AVIAN INFLUENZA VIRUSES IN POULTRY: EVALUATION OF ENVIRONMENTAL SAMPLES AND A DIVA STRATEGY BASED ON N1 AND N2 ELISAS

by

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(Under the Direction of Maricamen García)

ABSTRACT

Maintaining poultry free from Avian Influenza is essential to continued trade between countries. Although there are mechanisms to control influenza such as vaccination, it is important to understand the role of contaminated feces and litter in LPAI virus transmission. The infectivity of the viruses A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), A/turkey/Ohio/313053/04(H3N2) in contact with poultry litter and manure were evaluated. The viruses retained infectivity in manure and litter for 24hrs, and infectivity was directly related to the rate of shedding, moisture content and environmental temperature. Control of influenza can be done by vaccination and monitoring with rapid serological methods for differentiating infected from vaccinated animals. The ability of the N1-ELISA and N2-ELISA to discriminate vaccinated from subsequently challenged turkeys was tested. The N1-ELISA and N2-ELISA detected 50% and 33 % respectively, of vaccinated and infected turkeys. Overall N1-ELISA and N2-ELISA were effective and rapid assays to identify exposure to the challenge virus during a DIVA vaccination strategy.

INDEX WORDS: Avian Influenza, tenacity, poultry litter, Heterologous Neuraminidase DIVA strategy

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DEDICATION

Dedico essa tese a minha mae, por estar sempre presente mesmo distante; ao meu pai pelo apoio incondicional e por ser meu fa numero um (uns dia eu ganho, outro dia ele perde..), a Renata (*in memoriam*), a Fabiana, a Nara e ao meu marido (Ju, obrigada por tudo e mais um pouco..).

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Introduction

Poultry and poultry products are a major source of high-quality protein as human food and the per-capita consumption has been increasing around the world. Maintaining poultry free from pathogens, particularly Avian Influenza is essential to continue the trade of poultry and poultry products among countries (Swayne 2003b), and to prevent or control avian influenza outbreaks in poultry we need a better understanding of the disease, the transmission and shedding of the virus.

Avian influenza viruses are type A influenza virus belonging to the *Orthomyxoviridae* family. They are enveloped viruses with segmented single-negative-stranded RNA genomes (Lamb 2001). The AI viruses have been classified based on the antigenic profiles of the surface glycoproteins: at the moment 16 hemagglutinin (H1 through H16) and 9 neuraminidase (N1 through N9) subtypes have been identified (Causey 2008). Each virus has any combination of H and N subtypes, for a total of 144 possible different influenza virus subtype combinations (Alexander 1982; Causey 2008).

AIV can infect a wide variety of animals, from wild birds to mammals. However AIV is perpetuated in wild birds, predominantly in waterfowl like species of ducks and shorebirds, which are AI natural reservoir and can harbor all 16 hemagglutinin and all 9 neuraminidase subtypes (Munster et al. 2007; Webster et al. 1978). In these species of birds AIV naturally infect the intestinal tract and usually cause asymptomatic infections (Causey 2008; Webster et al. 1978)

Although poultry is not a natural host of AI, infections in poultry are current and can be differentiated in two distinct types based on the ability of these viruses to cause disease in chickens: the low pathogenic AIV (LPAI) and the highly pathogenic AIV (HPAI) (Alexander 2000).

The HPAI viruses are characterized by their high morbidity and mortality. Most of the highly pathogenic outbreaks of poultry have been of the H5 and H7 subtypes (Alexander 2000). Phylogenetic analysis of HPAIV indicates that such viruses do not constitute unique lineages but are derived from non-pathogenic lineages of AI (Brugh et al. 1991). The ability of LPAIV to mutate into HPAIV was illustrated in 1983-84 outbreak of HPAI in Pennsylvania and in 1994-95 outbreak in Mexico, where the abrupt emergence of HPAI subtype H5N2 immediately preceded by circulation of a non-pathogenic H5N2 strain (Alexander 2000; Kawaoka et al. 1988; Webster et al. 1987). A similar phenomenon occurred in 1999-2000 in Italy where outbreaks of LPAI H7N1 preceded a mutation, which then resulted in HPAI outbreaks (Alexander 2007).

The LPAIV have been shown to cause mild forms of the disease in commercial poultry characterized by low morbidity and no mortality. For LPAI infections in chickens, viral replication is primarily restricted to the respiratory and gastrointestinal tracts, while during HPAI infections replication occurs systemically (Spickler et al. 2008; Suarez 2000).

The mechanisms by which influenza viruses transfer from bird to bird, how frequently an infected bird can shed virus, or for how long the AIV can remain infective within an affected flock are poorly understood, some attempts were made to assess the transmissibility of LPAIV in domestic poultry and the results suggested that bird to bird transmission is extremely complex and depends on strain of virus, the species of birds, and environmental factors. (Alexander 2000, 2007; Alexander et al. 1978; Yee et al. 2009a). The ability of the AI viruses to spread is also

closely related to the amount of virus released from the respiratory or intestinal route by infected birds (Alexander 2000). In poultry LPAI virus replication is primarily restricted to the respiratory and gastrointestinal tracts (Spickler et al. 2008) and movement of contaminated poultry, in particular the daily mortality (McQuiston 2005), poultry products, and equipment is probably the main sources that facilitates LPAIV transmission between flocks (Swayne 2008a). Depopulation and movement of poultry litter has been indirectly associated with the failure to control the spread of LPAI H7N2 virus in commercial poultry (Henzler et al. 2003). Nevertheless, data about survivability of avian influenza viruses in the poultry house environment, particularly in poultry manure, are limited. The environmental persistence and shedding by specific pathogen free (SPF) chickens of H7N2 virus in chicken manure was investigated by Lu et al (Lu et al. 2003) showing that chickens paused shedding virus at two weeks post-infection (pi). However, manure sample remained positive for virus isolation over a 3-week period (31). The risk of fecally contaminated litter in LPAI virus transmission and the ability of LPAI viruses to remain infective in poultry feces have not been well defined. It is important to understand the mechanism of transmission of LPAI virus so this information can be used to prevent and control new outbreaks of the disease.

The most common way to control AI outbreaks is the culling of infected poultry, which has been consistently successful in previous outbreaks including the outbreak in Hong Kong in 1997 and the Netherlands in 2003 (Lipatov et al. 2004). Culling infected poultry causes the reduction of the viral load and reduces the likelihood of transmission to humans (Lipatov et al. 2004). However, the poultry industry has undergone substantial changes in the last twenty years, mainly resulting in shorter production cycles and greater animal densities per territorial unit, as a result mass depopulation led to very high costs and economic losses for the national and federal

governments, the stakeholders and ultimately for the consumers (Capua et al. 2003a). While the optimal method of eradication of influenza is the culling of poultry when the outbreak is widespread, this course of action may not be possible and alternative strategies have to be design (Lipatov et al. 2004).

In the United States, when H5 or H7 outbreaks occur, emergency control programs are activated and managed through the cooperative efforts of USDA, state authorities and the poultry industry. Control programs for LPAI outbreaks caused by viruses of other subtypes are managed by the poultry industry working in conjunction with state authorities. Elimination is usually the goal and the outcome of LPAI control programs, but the measures applied are typically less stringent than those used for H5 or H7 outbreaks and largely based on risk assessment and cost-benefit evaluation (Swayne 2003b). In other areas such as Mexico (Villarreal 2009), some Asian countries (Lipatov et al. 2004) and in the Middle East (Banet-Noach et al. 2007), the current strategy when dealing with influenza virus outbreaks in poultry is culling along with vaccination. Vaccination has potential benefits, such as the reduction of clinical signs of diseases, reduction of viral shedding and increase the resistance of birds to becoming infected (Capua et al. 2003a).

However, vaccination also has drawbacks, such as the effect of vaccination on international trade, and the effect that it has on serologic surveillance, since vaccinated birds cannot be distinguished from naturally infected birds. Losing the ability to do surveillance has been the main reason for countries as the US and the European Union to oppose vaccination as a routine tool in the AIV control (Capua et al. 2003a; Suarez 2005). Therefore there is a need to perform surveillance that allows the differentiation of naturally infected from vaccinated birds and to identify vaccinated birds that become infected (Suarez 2005). There are four different vaccination strategies approaches that allow differentiation of infected from vaccinated animals

(DIVA), the use of sentinel birds, the use of subunit vaccines, the detection of nonstructural protein NS-1 antibodies, and the detection heterologous neuraminidase antibodies when inactivated vaccines are utilized (Suarez 2005; Swayne et al. 2007; Zhao et al. 2005). Naïve sentinel birds are difficult to manage and locate within a flock, and the introduction of naïve birds may increase the risk of transmission of a AIV (Suarez 2005). Subunit vaccines carrying the HA and/or NA genes have shown to induce protection [61, 77, 78] and are suitable for a DIVA vaccination strategy, however they are difficult to license and costly to produce (Suarez 2005). Inactivated AI vaccines for poultry are mainly grown in embryonated chicken eggs and prepared from relatively cell-free whole viruses preparations. Because the NS-1 protein is not a virion associated protein, birds vaccinated with inactivated AI vaccines will not mount an immune response against NS-1 while naturally infected birds will (Suarez 2005; Tumpey et al. 2005; Zhao et al. 2005). However, AI poultry vaccines have residual NS-1 protein in its composition, making it difficult to use the NS-1 as a suitable DIVA strategy (Suarez 2005; Tumpey et al. 2005). Another disadvantage of using the NS-1 antibody response for DIVA vaccination strategy is the weak nature of the NS-1 antibody response observed in infected birds as compared to the nucleoprotein (NP) (Watson et al. 2009).

Another DIVA vaccination strategy option is the use of an inactivated vaccine with homologous HA and heterelogous NA to the circulating AIV strain. Vaccines with heterologous NA and homologous HA has been shown to effectively protect birds (Nayak et al. 2010; Suarez 2005). Using an NA heterologous vaccine, birds can be monitored for presence of antibodies against circulating NA serotype, and though differentiated infected birds from vaccinated, or vaccinated and infected. This strategy was applied successfully to control an HPAI outbreak in 2000 in Italy (Capua et al. 2003b, c; Capua et al. 2002).

The successful use of the heterologous NA DIVA vaccination strategy in Italy has increased the interest in its use; however this strategy is relatively untested and does have potential issues that have to be addresses. One potential problem of this particularly DIVA strategy is the detection of vaccinated-infected birds where low NA antibodies levels are expected particularly in birds with high HI titers that maintain low levels of infection and most-likely will not mount a significant antibody response against the heterologous NA. A second disadvantage of the heterologous NA DIVA vaccination strategy is the availability of vaccines and diagnostics assays for the different subtypes combinations. The neuraminidase inhibition (NI) is available but not suitable for screening a large number of samples and is not quantitative (Aymard-Henry et al. 1973; Van Deusen et al. 1983). The indirect immunofluorescent antibody (iIFA) has been utilized, however this test is not available to all NA subtypes, it can be difficult to interpret, and not suitable for high throughput serological screening (Suarez 2005). To overcome the disadvantages of the iIFA, indirect and competitive ELISA systems for the detection of N1, N2 and N3 have been developed (Liu et al. 2010).

In order to better understand the dynamics of AIV transmission, and facilitate control of AIV outbreaks the objectives of this thesis were: 1) to determine duration and extent of fecal shedding for LPAI H6N2, H5N2 and H3N2 viruses in experimentally infected poultry species (broilers, layers, quails and turkeys) and to the determine the infectivity of LPAI viruses in contaminated feces and litter. 2) In order to facilitate the establishment of DIVA vaccination strategies utilizing the NA heterologous approach we developed an indirect N2 ELISA and together with previously developed indirect N1 ELISA, the assays were evaluated for the ability to detect vaccinated and or challenged turkeys with H1N1 and H1N2 viruses. The assays sensitivity was compared to HI and NI in a heterologous NA DIVA strategy.

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Chapter 2

Literature Review

Avian influenza biology.

Avian influenza viruses belongs to the Genera *Mononegalavirus* the family *Ortomyxoviridae* and to the Influenza A group. AIV are enveloped viruses with a segmented single-stranded negative sense RNA genome, the viruses are classified based on the antigenic profiles of the surface glycoproteins: at the moment 16 hemagglutinin (HA1 through HA16) and 9 neuraminidase (NA1 through NA9) subtypes have been identified (Causey 2008; Lamb 2001). Each virus has any combination of HA and NA subtypes, for a total of 144 possible different influenza virus subtype combinations (Alexander 1982; Kawaoka et al. 1988). AIV can infect a wide variety of animals, from wild birds to mammals. However AIV is perpetuated in wild birds, predominantly in waterfowl like species of ducks and shorebirds, which are the natural reservoir of AI viruses and can harbor all 16 hemagglutinin and all 9 neuraminidase subtypes (Munster et al. 2007; Webster et al. 1978).

Virion structure

Avian Influenza viruses have a pleomorphic virion, with a lipid enveloped derived from the plasma membrane of the host cell. The envelope harbors three membrane proteins, rod-shaped spikes of Hemagglutinin (HA) glycoprotein, a mushroom-shaped spike of

Neuraminidase (NA) glycoprotein, the ratio of HA to NA varies but is usually 4:1 to 5:1. AIV also encodes a third integral membrane protein the Matrix protein 2 (Lamb 2001).

The viral Matrix protein 1 (M1) underlies the lipid membrane and associates with the ribonucleoprotein (RNP) complex. The RNP complex is formed by the viral RNA segment, the polymerase proteins (PB1 [polymerase basic 1], PB2 [polymerase basic 2], and PA [polymerase acid]) and the nucleoprotein (NP). The nuclear export protein (NEP) is also present in purified virions (Palese et al. 2007).

Viral Genome Organization

AIV contain eight segments of negative sense single- stranded RNA which encodes for 11 proteins. RNA segment 1 codes for PB2, segment 2 codes for PB1 and for PB1-F2, segment 3 codes for PA, segment 4 codes for HA, segment 5 codes for NP, segment 6 codes for NA, segment 7 codes for M1 and M2 and segment 8 codes for Non Structural (NS) protein 1 and Nuclear export protein (NEP) (Lamb 2001).

One interesting aspect of AIV replication is their ability to produce reassortant virus, in which different virus subtypes replicating in the same host cell exchange genome segments or partial segments. These reassortment events gives rise to novel virus strains, in the case of humans, these can occasionally evolve in pandemic strains because the population is immunological naïve to new reassortants. These events are the result of the segmented nature of the influenza genome (Lamb 2001).

Viral proteins

AIV contain encodes 11 proteins, 8 structural proteins and 2 non-structural proteins, and an accessory protein (PB1-F2) which is enconded only by some influenza viruses (Lamb 2001).

Protein basic 2. The protein basic 2 (PB2) is a component of the RNA dependent RNA polymerase complex (Digard et al. 1989; Toyoda et al. 1996). PB2 is required to snatch type I mRNA cap structures, the cap group is then used to prime the synthesis of viral mRNA (Blaas et al. 1982; Shi et al. 1996). PB2 has also been shown to be associated with host range restriction of influenza (Subbarao et al. 1993). PB2 interacts with PB1, and with the nucleoprotein (Biswas et al. 1998).

Protein basic 1. PB1 is the backbone of the viral RNA polymerase complex (Coloma et al. 2009), it is the viral RNA dependent RNA polymerase. PB1 exhibits nucleotides polymerization activity, and it is responsible for elongation of viral mRNA, of viral complementary RNA and viral genomic RNA (Nakagawa et al. 1996). PB1 interacts with PB2 and protein acidic (PA). PB1 contains the nuclear localization signal for the polymerase complex to enter the cell nucleus and start genome replication (Asano et al. 1997; Nath et al. 1990).

PB1-F2. PB1-F2 is a protein enconded by a spliced viral mRNA of segment 2 (Ghedin et al. 2005). It appears that this protein is an accessory protein, since some influenza virus lack this open reading frame (Palese et al. 2007). PB1-F2 is a pro-apoptotic protein, PB1-F2 interacts with inner and outer proteins from the mitochondrial membrane, inducing apoptosis by realizing cytochrome C (Chen et al. 2001). The PB1-F2 it is primarily expressed when virus infects monocytic cells (Chen et al. 2001), and this protein may be involved in targeting leukocytes to undergo apoptosis playing an important role in the downregulation of the host immune response to influenza virus infections (Zamarin et al. 2005).

Protein acidic. PA is the third subunit of the influenza RNA polymerase complex. The function of PA is not very well understood, but PA has been shown to be essential for viral replication (Nakagawa et al. 1996). PA has been described to have endonuclease activity (Sanz-Ezquerro et al. 1995), but the level of proteolysis does not seem to correlate with polymerase activity (Naffakh et al. 2001). It was suggested that the N-terminal region of PA s involved in multiple functions of the polymerase, including protein stability, endonuclease activity, cap binding, and promoter binding (Hara et al. 2006a).

