 influenza strain. This most famous and lethal pandemic named Spanish flu lasted from 1918 to 1919, and spread across Europe, Asia, and North America

(Taubenberger and Morens, 2006). This pandemic has been described as “the greatest medical holocaust in history”, and it is generally assumed that 50 million people died. The unusually severe disease killed from 20 to 2% of those infected, who were mostly young adults (20-40 yr old), and it is estimated that 2.5% to 5% of the world’s population at that time was killed. The complete coding of the sequences of all 8 viral RNA segments of the 1918 H1N1 virus was enabled with the recovery of the genomic RNA from archived formalin-fixed autopsy material and from frozen tissue from an influenza victim who was buried in permafrost in 1918 (Taubenberger, 2003). The 1918 virus according to this investigation was not a reassortant virus like those of the 1957 and 1968 pandemics, but more likely an entirely avian-like virus that adapted to humans. Research has shown that just 10 amino acid changes in the polymerase proteins differentiate the 1918 virus sequences from that of avian viruses, and that a number of the same changes have been found in recently circulating , highly pathogenic H5N1 viruses (Taubenberger et al., 2005). Later pandemic such as the 1957 Asian flu (H2N2 strain), the 1968 Hong Kong flu (H3N2 strain), and 2009 Swine flu (H1N1 strain), were not as devastating although it killed millions of people. In later pandemics antiviral drugs and antibiotics to control secondary infections were available and so may have helped reduce mortality compared to the 1918 Spanish flu.

The 2009 H1N1 pandemic is now known to involve a re-assorted virus produced from 2 kinds of porcine influenza, one of which is itself already a “triple-re-assortant” strain containing segments originating in human seasonal H3N2, AIV, and porcine influenza virus. As of August 1, 2010, more than 214 countries and overseas territories or communities worldwide have reported laboratory confirmed cases of pandemic influenza

H1N1 2009, including over 18,449 deaths (WHO, 2010). On August 10, 2010, the World Health Organization (WHO) International Health Regulations (IHR) Emergency Committee declared an end to the 2009 H1N1 pandemic globally. The U.S. Public Health Emergency for 2009 H1N1 Influenza expired on June 23, 2010.

The most important thing to remember when talking about pandemic influenza is that its severe form has little in common with seasonal influenza.

2.2 The biology of avian influenza virus

Influenza viruses are RNA viruses that make up 3 of the 5 genera of the family Orthomyxoviridae. The viral particles are about 80-120 nm in diameter and can be spherical or pleomorphic. There are 3 types of influenza viruses based on the molecular nature of its internal proteins, namely A, B, and C. Influenza A viruses are the most prevalent pathogens for both humans and animals. Only viruses of the influenza A genus are known to infect birds, and have been shown to cause devastating infections in poultry, which spread throughout the world. Influenza B viruses almost exclusively infect humans, causing significant flu-like illnesses, and is less common than influenza A viruses. B subtypes H3 and H2 are in human vaccines. Influenza C viruses infect humans, dogs and pigs and sometimes causing mild respiratory illness and are not thought to cause epidemics.

The influenza A viral genome consists of 8 segments of single negative strand RNA. Each RNA segment contains either 1 or 2 genes which code for a gene product (protein). Segments that codes for single gene products are: PB1, PB2, and PA proteins that constitute the viral polymerase; the hemagglutinin (HA) and neuraminidase (NA)

glycoproteins; and the nucleoprotein (NP) (Steinhauer, 1999). The two glycoproteins determine the subtypes of influenza A, and are associated with virulence, thus these proteins are targets for antiviral drugs. The HA protein is the most abundant and is responsible for binding of virus particles to sialic acid-containing cell surface receptors and, for mediating fusion of the virus to host cell membranes after endocytosis (Steinhauer, 1999). NA is involved in the release of new virions from infected host cells, by cleaving sugars that bind mature viral particles. Antibody responses to these 2 proteins form the basis for the division of influenza A viruses into their antigenic subtypes. To date, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been detected in birds and mammals worldwide.

New epidemic influenza A strains arise every 1 to 2 years by the introduction of selected point mutations within 2 surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). These permanent and usually small changes in the antigenicity of influenza A viruses are termed “antigenic drift” and are the basis for the occurrence of influenza epidemics. In addition, there is now evidence that multiple lineages of the same virus subtype can co-circulate, persist, and reassort in epidemiologically significant ways (Holmes et al., 2005)

In contrast to epidemics, pandemics are rare events that occur every 10 to 50 years. The 1918 pandemic was caused by a H1N1 virus of apparently avian origin ((Reid et al., 1999), whereas the subsequent pandemic strains – H2N2 in 1957 and H3N2 in 1968 – were reassortant viruses containing genes from avian viruses: 3 in 1957 (HA, NA, and the RNA polymerase PB1) and 2 (HA and PB1) in 1968 (Kawaoka et al., 1989). These major changes in the antigenicity of an influenza virus are called “antigenic shift”.

2.3 Natural hosts of avian influenza

Only viruses of the influenza A genus have been isolated from birds and termed avian influenza viruses (AIVs), but all 16 hemagglutinin (H1-H16) and all 9 neuraminidase (N1-N9) subtypes, and most of the possible H-N combinations have been detected or isolated from wild birds, particularly ducks, geese, and shorebirds (Fouchier et al., 2005). Since wild birds are considered the natural reservoir of influenza A viruses, they are thought to be the source of these viruses for all other animals. The highly pathogenic avian influenza (HPAI) viruses, which are restricted to the H5 and H7, are rarely isolated from wild birds. On the other hand, the low pathogenic (LPAI) viruses, which causes mild respiratory disease have extremely high isolation rates of about 11% for ducks and geese and around 2% for all other species (Alexander, 2007). LPAI viruses have been isolated from at least 109 wild bird species with 1.6 to 10% prevalence in ducks (Olsen et al., 2006).

AIVs from wild birds usually cause no disease; however, some subtypes (H5 and H7) may undergo mutation and become highly pathogenic, especially in more susceptible domestic species (Capua and Alexander, 2008). Influenza viruses that wild birds carry have been implicated in major human pandemics, including the most devastating "Spanish Flu" of 1918, which killed an estimated 20-50 million people around the world.

HPAI, subtype H5N1 has been occurring in domestic poultry in Southeast Asia since 2003, but it was only in 2005 when deaths of migratory birds due to H5N1 were reported. In April to June 2005, a large-scale outbreak of H5N1 infection where more than 6000 migratory birds were reported at the Qinghai Lake Nature Reserve in Qinghai Province,

China (Gall-Reculé et al., 2008), which is a protected nature reserve with no poultry farms in the vicinity. This was the first observation of sustained transmission within migratory fowls. In early August, 2005, Mongolia reported the death of some 90 migratory birds at two lakes in the northern part of the country (Brown et al., 2008).

AIV was reported as an etiological agent of an epizootic outbreak among Common Terns in South Africa in 1961 (Becker, 1967). The strain-specific antigen of this virus was found to be closely related to the Chicken/Scotland/1959, which caused an outbreak in chickens in Scotland. This relationship supported the hypothesis that migrating sea-birds such as Common Tern may transmit AIVs to domestic poultry.

HPAI H5N8 viruses emerged in China in 2010 and continued to spread to poultry in South Korea and Japan in early 2014, and to Germany, the Netherlands, UK, Italy and North America by late 2014. This unprecedented spread coincided with direction and timing of bird migration from Russia; however, the role of migrating birds in the global dispersal of H5N8 virus is unclear. By 2015, HPAI H5N8 virus has been detected in 16 migratory bird species of the orders Anseriformes, Charadriiformes and Gruiformes. In Germany, H5N8 was detected in common teal, mallar, and great black-backed gull. In the Netherlands, H5N8 was detected in two Eurasian wigeons (Verhagen et al., 2015).

2.4 Avian influenza virus testing and diagnostics

Detection of AIV virus infection may be accomplished by isolating the virus in embryonating eggs or cell culture or by detecting viral protein, viral RNA, or antibodies (Spackman Suarez, D. L. and Senne, D.A., 2008). The use of embryonated chicken eggs for the isolation of AIVs remains the gold standard for detection of the virus. It has been

the most sensitive and widely used approach. Most importantly, isolation of the virus is necessary for viral characterization. Although virus isolation (VI) has many advantages, it does present challenges for testing large numbers of clinical samples. One of the main problems is that specific pathogen free (SPF) embryonated eggs are perishable, expensive, and supply may be limited. Virus isolation also requires laboratory space with high levels of biosecurity, and takes days or weeks for results. Working with live influenza viruses requires additional precautions to prevent the virus from spreading outside the laboratory or cross contamination inside the laboratory. Furthermore, it should be noted that AIVs pose a risk of human infection.

In recent years, detection of influenza A virus nucleic acids has been used for rapid detection and diagnosis of influenza in domestic poultry and surveillance in wild birds (Spackman et al., 2002; Fouchier et al., 2005; Spackman, E., Suarez, D. L., and Senne, D.A., 2008). Real-time RT-PCR (RRT-PCR) detects viral RNA during both infectious and non-infectious phases, and has been found to be as sensitive as VI. A one-step RRT-PCR was developed for typing and subtyping AIVs using specific primers (matrix gene, H5 or H7 subtypes) and fluorescent Taqman probes (Spackman et al., 2002). Lastly, it has proven to be the best combination of sensitivity, ease of use, timeliness, and cost.

Antigen detection using commercially available antigen capture-ELISA (AC-ELISA) kits have been used successfully, although of lower sensitivity or specificity than VI and RRT-PCR. A test kit licensed for human use (Directigen Flu A test) has been used as a diagnostic test in poultry for several years (Spackman, E., Suarez, D. L., and Senne, D.A., 2008). The only immunoassay kit licensed for use in poultry in the US is the FluDetect test (Synbiotics Inc., San Diego, CA). These AC-ELISA kits use a monoclonal

antibody directed against the highly conserved AI nucleoprotein to bind viral antigen on a membrane or filter strip. Results are shown as a pattern or band on the membrane disk or paper strip following a chromatographic immunochemical reaction.

2.5 Avian influenza spread and transmission

Poor biosecurity and the migratory flight paths of wild birds are the two key points of how avian influenza spreads and how it should be controlled. It should also be remembered that poverty, poor human and veterinary infrastructure - as well as ignorance - also play a part.

AIVs can be transmitted among birds through direct contact with secretions from infected birds, especially feces or through contaminated feed, water, equipment, and human clothing and shoes. They are readily transmitted from farm to farm by the movement of domestic live birds, people, and contaminated vehicles, equipment, feed, and cages. Airborne transmission has not been noted as a means of AIV spread, although it was considered to have been possible in an HPAI outbreak in Frazer Valley, British Columbia in 2004 (Halvorson, 2008). However, attempts to detect airborne live virus outside poultry houses in that outbreak were not successful (Halvorson, 2008).

Swine has been considered to serve as an intermediate host in the transmission of AIVs to humans, but cases of direct transmission from domestic poultry to humans have been reported (Kaye and Pringle, 2005). The influenza viruses are easily spread by fomites and survive and spread in water. Illegal trade in wild birds without quarantine procedures are also a H5N1 transmission vehicle.