Hemagglutinin. Hemagglutinin (HA) is a trimeric type I membrane protein, which is responsible for viral binding to sialic acid receptors in host cells, internalization, and membrane fusion by the endosomal pathway after infection (Skehel et al. 2000). HA is also responsible for the determination of host range and tissue tropism. HA from avian virus isolates have a preference for receptors with a 2,3α-linked sialic acid , while mammals isolates have a preference for 2,6α-linked sialic acid (Nelson et al. 1993; Rogers et al. 1983). In humans, the 2,6α-linked sialic acid is dominant on epithelial cells in nasal mucosa, with 2,36α-linked sialic acid being occasionally detected, and its is believed that the low prevalence of SAα-2,3 Gal molecules, on the epithelial cells of the human upper respiratory tract decreases the susceptibility of humans to infection by avian influenza virus (Shinya et al. 2006). However, chicken and duck intestine predominantly express the 2,3 α-linked sialic acid receptor type across the epithelial lining of villi (Ito et al. 2000; Wan et al. 2006), while in chicken tracheal epithelium, 2,6 α-linked sialic acid is the dominant receptor type whereas in ducks the 2,3 α-linked sialic acid receptor is more abundant in the ciliated cells of the tracheal epithelium and it was found that the ratio of SAα2,6-Gal to SAα2,3-Gal in chicken trachea was approximately 10:1 whereas in duck the ratio was 1:20 (Nicholls et al. 2007).

Although the pathogenicity of AIV is a polygenetic trait (Bruns et al. 2007; Chen et al. 2001; Henklein et al. 2000), structural features in the HA glycoprotein had been identified as the primary determinant of viral pathogenicity (Bosch et al. 1981; Rohm et al. 1995; Scholtissek et al. 1977). The HA molecule exists as a homotrimer in its native form, and each monomer consists of two polypeptides known as HA1 and HA2 (Lamb 2001). The cleavage of HA into subunits HA_1 and HA_2 is an essential requirement for infectivity of the virus particle, and also plays a important role in the pathogenic properties of the viruses (Klenk et al. 1975). Most HPAI viruses possesses multiple basic amino acids at the HA cleavage site, making it accessible to a wide variety of intracellular proteases (Bosch et al. 1981; Webster et al. 2006), in contrast to LPAIV which possess only two basic amino acids at the HA cleavage site and are not susceptible to these proteases (Bosch et al. 1981). LPAIV HA are limited to cleavage by trypsin-like proteases and therefore their replication is restricted to the respiratory and intestinal tracts where such proteases are found (Alexander 2000). HA is the most abundant protein on the viral envelope and contains the epitopes recognized by major neutralizing antibodies (Lamb 2001).

Nucleoprotein. Nucleoprotein (NP) associates with viral RNA and the polymerase complex to form the ribonucleoprotein complex (RNP). Subunits of NP interact with each other and with RNA to from a helical structure (Lamb et al. 1981; Shi et al. 1996), NP also interact with Matrix 1 protein, PB1 and PB2 (Biswas et al. 1998; Ye et al. 1999; Ye et al. 1995).

Neuraminidase. NA is a class II membrane protein, with its N-terminus oriented toward the interior of the virus (Varghese et al. 1983). The NA protein can be divided into four main regions, including a short hydrophilic amino terminal tail, a hydrophobic transmembrane domain, a stalk region and a globular head that contains the enzymatic site for the protein

(Colman et al. 1983). The enzymatic active site, containing a number of conserved amino acid residues, is located at roughly the center of each unit (Gamblin et al. 2010).

The NA is responsible for cleavage of terminal sialic acid residues from cell surface and from viral glycoproteins, thus preventing viruses from binding to the infected cell surface and from aggregating with each other. Instead, the viruses are released infected cell to infect new cells and spread the infection (Palese et al. 1976; Palese et al. 1974).

The immune response to influenza surface glycoproteins NA, is mainly humoral, although the humoral response to HA is best characterized to protect against influenza infection (Webster et al. 1968), studies suggested that anti-NA antibodies afford some protection (McNulty et al. 1986; Webster et al. 1988). The NA glycoprotein is immunogenic, but not all antibodies generated against it can inhibit its function. Studies suggests that antibodies directed against the enzymatic site in the globular head region, as well as nonenzymatic globular head and stalk sites, produced NI activity and a reduction in disease mortality (Webster et al. 1988). It is not entirely clear why NI was produced from antibodies distant to the enzymatic site, but they may induce conformational changes in the tertiary structure of NA that affects its ability to bind substrate (Sylte et al. 2009). Several factors have limited the ability to study the role of NA-specific antibodies in the protection against influenza. First, the balance of humoral immune response to influenza glycoproteins is partial toward a HA response because there is approximately four times more HA than NA protein expressed on the surface of an infectious influenza virion (Webster et al. 1968), and the HA immunologically outcompetes NA in the priming of B and T cell responses in mice (Johansson et al. 1987), It is not clear if the effect seen in mice is conserved for all mammals and birds, but susceptible species tend to have higher serum levels of antibodies against HA compared to NA (Sylte et al. 2009).

The major anti-influenza drugs are NA inhibitors (Monto et al. 2006). Osetalmivir and zanamivir actas against influenza by binding to the NA active site and preventing virus release, this anti-influenza drugs do not prevent infection, but control infections by preventing influenza shedding from infected cells, causing accumulation of influenza virions on the apical surface in the infected cell (Moscona 2005). However, despite little anti-influenza drug usage worldwide, NA inhibitors resistant influenza was found, in Europe during the winter of 2007-2008 osetalmivir-resistant H1N1 account for the majority of influenza isolates in humans (Meijer et al. 2009).

Matrix protein 1. M1 is the most abundant protein localized within the virion. M1 is believed to make contact with the surface glycoproteins and with the RNPs complexes, forming a bridge between the inner core components and the envelope proteins (Nayak et al. 2004). M1 is absolutely required for viral assembly (Gomez-Puertas et al. 2000), it is believed that MI interacts with RNP complexes and NEP, therefore M1 is proposed to play a crucial role in viral assembly, by recruiting the viral components to the site of assembly at the cell membrane (Allen et al. 1980).

Matrix Protein 2. Matrix Protein 2 (M2) is a protein encoded by a splicing of segment seven mRNA (Martin et al. 1991). M2 is a type III integral membrane protein (Lamb et al. 1985). The protein forms a proton-selective ion channel, which is activated at acidic pH (Pinto et al. 1992). During virus entry, via receptor mediated endocytosis, M2 transports protons across the viral membrane, reducing the pH of the virion interior.(Betakova 2007; Bui et al. 1996), the acidic pH induces a dissociation of the M1 protein from the RNPs, which is essential to entry of RNP in the nucleus of the infected cell (Betakova 2007; Bui et al. 1996; Whittaker et al. 1996). M2 has also been implicated in stabilizing HA in the trans-Golgi network, the ion channel activity of M2

modify the acidic environment of the trans Golgi, preventing premature acid activation of newly HA (Betakova 2007; Ciampor et al. 1992). M2 is a specific target of the anti-influenza drugs amantadine and rimantadine, these inhibitors affects two steps of virus replication: virus uncoating and virus maturation (Betakova 2007).

Non structural protein 1. Non structural protein 1 (NS1) has numerous functions, it has been shown to bind to the poly-(A) tails of viral and cellular mRNAs, inhibiting their nuclear export (Qiu et al. 1994), to facilitate the availability of cellular mRNAs for the viral polymerase complex to snatch the cap. NS1 has been shown to inhibit the splicing of pre-mRNAs, with the exception of NS1 mRNA (Lu et al. 1994). NS 1 stimulates the transcription of the M1, NP and NA mRNA (Enami et al. 1994). NS 1 also plays a role in modulating the host immune response, by suppressing virus-induced host type I interferon response (Garcia-Sastre et al. 1998).

Nuclear export Protein. Nuclear export protein (NEP) is translated from a spliced viral mRNA of segment 8. NEP is responsible for export of RNPs from the nucleus to the cytoplasm (Ward et al. 1995).

Virus replication cycle

The replication cycle of AIV starts with attachment of the virus HA protein to terminal sialic acid residues of glycoproteins or glycolipids on the host cell surface (Wiley et al. 1987). The virion is than endocytosed (Matlin et al. 1981). The majority of AIV virions enter the cell through a clathrin-mediated pathway, however a clathrin and caveolin independent entry mechanism is also utilized by some AIV (Rust et al. 2004; Sieczkarski et al. 2002). The endocytic vesicle with the virion fuses with the early endosome, and then undergoes transport to

the perinuclear region, and finally mature into a late endosome with a low pH of 5 (Lakadamyali et al. 2003).

The low pH of the late endosome induce the activation of the ion channel M2, which allow protons to enter the core of the virion (Chizhmakov et al. 1996). The acidification of the core of the virion via the M2 channels induces a conformational change in M1, and M1 release its interaction with the RNPs complex. When the viron envelope fuses with the endosomal membrane the RNPs are released into the cytoplasm (Bui et al. 1996). The RNP is then actively transported to the nucleus via the nuclear localization signal of the nucleoprotein (Neumann et al. 2000).

Once in the nuclei, the negative sense vRNA is transcribed into mRNA (positive sense) by the RNP complex. PB2 binds the 5'-capped group of a cellular mRNA (Blaas et al. 1982; Ulmanen et al. 1981), the cellular mRNA is cleaved downstream of the cap by PA (Dias et al. 2009; Fodor et al. 2002; Hara et al. 2006b; Plotch et al. 1981; Yuan et al. 2009). The stolen cap will be used to prime the transcription of viral mRNA, and PB1 is responsible for the elongation of the mRNA (Nakagawa et al. 1996). The polyadenylated viral mRNA is transported from the nucleus to the cytoplasm and then is translated into viral proteins (Cheung et al. 2007).

The RNP synthesizes positive-sense complementary RNA and uses the cRNA as template to produces viral negative sense RNA genome (Lamb 2001). NP may play a role in switching the synthesis of mRNA to cRNA and vRNA, but the mechanism is not know (Biswas et al. 1998).

In the final stage of the infection, the progeny virions must be released from the host cell. The influenza vRNA are not packaged randomly (Duhaut et al. 1996; Fujii et al. 2003). There is

a selective process by which one copy of each vRNA segment is placed within a budding virion (Nakajima et al. 1977).

The M1 and NEP are thought to be the major mediators of the exit of the viral RNA genome from the nucleus to the budding region of the cytoplasm membrane (Martin et al. 1991). In the cytoplasma, M1 associates with the cytosolic face of the cell membrane (Zhang et al. 1996), docking the bound RNP in place at the site of budding. The structural proteins HA, NA and M2 are trans membrane proteins and are embedded in the plasma membrane during their synthesis (Wiley et al. 1987). The cytoplasmic tails of these proteins are necessary for their efficient packaging into virions (Mitnaul et al. 1996). After budding from the cell surface, the NA cleaves the sialic acid residues from the glycoproteins of the newly formed virions disrupting the sialic acid binding mediated by HA and releasing the virions to to infect neighboring cells (Palese et al. 1974).

Pathogenesis

AIV can infect a wide variety of animals, from wild birds to mammals. However AIV is perpetuated in wild aquatic birds (Alexander 2007). In these species of birds AIV naturally infect the intestinal tract, and replicate in the epithelial cells of the intestine, the virus may be shed in high concentrations in the feces (Munster et al. 2009; Webster et al. 1992). However, AIV infections in wild birds are usually asymptomatic (Causey 2008; Munster et al. 2009).

Although poultry is not a natural host of AI, infections in poultry are current (Alexander 2000). AI viruses vary in their ability to infect and cause disease in a host species (Swayne 2008a; Swayne 2006), and can be differentiated in two distinct types based on the existing World

Organization of Animal Health (OIE) criteria: the low pathogenic AIV (LPAIV) and the highly pathogenic AIV (HPAIV) (Alexander 2000; Swayne 2006). To be classified as HPAIV, the virus needs to meet one of two criteria: (i) an intravenous pathogenicity index (IVPI) > 1.2 or be lethal for 76% or more of intravenously inoculated susceptible chickens, or (Fujii et al.) AIVs of the H5 or H7 subtypes with amino acid sequence at the HA proteolytic cleavage site compatible with HPAIV. All other AIV are considered as LPAI (Swayne 2006).

The cleavage of HA into subunits HA_1 and HA_2 is an essential requirement for fusion of the viral envelope with the endosomal membrane, HPAIV possesses multiple basic amino acids inserts at the HA cleavage site, making it accessible to ubiquitous furin proteases (Bosch et al. 1981; Pantin-Jackwood et al. 2009; Webster et al. 2006), resulting in infection and lesions in many cell types in several visceral organs, including nervous system and the cardiovascular system (Pantin-Jackwood et al. 2009).

The HPAI viruses are characterized by their high morbidity and mortality. However, the mean death times vary between individual virus strain (Swayne 2006). Most of the highly pathogenic outbreaks of poultry have been of the H5 and H7 subtypes (Alexander 2000). Phylogenetic analysis of HPAIV indicates that such viruses do not constitute unique lineages but are derived from non-pathogenic lineages of AI (Brugh et al. 1991). The ability of LPAIV to mutate into HPAIV was illustrated in 1983-84 outbreak of HPAI in Pennsylvania and in 1994-95 outbreak in Mexico, where the abrupt emergence of HPAI subtype H5N2 immediately preceded by circulation of a non-pathogenic H5N2 strain (Alexander 2000; Kawaoka et al. 1988; Webster et al. 1987). A similar phenomenon occurred in 1999-2000 in Italy where outbreaks of LPAI H7N1 preceded a mutation, which then resulted in HPAI outbreaks (Alexander 2007).

LPAIV which possess only two basic amino acids at the HA cleavage site and are not

susceptible to furin proteases (Bosch et al. 1981) are limited to cleavage by host proteases such as trypsin-lyke enzymes and thus restricted to replication at sites in the host where such enzymes are found such as the respiratory and gastrointestinal tracts (Alexander 2000; Spickler et al. 2008).

The LPAIV have been shown to cause mild forms of the disease in commercial poultry characterized by low morbidity and no mortality in some instances complication with other respiratory diseases of poultry will severely increase the mortality in flocks infected with LPAIV (Pantin-Jackwood et al. 2009; Spickler et al. 2008).

Transmission

Wild birds from the order *Anseriformes* and *Charadriiformes* are the natural reservoir for AIV (Stallknecht et al. 2010b). The transmission of AIV in these orders and particular in ducks occur through fecal-oral route via contaminated water (Hinshaw et al. 1979; Stallknecht et al. 2010b). In support with this form of transmission, AIV have been isolate from surface water used by aquatic birds in Alberta (Hinshaw et al. 1980), Minnesota (Halvorson et al. 1985), and Alaska (Ito et al. 1995; Stallknecht et al. 2010a).

Although the fecal-oral route of transmission via contaminated water in wild birds aquatic environment is well recognized, and data on the tenacity of AIV in this aquatic environments still is limited (Stallknecht et al. 2009). The first study on persistence of AIV in water was conducted by Webster *et al (Webster et al. 1978),* where he demonstrated that AIV from the subtype H3N6 was detectable for 32 days at 4° C and 4 days at 22° C in non-chlorinated water mixed with infected feces. Other studies have evaluated the persistence of low pathogenic AIV (LPAIV) isolated from wild ducks in water using an experimental system

(Brown et al. 2009; Brown et al. 2007; Stallknecht et al. 2010a; Stallknecht et al. 1990a; Stallknecht et al. 1990b), where distilled water was contaminated with infected allantoic fluid, and the amount of virus in the contaminated water is titrated over time. Collectively, these studies demonstrated that survivability of AIV in water is inversely related with temperature, with AIV persisting for longer times in lower temperatures, over a year at 4°C and only for several days at 37° (Brown et al. 2009; Stallknecht et al. 2009). These studies also demonstrated that the survivability of AIV in water is dependent of the pH and salinity of the water, individual AIV isolates differ in their phenotypic abilities to remain infective under variable pH and salinity, however viruses are most stable between pH 7.4 and pH8.2, and between 0 or 15,000ppm sodium chloride conditions (Stallknecht et al. 2009).

The persistence of AIV in different types of water (distilled, normal saline water, and surface water) was evaluated by Nazir et al (Nazir et al. 2010), in this study it was shown that that AIV remained infective for a lower amount of time in surface water than in distilled water.

The transmissibility of LPAIV in domestic poultry also is poorly understood, and results from previous work, suggest that bird- to -bird transmission is extremely complex and depends on the viral strain, species of birds, and other environmental factors (Alexander 2007; Alexander et al. 1978; Alexander et al. 1986; Yee et al. 2009a). LPAIV transmission has been demonstrated by direct contact of susceptible poultry (chickens, quails, and turkeys) with infected poultry (Lu et al. 2004; Perez et al. 2003; Pillai et al. 2008; Yassine et al. 2007). Some examples of experimental contact transmission of LPAIV are the H9N2 duck isolate, H9N2 quail isolate that were successfully transmitted to naïve quails in direct contact with infected quails (Perez et al. 2003), the LPAIV H5N2 parrot isolate (Pillai et al. 2008), H6N2 chicken isolate (Yee et al. 2009a), H7N2 chicken isolate (Lu et al. 2004), H11N9 duck isolate (Li et al. 2010)

that were transmitted to naïve chickensthe LPAIV H5N2 parrot isolate (Pillai et al. 2008), the H3N2 turkey isolate (Yassine et al. 2007) were transmitted to naive turkeys. Although in transmission studies it is difficult to distinguished between aerosol and/or fecal transmission in some of the studies the separation of infected and contact birds has allowed to observe that both routes of transmission are important in the spread of AIV (Perez et al. 2003; Yee et al. 2009b).