Certain species of wild waterfowl and shorebirds (orders Anseriformes and Chadriiformes) can carry AIVs without exhibiting any clinical signs. High titers of virus occur in late-summer and thus readily detected, when birds leave their northern breeding areas than in birds migrating north from overwintering areas in the spring (Runstadler et al., 2007). A duck can excrete 10^8 embryo infectious doses per milliliter (EID_{50}/ml), and shed virus for 30 days (Webster et al., 1978). Thus, virus contamination of the water habitat, transmission via oral-fecal route, and movement of waterfowl and shorebirds provide the mechanisms for AIV survival and spread in nature (Halvorson, 2008). Cold environments will allow AIVs to survive for weeks at 4^0C or for months in a frozen state, but at 21^0C, the virus is usually inactivated in 7 days (Swayne and Halvorson, 2008). Thus, not only are wild waterfowl a main source virus, but also its environment can be an important means of its survival and spread.

Several factors can contribute to the spread of all AIVs including: movement of people and goods, marketing practices (live bird markets), farming practices and the presence of the viruses in migratory wild birds, insects, rodents, etc. (OIE.int)

2.6 Avian influenza prevention and control

Due to the natural existence and maintenance of LPAI viruses in a variety of wild aquatic birds, causing mostly asymptomatic infections, control of AI within the natural reservoir is of minimal consequence. However, prevention of wild bird-origin AI viruses into domestic poultry is of utmost importance for AI control strategies.

The most important component of all AI control plans is biosecurity, which is the prevention of exposure to disease agents such as AIVs, through management practices.

Preventing the introduction of AI by eliminating all contact between commercial poultry and wild birds, swine farms, and live bird markets (LBMs) is a common, routine and successful practice. The majority of AIVs that are enzootic in wild aquatic birds are unable to replicate efficiently in chickens. However, multiple subtypes of AIVs have been isolated from healthy and diseased chickens; and occasionally, these viruses have acquired high virulence (Webster et al., 1992). It is not known whether AIVs isolated from chickens were transmitted directly from aquatic birds, or if the viruses acquired expanded host-range capabilities by replication in other avian species, e.g. quail, prior to infection of the chicken. When an AI outbreak occurs in an area with a high population density, the application of rigorous biosecurity measures might not be possible, and the disease may spread very rapidly. In this case, especially when culling capacities might not be adequate, ring vaccination around infected farms can be taken to attempt to reduce disease spread. The expected results on the dynamics of infection are: reducing the susceptibility of infection (i.e. a higher dose of virus is necessary for establishing productive infection); and reducing the amount of virus shed into the environment (Capua and Marangon, 2005). A higher infective dose necessary to establish infection and fewer virus contaminating the environment as a result of vaccination, represents a valuable support to the eradication of AIV infection. Therefore, limited vaccination represents a tool to support eradication, and will be successful tool only if coupled with restriction and increased biosecurity. However, use of vaccines risks embargo of export markets.

AI vaccines have been used for decades to protect against low and high pathogenic strains of AIVs. Recent successful application of vaccines to control HPAI incorporated additional well-defined control measures that included early reporting, culling,

enforcement of strict biosecurity, closure of live bird markets, and compensation to farmers. Vaccination reduces virus shedding and, when properly applied, can be of significant benefit in controlling the rapid spread of the disease and reducing risk of human infection. According to the OIE, poultry receiving killed vaccines are not excluded from the export trade, although specific technical guidelines must be followed to ensure that the vaccine is being applied properly and monitored effectively (Lubroth et al., 2008). However, countries are not mandated to adhere to OIE regulations.

The use of vaccines has been limited by the impossibility of differentiating vaccinated/infected from vaccinated/non-infected animals (DIVA). The major concern is that through trade or movement of apparently uninfected animals or products, the disease could spread further or might be exported to other countries (Capua and Marangon, 2005). According to the United Egg Producers, USA egg farms are repopulating, but it could take a year or more before the US egg industry reaches the production levels it attained before the recent H5N2 2015 avian influenza outbreak in the Midwestern USA.

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CHAPTER III: EVALUATION OF FIELD AND LABORATORY PROTOCOLS USED TO DETECT AVIAN INFLUENZA VIRUSES IN WILD AQUATIC BIRDS

T. V. Dormitorio,* J. J. Giambrone,* K. Guo,† and G. R. Hepp‡

**Department of Poultry Science, 260 Lem Morrison Drive, Auburn University, Auburn, AL 36849; †Department of Microbiology, University of Colorado Denver School of Medicine, Aurora 80014; and ‡School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL 36849*

3.1 ABSTRACT

Careful selection and observance of standard field and laboratory protocols are critical for successful detection and characterization of avian influenza viruses (AIV) from wild birds. Cloacal swabs were collected from hunter-killed or nesting waterfowl and shorebirds from wildlife refuges in Alabama, Georgia, and Florida during 2006 to 2008. Swab samples were inoculated into embryonated eggs followed by hemagglutination (HA) test to determine presence of hemagglutinating agents. Antigen capture-ELISA (AC-ELISA) and real-time reverse transcription PCR (RRT-PCR) were used to detect AIV from both allantoic fluids (AF) and swab specimens of HA-positive samples. Hemagglutination inhibition test was used to detect Newcastle disease virus, a paramyxovirus that causes respiratory disease and can hemagglutinate red blood cells. It is also common in wild birds. The HA-positive AF were sent to the National Veterinary Services Laboratory for subtyping of the isolates. Out of 825 samples tested, 19 AIV and

3 avian paramyxovirus subtypes were identified by the National Veterinary Services Laboratory. Without egg passage, AC-ELISA did not detect virus, whereas matrix gene of 13 AIV were detected using RRT-PCR. When testing was done on AF, 14 were positive for influenza A by AC-ELISA and 20 by RRT-PCR. AC-ELISA did not detect influenza A when the HA titer was lower than 125, whereas RRT-PCR detected AIV from AF with HA titer as low as 4. The highest isolation rate was from Florida, where out of 109 samples analyzed, 14 AIV were detected by RRT-PCR from AF. Real-time reverse transcription-PCR was more sensitive, specific, and cost-effective than AC-ELISA. However, to avoid false negative results, testing should be performed on AF and not directly from cloacal swabs. Our procedures to detect AIV directly from cloacal swabs from wild birds need further optimization for improved sensitivity.

Key words: avian influenza virus, wild aquatic bird, real-time reverse transcription polymerase chain reaction, antigen capture-enzyme-linked immunosorbent antibody assay

3.2 INTRODUCTION

Wild aquatic birds are a natural reservoir of avian influenza viruses (**AIV**). Ecological studies have established them as a primary source of virus for domestic poultry as well as other animal species including humans (Slemons, *et al*., 1974; Webster, *et al*., 1992). AIVs from wild birds usually cause no disease; however, some subtypes (H5 and H7) may undergo mutation and become highly pathogenic especially in more susceptible domestic species (Capua and Alexander, 2008). All 16 hemagglutinin (**HA**) and 9 neuraminidase AIV subtypes, and most HA-neuraminidase combinations have been detected in wild birds, predominantly ducks, geese**,** and shorebirds (Fouchier *et al*.,

2005). Low pathogenic AIV have been isolated from at least 109 wild bird species with 1.6 to 10% prevalence in ducks (Olsen *et al*., 2006). Avian influenza viruses have been found to replicate in the intestinal tract of ducks and thus, viral contamination of the water habitat through shedding of virus in feces may play an important role in spreading type A influenza viruses (Slemons and Easterday, 1978).

Because most wild bird species considered to be AIV reservoirs are migratory, they are suspected of having a role in the spread of AIV. Surveillance studies on prevalence of AIV in wild birds have been conducted in North America (Slemons, *et al*., 1974; Stallknecht, *et al*., 1991; Hanson, *et al*., 2003; 2005; Krauss, *et al*., 2004; Dugan, *et al*., 2008; Ferro, *et al*., 2008; Stallknecht and Brown, 2008). With the recognition of the human health implications of AI, especially as a result of the spread of Asian H5N1 virus, AI infections have attracted the attention of veterinary and human scientists. Thus, there has been increased virus surveillance and establishment of AIV databases, not only in the United States, but around the world. There is a global effort to collect and characterize AIV to identify genetic mutations and antigenic properties (Capua and Alexander, 2008).

To establish a collaborative database to explain the natural history and ecology of AIV in wild birds, it is important to observe standard field and laboratory protocols (Runstadler *et al*., 2007). Maintenance of cold temperatures during sampling and transport, use of specific-pathogen-free or commercial eggs for virus isolation, and RNA extraction procedures are steps that require standardization. After virus detection, further viral characterization requires in-depth and standardized techniques.

Virus isolation in embryonating eggs is the most sensitive and widely used approach to demonstrate presence of AIV in clinical samples (Spackman *et al*., 2008). Moreover, viral isolates are available for further genetic and antigenic characterization. However, embryonated eggs are costly and perishable and supplies are limited. Since the viruses are amplified during virus isolation, there is also a concern of cross-contamination among samples and exposure of laboratory personnel to infectious virus (Spackman *et al*., 2008). Antigen capture-ELISA (**AC-ELISA**) using a commercially available kit licensed for human diagnostic use (Directigen Flu A test; Becton-Dickinson, 2004), has been used in wild bird surveillance and poultry for several years (Slemons and Brugh, 1997; Davison *et al*., 1998; Woolcock and Cardona, 2005; Runstadler *et al*., 2007). This test is easy to use, doesn't require expensive equipment, and provides results in 15 min (Spackman *et al*., 2008).

Avian influenza virus detection using real-time reverse transcription-PCR (**RRT-PCR**) has been used worldwide (Spackman, *et al*., 2002; Cattoli *et al*., 2004; Karlsson, *et al*., 2007; Runstadler *et al*., 2007). This technique is sensitive, specific, rapid, and has high-throughput (Suarez *et al*, 2006). Real-time reverse transcription-PCR can identify H5 and H7, which have the potential to become highly pathogenic, using specific primers and probes (Spackman, *et al*., 2002).

In this study, we isolated and characterized AIV and avian paramyxoviruses (**APMV**) from wild aquatic birds in Alabama, Georgia and Florida. We examined AC-ELISA and RRT-PCR for use in detection of AIV directly from swabbed material and allantoic fluids (**AF**) of hemagglutination-positive (**HA+**) samples. We showed that

successful AIV detection and isolation from wild aquatic birds was dependent on sampling and laboratory protocols.

3.3 MATERIALS AND METHODS

Swab Collection

Most sampling was conducted during the hunting season, which is in late September to early February 2005 to 2008. Sampling of non-migratory, nesting wood ducks (*Aix sponsa*) occured in the summer during the breeding and egg-laying season. Samples were from hunter-killed and nesting waterfowl from wildlife refuges in Alabama (geographic information system coordinates near 31° 58’ N, 85° 06’ W; 34° 34’ N, 86° 06’ W), Georgia (31° 59’ N, 85° 03 W), and Florida (30° 25’ N, 82° 47’ W). Data such as species, sex, age, habitat, and time of isolation were recorded. There were about 13 species of birds sampled, but mainly wood ducks (*Aix sponsa*), mallards (*Anas platyrhynchos*), ring-necked ducks (*Aythya collaris*), northern shovelers (*Anas clypeata*), hooded mergansers (*Lophodytes cucullatus*), American green-winged teals (*Anas crecca carolinensis*) and blue-winged teals (*Anas discors*; BWTE). Most sampling was conducted by Auburn employees, but some samples were collected by federal or state, or both, government wildlife employees, who placed samples in polystyrene foam coolers with cold packs and sent to Auburn via overnight mail.