It is believed that the ability of the LPAI viruses to transmit is closely linked to the amount of virus released from infected birds (Alexander 2007). However, the duration and extent of AIV shedding, particularly of LPAIV in infected poultry, is poorly understood and can be highly variable (Alexander 2000, 2007; Jackwood et al. 2010; Lu et al. 2003; Morales et al. 2009; Pillai et al. 2010b). Under experimental conditions LPAI viruses have been consistently detected in the respiratory tract of quails, while some viruses were occasionally isolated from the cloaca (Liu et al. 2003; Makarova et al. 2003; Sorrell et al. 2007) indicating that in quails shedding from the respiratory route is more prevalent, although some intestinal shedding occurs as well.

In chickens, replication of LPAIV is restricted to the respiratory and intestinal tracts as demonstrated by successful isolation from the oropharingeal and cloacal samples (Alexander 1982, 2000; Jackwood et al. 2010). However under experimental conditions some LPAIV are shed exclusively through the respiratory tract (Alexander 1982, 2000; Jackwood et al. 2010; Zarkov 2008), some LPAI virus show shedding restricted to the intestinal tract (Jackwood et al. 2010; Shan et al. 2010; Zarkov 2008) while some are shed through the respiratory and intestinal tract (Jackwood et al. 2010; Lu et al. 2004; Pillai et al. 2010b).

One factor that influences the spread and transmission of LPAIV from flock to flock during an outbreak of the disease is the movement of contaminated poultry, in particular the daily mortality (McQuiston 2005), poultry products, and equipment (Swayne 2008a).

Depopulation and movement of poultry litter has been indirectly associated with the failure to control the spread of LPAI H7N2 virus in commercial poultry (Henzler et al. 2003).

During outbreaks of AIV, there is a necessity to find alternative methods of disposing daily mortality and litter (Akey 2003; Swayne 2003b). On-farm composting is good choice, since composting is proved to inactivate AIV (Guan et al. 2009).

Transmission of AIV to humans. It was previously believed that adaptation in an intermediate host was necessary for AIV to be transmitted to humans (Scholtissek et al. 1977). However, poultry outbreaks of H5N1 in Hong Kong during 1997 and 1999 demonstrated that humans in direct contact with infected poultry acquired the virus indicating that adaptation in a mammalian host was not necessary (Bridges et al. 2002). Since 2003, there have been 372 cases of avian influenza virus in humans and 235 fatalities caused by H5N1 virus ((WHO) 2008). Most of the individuals infected have had direct contact with infected poultry (Bridges et al. 2002). Besides the Asian H5N1 lineage of viruses and other poultry adapted AI viruses have also infect humans. The H7N2, H7N3 and H7N7viruses were known to cause conjunctivitis in humans, and serologic studies provided evidence of subclinical human infection with the H7 subtype virus prevalent in live poultry markets (Causey 2008; Wentworth et al. 1997). Cases of influenza in humans has been associated to avian H9N2 (Fouchier et al. 2004), H7N3 (Tweed et al. 2004), H7N7 (Webster et al. 1992). When a influenza virus cross species, like in the case of H5N1, from commercial poultry to humans, it will often replicate, occasionally cause disease, but rarely the virus will transmit from human to human. However, a specie crossover may result in the introduction of a new hemagglutinin and/or neuraminidase subtypes into humans, causing an antigenic shift, which can result in a severe epidemic or pandemic, because the new host has no protective immunity to the newly recombinant influenza strain (Suarez 2000). This type of

antigenic shift has already occurred in human populations three times (Alexander et al. 1978; Spackman et al. 2007), in 1957, 1968 and in 1977. Genetic and biochemical studies concluded that the 1957 and 1968 human pandemic strains arose by reassortment, the 1957 Asian H2N2 obtained its HA, NA and PB1 genes from an avian virus and the remaining five genes from the preceding human H1N1 virus (Alexander et al. 1978). The 1968 strain contained the HA and PB1 genes from an avian influenza and the other 6 genes from the H2N2 human strain circulating during early 1968 (Alexander et al. 1978).

In April 2009, a new influenza A (H1N1) virus emerged among humans in California and Mexico, quickly spreading worldwide through human-to-human transmission, and generating the first influenza pandemic of the 21st century (Scalera et al. 2009). The virus was found to be antigenically unrelated to human seasonal influenza viruses but genetically related to viruses known to circulate in pigs. Molecular studies of the new A (H1N1) 2009 pandemic virus genome showed that it was derived from several viruses which had been circulating in pigs for years, namely the North American H3N2 triple-reassortant (see details below), the classical swine H1N1 lineage, and the Eurasian 'avian-like' swine H1N1 virus (Brown 2000; Girard et al. ; Pensaert et al. 1981). Initial transmission of the pandemic A (H1N1) 2009 virus to humans is believed to have taken place at least several months before recognition of the first outbreak (Garten et al. 2009; Girard et al.). Surprisingly however, there has been no evidence so far that pigs have played any role in the epidemiology or in the worldwide spread of the virus in human populations (Vallat 2009). On June 11, 2009, the World Health Organization raised the pandemic alert to level 6, in view of the number of countries and regions which officially reported A (H1N1) 2009 influenza cases in their communities. The virus was spreading rapidly around the world and appeared to affect primarily children and young adults as well as those with an

underlying lung or cardiac disease condition (Peiris et al. 2009). The need for a specific vaccine was recognized in view of the continued outbreaks of severe human infections and the risk of a possible increase in pathogenicity and/or acquisition of antiviral resistance of the A (H1N1) 2009 virus through eventual reassortment. Vaccine development was promptly initiated in collaboration between the World Health Organization, Health Ministers and National Health Agencies, and the vaccine industry (Girard et al.).

Diagnostics techniques

Several diagnostic tests can be used for detection of avian influenza, the initial diagnosis is preferably made by virus isolation. Serologic detection of infection in the host is important for surveillance.

Viral isolation. Virus isolation is the gold standard in the diagnosis of AIV. Virus isolation can be performed in either tissue culture, MDCK cells, or in SPF embryonated chicken eggs, been the latter the most frequently for avian influenza. In this procedure eggs are inoculated with 100-200 µl of a sample via the allantoic sac route. The eggs are incubated at 37°C for 4-7 days and then allantoic fluid are collected and tested for the ability to agglutinate erythrocytes in a hemagglutination assay (HA) (Swayne 2008b). After virus isolation is done, additional testing must be performed to confirm the identity of the virus.

Virus isolation in embryonated eggs is recognized as the standard method of isolation by OIE; however virus isolation is time consuming, expensive, and not suitable for high-throughput screening of a large volume of samples.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RT-PCR procedures have been developed to aid in the detection of AIV directly from clinical samples to accelerate the

diagnosis and to allow high-throughput screening, In addition, RT-PCR facilitates assessment of H5 and H7 pathotypes based on the HA1 cleavage sequence (Spackman et al. 2002). The test have four steps, the isolation of RNA, conversion of RNA to cDNA by the use of reverse transcriptase, amplification of the cDNA by multiple cycles of amplification with AIV specific primers, and analyses of the PCR product. AIV are tipically cleared from the upper respiratory tract during the first three days post-infection, therefore there is only a small window in which to collect sample that are potentially positive for virus (Lu et al. 2003).

Antigen Capture to Detect AIV antigen. Detection of AIV in samples can be done by demonstrating the presence of specific AIV antigens with colorimetric assays, this assay was first developed to rapidly detected influenza A virus in humans, the test detects type-specific influenza A antigen via enzyme-conjugated monoclonal antibodies (Ryan-Poirier et al. 1992). There are commercial licensed test kits available to use in humans (Directigen Flu A®, Becton Dickinson Microbiology Sutems, Sparks, MD; NOW Flu A®, Binax, Portland, ME; Flu Detect ®, Synbiotics, San Diego, CA) (Swayne 2008b).

There are also tests that detects AIV antigen in a solid-phase, flow-through ELISA, this assay can detect AIV in allantoic fluid or directly in clinical specimens (Swayne 2008b). Antigen capture tests are intended for use on-farm site tests for avian influenza infection outbreaks, as a rapid screening of a flock (Suarez et al. 2003; Swayne 2008b).

Antibody detection tests. There are many antibody detection tests available, the detection of antibodies produced by a host in response to AIV infection can be long-lasting (Dlugolenski et al. 2010).

Hemmaglutination Inhibition assay (HI). A serum sample is determined to have antibodies to a particular AIV strain if this serum sample is able to inhibit the agglutination of erythrocytes by

the viral strain. In this test only the HA subtype can be determined (Swayne 2008b; Wu et al. 2007). The HI assay usually requires the use of infectious virus, and the test has to be conducted using certain safety precautions in specialized facilities (Swayne 2008b).

Agar Gel Precipitation Test (AGPT). The AGPT uses formalin-inactivated antigen prepared from the chorioallantoic membranes of embryonated chicken eggs infected with AIV. The antigen is placed in a well in a sheet of agar. Serum samples and controls are placed in different well in the same sheet of agar gel surrounding the antigen. If serum samples have antibodies against the AIV nucleoprotein, a visible precipitate of antigen-antibody complexes will form in the agar. This test can identify positive serum for any type of A influenza virus in avian and mammalian sera (Beard 1970a, b).

Neuraminidase Inhibition assay (NI). The NI test allows AIV to be differentiated on the basis of the antigenic character of the Neuraminidase glycoprotein of the virus. The neuraminidase inhibition test relies on the presence of neutralizing antibodies that inhibits the enzymatic activity of the neuraminidase. The neuraminidase inhibition test is relatively expensive and requires more time to complete when compared to the hemagglutination inhibition test (Swayne 2008b; Van Deusen et al. 1983; Webster et al. 1972).

Enzyme Linked Immunosorbent Assay (ELISA). The ELISA can be carried out on the bench top as it usually does not need to utilize infectious virus. The method consists of coating a 96-well plate with a viral protein antigen. There are commercially available ELISA kits which use NP as protein antigen which is conserved among all influenza A viruses (Wu et al. 2007). Different viral antigens can also be used in the same format test, if HA or NA is used as the protein antigen, we may determine if a sample contains antibodies to a particular subtype of AIV.

In a l indirect ELISA, the coated plate is incubated with the serum samples followed by incubation with an enzyme-tagged conjugate which specifically binds antibodies of the species from which the sera were taken (Wu et al. 2007). Upon the addition of a substrate, a colorimetric change is observed in wells that contain the conjugate bound to serum antibodies, which are in turn bound to the viral antigen. One limitation of this assay is that it is species-specific.

Control of AIV

The most common way to control AI outbreaks is the culling of infected poultry, which has been consistently successful in previous outbreaks including the outbreak in Hong Kong in 1997 and the Netherlands in 2003 (Lipatov et al. 2004). Culling infected poultry causes the reduction of the viral load and reduces the likelihood of transmission to humans (Lipatov et al. 2004). However, the poultry industry has undergone substantial changes in the last twenty years, mainly resulting in shorter production cycles and greater animal densities per territorial unit, as a result mass depopulation led to very high costs and economic losses for the national and federal governments, the stakeholders and ultimately for the consumers (Capua et al. 2003a). While the optimal method of eradication of influenza is the culling of poultry when the outbreak is widespread, this course of action may not be possible and alternative strategies have to be design (Lipatov et al. 2004).

In the United States, when H5 or H7 outbreaks occur, emergency control programs are activated and managed through the cooperative efforts of USDA, state authorities and the poultry industry. Control programs for LPAI outbreaks caused by viruses of other subtypes are managed by the poultry industry working in conjunction with state authorities. Elimination is usually the goal and the outcome of LPAI control programs, but the measures applied are typically less

stringent than those used for H5 or H7 outbreaks and largely based on risk assessment and cost-benefit evaluation (Swayne 2003b). In other areas such as Mexico (Villarreal 2009), some Asian countries (Lipatov et al. 2004) and in the Middle East (Banet-Noach et al. 2007), the current strategy when dealing with influenza virus outbreaks in poultry is culling along with vaccination. Vaccination has potential benefits, such as the reduction of clinical signs of diseases, reduction of viral shedding and increase the resistance of birds to becoming infected (Capua et al. 2003a). However, vaccination also has drawbacks, such as the effect of vaccination on international trade, and the effect that it has on serologic surveillance, since vaccinated birds cannot be distinguished from naturally infected birds. Losing the ability to do surveillance has been the main reason for countries as the US and the European Union to oppose vaccination as a routine tool in the AIV control (Capua et al. 2003a; Suarez 2005). Therefore there is a need to perform surveillance that allows the differentiation of naturally infected from vaccinated birds and to identify vaccinated birds that become infected (Suarez 2005).

Vaccination against AIV. The majority of the current licensed influenza vaccines are in inactivated antigen preparations (Ellebedy et al. 2009). All vaccines containing inactivated influenza virus vaccines work primarily through generation of antibodies directed against the hemagglutinin protein. There are three formulations of inactivated virus vaccines for influenza. The first formulation is the whole virion, this type of vaccine has been utilized since the 1940's, a different formulation is composed of a split virion which is formulated by disrupting whole viral particles with detergents. The last and final formulation is a subunit composition which is developed by enriching viral surface glycoproteins following disruption of viral particles (Ellebedy et al. 2009). Current research has given emphasis on the development of alternative vaccine strategies with a rapid vaccine production. Methods for the development of new

influenza vaccines are currently being investigated, especially the use of reverse genetic systems to develop new vaccines (Hoffmann et al. 2000). Reverse genetics allows for the generation of very specific live vaccine formulations. Reverse genetics does not allow for quicker preparation of vaccine stocks, but does allow for adequate preparation of seed strains (Ellebedy et al. 2009) (Wood 2001).

There are some potential advantages of live attenuated viruses versus inactivated virus vaccines. First live attenuated vaccines mediate both neutralizing antibody production and a cell mediated response. Cell mediated immunity is vitally important because aids in targeting of more conserved regions of the virus (Thomas et al. 2006). Much like inactivated vaccines live attenuated vaccines most often require multiple doses.

DNA vaccines are also a new technology being utilized for formulation of new vaccines. DNA vaccines are currently being investigated in use for influenza viruses (Ellebedy et al. 2009). DNA vaccines are relative cheap, safe, and rapid for use in humans. The one drawback to influenza DNA vaccines is the poor immunogenicity that they confer in humans (Drape et al. 2006), however DNA vaccines do not seams to be fit to use in a high population chicken flock. Recombinant fowl pox vaccines have been tested containing the H5 and NP genes of influenza virus and have shown protective efficacy in poultry (Doherty et al. 1989).

Vaccination has potential benefits, such as the reduction of clinical signs of diseases, reduction of viral shedding and increase the resistance of birds to becoming infected. However, vaccination also has drawbacks to use, first is the cost of vaccination since the value of individual birds is low, the cost of the vaccine and its administration can be impediment to its use, second the effect of vaccination on international trade, and third the effect that it has on serologic surveillance, since vaccinated birds cannot be distinguished from naturally infected

birds. This loss of the ability to do surveillance has been a major inhibitors for use of vaccination in AIV control (Suarez 2005; Swayne 2003a).

Differentiation of infected from vaccinated animal (DIVA) vaccination strategies. Vaccination is an important tool in control of AIV, but for trade and surveillance purposes, it is important to differentiate naturally infected and vaccinated birds and also to identify vaccinated birds that become infected (Suarez 2005). There are four different approaches for DIVA vaccination, the use of sentinels, the use of subunit vaccines, the nonstructural protein NS-1 strategy and the heterologous neuraminidase strategy (Suarez 2005; Swayne et al. 2007; Zhao et al. 2005).

Sentinels. Unvaccinated, sentinels birds are placed within a flock of vaccinated birds. The sentinel birds are tested for exposure to avian influenza, usually by standard serologic tests. The disadvantage of this strategy is the management of the sentinel birds (to distinguish the sentinels from vaccinated) and the concern that these naïve birds may increase the risk of infection in the flock (Suarez 2005).

Subunit vaccines. It is possible to protect birds by having only the HA and NA proteins in a vaccine (Lee et al. 2005; Nayak et al. 2010; Nayak et al. 2009), however the vaccinated birds will not develop antibodies to the internal proteins of the virus (Suarez 2005). The subunit vaccines also have disadvantages, the biggest issue with the vactored vaccines is regulatory, the licensing is more difficult to obtain (Suarez 2005).

NS-1 strategy. NS-1 protein is produced in high amounts in infected cells, but is not present in the virion. The inactivated vaccines are usually made of whole viral particles, therefore vaccinated birds will not mount an immune response against NS-1, and naturally infected birds will (Suarez 2005; Tumpey et al. 2005; Zhao et al. 2005). The disadvantage of this strategy is

that a vaccine can have residual NS 1 protein in its composition, and this makes more difficult to use the NS-1 DIVA strategy (Suarez 2005; Tumpey et al. 2005).