Experimental Design

A cloacal swab was collected from each bird by swirling a sterile Dacron swab with polyester shaft (14-959-90; Fisher Scientific, Hampton, NH) around the interior mucosa of the cloaca. Swabs were placed in a tube containing 2 ml virus-transport medium (brain-heart infusion broth supplemented with 10,000 IU/ml of penicillin G and 10 g/ml

of streptomycin sulfate), kept cold in the field with wet ice or frozen in dry ice and transferred to a -70°C freezer within 5 h. Frozen tubes containing cloacal swabs in transport medium were thawed and centrifuged at 2000 x g for 10 min. The cotton-tipped swab was discarded and the supernatant was aliquoted into three vials for use in egg embryo inoculation (aliquot 1), AC-ELISA (aliquot 2) and RRT-PCR (aliquot 3) as shown in Figure 1. Egg embryo inoculation was accomplished first, and after HA+ samples were identified from AF, the corresponding aliquots were then tested by AC-ELISA and real-time reverse transcription-PCR. Thus, AC-ELISA and real-time reverse transcription-PCR were used to detect AIV only from HA+ samples, both from swab material and AF. HA+ AF were also tested for presence of Newcastle disease virus (NDV) by hemagglutination inhibition (HI) test. Allantoic fluids that were found positive for the matrix gene of AIV were further tested if they were of the H5 and H7 subtypes by RRT-PCR, and then sent to the National Veterinary Services Laboratories (**NVSL**), Ames, Iowa for virus identification.

Virus Isolation in Embryonated Eggs

Aliquot 1 of each of the 825 samples was inoculated into 10-day-old embryonating specific-pathogen-free or commercial chicken eggs following standard egg inoculation procedures via allantoic route (Senne, 1998)**.** Samples were thawed quickly in a 37°C water bath, vortexed for 1 min, and then centrifuged at 2000 x g for 10 min. Four eggs were inoculated with 150µl (per egg) of each swab sample. Eggs were incubated at 37°C and 62% RH. After 48 hours of incubation, eggs were chilled overnight and AF was harvested (Hitchner, 1980).

Hemagglutination and HI Tests

Hemagglutination (HA) titers in AF were determined with chicken erythrocytes by standard procedures (Alexander, 199**7).** The HA+ were identified by adding one drop (duplicate) of AF from each egg into a labeled 96-well plate. Fifty microliters of 0.5% chicken red blood cells (**CRBC**) was added to each well and after 45 min of incubation at room temperature, results were recorded as positive (HA+) when CRBC were hemagglutinated (formed in a latticework, giving a solid pink appearance to the entire well). Wells that had pelleted CRBC, which ran in a tear-drop shape upon tilting the plate 45°, were negative. Allantoic fluid from HA+ eggs was harvested and the HA titers determined. HA+ AF were tested for **NDV** by HI (Thayer & Beard, 1998) using NDV antisera (Charles River SPAFAS Inc., North Franklin, CT).

Antigen Capture Enzyme Linked Immunosorbent Assay

Type A influenza viruses were identified using a commercially available AC-ELISA kit (Directigen Flu A assay, Becton Dickinson). Instructions of the manufacturer were followed to perform the test. Attempts to detect AIV were done directly on original swab specimens and AF that were HA+.

Real-time Reverse Transcription Polymerase Chain Reaction

The HA+ AF were tested for AIV (matrix, H5, and H7 genes) by RRT-PCR using specific primers and probes developed by (Spackman *et al*., 2002). Same reagents and procedures were used to detect AIV directly from swab specimens, except that TRIzol reagent (Invitrogen Corp., Carlsbad, CA) was used to extract RNA instead of RNeasy kit (Qiagen Inc., Valencia CA) used for AF.

RNA extraction. Ribonucleic acid from AF was extracted with the RNeasy kit following the instructions of the manufacturer. Briefly, 500 µL of AF was mixed with 500 µL of kit-supplied RLT buffer (Qiagen Inc., Valencia, CA) and the entire sample was applied to the RNeasy spin column. The column was washed with buffers and then RNA was eluted in 50 µL of nuclease-free water and 5 µL per RRT-PCR reaction was used for template. Extraction of RNA directly from swabs was accomplished by adding 750 µL of Trizol reagent to 250 µL of the thawed aliquot of each HA+ sample. After a series of chloroform, isopropanol and ethanol additions followed by incubations and centrifugations, the pelleted RNA was hydrated in 50 µL nuclease-free water. Five microliters per RRT-PCR reaction was used for template.

Primers and probes. Published primers and probes targeting the matrix, H5 and H7 genes of type A influenza virus were used, namely: forward primer M+25, reverse primer M-124, probe M+64; forward primer H5+1456, reverse primer H5-1685, probe H5+1637; and forward primer H7+1244, reverse primer H7-1342, probe H7+1281 (Spackman *et al*., 2002)

PCR. The One-step RT-PCR kit (Qiagen Inc.) was used to make reaction mixtures containing: 4 µL of 5x buffer, 3.75 mM of $MgCl_2$ (Promega, Madison, WI) 325 µM of deoxynucleoside triphosphate (each), 10 pmol of each forward or reverse primer, 6.5 U of RNase inhibitor (Promega), 0.8 µL of enzyme mix, and 0.15 µM of probe. The PCR reactions were performed in a Lightcycler (Roche Applied Science, Indianapolis, IN) with a 20 µL volume, containing 5 µL viral RNA sample and 15 µL of reaction mixture, using the following protocol: 30 min at 50°C, 15 min at 95°C; followed by 45 cycles at

94°C for 0 s and at 60°C for 20 s (matrix); 40 cycles at 94°C for 0 s, at 57°C for 20 s, and 72°C for 5 s (H5); and 40 cycles at 94°C for 0 s and at 58°C for 20 s (H7).

The RRT-PCR results are reported as positive or negative (amplification or no amplification) for the target gene, with a corresponding crossing point (**Cp**) value, which represented the point at which amplification of DNA is detected above background fluorescence (Lightcycler 2.0 Instrument Operator's Manual). A sample with a lower initial concentration of target DNA required more cycles to reach the Cp. Samples with a higher concentration required fewer cycles.

AIV Subtyping

The HA+ AF were submitted to NVSL for subtyping of the isolates. When there was difficulty subtyping, (e.g., reaction to more than 1 HE subtype), a limiting dilution study was conducted and the highest dilution exhibiting HA was resubmitted to NVSL.

Second Passage in Embryonating Eggs

Five RRT-PCR-positive and 10 negative samples (first passage) from the first 2 yr of sampling (713 samples) were used to determine the effect of egg passage on AIV detection. These samples were repassed on 10-d-old embryonating eggs following procedures as in the initial pass. The HA titers, AC-ELISA and RRT-PCR results of egg passage1 and passage2 were compared.

3.4 RESULTS AND DISCUSSION

Out of 825 swab samples inoculated into embryonated eggs, 21 were HA+ (Table 1). Real-time reverse transcription-PCR and AC-ELISA were used to detect or identify AIV from AF and original swab material of these HA+. Subtypes of the virus isolates were determined by NVSL. Newcastle disease virus and other APMV were identified by HI

test and NVSL. When testing was done on AF, RRT-PCR revealed 20 positive results for the matrix gene of AIV, with Cp ranging from 12 to 40. None of the matrix positives were H5 or H7. ELISA tests of the same HA+ allantoic fluids gave only 14 positives. Furthermore, AC-ELISA could not detect AIV when the HA titer was lower than 128. In contrast, RRT-PCR detected AIV from AF having an HA titer as low as 4, although the Cp was high (40), indicating that the concentration of the virus was low.

During the first year of sampling (2006), only one virus (H10N7) was isolated. At this time, procedures and techniques were still being developed. In the second year, more viruses were isolated, although there was difficulty in determining its subtypes. The NVSL reported that all submitted samples except AL381 were AIV by PCR and positive for N1 by subtyping, but the HA could not be subtyped. Limiting dilution studies were conducted on five samples (AL121, AL167, AL253, AL299 and AL253). It took one experimental run to determine the highest dilution that exhibited HA for the 4 samples, but took several for AL381. On first egg passage for AL381: HA titer was high (2048); AIV matrix gene positive by RRT-PCR (Cp=40); and NDV+ by HI (Table 1). On further passages, there was difficulty determining the end point of agglutination. Two samples (AL381a and AL381b) of the fourth passage (10^9 dilutions), were re-submitted to NVSL. The virus H1N1 was identified from AL381a, and APMV-1 from AL381b. It was found that AL381 was the only virus-positive sample from those collected and sent via overnight mail by wildlife employees. This sample came from an American widgeon, which was found dead in a wildlife refuge in North Alabama. These results showed that detection and subtyping of AIVs may be difficult when there is a mixed infection with APMV.

Table 1 showed that 15 samples (out of 109 swabs) were positive for AIV or APMV or both, from one sampling day (9/22/07). These samples were included in the third sampling year (2008) batch, where we were more trained and careful with sampling and laboratory techniques. The hosts of the virus isolates were all BWTE from Lake City, Florida. Based on testing of the 15 HA+ samples, AIV detection rate from AF by PCR was 93% (14/15) and 73% (12/15) by AC-ELISA. The RRT-PCR detection directly from swabs was also high (80%), whereas AC-ELISA has not yet been done on these swabs. In addition, H3N8, H4N6, H4N8, APMV-1 and APMV-4 were identified by NVSL from AF of these samples. These higher isolation rates may be attributed to the use of dry ice to freeze the samples in the field, which was not done in the previous two yr. Other factors that may have contributed to the higher isolation rate are: 1) time or location of sampling; 2) bird species or age; and 3) improved sampling and laboratory techniques. It is interesting to note that 97% of the samples collected in September were BWTE, whereas on another sampling time (1/21/06) conducted on this site, more green-winged teals were sampled versus BWTE (40:1) and there were other species such as ruddy duck (*Oxyura jamaicensis*), northern shoveler, *etc*.

One HA+ sample (FL20) had an HA titer of 256, but was negative for all other tests except for HI. This sample was not submitted to NVSL because following the experimental flowchart in Figure 1; only samples that were found positive by RRT-PCR were considered for subtyping. Since this sample was positive for NDV with a titer of 480, this virus could be the hemagglutinating agent and not AIV.

None of the AIV isolates were detected by AC-ELISA when testing was done directly on cloacal swabs. In contrast, 13 were detected by RRT-PCR from the swabs,

although the Cp were high (>22). It was noted that the cloacal specimens were inadequately absorbed in the membrane of the AC-ELISA device. The manual of the manufacturer suggested dilution of the specimen with saline, but this was not done. Possible reasons for failure of RRT-PCR to detect AIV directly from swabs (RRT-PCR-1) when it could from AF (RRT-PCR-2) may be inhibitory substances in the cloacal swabs, low or undetectable virus concentration, or loss of virus during RNA extraction.

Limiting dilutions need to be done on AF (FL52 and FL108) that were RRT-PCR positive, but no AIV was identified by NVSL. Through the use of subtyping and HI tests, APMV-1 and APMV-4 were identified or detected in these samples. It was noted that Cp of these AF were higher compared with others within this sampling group, plus they were AC-ELISA-negative. Co-infection with APMV may have masked AIV detection by RRT-PCR or AC-ELISA, or APMV might have produced a higher titer than AIV in chicken embryo culture; thus, they were the ones picked up and identified by NVSL.