Heterologous NA strategy. The heterologous NA strategy requires a vaccine with homologous HA and heterelogous NA from the circulating AIV strain, although antibodies to both HA and NA subtype can be protective, antibodies to the HA are the most important (Nayak et al. 2010; Suarez 2005). This strategy was proposed more than 20 years ago (Beard 1986; Suarez 2005), but was applied successfully only recently to control an HPAI outbreak in 2000 in Italy (Capua et al. 2003b, c; Capua et al. 2002).

In the 2000 outbreak in Italy, turkeys were vaccinated with and H7N3 vaccine, some flocks were monitored for evidence of infection using sentinels birds tested each 45 days for evidence of infection (Suarez 2005), some other flocks were monitored periodically for antibodies to the NA subtype 1 (the NA subtype from the circulating challenge virus- H7N1) using an indirect immunofluorescence assay (Capua et al. 2003d; Suarez 2005). The same strategy was used a second time in Italy to control an outbreak of low pathogenic H7N3, birds were vaccinated with H7N1 and monitored using an indirect immunofluorescence assay (iIFA) to detect antibodies against the NA subtype 1 and an iIFA to detect antibodies against NA subtype 3 (Cattoli et al. 2006; Suarez 2005). In the US, the DIVA strategy has been used on limited basis. In 2003, three layers farms in Connecticut were having a low pathogenic H7N2 avian influenza infection. Vaccination was used to control the outbreak, birds were first vaccinated with a H7N2 vaccine, eventually a heterologous NA H7N3 vaccine was used. The evaluation of the DIVA strategy was not possible because the first round of vaccination (homolgous HA and NA) controlled the outbreak (Suarez 2005).

The successful use of this DIVA strategy in Italy has increased the interest in its use, however this strategy is relatively untested and does have potential issues that have to be addresses. One potential problem of this particularly DIVA strategy is the sensitivity of the serologic testing, since birds with good HI titers should have greater resistance to virus infection, and may not have enough virus replicating to stimulate an antibody response against the heterologous NA. The second problem is the availability of appropriate vaccine strain and the availability of the specific diagnostic test, and the third issue with the heterologous NA strategy is the availability of diagnostics, NI test is available but not suitable to screen a large number of samples, iIFA test is not available to all NA subtypes and it can be difficult to interpret the test (Suarez 2005). To overcame the disadvantages of the iIFA, indirect and competitive ELISA systems for the detection of N1, N2 and N3 have been developed (Liu et al. 2010).

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Chapter 3

Shedding and Fecal Contamination Associated with Low Pathogenic Avian Influenza Viruses (LPAIV) in Commercial Layers, Broilers, Turkeys and Quails

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Abstract

Information on transmissibility of avian influenza viruses among poultry and humans exposed to poultry house environment is limited. In this study, intestinal shedding and tenacity of the virus in feces of poultry infected with virus A/Ck/CA/431/00(H6N2), duck isolate A/Mallard/MN/355779/00(H5N2), and turkey isolate A/turkey/Ohio/313053/04(H3N2) were evaluated in chickens, turkeys and quails. All three viruses were detected in chickens (layers and broilers); only the H6N2 isolate was shed by turkeys and quails. The highest viral titers for the H6N2 virus were observed in chickens. The H6N2 isolate retained infectivity in chicken feces for 24 hrs and in turkey feces for 12 hrs. Low intestinal shedding of the H6N2 virus by quails did not allowed to evaluate the tenacity of this virus in quail feces. Results obtained from this study clearly indicated that shedding of LPAI viruses is an extremely intricate process dependent on environmental factors, the host, viral strain, and the degree of adaptation of the virus to the host. In this study the infectivity of LPAI virus in feces was directly related to the rate of virus shed through the fecal route and the moisture content of feces as well as the environment temperature played important roles.

Introduction

Avian influenza viruses (*type A influenza virus*, *Orthomyxoviridae*) are enveloped viruses with segmented single-negative-stranded RNA genome (Lamb 2001). Type A Influenza viruses are classified on the antigenic profiles of the surface glycoproteins that include 16 hemagglutinin (H1 through H16) and nine neuraminidase (N1 through N9) subtypes, for a total of 144 possible different influenza virus subtype combinations (Causey 2008).

Avian Influenza virus (AIV) can infect a wide variety of animals, including birds and mammals, wild birds predominantly ducks and shorebirds, are the reservoir for all 16 hemagglutinin and all 9 neuraminidase AIV subtypes (Munster et al. 2007; Webster et al. 1978). Although commercial poultry is not the natural host of AIV, infections occur frequently (Alexander 2000) and can be associated with two pathologic outcomes; low pathogenic AI (LPAI) characterized by a mild respiratory disease with low morbidity and no mortality, and the highly pathogenic AI (HPAI) characterized by severe respiratory disease accompanied with high morbidity and mortality (Spickler et al. 2008).

The duration and extent of AIV shedding, particularly of LPAIV in infected poultry, is poorly understood and can be highly variable (Alexander 2007; Jackwood et al. 2010; Morales et al. 2009; Pillai et al. 2010a). Likewise, the transmissibility of LPAIV in domestic poultry also is poorly understood, and results from previous work, suggest that bird- to -bird transmission is extremely complex and depends on the viral strain, species of birds, and other environmental factors (Alexander 2007; Alexander et al. 1978; Alexander et al. 1986; Yee et al. 2009a). It is believed that the ability of the LPAI viruses to transmit is closely linked to the amount of virus released from the intestinal route of infected birds (Alexander 2007). In poultry

LPAI virus replication is primarily restricted to the respiratory and gastrointestinal tracts (Spickler et al. 2008), and movement of contaminated poultry (daily mortality) (McQuiston 2005), poultry products, and equipment is probably the main sources that facilitates LPAIV transmission between flocks (Swayne 2008a). Depopulation and movement of poultry litter has been indirectly associated with the failure to control the spread of LPAI H7N2 virus in commercial poultry (Henzler et al. 2003). However, the role of contaminated litter in LPAI virus transmission and the ability of LPAI viruses to remain infective in poultry feces have not been well defined. The objectives of this study were to determine duration and extent of fecal shedding for LPAI chicken (H6N2), duck (H5N2) and turkey (H3N2) viruses from experimentally infected layers, broilers, turkeys and quails and to the determine the duration of LPAI viruses infectivity in feces from these poultry species.

Material and methods

Viruses. Three viruses were used in this study, the poultry adapted virus A/Ck/CA/431/00(H6N2) first isolated from a layer flock in California and characterized as low pathogenic (Webby et al. 2002), duck isolate A/Mallard/MN/355779/00(H5N2) (Costa et al. 2010a), and low pathogenic A/turkey/Ohio/313053/04(H3N2) originally isolated from a 34 week old turkey breeder flock (Tang et al. 2005). Viruses were propagated in 10-day-old specific-pathogen-free (SPF) chicken embryos (CE) as previously described (Swayne 2008b). The second CE passage of each of the viruses was utilized to inoculate birds.

Experimental design. Four separate experiments were performed using commercial layers, broilers, turkeys, and quails (*Cuturnix cuturnix japonica*). All birds were obtained from commercial sources at 1-day of age and raised in floor pens located in our facilities at the Poultry

Diagnostic and Research Center (PDRC, University of Georgia). For each species, 24 birds were equally distributed in four stainless steel negative pressure- HEPA filtered, isolator units (PDRC). Birds were fed a standard diet and water *ad libitum* and kept with light for a period of 12 hours daily. Layers, broilers and turkeys were infected at three weeks of age and quails at eight weeks of age. All birds were inoculated intranasally with viral stocks of H6N2, H5N2, and H3N2 viruses at a total dose of $10^{7.2}$ EID_{50} per bird in a 200 µl volume. Six birds were inoculated intranasally with 200µl of non-infected allantoic fluid as negative controls. Three pools of cloacal swabs (two swabs per pool), and three samples of fresh fecal droppings (0.5 gr) were collected from each unit at 3, 7, and 11 days post-inoculation (PI) to determine viral titer. Fecal samples collected at 3 days PI in three open Petri dishes, were placed inside the units away from the birds, and at 0, 12, 24, and 36 hours post-placement fecal samples were collected to determine viral titers as described below. Also droppings were collected at 0, 12, 24, and 36 hours post-placement to determine the moisture content of the droppings. Briefly, fecal droppings were weighted (first weight), placed in oven at 70°C for 24hrs, and then weighted a second time (second weight). Moisture content per sample was determined using the formula: % moisture= 100- (second weight/first weight *100).

Cloacal and fecal samples were transported in brain-heart infusion (BHI- Sigma Alderich) media containing 10000u/L penicillin-G potassium (Fisher Scientific), 250g/L gentamycin sulfate (Fisher Scientific), 25mg/L of amphotericin B (Fisher Scientific), 500g/L kanamycin (Sigma-Aldrich) and 1g/L of streptomycin sulfate (Sigma-Aldrich). Blood was collected at one-week prior to inoculation and 11 days post-inoculation (PI) when all birds were humanely euthanized. Hemagglutination inhibition (HI) titers were determined for all serum samples as described below.

Sample processing for virus titration. After collection, cloacal swab pools were vortexed and stored at -80^{0}C for virus titration in embryonated SPF CE. Fecal droppings were resuspended by vortex for 30 minutes at room temperature, and centrifuged for 5 minutes at 4,000 rpm. After centrifugation, the supernatant was collected and filtrated through a 0.45μm pore size syringe filter (Whatman). The supernatant was then inoculated in embryonated CE for virus titration.

Virus titration. Virus titers from cloaca and fecal samples were determined in 9 to 11 day old embryonated SPF CE. Briefly, undiluted (200 μl) and a 10^{-1} to 10^{-7} serial dilution of the processed cloacal and feces samples were inoculated in the allantoic cavity of embryonated CE, in triplicates. After inoculation, eggs were incubated for four days and candled daily to monitor mortality rates. On the fourth day, surviving embryos were stored at 4°C for 3 to 6 hours. Allantoic fluid from each embryo was collected and tested for hemagglutinin (HA) activity (Swayne 2008b; Thayer 2008), and samples with an HA titer > 2 were confirmed as AIV by HI as described below. Viral titers were estimated by Reed and Muench (Reed 1938) and expressed as embryo infectious dose $(EID)_{50}$, the mean titer per treatment was estimated from three independently collected samples.

HI assay. The HI assay was utilized for confirmation of AIV in HA positive cloacal and fecal samples; for this purpose, hyperimmune sera against A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/ Ohio/313053/04(H3N2) were produced in chickens. Briefly, three groups of chickens were immunized intravenously with $10^{7.5}EID_{50}$/ml of each virus. Three weeks after the first immunization chickens were boosted intravenously with the same dose and two weeks after boost inoculation blood samples were collected (Pillai et al. 2008). Using four HA units and two-fold dilutions of respective hyperimmune sera the HI assay

was performed as previously described (Swayne 2008b; Thayer 2008). HI assay was also used to determine AIV HI antibody titers from infected layers, broilers, turkeys and quails. Sera with HI titers ≥ 4 $\log_2$ were considered positive as previously described ([Anon] 2005).

Statistical analysis. To test if intestinal viral shedding of H6N2, H5N2 or H3N2 differ between layers, broilers, turkeys, and quails, or differences in intestinal shedding among viral strains exits among the poultry species tested, viral titers from feces and cloaca samples collected at 3, 7, and 11 days PI were compared using repeated measures ANOVA (PASW statistics 18 program. SPSS, Illinois). To test the tenacity of LPAIV, mean viral titers of H6N2, H5N2, and H3N2 shed in feces by infected layers, broilers, turkeys and quails were compared at 0, 12, 24 and 36 hours post subsequent placement. Repeated measures ANOVA was utilized to determined differences in viral titers over time among the different poultry species (PASW Statistics 18, SPSS, Illinois). To determine if differences in HI antibody titers against H6N2, H5N2, and H3N2 were observed in sera from the different poultry species HI titers were compared using T student test (PASW Statistics 18, SPSS, Illinois).

Results

Serology. All serum samples collected at one week prior to inoculation and from sham inoculated birds tested negative for HI antibodies. HI antibody titers were detected in all inoculated birds (Figure 1). No significant differences ($p < 0.05$) were observed among HI titers elicited by H6N2, H5N2, and H3N2 infections among the different commercial poultry species (Fig. 1).

Viral shedding. Virus isolation was successful on cloacal swabs and feces collected on day three PI but no viruses were isolated from samples collected at days 7 and 11 PI. On day three

PI no significant differences ($p < 0.05$) were observed between viral titers recovered from cloaca swabs or fecal samples independently of the poultry species tested or viral isolate. Layers and broilers shed the H6N2, H5N2, and H3N2 viruses in significantly higher titers ($p < 0.05$) than turkeys and quails. The H6N2 virus was recovered from feces and cloacal samples of broilers, layers, turkeys and quails and it was shed in significantly higher titers ($p < 0.05$) by broilers and layers. No viral shedding was detected in cloacal or feces of turkeys and quails infected with H5N2 and H3N2 (Table 1 and 2).

Viral titers of H6N2, H5N2 and H3N2 in poultry feces. Mean viral titers of H6N2, H5N2 and H3N2 in feces collected at day 3 PI and subsequent 0, 12, 24 and 36 hours post-placement are shown in Figure 2. Viral titers of H6N2 were numerical higher at each time point in layers, followed by broilers, turkeys and quails. However, no significant differences were observed in viral titers recovered over time in feces from chickens, turkeys or quails (Fig. 2a). Analysis of H5N2 and H3N2 viral titers over time in feces of layers and broilers are shown in Figures 2b and 2c. Different from the H6N2 virus, the viral titers of H5N2 and H3N2 were numerically similar for layers and broilers and no significant differences ($p < 0.05$) were observed in viral titers over time (Fig 2b and 2c). The moisture content of feces samples was evaluated at different time points. Samples showed average moisture content of 73.02% at 0 hrs, 60.27% at 12hrs, 34.03% at 24hrs and 22.18% at 36hrs post-collection.

Discussion

It is known that replication and shedding of AI viruses are host, viral strain, and inoculation route dependent (Alexander 2007; Jackwood et al. 2010; Morales et al. 2009; Pillai et al. 2010a). In chickens, replication of LPAIV is restricted to the respiratory and intestinal tracts as

demonstrated by successful isolation from the oropharingeal and cloacal samples (Jackwood et al. 2010; Morales et al. 2009; Spickler et al. 2008). However under experimental conditions some LPAIV are shed exclusively through the respiratory tract (Jackwood et al. 2010; Zarkov 2008), while others showed shedding restricted to the intestinal tract (Jackwood et al. 2010; Shan et al. 2010; Zarkov 2008). Because our objective was to better understand the risk of AIV transmission in the poultry environment via contaminated feces, intestinal shedding by chickens, turkeys and quails of chicken adapted A/Ck/CA/431/00(H6N2), wild bird isolate A/Mallard/MN/355779/00(H5N2), and turkey isolate A/turkey/Ohio/313053/04(H3N2) were evaluated. Similar to other studies (Alexander 2007; Jackwood et al. 2010; Morales et al. 2009; Pillai et al. 2010a) the level of cloaca shedding and fecal contamination was highly variable and significantly influenced by host and viral strains. In this study regardless of the viral strain, turkeys and quails shed lower titers than broilers and layers, and independently of the host while fecal contamination with A/Ck/CA/431/00(H6N2) virus was significantly higher than H5N2 and H3N2. Reports of cloacal titers of LPAI H5 viruses from wild birds and turkeys, shed by chickens (Pillai et al. 2010b) and LPAI viruses, of the H4, H6 and H9 subtypes, from wild birds origin (9) were comparable to those detected from feces and cloacal samples of chickens infected with chicken H6N2, mallard H5N2 and turkey H3N2 isolates. In agreement with Morales et al (14) study, where shedding of H4, H6, and H9 wild bird origin viruses in turkeys was minimal, in this study shedding of the mallard H5N2 and turkey H3N2 isolates was not detected in turkeys. On the other hand, these results differ from Pillai et al., 2010 (Pillai et al. 2010b) where wild bird isolates of the H5 subtype where consistently shed by turkeys, and Pillai et al 2009 (Pillai et al. 2009) where turkey H3N2 isolate was detected two and four days PI in the cloaca of 26 week old turkeys. The potential of quails to serve as an intermediate host in the spread AI

viruses to poultry has been studied (Sorrell et al. 2007), without any adaptation AI viruses from wild birds showed to efficiently replicate in the respiratory tract with limited cloaca shedding in quails (Liu et al. 2003; Makarova et al. 2003; Sorrell et al. 2007). In this study neither the H5N2 mallard virus, or the H3N2 turkey virus were shed by quails. On the other hand, in this study the poultry adapted A/Ck/CA/431/00(H6N2) virus was detected in the cloaca of quails. In a recent study (Yee et al. 2009a) the A/CK/CA/1772/02(H6N2) virus that belongs to the California H6N2 group of viruses showed to effectively transmit from chickens to quails via aerosol and possibly via fomites.