During the first two yr of sampling, out of 713 swabs, 16 were HA+. Six (GA26, AL121, AL167, AL253, AL299, AL381) of the AF were positive for the matrix gene of AIV by RRT-PCR, with Cp ranging from 19 to 40 (Table 1). Avian influenza was detected directly from the swab by RRT-PCR from only 1 sample (AL167). Because the Cp were high and there was difficulty with subtyping, an experiment was conducted to determine the effect of egg passage on AIV detection from AF. Following the flowchart in Figure 1, HA+ that were negative by RRT-PCR were included in the samples tested in the experiment. Results for 3 samples (GA26, AL121 and AL299) are reported in Table 2. There were problems with data obtained from samples AL253 and AL381, and thus were not included in the table. Samples that were negative by RRT-PCR test on the first

passage remained negative after repassage and therefore were likewise not included. Results presented on Table 2 showed that amplification of the virus as a result of passage occurred and resulted in increased HA titer. As a consequence of an increase in HA titer, AF (GA26 and AL299) that were negative by AC-ELISA on the first passage, tested positive on the second. The effect of egg passage on AIV detection by RRT-PCR was lowered Cp, indicating that there was an increase in the detectable virus. Therefore, a second passage into embryonated eggs is helpful in confirming presence of AIV in samples that are AC-ELISA-negative and RRT-PCR-positive but with a high Cp. However, for detection purposes, it is not productive to do a second passage on samples that are negative by RRT-PCR on initial passage.

It was found that RRT-PCR was more sensitive than AC-ELISA in detecting AIV from swabs and AF. However, AC-ELISA assay using the Directigen kit has advantages. This kit is commercially available, needs no additional reagents or expensive equipment, and provides results in 15 minutes. It is simple to use and unlike RRT-PCR, testing can be performed without intensive training and molecular expertise. Moreover, this kit was developed for the rapid diagnosis of type A influenza infections in humans using nasopharyngeal specimens and therefore may not be best for use in AIV detection in healthy wild birds, especially when used on cloacal specimens. Sensitivity of detection from cloacal swabs may be enhanced by pre-treatment of samples such as filtering to remove particles which clog the membrane on the ColorPAC device (Becton-Dickinson, Sparks, NJ). Other licensed AC-ELISA or antigen detection kits for AIV are commercially available and maybe sensitive or convenient, or both, for use with AIV detection in wild birds.

Results showed that more work is needed to optimize our RRT-PCR procedures for use in detecting AIV directly from cloacal swab material without passage in embryonated eggs. Identification of PCR inhibitors and more efficient RNA extraction procedures, such as use of robotics are some of the factors that need to be explored.

We detected and characterized AIV and APMV from wild aquatic birds of several wildlife refuges in the southeastern states. It was found that RRT-PCR was more sensitive, cost-effective, and specific than AC-ELISA for the detection of AIV in wild aquatic birds. It could detect AIV from AF with HA titer as low as 4. Samples that are positive for AIV, with a high Cp (<32) and were mixed infections with APMV, required further limiting dilution to separate the viruses. Our procedures to detect AIV directly from cloacal swabs need to be further optimized for improved sensitivity.

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Table 3.1. Detection and isolation of avian influenza virus (AIV) and avian paramyxovirus (APMV) from cloacal swabs of hunter-killed wild birds[1]

Sample ID[2]	Sampling Date	Host[3]	HA titer	AC-ELISA	RRT-PCR-1[4] (Cp[5])	RRT-PCR-2[6] (Cp)	HI (NDV titer)	Subtype(s)
GA26	1/7/06	NSHO	64	negative	negative	36	negative	H10N7
AL121	1/6/07	RNDU	256	positive	negative	24	negative	H1N1, H1N4
AL167	1/6/07	BWTE	256	positive	34.2	19	negative	H1N1
AL253	1/20/07	MALL	512	positive	negative	20	negative	H1N1
AL299	1/27/07	AMWI	4	negative	negative	40	negative	H1N1
AL381	1/21/07	AMWI	2048	negative	negative	40	+ (640)	H1N1, APMV-1
FL20	9/22/07	BWTE	256	negative	negative	negative	+(480)	ND[7]
FL21	9/22/07	BWTE	512	positive	27.4	12.4	negative	H3N8
FL28	9/22/07	BWTE	1024	positive	38.4	28.0	negative	H3N8
FL36	9/22/07	BWTE	1566	positive	33.6	22.4	negative	H3N8
FL43	9/22/07	BWTE	2048	positive	33.8	26.7	negative	H4N6
FL52	9/22/07	BWTE	384	negative	40.0	32.2	+(1280)	APMV-1
FL55	9/22/07	BWTE	1024	positive	22.9	13.8	negative	H3N8
FL64	9/22/07	BWTE	1536	positive	negative	16.8	negative	H4N6
FL77	9/22/07	BWTE	1536	positive	negative	14.2	negative	H3N8
FL93	9/22/07	BWTE	128	positive	36.3	19.6	negative	H3N8
FL101	9/22/07	BWTE	128	positive	28.9	19.3	negative	H4N8
FL102	9/22/07	BWTE	1024	positive	34.2	18.7	negative	H3N8
FL105	9/22/07	BWTE	128	positive	36.6	15.6	negative	H3N8
FL107	9/22/07	BWTE	128	negative	40.0	15.9	negative	H4N8
FL108	9/22/07	BWTE	64	negative	38.4	32.3	+(10)	APMV-4

[1]Out of 825 virus isolation samples, 21 were hemagglutination-positive (HA+) in allantoic fluid and further characterization of these 21 samples is shown. Hemagglutination (HA) titer, antigen capture-ELISA (AC-ELISA), real-time reverse transcription-PCR (RRT-PCR) of the AIV matrix gene, HA Inhibition (HI) Newcastle disease virus (NDV) titer, and subtyping test results. All tests except RRT-PCR-1 were conducted on allantoic fluids collected from swab inoculated embryonating eggs (passage1). RRT-PCR-1 tests were done directly on swabs. The AC-ELISA test results done directly on swabs; of these, 21 HA+ samples were all negative and were not included in the table.

[2]ID = sample indentification.

[3]NSHO = northern shoveler; RNDU = Ring-necked duck; MALL = mallard; BWTE = blue-winged teal; AMWI = American wigeon.

[4]RRT-PCR-1 = AIV detection directly from swab by RRT-PCR.

[5]Cp = crossing point.

[6]RRT-PCR-2 = AIV detection from allantoic fluids of swab-inoculated embryonating eggs by RRT-PCR.

[7]ND = not determined

Table 3.2. Effect of egg passage (P1 vs. P2) on hemagglutination (HA) titer, antigen capture-ELISA, and real-time reverse transcription-PCR (RRT-PCR; matrix gene) detection assays on allantoic fluid of swab inoculated embryonated eggs[1].

Sample ID[1]	HA titer P1/P2[3]	AC-ELISA P1/P2	RRT-PCR (Cp[4]) P1/P2
GA26	64/256	-/+	36/24
AL121	256/1024	+/+	24/17
AL299	4/640	-/+	40/17

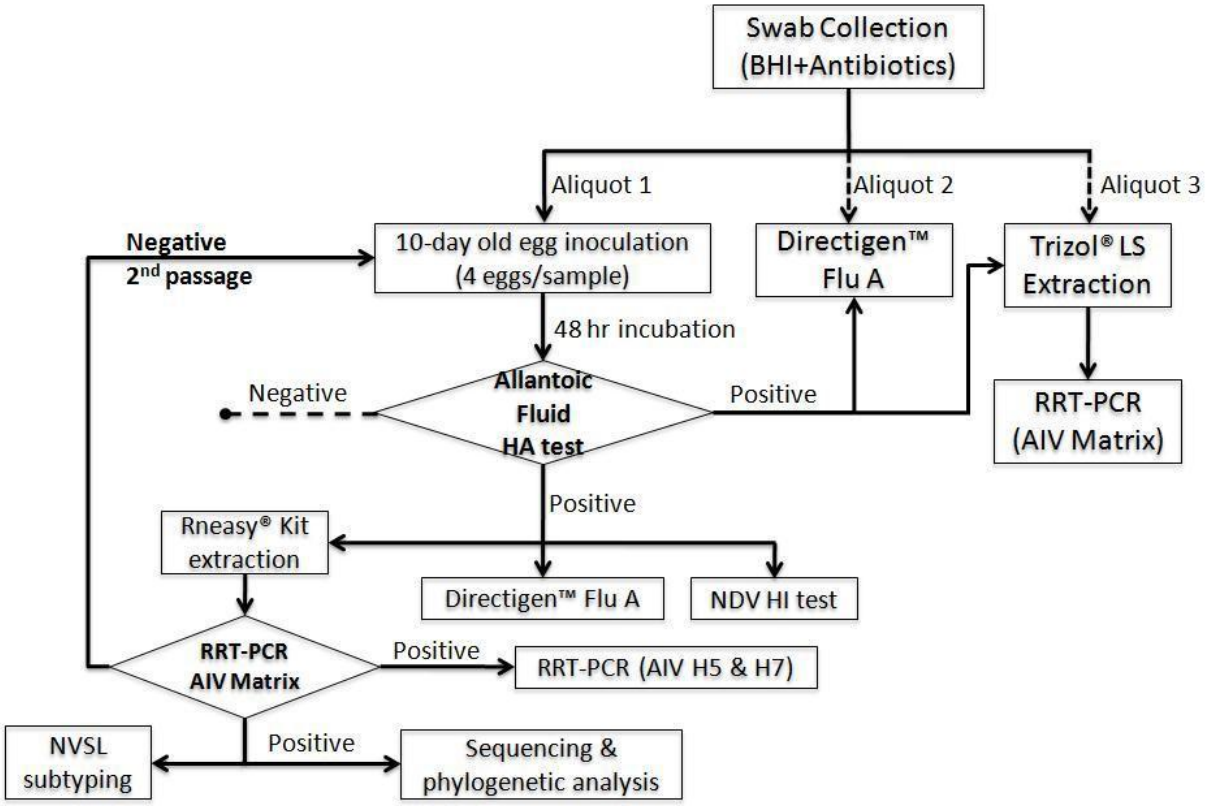
[1]Five RRT-PCR positive and 10 negative samples on first passage were included in the experiment. Results for 3 samples are included in the table.

[2]ID = sample indentification.

[3]P1/P2 = passage 1/passage 2

[4]Cp = crossing point

Figure 3.1. Flowchart of experimental procedures used to detect avian influenza viruses in wild aquatic birds. After hemagglutination positive (HA+) samples were identified from allantoic fluids of inoculated eggs (Aliquot 1), the corresponding aliquots (Aliquot 2 and Aliquot 3) were then tested by AC-ELISA and RRT-PCR.



CHAPTER IV: DETECTION AND CHARACTERIZATION OF AVIAN INFLUENZA VIRUSES AND OTHER AVIAN PARAMYXOVIRUSES FROM WILD WATERFOWL IN PARTS OF THE SOUTHEASTERN UNITED STATES

T. V. Dormitorio,* J. J. Giambrone,*[2] K. Guo,* and G. R. Hepp†

**Department of Poultry Science, 260 Lem Morrison Drive, and †School of Forestry and Wildlife Sciences, Alabama Agriculture Experiment Station, Auburn University, Auburn 36849-5416.*

4.1 ABSTRACT

Cloacal swabs were taken from migratory hunter-killed, non-migratory, nesting waterfowl and migratory shorebirds from wildlife refuges in Alabama, Georgia, and Florida during 2006 to 2008. Samples were processed in embryonated eggs followed by hemagglutination (HA), Directigen, and real-time reverse transcription-PCR tests. Sequence analysis of the hemagglutinin (H) gene of the H10N7 Alabama isolate revealed that it was closely related (98%) to recent isolates from Delaware and Canada, but only 90% related to an H10N7 isolated 30 yr ago. Four isolates had 94 to 97% similarity to published H1N1 isolates including one from swine. No H5 or H7 isolates were found. One sample was highly pathogenic in embryos, produced a high HA titer, and was positive for both avian influenza (AIV) and Newcastle disease virus (NDV) or avian paramyxovirus-1 (AMPV-1). In recent (2008) sampling, more (14%) AIV, AMPV, or both were isolated than in 2006 to 2007 (1% isolation rate). The higher isolation rate during 2008 may be attributed to optimized sample collection, storage in dry ice, new egg incubator, healthier eggs, time or habitat for isolation, species sampled, migratory status of birds, and more experience with detection procedures. An additional egg passage resulted in increased viral titer; however, no HA negative samples

became HA positive. The chance of transmission of APMV or low-nonpathogenic AIV from wild waterfowl to commercial poultry is possible. However, the chance of transmission of H5 or H7 AIV isolates from waterfowl to commercial farms in Alabama, Georgia, or Florida is unlikely. Therefore, continual testing of these birds is justified to insure that H5 or H7 AIV are not transmitted to commercial poultry.

Key words: avian influenza virus, paramyxovirus, wild waterfowl, real time-reverse transcription polymerase chain reaction, phylogenetic analysis

4.2 INTRODUCTION

Wild waterfowl are a natural reservoir of avian influenza A viruses and have been implicated as a source of virus for domestic birds as well as other animal species including humans. In addition, avian paramyxovirus (APMV) is routinely found in wild birds. All of the 16 hemagglutinin (H) and nine neuraminidase (N) influenza virus (AIV) subtypes, and most H-neuraminidase combinations have been detected in bird reservoirs, predominantly ducks, geese and shorebirds (Fouchier, *et al*, 2005). The AIV usually replicate in the intestinal tract in these birds, cause no disease, and are spread by fecal contamination of the water habitat. Some APMV-1 (Newcastle disease virus; NDV) are pathogenic in chickens.

Avian influenza virus was reported as an aetiological agent of an epizootic among common terns in South Africa in 1961 (Becker, 1966). The strain-specific antigen of this virus was closely related to the Chicken/Scotland/1959, which caused an outbreak in chickens in Scotland (Becker, 1967). This supported the hypothesis that migrating sea-birds such as the Common Tern may transmit AIV to domestic poultry. Another case of lethal AIV infection in wild aquatic birds occurred in late 2002 when H5N1 caused deaths of many resident avian species, including waterfowl and greater flamingo in 2 Hong Kong parks

(Sturm-Ramirez, *et al*., 2004). The most noteworthy AIV outbreak happened in April 2005 when more than 6,000 migratory wild birds died of H5N1 infection at Qinghai Lake Nature Reserve (protected nature reserve with no poultry farms in the vicinity) in China (Wang, *et al*., 2008). This is the first observation of sustained transmission within migratory fowls. In addition, low pathogenic AIV have been isolated from 109 wild bird species with 1.6 to10% prevalence in ducks (Olsen, *et al.* 2006).

We reported the isolation and characterization of AIV and APMV from wild waterfowl from parts of the Southeastern United States. This is important because Alabama, Georgia, and Florida produce 20% of commercial poultry in the United States. Poultry in these areas are highly concentrated and are reared close to wildlife refuges. In addition, the isolation of any H5 or H7 isolate in these flocks will result in the temporary loss of importation into many foreign countries. These states export approximately 25% of their production to other countries.

4.3 MATERIALS AND METHODS

Swab Collection

Samples were from hunter-killed and trapped-nested waterfowl from wildlife refuges in Alabama, Georgia and Florida. A cloacal swab was collected from each bird and placed in a vial containing 2 ml virus-transport medium (Brain-heart infusion broth supplemented with 10000IU/ml of penicillin G and 10 g/ml of streptomycin sulfate; Sigma-Aldrich Inc., St. Louis, MO). Bird data such as species, sex, age, habitat, and time of isolation were recorded. Most sampling was done by Auburn employees. These samples were placed in vials and stored in wet ice or dry ice, and transferred to a -70°C freezer within 5 h. Some samples were

collected by federal or state government wildlife employees and these were placed in Styrofoam coolers with cold packs and sent to Auburn via overnight mail.

The majority of sampling was during the hunting season, which is between late September to early February of 2006, 2007, and 2008. Sampling of nonmigratory, nesting wood ducks occurred in the Summer during the breeding and egg laying season.

Virus Isolation

Standard virus isolation procedures were accomplished using 10-day-old embryonating specifi-pathogen-free or commercial chicken eggs (Slemons *et al*., 1973). Four eggs inoculated with 150µL (per egg) of original specimen were propagated for each sample. After 48 hr of incubation, the eggs were chilled overnight and allantoic fluids (AF) harvested (Hitchner, 1980).

Hemagglutination and Hemagglutination Inhibition (HI) tests

The hemagglutination (HA) titers in AF were determined with chicken erythrocytes by standard procedures (Alexander, 1979). The HA-positive AF were tested for NDV by hemagglutination-inhibition (HI) test using NDV antisera.

Directigen Flu A Test

Type A influenza viruses were identified using a commercially available antigen capture-ELISA (Directigen Flu A assay, Becton Dickinson Microbiology Systems, Cockeysville, MD). Due to the high cost of the kit, only HA-positive samples were tested and only for the 2006-2007 years.

Real-Time Reverse Transcription-PCR and AIV Subtyping

The HA-positive AF were tested for AIV (matrix, H5 and H7 genes) by real-time reverse transcription-PCR (RRT-PCR) using specific primers and procedures (with modifications)

developed by Spackman, E., *et al* (2002). Subtyping of AIV and APMV was done by the National Veterinary Services Laboratories (NVSL), Ames, Iowa.

Reverse Transcription-PCR

Viruses were characterized by nucleotide sequence analysis of the AIV H gene. A 2-step reverse transcription-PCR amplified the H segment of each AIV isolate (Hoffman, *et al.,* 2001).

Sequencing

The PCR products were run on a gel, and H fragments (~1800bp) were excised and purified using the Wizard PCR Preps DNA purification system (Promega Co., Madison, WI). Purified PCR products served as templates for automated sequencing by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at Auburn University Genetic Analysis Lab.

Phylogenetic Analyses

Sequences generated by the ABI Genetic Analyzer were entered into the Vector NTI software (Invitrogen, Carlsbad, CA) for phylogenetic analyses. Percent identity and sequence alignment were compared with published isolates. Their H genes were sequenced, and regions of similarity with other AIV isolates were performed using the BLAST tool of GenBank. A phylogenetic tree was generated with Mega2 program by neighbor-joining method with 1,000 bootstrap replicates. A dendrogram was drawn using PHYLO_WIN (Galtier *et al*., 1996).

4.4 RESULTS AND DISCUSSION

Out of 704 swabs collected during September 2006 to February 2007, 16 samples agglutinated red blood cells. From these 16 HA-positive samples, 7 AIV and 1 APMV-1

were identified (Table 1). No samples from nonmigratory, nesting ducks, migratory shorebirds, or samples shipped overnight were positive for AIV or APMV. No AIV was detectable directly from cloacal swabs by antigen capture-ELISA, but one AIV was detected by RRT-PCR. Allantoic fluid from HA negative for the first egg passage were re-passed in eggs for additional HA testing. Although an additional egg passage increased HA titer and lower crossing point, no HA-negative sample became positive.

Initially, there was difficulty in determining the subtypes of the viruses found in 5 samples. Additional passage in embryos and limiting dilution experiments were conducted. After resubmitting samples to NVSL, H1N1 were identified. Sample 381 had both H1N1 and NDV, confirming gene sequencing analysis, RRT-PCR, and HI-positive results for both AIV (H1N1) and NDV.

Figure 1 showed the H sequence of isolate 26 [A/Northern shoveler/AL/26/2006 (H10N7)] compared with other published H10N7 isolates. The Alabama isolate was closely related (98%) to recent isolates from Delaware and Canada, but only 90% related to an H10N7 isolated 30 yr ago. The Alabama isolates may have evolved from older isolates, producing changes as they circulated in ducks over the years.

Figure 2 showed that the 4 isolates from Alabama (121, 167, 253, and 299) had 94-97% similarity to published H1N1 isolates including one from swine [A/swine/Saskatchewan/ 18789/02]. These findings were confirmed by the NVSL, which reported H1N1 subtypes from these samples, as shown in Table 1.

Table 2 showed results from swabs collected during the early teal hunting season (September 2007) in a former Florida Potash mine (Potash Corp. Wildlife Refuge, White Springs, Florida). Out of 109 swabs tested so far, 15 were HA positive, all were either AIV

or AMPV, and were isolated from all teals. We are currently analyzing the remaining 700 samples from the recent (September 2007 to February 2008) hunting season in Alabama, Georgia, and Florida, for HA positive viruses. We are also looking at separately analyzing the AIV isolates from green-winged-teals and blue-winged-teals because they most likely originated from different breeding areas: green-winged-teals (eastern Canada) and blue-winged-teals (prairie Canada).

The higher isolation rate in 2007-2008 (14 vs 1%) may be due to improved sampling, transportation in dry ice, use of new egg incubator, month of the year, species of birds, habitat, migratory status, use of more viable commercial eggs compared with specific-pathogen-free, and more experience with the procedures.

Three of the 15 isolates were NDV (APMV-1) or APMV-4, 8 were H3N8, 2 were H4N6, and 2 were H4N8. Because NDV and other paramyxoviruses were isolated, there is a possibility of transmission of these viruses to commercial poultry. As of yet, no attempt has been made to examine their pathogenicity in chickens. The APMV-4 are not pathogenic for chickens.

No H5 or H7 isolates were detected. Therefore, the chance of transmission of these isolates from waterfowl to commercial farms in Alabama, Georgia, or Florida is unlikely. However, continual testing of these birds is justified to ensure they they do not transmit H5 or H7 to commercial poultry.

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Table 4.1. Hemagglutination (HA) titer, Directigen, real-time reverse transcription-PCR (RRT-PCR; matrix gene) assays, hemagglutination inhibition (HI) testing, and subtyping of viruses from 2006 to 2007.

Sample ID[1]	HA titer	Directigen	RRT-PCR (Cp[2])	HI (NDV[3])	Subtype
26	64	negative	36	negative	H10N7
121	256	positive	24	negative	H1N1, H1N4
167	256	positive	19	negative	H1N1
253	512	positive	20	negative	H1N1
299	4	negative	40	negative	H1N1
381	2048	negative	40	positive	H1N1, AMPV1[4]

[1]ID = identification number.

[2]Cp = crossing point.

[3]NDV = Newcastle Disease virus.

[4]APMV = avian paramyxovirus.

Table 4.2. Hemagglutination (HA) titer, real-time reverse transcription-PCR (RRT-PCR; matrix gene)assay, hemagglutination inhibition (HI) testing, and subtyping of viruses from 2007 to 2008.

Sample ID[1]	HA titer	RRT-PCR (Cp[2])	HI (NDV[3])	Subtype
20	256	Negative	+ (480)	ND[4]
21	512	12.4	Negative	H3N8
28	1024	28.0	Negative	H3N8
36	1566	22.4	Negative	H3N8
43	2048	26.7	Negative	H4N6
52	384	32.2	+ (1280)	APMV1[5]
55	1024	13.8	Negative	H3N8
64	1536	16.8	Negative	H4N6
77	1536	14.2	Negative	H3N8
93	128	19.6	Negative	H3N8
101	128	19.3	Negative	H4N8
102	1024	18.7	Negative	H3N8
105	64	15.6	Negative	H3N8
107	128	15.9	Negative	H4N8
108	64	32.3	+ (10)	APMV4

[1]ID = identification number.