The infectivity of LPAI viruses in aquatic habitats has been shown to be inversely proportional to water temperature and salinity (Brown et al. 2009; Nazir et al. 2010; Stallknecht et al. 1990a). In this study the infectivity of LPAI shed in feces kept at ambient temperatures ranging from 21 to 25 °C was 24 hours (1 day). The limited period of infectivity of LPAI viruses H6N2, H5N2, and H3N2 in feces greatly differs from infectivity of LPAI viruses in water at temperatures of 30 and 20 °C was 5 to 9 days, respectively (Nazir et al. 2010). The short infectivity of LPAI virus in feces observed can be attributed to the temperature in range in the experimental unit (from 21 to 24 °C), and the decrease in moisture content of feces over the 36 hours examination period. . In this study fecal droppings were placed in open containers with no humidity control and by 24 hours the humidity was not sufficient to keep the feces moist resulting in viral inactivation. Fecal material from mallard ducks infected with A/duck/Memphis/546/74 remained infective for four days at 22°C when mixed with river surface water (Webster et al. 1978). Watery feces from HP A/CK/Pennsylvania/1370(H5N2) infected layers collected at day 3 PI and without any humidity control at 25°C, virus infectivity was retained for 24 hours, when the humidity was increased the virus retained infectivity for up to 48 hours (Beard 1984). Indicating that increasing

the environment humidity extended the infectivity of the H5N2 HPAI virus in feces by 24 hours. We speculate that under controlled humidity the A/Ck/CA/431/00 (Webby et al. 2002) isolate may have retained its infectivity in feces for probably 48 hours as previously reported for HP poultry adapted A/CK/Pennsylvania/1370(H5N2). Overall this study shows that shedding and survivability of LPAIV is an extremely intricate process dependent on environmental factors, host, viral isolate degree of adaptation to the host, and viral tropism in that particular host.

Acknowledgement

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Figure 3. 1. Hemagglutination inhibition titers in layers, broilers, quails and turkeys at day 11 post-inoculation. HI titer shown as Log 2 for individual birds.

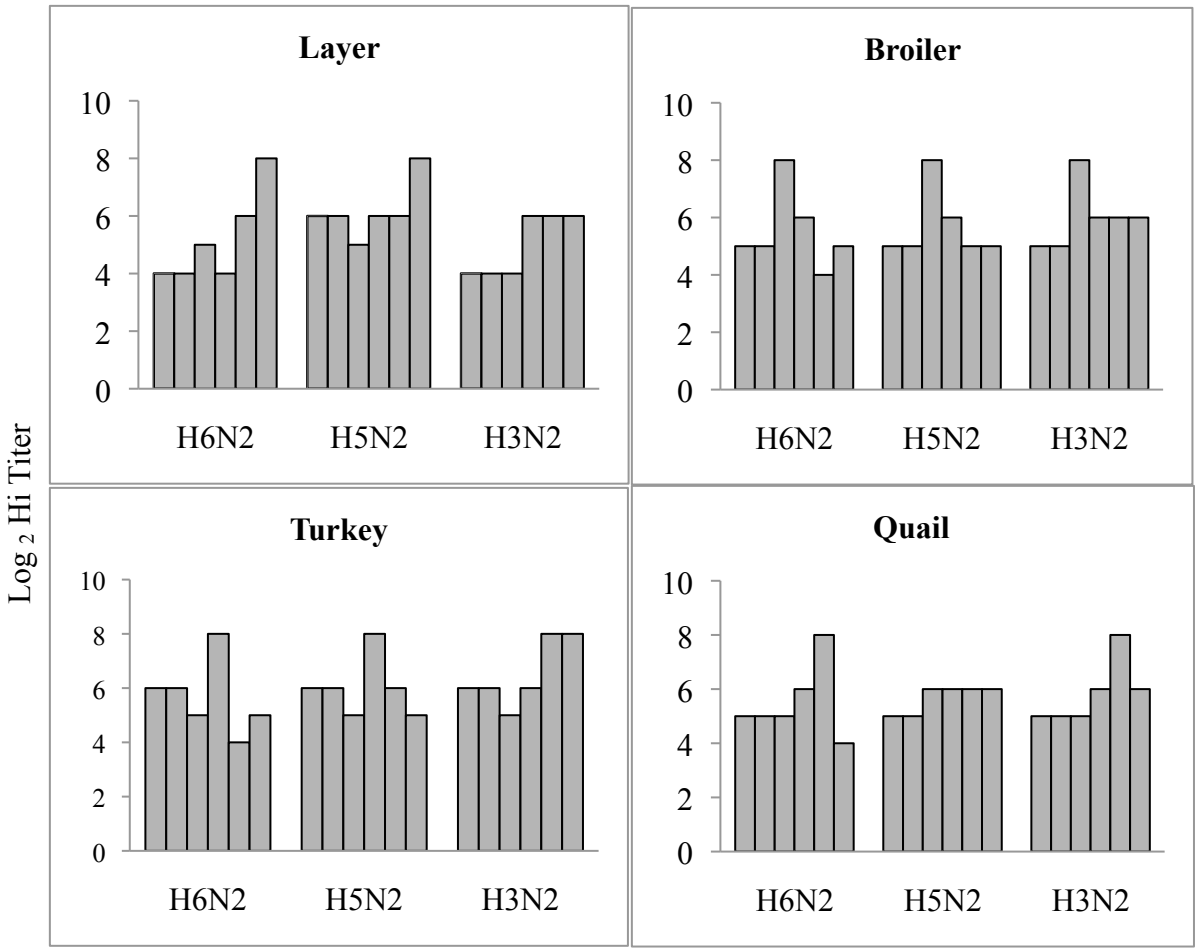


Table 3.1. Cloacal shedding of H6N2, H5N2, and H3N2 three days post-inoculation.

	H6N2	H5N2	H3N2
Layers	$4.42^{1\ A,a}(2.50)^{2}$	$1.50^{A,b}(0)$	$1.33^{A,b}(0.14)$
Broilers	$3.00^{A,a}(2.60)$	$1.50^{A,a}(0.87)$	$0.50^{A,a}(0.87)$
Turkeys	$0.47^{C,a}(1.39)$	$0.00^{B,b}(0)$	$0.00^{B,b}(0)$
Quails	$0.80^{C,a}(0.81)$	$0.00^{B,b}$ (0)	$0.00^{B,b}(0)$

[1]Average titer of three cloacal swab pools (two swabs) expressed as $\log_{10}$ EID_{50}.
[2] Standard deviation

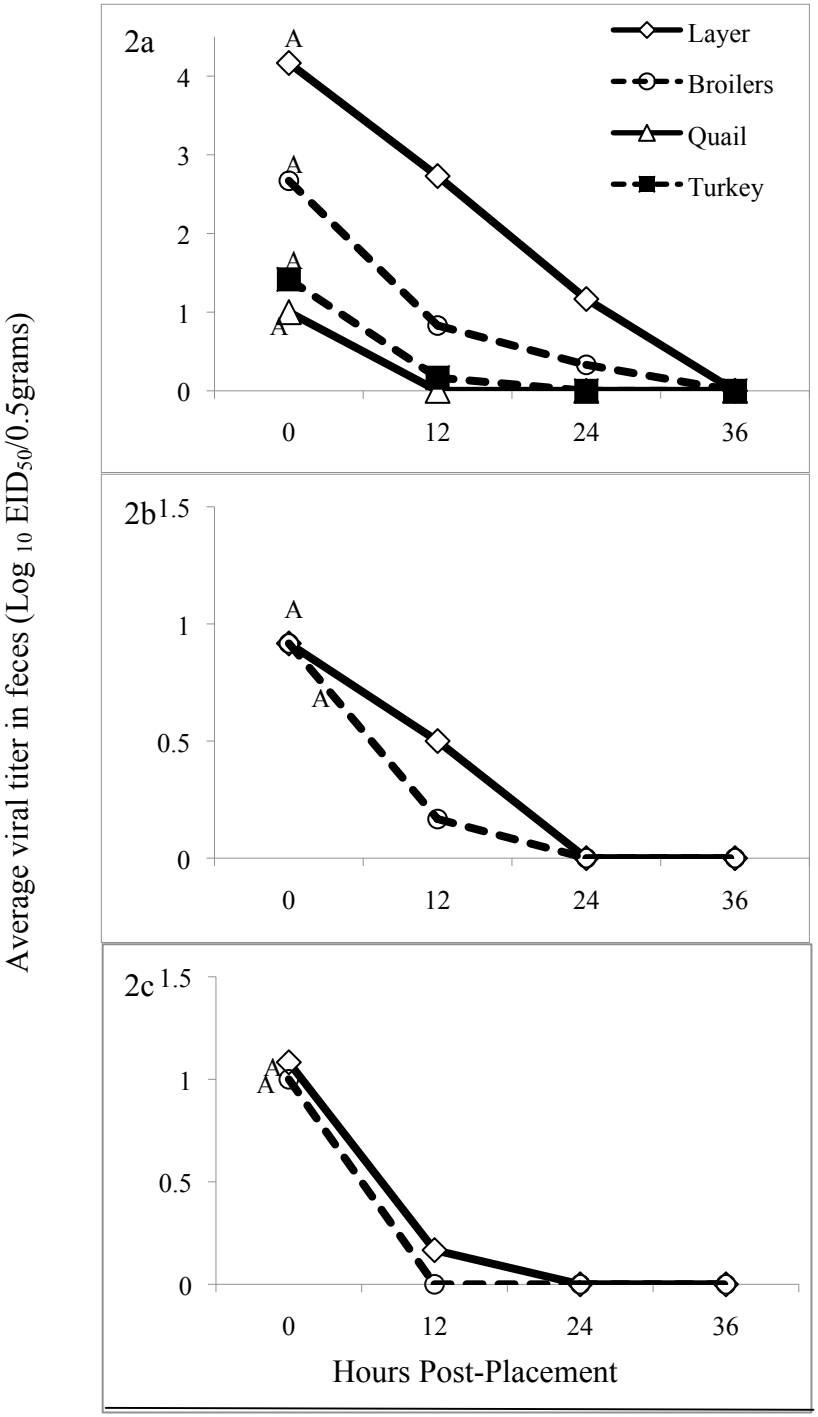
[A,B] Average titer with the same upper case were not significant different between the different species compared when infected with the same LPAI virus.
[a,b] Average titer with the same lower case showed no significant difference between the three LPAI virus compared in the same poultry species.

Table 3.2. Detection of virus in fecal droppings of H6N2, H5N2, and H3N2 three days post-inoculation

	H6N2	H5N2	H3N2
Layers	$4.17^{A,a}$[1](0.58)[2]	$0.92^{A,b}$(1.59)	$1.08^{A,b}$(0.95)
Broilers	$2.17^{A,a}$(0.58)	$0.92^{A,b}$(1.54)	$1.00^{A,b}$(0.87)
Turkeys	$1.42^{B,a}$(0.87)	$0.00^{B,b}$(0)	$0.00^{B,b}$(0)
Quails	$1.00^{B,a}$(0.14)	$0.00^{B,a}$(0)	$0.00^{B,a}$(0)

[1]Average titer of three fecal droppings (0.5grams each) expressed as $\log_{10}$ EID_{50}.
[2] Standard deviation
[A,B]Average titer with the same upper case were not significant different between the different species compared when infected with the same LPAI virus.
[a,b] Average titer with the same lower case showed no significant difference between the three LPAI virus compared in the same poultry species.

Figure 3.2. Tenacity of H6N2, H5N2, and H3N2 in feces from broilers, layers, turkeys, and quails. Mean viral titers (log_{10} EID_{50}/0.5 grams of feces) of (a) H6N2, (b)H5N2, and (c)H3N2 in feces over time (0, 12, 24, and 36 hours post-placement). Lines representing the different poultry species with the same upper case letter indicates no significant difference in tenacity of the LPAI viral strain.



Chapter 4

Survivability of Low Pathogenic Avian Influenza (LPAI) Viruses in Poultry Litter

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Abstract

To predict the risk of human infections associated with exposure to the poultry environment further knowledge on the survivability of LPAI viruses in this environment is necessary. The infectivity of the viruses A/Ck/CA/431/00(H6N2), duck isolate A/Mallard/MN/355779/00(H5N2), and turkey isolate A/turkey/Ohio/313053/04(H3N2) at 4 and 25 °C in contact with three types of poultry litter (wood shavings, shavings plus gypsum and shavings plus peanut hulls) from commercial flocks was evaluated. The LPAI viruses retained infectivity for at least 48 hours at 4°C and for 24hrs at 25°C in litter made of wood shavings and shavings plus peanut hulls, in litter made of shavings and gypsum viral isolates retained infectivity for up to 72hrs. The infectivity of A/Ck/CA/431/00(H6N2) virus in litter when shed by infected layers, broilers, and turkeys was retained for 24 hours in the three contaminated types of litter.

Introduction

Avian influenza viruses (**AIV**) are type A influenza viruses from the *Orthomyxoviridae* family (Lamb 2001). AIV can infect a wide variety of animals, from wild birds to mammals. Waterfowl like species of ducks and shorebirds are the natural host and reservoir of this diverse group of viruses. Type A Influenza viruses are classified on the antigenic profiles of the surface glycoproteins that include 16 hemagglutinin (H1 through H16) and nine neuraminidase (N1 through N9) subtypes, for a total of 144 possible different influenza virus subtype combinations (Causey 2008).

Although poultry is not the natural host of AIV, accidental spread to poultry occurs frequently (Alexander 2000), resulting in two distinct types of AI infections, the low pathogenic (**LPAI**) virus infection characterized by a mild respiratory disease with low morbidity and no mortality and the highly pathogenic (**HPAI**) virus infection characterized by severe respiratory disease accompanied with high morbidity and mortality(Spickler et al. 2008). The mechanisms by which AIVs from different or the same subtypes are transmitted, how frequently an infected bird can shed virus, or for how long the host can remain infective is extremely variable and depends on the viral strain, the species of bird infected, and a variety of environmental factors (Alexander 2007; Makarova et al. 2003; Pillai et al. 2010b; Yee et al. 2009a). In particular viral transmission is intimately related to the amount of virus released from the respiratory or intestinal tract of infected birds (Alexander 2007). Because of the high virus concentration in the respiratory tract, aerosol transmission is important in the spread of the disease, however the movement of infected poultry, poultry by-products and management of poultry wastes made mechanical transmission a main contributor in the circulation of the disease (McQuiston 2005; Swayne 2008a). Among wastes generated by the poultry industry, litter is the most significant in

volume and value (Bernhart et al. 2009; Coufal et al. 2006), and its disposal and management is vital in the control of infectious diseases. Experimentally it has been established that composting of poultry wastes contributes to the rapid killing of AI and ND viruses (Guan et al. 2009; Kinde et al. 2004), and on farm composting for was utilized for emergency disposal of carcasses during the LPAIV H7N2 outbreak (Akey 2003; Wilkinson 2007) very limited information exists regarding survivability of AI viruses in poultry litter. Lu et al., 2003 found that the infectivity of LPAI A/Ck/PA/3779-2/97(H7N2) viral stocks when mixed with litter from commercial layers at an ambient temperature of 15 to 20 °C was more than two days, and 24 hours at 30 to 37 °C. Using a similar protocol as the previously described by Lu et al., 2003, the first objective of this study was to determine the survivability of LPAI A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/Ohio/313053/04(H3N2) viral stocks when in contact with different types of poultry litter at 4 and 25 °C. Secondly, in order to mimic field conditions, the infectivity of these LPAI viruses in three types of litter when shed by infected layers, broilers, turkeys and quails was determined.

Material and methods

Viruses. Three viruses were used in this study, the poultry adapted A/Ck/CA/431/00(H6N2) originally isolated from a layer flock in California between 2000 and 2001 (Webby et al. 2002), duck isolate A/Mallard/MN/355779/00(H5N2) originally isolated from a cloacal swab of a mallard (Costa et al. 2010b), and turkey isolate A/turkey/ Ohio/313053/04(H3N2) originally isolated from a 34 week old turkey breeder flock in Ohio (Tang et al. 2005). To conduct experiments, viruses were propagated in 10-day-old specific-pathogen-free (**SPF**) embryonated chicken eggs (**CE**) as previously described (Swayne 2008b).

The second embryonated CE passage of each virus was utilized to spike litter samples in the laboratory and to infect chickens, turkeys and quails.

Survivability of A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/ Ohio/313053/04(H3N2) viral stocks in different types of broiler litter. A total of 0.5 grams of broiler litter from a local commercial flocks made of pine shavings (**shavings)**, pine shavings plus gypsum (**gypsum**), and pine shavings plus peanut hulls (**peanut hulls**) were inoculated with 1 ml of H6N2, H5N2, and H3N2 viral stocks in allantoic fluid at a titer of $10^{7.5}EID_{50}$/ml, mixed and placed in snap cap tubes. As a negative control 0.5g of the different litters were inoculated with uninfected allantoic fluid; and as a positive control distilled water (900µl) was inoculated with 100µl of H6N2, H5N2, and H3N2 viral stocks ($10^{7.5}EID_{50}$/ml). Tubes were incubated at 25°C and 4°C, three samples of litter (0.5 grams/sample) incubated at 25°C were collected at 0, 24, 48, 72 and 96 hrs, and three samples of litter incubated at 4°C were collected at 0, 24 and 48 hrs for virus isolation and titration as described above.