[2]Cp = crossing point.

[3]NDV = Newcastle Disease virus.

[4]ND = not determined.

[5]APMV = avian paramyxovirus.

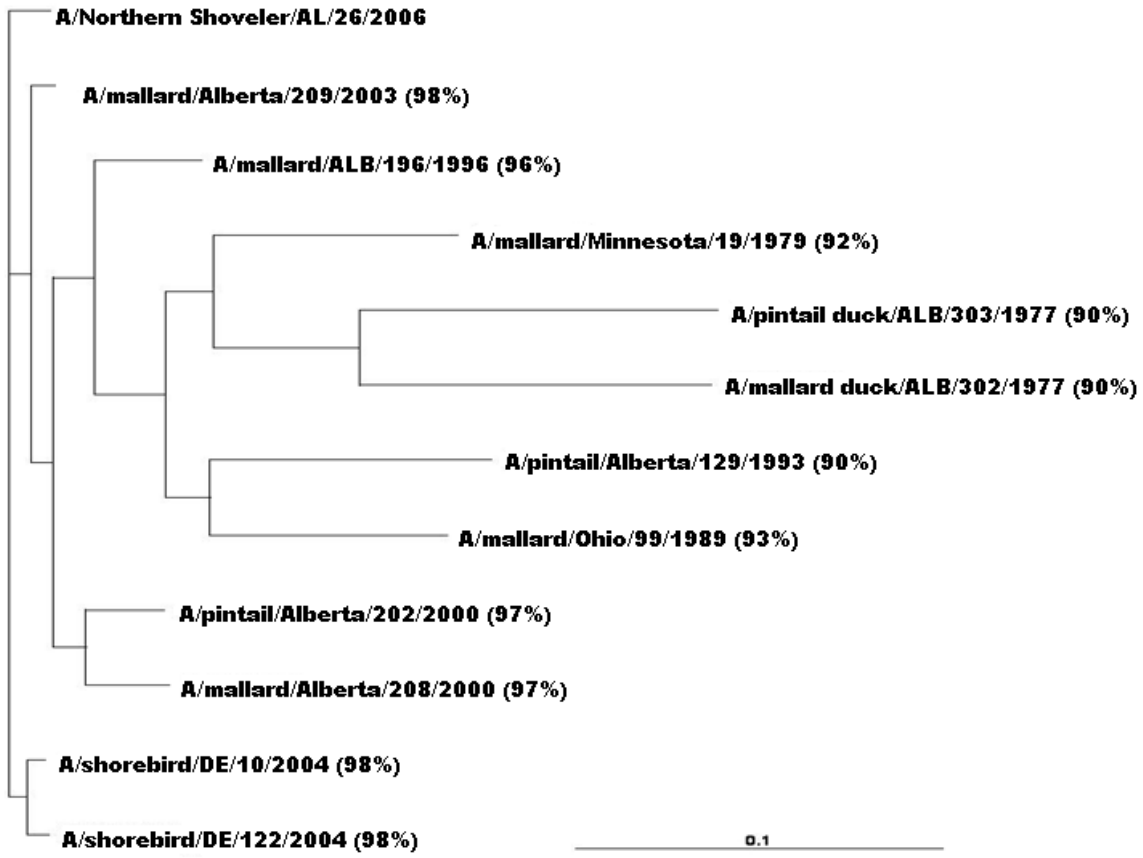

Figure 4.1. Phylogenetic analysis of the hemagglutinin (H) gene of of A/Northern Shoveler/AL/26/2006 in comparison with other published H10N7 isolates. Numbers in parentheses are percent identities with of A/Northern Shoveler/AL/26/2006.

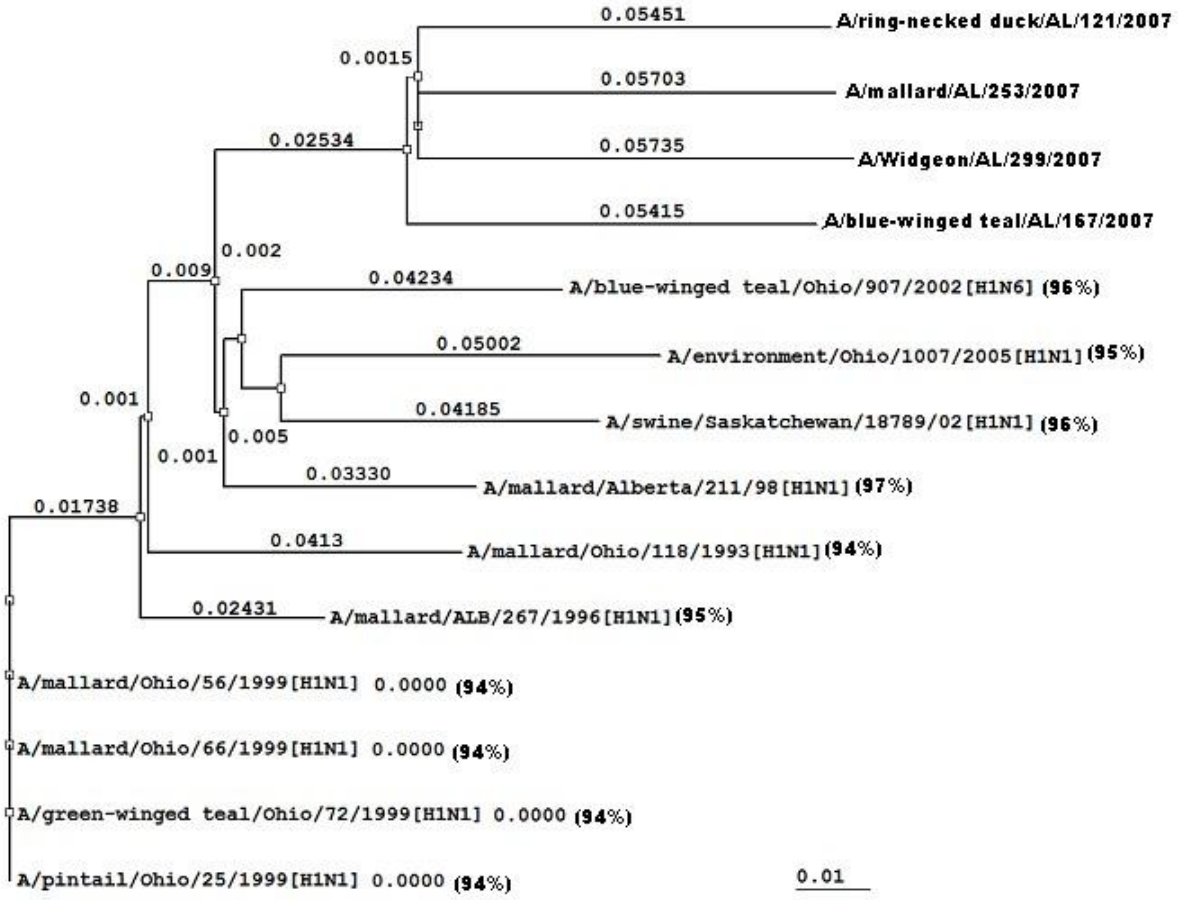

Figure 4.2. Sequence comparison of the complete hemagglutinin (H) gene of 4 Alabama isolates with those of previously published avian influenza virus isolates.

CHAPTER V: LITTER COMPOSTING PROCEDURES FOR INACTIVATION OF AVIAN INFLUENZA VIRUS IN EGGS

5.1 ABSTRACT

This study examined optimal litter composting conditions for the rapid inactivation of avian influenza virus (AIV) in eggs. AIV infected and non-infected eggs were placed into boxes that were then placed either in the middle of a litter compost pile or outside the compost pile. Hourly temperatures were monitored for 72 hours using data loggers. The compost pile consisted of used broiler litter containing pine wood shavings, feathers, insects, feed and manure. It was 1.2 m high by 3 m wide and water was added to obtain approximately 40% moisture. Twenty-four hours after compost set-up, seven boxes containing AIV infected and non-infected embryonated chicken eggs (ECEs) were placed in the positions described above and were removed after 24, 30, 36, 48, 54, 60, and 72 hours. At those designated times the allantoic fluid (AF) from each egg was inoculated into ECEs for determination of AIV survival by the hemagglutination (HA) test. Trial 1 showed that there was 62.5% AIV survival at 48 hours of composting, however, the virus was eliminated after 54 hours. In contrast, no viable virus was re-isolated from the eggs after 24 hours of composting in the 2nd trial when the internal compost temperature reached 57^{0}C. The rate of temperature increase and peak, varied upon the area within the compost pile where the eggs were located. This could account for differences in virus survival between trials. Results showed that optimum composting conditions and procedures are required to eliminate AIV in eggs within 24 hours. Litter should be

adequately moistened and thoroughly mixed, so that all areas of the compost pile will attain maximum temperature. It is also necessary to bury the infected eggs in the middle of the compost pile since temperature on the top only reached 34^0C. At 30^0C room temperature, AIV survived for 3 days. Using these results, a more effective composting strategy to rapidly inactivate AIV infected eggs can be implemented.

5.2 INTRODUCTION

AI outbreaks continue to occur resulting in economic loss world-wide from morbidity, mortality, reduced growth rate, lowered egg production/shell quality, culling of infected flocks, shutting down of live bird markets, and vaccination. In addition, AIV infections have resulted in trade sanctions against countries and consumers have often stopped eating poultry products from fear of becoming infected with AIV (Swayne, D. E. *et al*, 2013).

To control AI, most countries implement "stamping out" policies established by the OIE, by which all poultry that are potentially exposed to the virus are killed and disposed of in an efficient and humane manner (OIE, 2006). Inactivation of AIV in organic material including litter, carcasses, and eggs and the removal of this matter must be done as soon as possible to prevent the spread of the virus.

Methods for pathogen inactivation in litter, carcasses, eggs, and other organic matter include: windrow composting, incineration, burial, rendering, landfill disposal, and alkaline hydrolysis. In-house windrow composting is the fastest and most environmentally acceptable pathogen inactivation method and is a commonly used management practice to prevent the spread of infectious diseases in the USA (Bautista, D. A. 2008; Wilkinson, K. G. 2007). It has been shown to reduce or eliminate bacterial,

viral, and protozoan loads between grow-outs: due to thermal inactivation, exposure to high ammonia levels, and to antimicrobial action of heat-tolerant bacteria (Macklin, K. S. *et al*, 2006). Coliforms are inactivated by composting litter at 54°C-64°C in 3.5-6.5 days (Bautista, D. A., 2008). Adenovirus and AIV were inactivated in carcasses after composting for 10-12 days (Senne, D. A. *et al*, 1994). AIV was inactivated in chicken manure, litter, and feed at 3 days when the temperature in the compost pile reached 47°C and in carcasses, liver, breast meat, and broken and whole eggs after composting by 7 days, when the temperature reached 54°C (Guan, J. C. *et al*, 2008). To be a viable method for poultry carcass, eggs, and litter disposal, the compost process must inactivate pathogenic microorganisms completely before removal from the premise. Composting of contaminated litter and carcasses destroys pathogens in poultry houses, thus reducing the potential of disease spread. This present study determined optimum procedures needed to inactivate AIV in eggs during litter composting.

5.3 MATERIALS AND METHODS

An AIV-H1N1 isolate (A/Blue-winged teal/AL/167/2007) (Dormitorio, T. V. *et al*, 2009) was propagated in egg embryo culture and then used to infect ten-day-old ECE for composting. Each egg was inoculated with 0.15 ml of virus suspension via the allantoic sac, and then incubated for 48 hrs to allow viral propagation before use for the composting trials. In trial 2, the initial virus titer in each egg for composting was 2096 HA units. After each composting trial, the AFs from the eggs were harvested and then re-inoculated into ECE for determination of AIV survival using the HA test. A preliminary run was performed to establish composting mechanics and setup, after which two experimental trials were conducted.

For all the trials, used chicken litter consisting of pine wood shavings, feed, feather, insects, and chicken manure was utilized to create a compost pile. Water was added to the chicken litter to achieve litter moisture of approximately 40%, which had been determined to be optimal for composting (Macklin, K. S. *et al*, 2006). The compost pile, 1.2 meters high and 3 meters wide was placed on the floor of a concreate pathogen isolation building (Figure 5.1). Twenty four hours after compost was initiated, the upper half of the pile was removed, then 7 plastic boxes (23 cm x 36 cm) containing 4 AIV-infected and 4 non-infected (inoculated with phosphate buffered saline) eggs, were placed on this newly exposed area and immediately covered with composting litter. The boxes were lined in a row at the center portion of the pile. Each box was labeled (left to right): 24, 30, 36, 48, 54, 60, and 72, which corresponded to the number of hours it was to be composted (Figure 5.1). Each box was withdrawn using attached strings at the designated hour. Another set of seven similar boxes containing infected and non-infected eggs were placed on a table near the compost pile, to determine survival of AIV outside the compost (non-composted eggs). Non-composted boxes were collected at the same time corresponding composted eggs were taken.

Five temperature data logging probes were utilized to monitor hourly temperatures. These probes were placed in the following sites: 1) each of three boxes (24, 48, and 72hrs) that were buried in the compost pile, 2) on top of the compost pile, and 3) room where the study was conducted in. After each trial, the AFs from composted and non-composted eggs were inoculated unto 10-day old ECEs, and then incubated at 37.5^{0}C, 60% humidity for 48 hours to allow surviving virus to multiply. AFs from these eggs were tested by HA to determine AIV survival (Swayne, D. E. *et al*, 2008). Rate of

survival was measured by the number of wells on a plate per egg sample, that were positive for HA activity.

5.4 RESULTS AND DISCUSSION

Preliminary litter composting data (data not presented) showed that 6% of embryonated eggs inoculated with AIV and composted for 72 hours still contained viable virus. Efficiency of this composting trial was not known since temperatures were not recorded. Therefore, an improved compost setup was made by thoroughly and evenly mixing used litter with water to obtain optimum moisture content of approximately 40%. From this setup, the compost pile was created and its temperature monitored. The compost pile was "incubated" for 24 hours to allow generation of optimum temperatures for use in subsequent trials.

Results of HA testing in trial 1 (T1) indicated that AIV was 62.5% viable after 48 hours, but was totally inactivated after 54 hours (Figure 5.2). Temperature readings from probes placed in the middle-center of the compost pile at this time and beyond were 57.5°C and above. The speed at which temperatures rose and when the temperature reached their peaks was dependent upon the site of the compost pile where a box containing the eggs was buried. For example, as illustrated in Figure 5.3, after 10 hours of composting, the probe placed in the left-center (T1) registered a temperature of 31.5°C, whereas the one in the right-center (T1) was 40°C. Although it peaked at 45.5°C, heat generation on the far-left side of the pile where Box-24 was placed was slow to rise compared to those in the middle and far right. Figure 5.2 showed that with T1, there was a 100% virus survival of the eggs placed on 24, 30 and 36 hr boxes. It is possible that the

areas of the compost pile, where these eggs were buried, did not attain temperatures high enough to inactivate the virus quickly.

In contrast to Trial 1, AIV in eggs in trial 2 (T2) was fully eliminated within 24 hours of study initiation (Figure 5.2). Temperatures in all boxes with probes were higher and reached peak temperature (66°C) faster compared to those in Trial 1 (Figure 5.3). Temperature in the far-left center box of Trial 2 rose from 25°C to 43°C in one hour and reached a high of 66°C (was only 44^0C for Trial 1) after 24 hours. Maximum temperatures in the probes placed on top of compost pile and outside the pile (compost room) were 34°C and 29°C, respectively (Figure 5.3). As expected, AIV-infected eggs placed outside the compost (non-composted) still contained viable virus after three days in both trials. Therefore, it is imperative that AIV infected eggs be buried in the center of the compost pile where the temperature increase is quicker and at maximum, as long as other required components of composting is optimal.

Data from a prior study showed that the virus was destroyed in AIV infected whole and broken eggs, breast meat, and liver when placed in the top or middle of a compost pile for 7 days when the temperature reached 56^0C (Guan, J. C. *et al*, 2008). Other work showed that the virus can survive in whole non-composted eggs for 7 days (Bendfelt, E. S. *et al*, 2009); or at least 21 days if the temperature is not above 30^0C (Guan, J. C. *et al*, 2008). Other factors that influence AIV survival for a longer time (7 days) in whole eggs compared to our study (1 day) could be due to differences in the subtype and titer of the initial virus, composting conditions including type of litter, % moisture of the litter, organic matter in the litter, and position of the eggs in the pile. The proper amount of air, water, nutrients, and carbon must be balanced to allow the composting process to initiate

and continue at a rate sufficient to produce adequate heat to reduce the level of pathogens in the organic matter (Macklin, K. S. *et al*, 2006).

Results of the present experiment indicated that optimum composting conditions and procedures are required to inactivate AIV in eggs within 24 hours. Litter should be thoroughly mixed and have enough moisture, so that all areas of the compost pile will achieve maximum heat. AIV was inactivated in eggs after 24 hours of composting when the temperature reached 66^0C. Therefore, composting period for AIV-infected eggs could be reduced, assuming the proper composting procedures are initiated and internal composting temperature of the pile where the eggs are buried is at least 57^0C. Regardless of the time allowed for the samples to be composted, samples should be collected and tested to verify that the virus has been inactivated.

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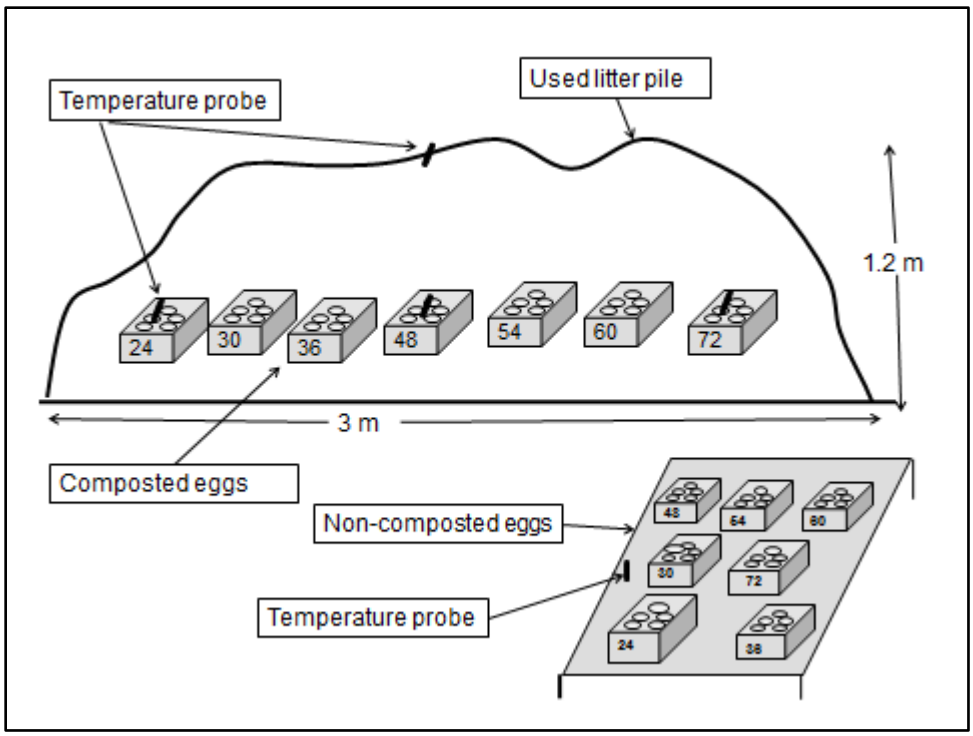


Figure 5.1. Compost layout of Trial 2.

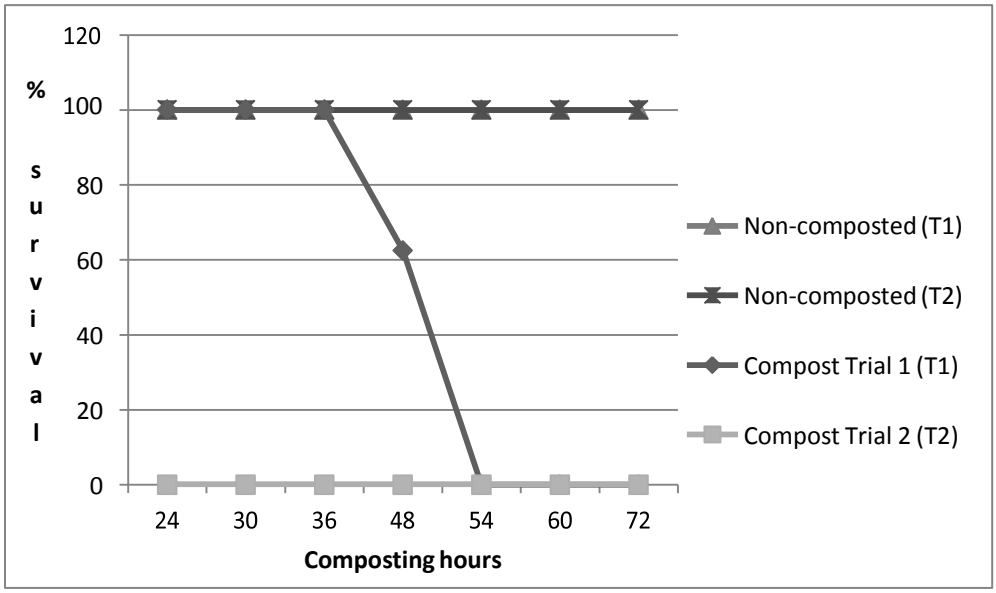


Figure 5.2. The rate (%) of survival of AIV in composted or non-composted eggs.