***Experimental infection of chickens, turkeys and quails with* A/Ck/CA/431/00(H6N2).** The H6N2 chicken adapted virus was selected for this experiment based on a previous study where this virus was characterized by its elevated shedding via the intestinal route of chickens (Reis et al unpublished data). Two experiments were performed, one to evaluate the infectivity of A/Ck/CA/431/00(H6N2) shed by layers into different types of litter (shavings, gypsum, peanut hulls), and a second experiment to evaluate the infectivity of A/Ck/CA/431/00(H6N2) shed by layers, broilers, turkeys and quails into shavings based litter. Briefly, 18 three week-old layers were inoculated intranasally with A/Ck/CA/431/00(H6N2) virus at a dose of $10^{7.2}EID_{50}$ per bird in a volume of 200µl, and six layers were inoculated intranasally with 200µl of non-infected allantoic fluid as negative controls. Infected and non-infected layers were distributed into four

stainless-steel, polycarbonated, negative pressure, HEPA filtered, isolator units with filtered-air at the Poultry Diagnostic and Research Center (PDRC, University of Georgia). A bed of shavings, gypsum, and peanut hulls (15.4 pounds/unit) from commercial broiler flocks was added to each unit. The birds were fed a standard diet and water *ad libitum*. Three days post infection (**PI**), three pools of cloacal swabs (two swabs per pool) were collected from each treatment group and transported in brain-heart infusion (**BHI**- Sigma Alderich) with antibiotics; litter from infected and non-infected layers was collected and placed in three different containers inside each unit. At 0, 12, 24, 36, and 48 hours post placement ten grams of litter were collected from each container in each unit, resuspended in 30 ml of BHI (Sigma Alderich) with antibiotics and processed for virus isolation and determination of viral titers in embryonated CE following procedure described above. In the second experiment, six 3 week-old layers, broilers, turkeys and six 8 week-old quails were inoculated with A/Ck/CA/431/00(H6N2), while six 3 week old layers, broilers, turkeys and 8 weeks old quails were inoculated with non-infected allantoic as described above. Infected and non-infected birds were distributed into eight isolator units prepared with a bed broiler pine-shaving litter (15.4 pounds/unit). At three days PI, three pools of cloacal swabs were collected, and litter from infected and non-infected birds were distributed in three open containers that remained inside each unit during the experiment. At 0, 12, 24, and 36 hours post-placement 10 gr of litter were collected from each container and resuspended in 30 ml of BHI (Sigma Alderich) with antibiotics and processed as described above for viral isolation and titration.

Sample processing for viral Isolation and titration.

Briefly 0.5 grams of litter from the in vitro experiments and 10 grams of litter from the experimentally infected birds were mixed with 2 and 30 ml of brain heart infusion media (BHI,

Sigma-Aldrich), respectively. BHI media contained 10000u/L penicillin-G potassium (Fisher Scientific), 250g/L gentamycin sulfate (Fisher Scientific), 25mg/L of amphotericin B (Fisher Scientific), 500g/L kanamycin (Sigma-Aldrich) and 1g/L of streptomycin sulfate (Sigma-Aldrich). Tubes were mixed by vortex for 30 minutes at room temperature, followed by centrifugation at 4,000 rpm for 5 minutes. Supernatants were collected and filtrated through a 0.45µm pore size syringe filter (Whatman), and filtrates were used for virus titration in embryonated CE. Cloacal swab pools were collected and resuspended in BHI and antibiotics as described above, after collection tubes were vortexed and stored at -80^0C for virus titration. Virus titrations were done in 9 to 11 day old SPF embryonated CE. Briefly, undiluted (200 µl) and a 10^{-1} to 10^{-7} serial dilution of the processed samples were inoculated in the allantoic cavity of embryonated CE, in triplicates. After inoculation, eggs were incubated for four days and candled daily to monitor mortality rates. On the fourth day, surviving embryos were stored at 4°C for 3 to 6 hours. Allantoic fluid from each embryo was collected and tested for hemagglutinin (HA) activity (Swayne 2008b; Thayer 2008). Samples were considered positive in samples were HA titer was higher than 2. To confirm that the HA activity was caused by AIV viruses, samples with positive HA activity were tested by the hemagglutination inhibition (**HI**) assay. Viral titers were determined using Reed and Muench (Reed 1938), and expressed as embryo infectious dose $(EID)_{50}$. Mean titer per treatment was estimated from three independently collected samples.

Hemagglutination Inhibition (HI) assay.

The HI assay was used for confirmation of isolated viruses and for serologic testing of inoculated birds. To confirm the presences of AIV in HA positive samples hyper-immune sera against chicken H6N2, duck H5N2 and turkey H3N2 isolates were produced. Briefly three groups of

seven day-old chickens were immunized intravenously with $10^{7.5}EID_{50}$/ml of each virus. Three weeks after the first immunization, chickens were boosted intravenously with similar doses of each virus. Fifteen days after the second immunization, sera were collected and used in the HI assay to confirm presence of AIV antibodies following protocols previously described (Swayne 2008b; Thayer 2008). Hemagglutinin inhibition antibody titers on sera from infected layers, broilers, turkeys and quails with HI titers ≥ 4 $\log_2$ were considered positive (Thayer 2008).

Statistical Analysis. Linear regression models were used to demonstrate the infectivity of the tested LPAI viruses in different types of litter. The viral titers ($\log_{10}$) of H6N2, H5N2, and H3N2 in litter at different time points were utilized to create linear regression equations (PASW statistics 18. SPSS, Chicago, Illinois). Differences in infectivity was statistically evaluated by comparing viral titers over time using the repeated measure ANOVA (PASW statistics 18. SPSS, Chicago, Illinois).

Results

Infectivity of A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/ Ohio/313053/04(H3N2) viral stocks in broiler litter

Viral stocks of H6N2, H5N2, and H3N2 isolates with an initial titer of $10^{7.5}EID_{50}$/ml were incubated with different types broiler litter, collected from either commercial or experimentally kept broilers, at 4°C for 48 hours. Viral titers for the H6N2, H5N2, and H3N2 isolates at 0, 24, and 48 hours are shown in figures 1a, 1c, and 1e, respectively. At 4°C no significance difference ($p < 0.05$) in viral titers was observed among H6N2, H5N2, and H3N2 isolates when incubated in the same type of litter or when the same isolate was incubated in different types of litter (Table 1). At 25°C viral titers for H6N2, H5N2, and H3N2 isolates are

shown in figures 1b, 1d, and 1f, respectively. Titers for the three viral isolates were detected in shavings and peanuts hulls broiler litter for 48 hrs PI and for 72 hrs in gypsum litter. At 25°C viral titers over time for the three viral isolates was significantly lower ($p < 0.05$) in shavings and peanuts hulls than in the gypsum litter as indicated by the slope differences in the linear regression model (Table 1). Forty eight hours PI, at 4°C, viral titers detected in distilled water were no significant different to those found in the gypsum litter ($p < 0.05$ data not shown). While ninety-six hours PI, at 25°C, average viral titers of 3.75 $\log_{10}$ were detected in water while no viral titers were detected in any of the litter treatments (Figure 1b). No virus was isolated from non-infected litter (data not shown).

Viral titer in cloacal swabs.

Average viral titers in cloacal swab pools of H6N2 infected layers placed in contact with pine shavings, gypsum and peanut hulls litter ranged from 4.33 to 4.42 $\log_{10}$ (data not shown). The average viral titers in cloacal swabs pools from layers, broilers, turkeys and quails infected with H6N2 isolate were 4.40, 4.5, 2.28 and 0.42 $\log_{10}$, respectively (data not shown).

HI Antibody Titers.

At 11 days PI H6N2 infected layers, broilers, turkeys and quails showed HI titers ranging from 4 $\log_2$ to 8 $\log_2$ confirming infection of the different poultry species (data not shown). None-infected birds showed no HI titers by the end of the experiment indicating no transmission to the negative controls.

Infectivity of H6N2 virus shed by layers in different types of litter.

Ten grams of litter contaminated with fecal droppings from H6N2 infected layers was collected at 3 days PI (or 0 hours post placement). An average viral titer of $10^{1.83}$ EID_{50} was detected in shavings and peanut hulls litters and an average viral titer of $10^{1.5}$ EID_{50} was detected in gypsum

litter. Viral titers were detected at 12 and 24 hours but not at 36 and 48 hours post-placement in the three bed litters tested (Figure 2). No significant differences ($p < 0.05$) were observed in decrease of viral titers over time for the H6N2 isolate when in contact with shavings, peanut hulls and gypsum types of litter as shown by the slope differences in the linear regression model (Table 2).

Survivability of H6N2 virus shed by layers, broilers and turkeys in pine shavings litter.

Pine shavings litter contaminated with fecal droppings of H6N2 infected, layers, broilers, turkeys and quails were collected at 3 days PI (or at 0 hours post placement). The average H6N2 virus titer detected in shavings litter in contact with infected layers and broilers was $10^{1.80}$EID $_{50}$/10grs and $10^{1.50}$EID $_{50}$/10grs in shavings litter in contact with infected turkeys (Figure 3). No virus was detected in shavings litter in contact with infected quails. Viral titers were only detected at 12 hrs post-placement of litter in contact with infected layers, while at 24 hours an average titer of $10^{0.5}EID_{50}$ in litter contaminated with infected layers, broilers, and turkeys (Figure 3). By 36 hours post-placement no virus was detected in litter contaminated with infected layers, broilers, or turkeys. The lack of virus isolation from litter in contact with infected broilers and turkeys at 12 hours post-placement most likely reflects a collection error. No significant differences in viral titers of the H6N2 virus over time at 3 days PI when in contact with shavings litter as shown by the slope differences in the linear regression model (Table 3). No significant differences ($p < 0.05$) were observed in the decrease of viral titers over time shed by layers, broilers or turkeys when in contact with shavings litter as shown by the slope differences in the linear regression model (Table 2).

Discussion

The survivability of A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), and A/turkey/Ohio/313053/04(H3N2) LPAI viral stocks was evaluated by determining viral titers over time after exposure to broiler litter using an in vitro experimental model. The results of this model indicated that similar to the results obtained for aquatic habitats (Brown et al. 2009; Davidson et al. 2010; Nazir et al. 2010; Stallknecht et al. 1990a; Stallknecht et al. 1990b) temperature greatly impacted the infectivity of LPAI viruses in all three types of litter tested. In contrast to viral survivability results obtained in water (Brown et al. 2009), in litter, at 4°C no differences in infectivity were observed among the three viral isolates tested during the 48 hour period tested, based on the long periods of infectivity observed in water it can be speculate that at 4°C the survivability of viral isolates in litter will be more than 48 hours. At 25°C viruses retained infectivity for 24 hours in litter made of shavings and shavings plus peanuts hulls and for 72 hours in litter composed of shavings plus gypsum. The longer infectivity showed by the three viral isolates in the gypsum litter was due to the hydrophobicity of the litter gypsum component that did not allowed the allantoic fluid to be completely absorb and consequently the virus was not as effectively inactivated as with the shavings and shavings plus peanut hulls litter treatments. Reports on survivability of pneumovirus (Velayudhan et al. 2003), velogenic NDV (Bankowski et al. 1975), and LPAI A/Ck/PA/3779-2/97(H7N2) (Lu et al. 2003) viral stocks when in contact with litter at ambient temperatures ranging from 21 to 25 °C, estimated viral inactivation to take three, four, and seven days, respectively. Its been shown that pH, and salinity are important factors that influence the survivability of LPAI viruses in water (Brown et al. 2009; Stallknecht et al. 1990b; Webster et al. 1978). A pH measure of 7.4 to 8.2 was determined to be the optimal range for stability of LPAIV in water (Brown et al. 2009). The pH

of shavings, shavings plus gypsum and shavings plus peanut hulls in used broiler litter after the addition of the allantoic fluid viral stocks ranged from 8.0 to 8.4. Therefore its proximity to the optimal pH in water suggests that pH was not a factor that influenced the inactivation of the H6N2, H5N2 and H3N2 LPAI viruses after 24 hours of contact with litter. One possibility to explain the lost of infectivity of LPAI viral stocks by 24 hours is that the allantoic fluid added to samples may promote the bacterial growth at high temperatures, resulting in decreased viral titers due to viral inactivation by bacterial metabolites or removal of viral particles by adherence to bacteria (Nazir et al. 2010). In order to better mimic field conditions the survivability of LPAI A/Ck/CA/431/00(H6N2) in litter contaminated with infected layers, broilers, and turkeys was evaluated. There was no difference in infectivity of the H6N2 virus in the different types of litter in contact with infected layers, boilers, or turkeys indicating that neither the type of litter bed nor the source of fecal contamination had an impact in the ability of the H6N2 LPAI virus to retain infectivity. Lu et al (2003) described positive viral isolation of LPAIV subtype H7N2 from manure of infected SPF chickens for up to 21 days after challenge. However, manure remained in contact with infected birds that could be re-introducing virus into the manure. In contrast in this study the H6N2 retained infectivity for a period of 24 hours in poultry litter out of contact with infected animals, and therefore no more infective virus was shed into the litter during the time periods tested. One important factor that most likely influenced the shorter infectivity of the H6N2 virus in contaminated litter was the decreased moisture over the 36 hours period during examination of the litter viral content. In this study infected litter was placed in open containers with no humidity control. Moisture content was shown to be very important factor that prolonged the infectivity of HP A/CK/Pennsylvania/1370(H5N2) in feces (Hemmes et al. 1960). The moisture content in the shavings and shavings plus peanut hulls decreased from 31 to

7.7% and the gypsum litter moisture content decreased from 19 to 8% in a 36 hour period (data not shown), the decrease moisture content of the litter most likely caused viral inactivation by 24 hours. Even though in this study LPAI viruses retained infectivity in litter for only 24 hours, biossecurity measures, disinfection of chicken houses, and proper handling of poultry by products, such as the composting of carcasses and windrow composting of litter during AI outbreaks should be implemented, particularly in lower temperature climates.

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Table 4.1. Linear regression models of H6N2, H5N2 and H3N2 in different types of litter

		Shavings		**Shavings plus gypsum**		**Shavings plus peanuts hulls**	
Virus	Temperature (°C)	Linear regression model1	R2	Linear regression model	R2	Linear regression model	R2
H6N2	4	y = 6.62 - 1.04xA	0.964	y = 7.04 – 0.712xA	0.894	y = 5.15 – 0.625xA	0.683
H6N2	25	y= 5.97 – 3.03xB	0.968	y = 7.17 – 1.77xC	0.989	y = 5.38 – 2.63xB	0.982
H5N2	4	y = 6.34 - 0.683xA	0.786	y = 6.94 – 0.667xA	0.873	y = 5.08 – 0.583xA	0.653
H5N2	25	y = 6.19 - 3.07xB	0.991	y = 6.88 – 1.74xC	0.992	y = 5.32 – 2.63xB	0.982
H3N2	4	y = 6.38 – 0.817xA	0.914	y = 6.92 – 0.617xA	0.851	y = 5.13 – 0.625xA	0.893
H3N2	25	y = 6.10 – 3.03xB	0.993	y = 6.83 – 1.77xC	0.986	y = 5.33 – 2.63xB	0.994

y= $\log_{10}EID_{50}$; x= persistence in hours; A,B,C Different upper case indicates significant differences in slopes between models.

Figure 4.1. Survivability of LPAIV in different types of litter evaluated in vitro. Survivability of LPAIV H6N2 (A and B), H5N2 (C and D) and H3N2 (E and F) are shown as the average viral titer $\log_{10}$ EID_{50} from 0 to 48 hrs post-collection at 4°C (A, C and E) and from 0 to 96 hrs post-collection at 25°C (B, D and F).