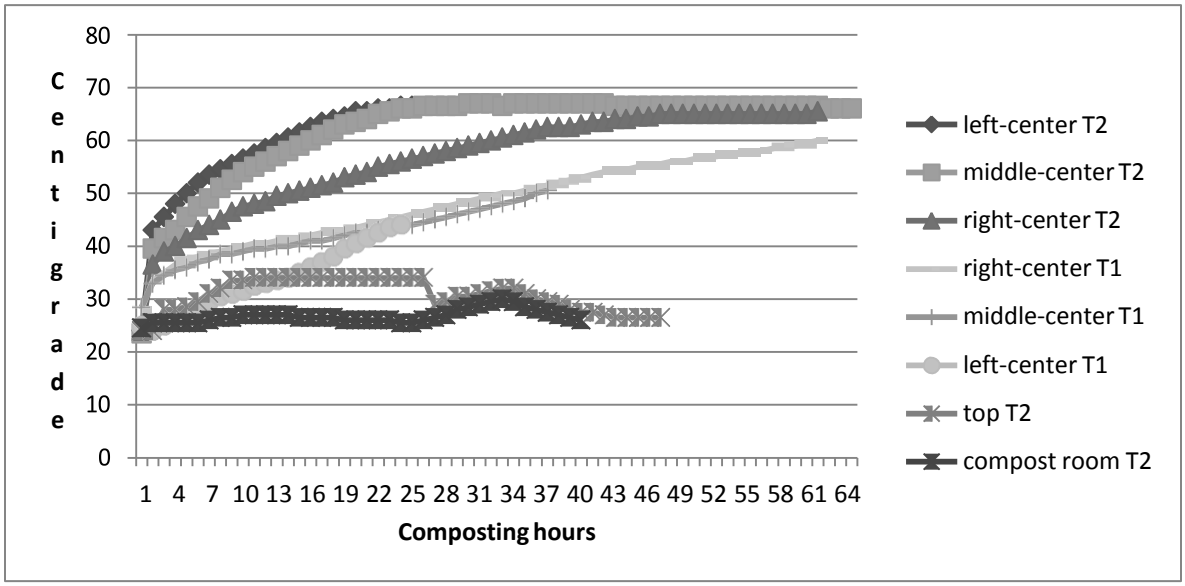


Figure 5.3. Temperature (°C) readings from probes placed at different sites of the compost pile and composting room for Trial 1(T1) and Trial 2 (T2).

DISSERTATION CONCLUSIONS

Based on available epidemiological data, migrating wild aquatic birds are the primary reservoir of influenza A viruses, and could be the initial source of virus, which can be passed on through contact with resident waterfowl or domestic poultry. In these birds, influenza viruses usually replicate in the intestinal tract, cause no disease, and are spread by fecal contamination of the water habitat. The majority of influenza viruses that are enzootic in wild aquatic birds are unable to replicate efficiently in chickens. However, multiple subtypes of AIVs have been isolated from healthy land-based poultry including chickens, turkeys, quail, guinea fowl, and chukar, without overt clinical signs. Occasionally, AIVs circulating in land-based poultry can increase their virulence rapidly, and cause important economic losses due to mortality, as well as trade restrictions. In addition, wild birds have been shown to introduce novel influenza gene segments into a population, that when reassorted with existing viruses, can generate a dissimilar virus with different antigenic and other biological characteristics.

AIVs and APMVs were detected and isolated from swabs taken from waterfowl of wildlife refuges in southeastern USA. Sampling sites were: Eufaula National Wildlife Refuges (Molnar, Kennedy, and Bradley units in AL and GA); PCS-Potash mine, White Springs, FL; Racoon Creek, Crow Creek Refuge, Stevenson, AL; TN River Lake Guntersville, Scottsboro, AL; and Daphne, AL. Out of 1260 samples used for virus isolation, we isolated 19 AIVs, three APMVs (APMV-1, APMV-4), and one had a mixture of two viruses. AIV subtypes were: H10N7 (1); H1N1 (5); H1N4 (1); H3N8 (8);

H4N6 (2); and H4N8 (2). Wild bird species sampled were commonly Wood Ducks (WODU), Northern Shovelers (NSHO), Ring-necked Ducks (RNDU), Gadwalls (GADW), Mallards (MALL), American Wigeons (AMWI), Hooded Mergansers (HOME), and Blue-winged Teals (BWTE)/Green-winged Teals (GWTE). We have submitted these data from the isolates to a national database that incorporates and tracks all avian influenza data collected from wild birds in the USA. The data collected in this system has been used by scientists to develop a better understanding of the movement of avian influenza viruses among wild and domestic animals, improve risk analyses, and target monitoring strategies to track the avian influenza spread.

AC-ELISA and RRT-PCR were examined for use in detection of AIV directly from swabbed material and allantoic fluids of HA+ samples. It was shown that successful AIV detection and isolation from wild aquatic birds was dependent on sampling and laboratory protocols. The highest isolation rate (14%) was obtained from one sampling day in Lake City, FL, wherein at this time laboratory personnel were more experienced with sampling and laboratory techniques. Other factors that may have contributed to the higher isolation rate are: 1) use of dry ice to freeze samples in the field; 2) time or location of sampling; and 3) bird species or age. It is interesting to note that 97% of the samples collected on this sampling date were BWTE, whereas on another sampling time (1/21/2006) conducted on this site, more GWTE were sampled versus BWTE. It was concluded that RRT-PCR was more sensitive, cost effective, and specific than AC-ELISA (Directigen Flu™ A test; Becton Dickinson, 2004) for the detection of AIV in wild birds. It could detect AIV from AF with an HA titer as low as 4, whereas AC-ELISA did not detect virus when HA titer was lower than 125. However, to avoid false-

negative results, testing should be done on AF and not directly from cloacal swabs. Moreover, samples that were RRT-PCR positive, but with a high Cp (>32), had either AIV in low concentration, APMV, or both. In a mixed infection, several limiting dilutions and passages were required to separate the viruses, since presence of APMVs may have interfered with AIV detection, and thereby resulted in false positive AIV RRT-PCR results.

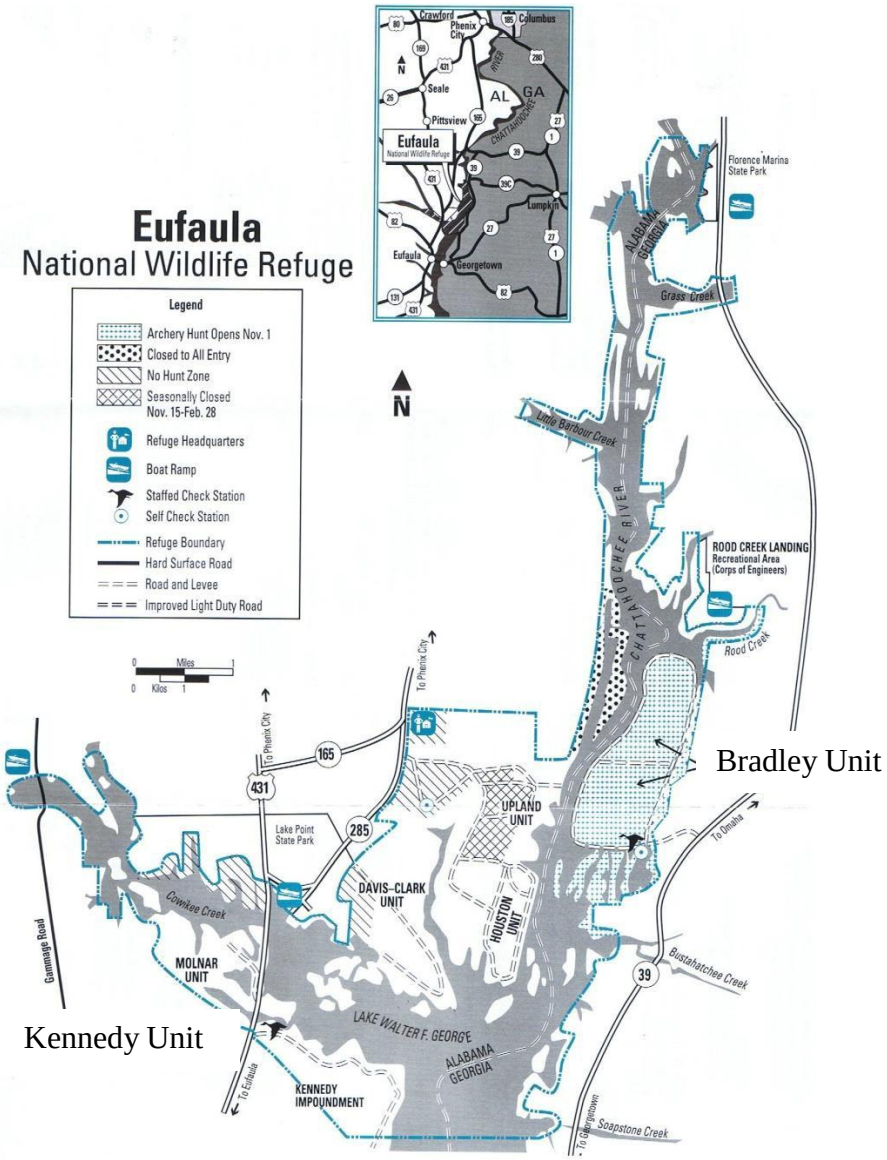
The H gene sequence of the AL H10N7 isolate was closely related (98%) to more recent isolates from Delaware and Canada, but only 90% related to an H10N7 isolated 35 years ago. Sequencing analysis of the H gene of four H1N1 isolates from AL revealed that they had 92-97% similarity with published H1N1 isolates including one from swine. The pandemic (2009) H1N1 influenza viruses had 65-75% relatedness to these isolates (data not reported). Results of composting experiment on survival of AIV-H1N1 in eggs indicated that optimum conditions and procedures are required to eliminate AIV in eggs. No viable virus was re-isolated from the AIV-infected eggs after 24 hours of composting when the internal compost temperature reached 57^0C. At 29^0C room temperature, the virus survived for 3 days. These results can be used to develop management strategies that can inactivate AIV in eggs laid by infected hens.

No H5 or H7 AIVs were isolated from wild waterfowl in Alabama, Georgia, and Florida during 2006 to 2011. However, because of the recent HPAI outbreaks in the Midwest, there is a significant need for testing wild birds as they migrate. Early detection of AIV, especially the subtype (H5N2) that caused the devastating outbreak, will help prevent possible infection and /or spread of these highly pathogenic (HP) strains to the U.S. poultry and swine industries. More importantly, it will provide early warnings of HP

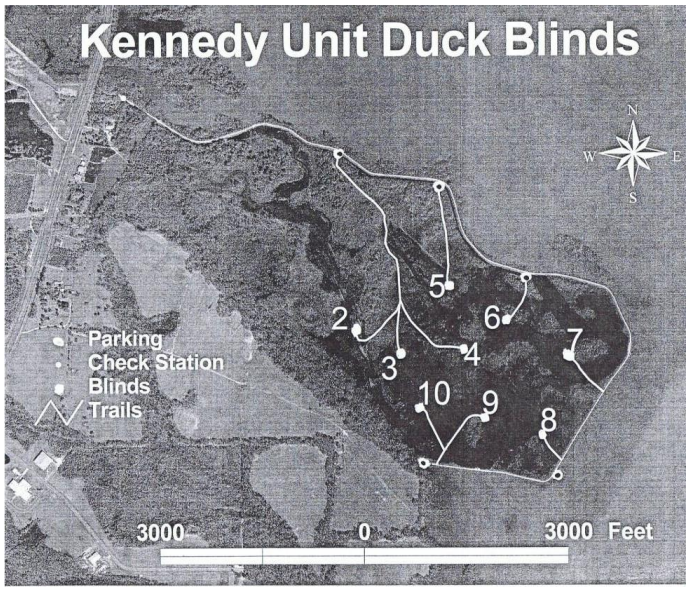
AIV presence in the concentrated area of the poultry and swine industries; thus advising companies and farmers to further strengthen or enforce more stringent biosecurity measures to prevent spread of the virus into commercial poultry and swine populations as well as to humans.

APPENDIX

Eufaula National Wildlife Refuge Map (Alabama and Georgia)



Eufaula National Wildlife Refuge, Kennedy Unit Duck Blinds



PotashCorp- White Springs, Florida Wildlife Management Area