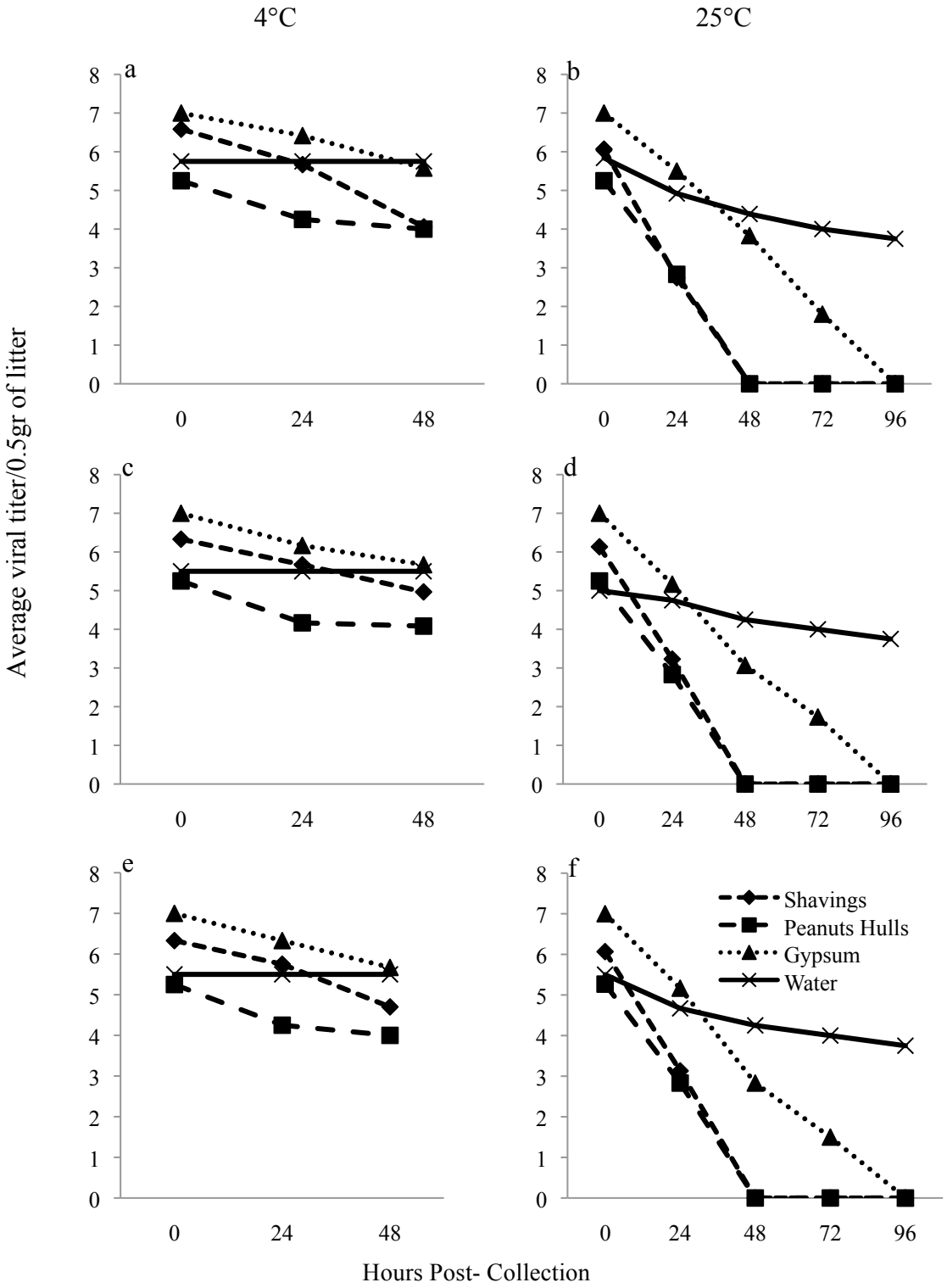

Figure 4.2. **Infectivity of A/Ck/CA/431/00(H6N2) shed by layers in different types litter**. Average viral titers from 0 to 48 post-collection are shown as shown as $\log_{10}$ EID_{50}.

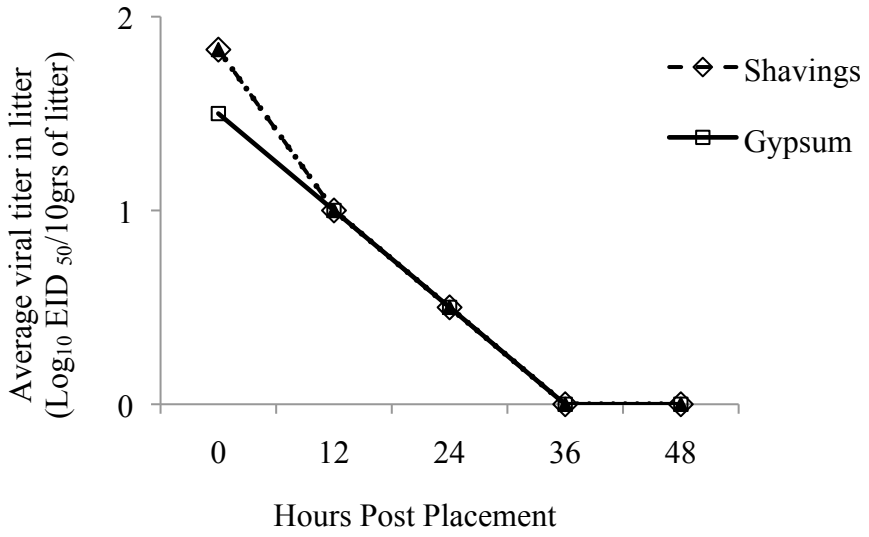

Table 4.2. Linear Regression models for persistence of H6N2 shed by layers in different types of Litter.

Litter Type	Virus	Species	Linear regression model1	R2
Shavings	H6N2	Layer	y = 1.93 – 0.0542xA	0.466
Shavings plus Peanuts Hull	H6N2	Layer	y = 1.93 – 0.0542xA	0.466
Shavings plus Gypsum	H6N2	Layer	y = 1.50 – 0.042xA	0.556

y= $\log_{10}EID_{50}$; x= persistence in hours; A,B Different upper case indicates significant differences in slopes between models.

Figure 4.3. Survivability of A/Ck/CA/431/00(H6N2), shed by layers, broilers and turkeys, in litter made of shavings. Average viral titers from 0 to 36 post-collection expressed as $\log_{10}$ EID_{50}.

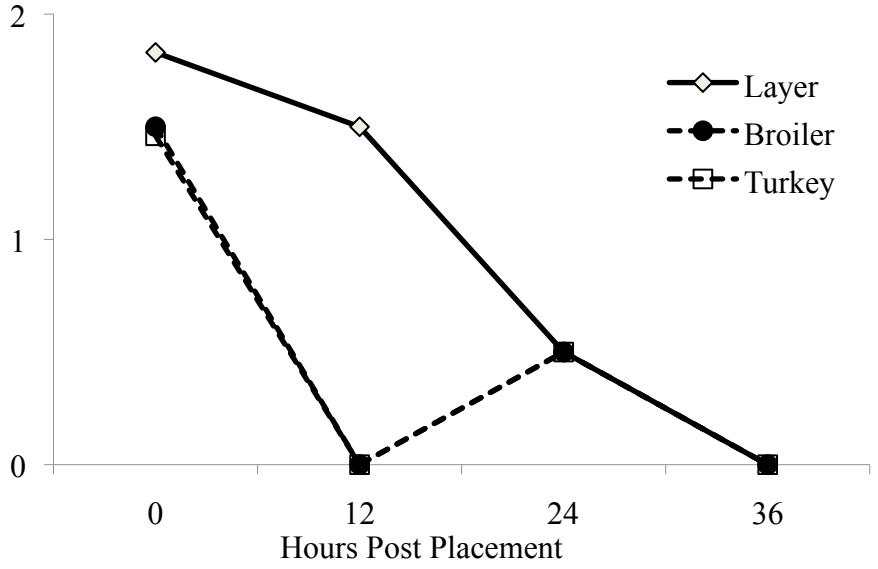

Table 4.3. Linear regression models for persistence of H6N2 shed by layers, broilers and turkeys in litter.

Virus	Species	Linear regression model1	R2
H6N2	Layer	y = 1.93 – 0.0542xA	0.466
H6N2	Broiler	y = 1.10 – 0.033xA	0.400
H6N2	Turkey	y = 1.10 – 0.033xA	0.400

y= $\log_{10}EID_{50}$; x= persistence in hours; A,B Different upper case indicates significant differences in slopes between models.

Chapter 5

Evaluation of Neuraminidase (NA) Subtypes 1 and 2 ELISAs for Detection of Avian Influenza Vaccinated/Infected Turkeys Using an NA Heterologous Vaccination Strategy

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Abstract

The development of a serological assay that allows differentiation between vaccinated/infected and vaccinate/non-infected; can aid the control of AIV in a vaccinated population. In this study we present the establishment of N2-ELISA and evaluated the ability of N1-ELISA and N2-ELISA in correctly identify infected birds. The sensitivity and specificity of N1-ELISA was 0.88 and 0.94 respectively, while the sensitivity and specificity of N2-ELISA was 0.90 and 0.94 respectively, as compared to NI assay. The N1-ELISA was able to identify 33% of H1N2vaccinated/H1N1infected turkeys, while N2-ELISA was able to identify 50% of H1N1vaccinated/H1N2challenge turkeys. Overall, the N1-ELISA and N2-ELISA showed a lower sensitivity than NI, but it was demonstrated that screening of NA antibodies by ELISA was an effective and rapid assay to identify exposure to the challenge virus during a DIVA vaccination strategy, however, the NA-ELISA should be utilized and interpreted as a flock assay.

Introduction

Poultry and poultry products are a major source of high-quality protein and the per-capita consumption has been increasing around the world. Maintaining poultry free from pathogens, particularly AI is essential to continue the trade of poultry products worldwide (Swayne 2003c). Avian influenza (AI) viruses are type A influenza virus belonging to the *Orthomyxoviridae* family. They are enveloped viruses with segmented single-negative-stranded RNA genome (Lamb 2001) and are classified in 16 hemagglutinin (H1 through H16) and 9 neuraminidase (N1 through N9) subtypes (Causey 2008). AIV infections in poultry can be differentiated in two distinct types based on the ability of these viruses to cause disease in chickens: the low pathogenic AIV (LPAI) and the highly pathogenic AIV (HPAI). The HPAI viruses are characterized by their high morbidity and mortality. Most of the highly pathogenic outbreaks of poultry have been of the H5 and H7 subtypes (Spickler et al. 2008).

The preferred control measures to control H5 and H7 AI outbreaks is the culling of infected poultry, which has been consistently successful in previous outbreaks including the outbreak in Hong Kong in 1997 and the Netherlands in 2003 (Lipatov et al. 2004). However, in cases where low pathogenic AI is endemic as in Mexico the H5N2 (Villarreal 2009), and in the Middle East the H9N2 (Banet-Noach et al. 2007), the current strategy to control avian influenza is biosecurity together with vaccination. Vaccination has potential benefits, such as the reduction of clinical signs of diseases, reduction of viral shedding and increases resistance of flocks be re-infected (Capua et al. 2003a). However, vaccination also has drawbacks, vaccination with inactivated vaccines hinders serological surveillance to identify infected flocks, since vaccinated flocks cannot be distinguished from naturally infected birds, subsequently the inability to

identify infected flocks severely affects international trade of poultry products (Suarez 2005; Swayne 2003a).

Therefore, it is important to differentiate naturally infected from vaccinated birds and also to identify vaccinated birds that become infected (Suarez 2005). For AI four different approaches for differentiation of infected from vaccinated animals (DIVA) vaccination strategies have been suggested: 1) the use of sentinels (Suarez 2005) 2) the use of subunit vaccines expressing one or both the HA and NA antigens and screening for nucleoprotein antibodies to identify infected birds (Pavlova et al. 2009; Swayne et al. 2000), 3) use of inactivated vaccines and screen for nonstructural (NS)-1 antibodies to detect infected flocks (Avellaneda et al. ; Tumpey et al. 2005; Zhao et al. 2005), 4) the heterologous neuraminidase strategy, which requires a vaccine with homologous HA and heterologous NA from the circulating AIV, infected flocks are identified by the presence of NA antibodies to the challenge virus (Suarez 2005; Swayne et al. 2007; Zhao et al. 2005). Although antibodies to both HA and NA subtype can be protective, antibodies to the HA are the most important in protection (Nayak et al. 2010; Suarez 2005). The heterologous NA strategy was proposed more than 20 years ago (Beard 1986) but was first applied successfully to control the H7N1 HPAI 2000 outbreak in Italy by vaccinating turkeys with an inactivated H7N3 vaccine and monitoring for NA subtype 1 antibodies using an indirect immunofluorescent assay (iIFA) (Capua et al. 2003b, c; Capua et al. 2002). A similar strategy was utilized a second time in Italy to control an outbreak of low pathogenic H7N3, birds were vaccinated with inactivated H7N1 and monitored using iIFA to detect antibodies against the NA subtypes 3 and 1 (Cattoli et al. 2006; Suarez 2005). The successful use of the heterologous NA DIVA strategy in Italy increased the interest in its use. Although the use of iIFA was successful in detecting infected birds, this types of fluorescent

based assays are not suitable for high-throughput screening of sera samples, requires fluorescent microscope, and depends on individual interpretation skills that in some cases is difficult (Suarez 2005). Another diagnostic assay that has been useful for the DIVA NA approach is the neuraminidase inhibition (NI) assay which relies in the detection of the neuraminidase enzymatic activity of viable virus that can be measure by a colorimetric substrate as positive or negative (Van Deusen et al. 1983) or by a fluorescent substrate where the reaction can be quantified (Avellaneda et al.). Similar to the HI assay the NI assay requires the propagation and handling of infectious virus, therefore is not applicable for the use in laboratories worldwide, To overcome the disadvantages of the iIFA and NI tests, indirect ELISA systems to detect antibodies to the NA subtype 1 (Liu et al., 2010), NA subtype 2 (Kwon et al., 2009) as well as competitive ELISA systems for the detection of N1, N2 and N3 (Kim et al. 2010; Kwon et al. 2009; Liu et al. 2010; Moreno et al. 2009) have been developed. Overall the indirect N1 and N2 were capable to detect infected birds, however the sensitivity if the indirect N1 ELISA was reduced compared to NI (N1) (Liu et al., 2010). Competitive ELISAs showed increased sensitivity when compared to indirect ELISA (Kim et al., 2010) and to NI assay (Moreno et al., 2009). However, the ability of the indirect and competitive ELISAs to identify vaccinated/infected birds was not clearly evaluated in these studies. The main objective of this study was to evaluate the ability of indirect N1 and N2 ELISAs to identify experimentally vaccinated/infected turkeys using heterologous NA DIVA vaccination strategy. To accomplish this objective we developed an N2 indirect ELISA using a recombinant purified N2 protein, and together with the indirect N1 ELISA (Liu et al., 2010) evaluated the sensitivity and specificity of these assays as compared to the NI assay, and to evaluate the ability of the NA-ELISAs to detect infected, vaccinated, and vaccinated/infected turkeys.

Material and methods

Viruses. Three viruses were used in this study, a A/turkey/Ohio/313053/04(H3N2) originally isolated from a 34 week old turkey breeder flock (Tang et al. 2005), a A/turkey/Missouri/24093/1999 (H1N2), isolated from turkey breeders with a sudden drop in egg production (Suarez et al. 2002), and the 1988 H1N1 turkey virus of swine origin. Viruses were propagated in 10-day-old specific-pathogen-free (SPF) chicken embryos (Tweed et al.) as previously described (Swayne 2008b). The second CE passage of each of the viruses was utilized to infect birds and to prepare inactivated vaccines.

Cloning, expression, and propagation of recombinant N2 protein. The coding sequence of the neuraminidase-2 gene of Influenza A/turkey/Ohio/313053/04(H3N2) was amplified using concensus primers for the N2 gene forward 5'-CTA GCT TCA TTT ACA ATG G-3' and reverse: 5'-TTATATAGGCATGAGATTGATATCC-3'. The PCR product was gel purified (QIAquick gel extraction kit, Qiagen, Valencia CA) and cloned into the plasmid pCR2.1-TOPO using the Topo TA cloning kit (Invitrogen, Carlsbad, CA). The isolated recombinant plasmids were sequenced to confirm N2 sequence insert. Based on the sequence of the plasmid, primers were designed, the forward primer (5'-ACG GAA TTC ATG GAC ATT TTT AGA ATT TGC TCT CTA ATC GTG ATC GCG ATG TGC GCG ACC GGT AGC GAC ACG ACT ACT GTA ACA TTG CAT TTC AGG C-3') replaced the natural signal peptide of the N2 protein by a signal peptide from the structural efp protein from *Leucania separata Nucleopolyhedrovirus (LsNPV)* and the reverse primer (5'-CGT TCT AGA CTA ATG GTG ATG GTG ATG GTG ACT TCT TCT ACT GTT TGA GCT CCA CCA TAC-3') introduced a coding sequence for a C-terminally added 6xHis oligopeptide. Restriction sites facilitated cloning in the pFastBacDual (Invitrogen, Carlsbad, CA) and placed the manipulated N2 gene downstream of the baculovirus

polyhedrin promoter. Inserts of recombinant plasmids were sequenced to confirm identity (pFAST-TKN2). Plasmid DNA was used to generate recombinant baculovirus using the Bac-to-Bac system (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer. The recombinant N2-bacmid was then amplified and purified and used to transfect Spodoptera frugiperda (Sf9) insect cells in serum-free medium (SFM) using Cellfectin (Invitrogen). Recombinant baculovirus (TKN2-Bac) was harvested at 6 days post-transfection. To generate a viral stock the recombinant virus was plaque purified and subsequently propagated in Sf9 cells suspension cultures (5.0 x 10^5 Sf-9 cells/ml) for 72 hours incubation at 28 °C under agitation. The viral stock was titrated by plaque assay following the procedure provided by the manufacturer.

Immunofluorescence analysis (IFA). Expression of recombinant N2-His was analyzed by IFA using anti-6xHis monoclonal antibody (BD Bioscience, Franklin, Lakes, NJ), and sera from chickens infected with A/turkey/Ohio/313053/04(H3N2) to determine if chicken antibodies recognized the recombinant N2-His protein. Briefly, 5x10^5 SF-9 cells were infected at MOI of 0.5, 24hrs post infection (PI), the medium was removed, -20°C ethanol was added to cells, and cells were incubated at room temperature for 30 minutes. Cells were rehydrated with 5% calf serum in PBS for 10 minutes. Subsequently, infected and non-infected cells were incubated at room temperature for 1 hour with anti-6xHis monoclonal antibody (Clontech, Mountain View, CA) at a 1: 500 dilution in Phosphate buffered saline (PBS), or 1:100 chicken sera against A/turkey/Ohio/313053/04(H3N2). The cells were washed 3 times with PBS. After washes the cells were incubated for 1 hr at room temperature with a 1:300 dilution of HPR- conjugated-anti-mouse antibody (KPL, Gaithersburg, MD) or a 1:300 dilution of HPR- conjugated- anti-

chicken antibody (KPL, Gaithersburg, MD). Cells were washed three times and overlaid with DABCO-glycerol diluted 1:1 with PBS.

N2-His protein purification. Several batches of N2-His protein were produced using Sf9 cell suspension culture supernatants (60 to 100 ml) infected at an M.O.I. of 2 with the N2-Bac virus at a cell density of $1x10^6$cells/ml. At 72h p. i. the supernatants were collected and centrifuged at 10,000 rpm for 5 minutes to remove the cells. The N2-His was purified from the clarified supernatants by immobilized metal affinity chromatography (IMAC) following the protocol recommended by the manufacturer (Clontech, Mountain View, CA). Briefly, 10 ml of the obtained supernatants were loaded onto the Talon resin column and mixed. The column was washed with 1x washing buffer three times. N2-His protein was eluted with 300mM imidazole elution buffer provided by the manufacturer. The purity and yield of N2-His protein was evaluated in Coomassie (Pierce, Rockford, IL) stained SDS10%PAGE gels, the concentration of N2-His protein was measured using the BCA Protein Assay (Pierce, Rockford, IL).

Western Blot analysis. Purified recombinant N2-His was analyzed by western blot using an anti-6xHis monoclonal antibody (BD Bioscience, Franklin, Lakes, NJ) and sera from chickens infected with A/turkey/Ohio/313053/04(H3N2) to determine if chicken antibodies recognized the same size protein as the anti-6xHis monoclonal antibody. Briefly, 20 μl of purified N2-His protein were separated by SDS-10%PAGE and transferred onto nitrocellulose (NC) membranes (Bio-Rad, Hercules, CA). The membrane was blocked in a Tris-buffered saline (TBS) (Thermo Scientific, Rockford, IL) containing 5% bovine serum albumin at 4°C overnight. Subsequently, the membrane was incubated at 37°C for 1 hour with anti-6xHis monoclonal antibody (Clontech, Mountain View, CA) at a 1: 10,000 dilution in a 3% BSA-TBS buffer. The membrane was washed 3 times with TBS-0.05% Tween 20. After washes the blot was incubated for 1 hr at 37

°C with a 1:2000 dilution of HPR- conjugated- anti-mouse antibody (KPL, Gaithersburg, MD). After washing of the membrane bound antibodies were visualized by enhanced chemoluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) following the manufacturer's recommendations and documented by exposure to medical x-ray film (Amersham Hyperfilm, GE Healthcare, Buckinghamshire, UK). Western blots was also used to analysis purified N2-His protein with sera from chickens infected with A/turkey/Ohio/313053/04(H3N2), and A/GF/MA/14081-11/02(H7N2). To determine if chicken antibodies recognized the recombinant N2-His protein. Chicken serum was diluted 1:1000, and secondary HPR- conjugated- anti-chicken antibody (KPL, Gaithersburg, MD) was utilized at a 1:10,000 dilution following the procedure described above.

N2-ELISA optimization. Different antigen concentrations were tested against serial dilutions of positive and negative control sera in a checkerboard titration (CBT) fashion. Two fold-serial dilutions of the N1-His antigen, ranging from 500 ng to 4 ng, were performed in polystyrene flat bottom 96 well plates (Fisherbrand, Pittsburg, PA) with 1X coating buffer (KPL, Gaithersburg, MD). Two fold serial dilutions (1:25 to 1:25,600) of chicken serum raised against A/turkey/Ohio/313053/04(H3N2) and negative chicken sera were utilized for the assay optimization. Briefly, diluted N2-His antigen was incubated with 1X coating buffer at 4ºC overnight. The plate was washed three times with 1X wash buffer (KPL, Gaithersburg, MD), incubated with with 1% BSA buffer and incubated with serial dilutions of the positive and negative control sera at 37ºC for 1 hour followed by three washes. The plate was then incubated with HRP-goat anti-chicken antibody conjugate at a 1:1000 dilution (KPL, Gaithersburg, MD) followed by three washes. The colorimetric reaction was developed with peroxidase substrate solution for 15 minutes and terminated with stop solution (KPL, Gaithersburg, MD). The

reactions were read at 405 nm in an ELx808 plate reader (Bio-Tek, Winooski, VT). The optical density (OD) values obtained were compared to determine the optimal antigen concentration that produced the lowest background and the best positive control titration curve. Using the optimal antigen concentration and serum dilution a total of 210 field serum samples, confirmed as avian influenza negative by AGPT were tested in triplicates by the N2-ELISA. A negative cut-off value was determined as the mean OD + 2SD. All serum samples tested by the N2-ELISA were run in triplicate. To account for the assay variability the sample to positive (Sp) ratio was calculated for all sera tested. Samples with an average Sp value larger than the cut-off OD value were considered positive for N2 antibodies.

N2-ELISA Specificity. The specificity of the N2-ELISA was evaluated by testing panels of reference chicken sera raised against N1 to N9 AI neuraminidase subtypes produced in the National Veterinary Service Laboratory (NVSL, Ames, IO) and the Southeast Poultry Research Laboratory (SEPRL, ARS, USDA, Athens. GA). In addition, antisera raised against different pathogens of poultry, such as infectious bronchitis virus (IBV) serotypes (Arkansas, DPI, Del-072, GA98), Newcastle disease virus (NDV), Avian Adenovirus type 1, Reovirus, infectious laryngotracheitis virus (ILTV), *Mycoplasma gallisepticum* and *M. synoviae* (MS), were tested by N2-ELISA.

Production H1N2 and H1N1 inactivated vaccines. A/turkey/Missouri/24093/1999 (H1N2) and the 1988 H1N1 turkey virus of swine origin were grown in 10-day old SPF CE, infected allantoic fluid was collected three days post-infection and inactivated with 0.1% beta-propionalactone (BPL) (Sigma, St Louis, MO) (Pillai et al. 2008). To confirm efficient of viral inactivation BPL treated allantoic fluid was inoculated in embryos and allantoic fluid was from embryos was tesred for HA activity 5 days post infection. Oil-emulsion inactivated vaccines were prepared as

previously described (Stone et al. 1997) and administered subcutaneously at 1 day and 3 weeks of age.

Experimental design. Turkeys were obtained from a commercial source at 1-day of age, when blood samples were collected to serologically confirm that turkeys were negative for AI. Turkeys were raised in floor pens located in our facilities at the Poultry Diagnostic and Research Center (PDRC, University of Georgia) for five weeks and fed a standard diet and water *ad libitum*. At one day of age 20 turkey pullets were vaccinated with H1N1 inactivated vaccine (H1N1Vx), 20 turkeys pullets were vaccinated with inactivated H1N2 (H1N2Vx), and twenty turkeys remained not vaccinated (NVx). At 3 weeks of age the vaccinated turkeys received a second vaccination. One and three weeks after the second vaccination blood samples were collected from vaccinated and non-vaccinated turkeys for serological evaluation. At five weeks of age, each group of turkeys (H1N1Vx, H1N2Vx and NVx) was separated into three stainless steel negative pressure-HEPA filtered, isolator unit (PDRC), six turkeys per unit, and each unit received a different challenge treatment. At six weeks of age units holding H1N1Vx, H1N2Vx, Nvx turkeys were inoculated intranasally with H1N1 virus at a dose of $10^{7.2}EID_{50}$ per bird in a 200 µl volume and were identify as H1N1Vx-H1N1Ch, H1N2Vx-H1N1Ch, and NVx-H1N1Ch treatments. Similarly at six weeks of age a second set of units holding H1N1Vx, H1N2Vx, Nvx turkeys were inoculated intranasally with the H1N2 virus at a total dose of $10^{7.2}EID_{50}$ per bird in a 200 µl volume and were identified as H1N1Vx-H1N2Ch, H1N2Vx-H1N2Ch, and NVx-H1N2Ch treatments. The remaining three groups of H1N1Vx, H1N2Vx, Nvx turkeys were sham inoculated intranasally with 200µl of non-infected allantoic fluid and identified as H1N1Vx-NCh, H1N2Vx-Nch and NVx-NCh treatments. At 3 days post- infection (PI) oropharingeal, cloacal swabs, and blood samples were collected from each bird. Oropharingeal and cloacal

swabs were transported in brain-heart infusion (BHI- Sigma Alderich) media containing 10000u/L penicillin-G potassium (Fisher Scientific), 250g/L gentamycin sulfate (Fisher Scientific), 25mg/L of amphotericin B (Fisher Scientific), 500g/L kanamycin (Sigma-Aldrich) and 1g/L of streptomycin sulfate (Sigma-Aldrich) and were used for confirmation of challenge virus replication by reverse transcriptase real time polymerase chain reaction with primers and probe specific for the matrix protein (Spackman et al. 2003). At 7, 15 and 21 days PI blood samples were collected from each bird.

RNA extraction and Reverse Transcriptase Real Time PCR. Viral RNA from oropharingeal and cloacal swabs were extract using the Mag Max 96 AI-ND kit (Ambion, Austin, TX) following manufacturers recommendations and the addition of two wash steps using a NaCl 2M ph 4 with 0.5M EDTA. The reverse transcripate RT-PCR for the Matrix gene was performed with the AgPath-ID One step Kit (Ambion, Austin, TX) following the manufacturers recommendations. Amplification reactions were performed in the SmartCycler (Cepheid, Sunnyvale, CA) using program previously described by Spackman et al., 2002. Samples were considered positive if they had a ct value ≤35.

Serological analysis. The antibody levels in serum samples collected from turkeys were evaluated by Hemagglutinin inhibition assay (HI), Neuraminidase inhibition assay (NI), N1 indirect ELISA (N1-ELISA) and N2 indirect ELISA (N2-ELISA). **HI assay**. The HI assay was performed using four HA units of inactivated H1N1 or H1N2 viruses and two-fold dilutions of sera sample as previously described. Sera with HI titers ≥ 4 $\log_2$ were considered positive (Swayne 2008b; Thayer 2008). **NI assay**. The NI assay was performed as described before (Van Deusen et al. 1983) in a 96 well plate format. Briefly, sera samples were inactivated serum at 56°C for 30 minutes two-fold dilutions of sera were incubated with previously titrated

inactivated H1N1 orH1N2 influenza antigen for one hour. After incubation, a 12.5mg/ml of feutin (Sigma- Aldrich, St. Louis, MO) was added, the plate was incubated at 37°C for 3 hrs. At the end of incubation periodate reagent (11.2%) was added to each well, and the plate was incubated at room temperature for 20 minutes, after incubation 25µl of 0.5g/ml arsenite (Sigma-Aldrich, St. Louis, MO) was added to each well and a dark brown color developed, plates were then shaked until the dark brown color in the wells faded. Subsequently, a 0.6% solution of thiobarbituric acid (Sigma- Aldrich, St. Louis, MO) was added to each well, and subsequently the plate was incubated in a water bath at 56°C for 30 minutes. Sera samples that caused a reduction in pink color of the medium in the well were considered positive for the presence of NA antibodies. Sera samples with NI titer ≥ 8 $\log_2$ (Aymard-Henry et al. 1973). **N1-ELISA**. The N1-ELISA was performed as described by Liu et al (Liu et al. 2010), and samples with Sp ratio higher than 0.222 were considered positive. **N2-ELISA**. The N2-ELISA was performed using 200ng/well of purified recombinant N2 protein and sera samples working dilution was 1:50, and samples with S/p ratio > 0.222 were considered positive.

Statistical analysis. The sensitivity of the N1 ELISA relative to the NI-N1 assay was calculated as the proportion of NA-ELISA positive samples within the H1N1Vx/H1N1Ch, H1N2Vx/H1N1Ch, and NVx/H1N1Ch groups (collected pre and post challenge), samples from NVx/H1N1Ch and H1N2Vx/H1N1Ch groups (collected post-challenge) and confirmed positive by either the NI-N1 assay. Similarly the sensitivity of the N2 ELISA relative to the NI-N2 assay was calculated as the proportion of NA-ELISA positive samples within the H1N2Vx/H1N2Ch, H1N1Vx/H1N2Ch, and NVx/H1N2Ch groups (collected pre and post challenge), samples from NVx/H1N2Ch and H1N1Vx/H1N2Ch groups (collected post-challenge) and confirmed positive by either the NI-N2 assay.

The specificity of the N1-ELISA relative to the NI-N1 assay was calculated as the proportion of N1-ELISA negative samples within the H1N2Vx-H1N2Ch, H1N2Vx-Nch, NVx-H1N2Ch, NVx-NCh (collected pre and post-challenge), H1N2Vx-H1N1Ch and NVx-H1N1Ch (collected pre-challenge) and confirmed negative by the NI-N1 assay. The specificity of the N2-ELISA relative to the NI-N2 assay was calculated as the proportion of N2-ELISA negative samples within the H1N1Vx-H1N1Ch, H1N1Vx-Nch, NVx-H1N1Ch, NVx-NCh (collected pre and post-challenge), H1N1Vx-H1N2Ch and NVx-H1N2Ch (collected pre-challenge) and confirmed negative by the NI-N1 assay. The overall agreement between tests was calculated as the proportion of samples with concordant results out of the total number tested, and kappa statistics were calculated to estimate the agreement between tests beyond that which would be expected to occur due to chance alone.

Results

N2 protein expression and purification. Following the same strategy that resulted in expression of a secreted AI N1 protein (Liu et al. 2010) the coding sequence of the neuraminidase-2 genes of Influenza A/turkey/Ohio/313053/04(H3N2) was modified by replacement of the predicted signal peptide sequence with the signal peptide sequence of *Leucania separata* nucleopolyhedrovirus (LsNPV) to generate secreted N2-protein. Expression of recombinant N2-His was confirmed by iIFA (Figure 1a, 1b, 1c and 1d). As expected the manipulated N2- protein was secreted into the supernatant of N2-Bac infected Sf9 cultures and could be purified by IMAC. The purified protein appeared as a single band with a relative molecular weight of approximately 70 kDa as estimated from migration in SDS-PAGE relative to molecular weight standards (Fig. 1e). Absence of other protein species besides purified N2-

His in Coomassie-stained gels ensured sufficient purity of the preparation. In Western blots purified N2-His was detected by the anti-6xHis mab (Fig. 1, lane 1), as well as by polyclonal sera from chickens that had been vaccinated with Influenza A/turkey/Ohio/313053/04(H3N2), and A/GF/MA/14081-11/02(H7N2) (Fig. 1e lanes 2 and 3).

N2-ELISA Optimization. The N2-ELISA was optimized by check-board titration using different dilutions of N2-protein and different dilutions of H3N2 polyclonal chicken sera. Based on the checkboard titration, the optimal N2 protein concentration utilized in the ELISA was determined at 200 ng/well and sera samples dilution of 1:50 provided the optimal optical density (OD) ratios between positive and negative controls, and the lower background readings (data not shown). Using the established protein concentration and sera dilution, a total of 210 chickens and 200 turkeys AI negative serum samples from commercial flocks were tested by the N2-ELISA, and a sp ratio ≥0.222 (2SD) was established as a cutoff value for the assay. The ability of the N2-ELISA to discriminate among AI subtypes was determined with anti-sera against-subtype N1 through N-9 (Fig. 2). While the N2 anti-sera yield Sp ratios ranging from 0.269 to 1.354. With the exception of one N5 anti-sera that yielded an Sp ratio above the cutoff value, anti-sera against N1-N4 and N6-N9 yielded Sp ratios lower than the cut-off value of 0.222, therefore did not cross reacted with the N2 antigen, The IBV, NDV, ILTV, Adenovirus, Reovirus, and MG/MS antisera yielded Sp ratios lower than 0.222, and therefore no cross reaction with the N2-ELISA was observed (data not shown).

Hemagglutinin and neuraminidase antibodies in H1N2 and H1N1 vaccinated turkeys. Sera collected from turkeys before and after vaccination were tested by HI, N1-NI, N2-NI, N1-ELISA and N2-ELISA. Sera samples, collected previous to vaccination were negative in all five AIV serological assays (data not shown). Percentage of turkeys vaccinated with H1N2 and positive by

HI, N2-NI and N2-ELISA are shown in figure 3a. One week post second vaccination with H1N2 vaccine 100% (18/18) of the turkeys had antibodies against the H1 hemagglutinin, 61% (11/18) have antibodies against N2 as determined by N1-N2, while 39% (7/18) of the samples were detected positive for N2 antibodies by the N2-ELISA (Fig. 3a). However at three weeks post-second vaccination, HI and N2-NI were able to detect AIV antibodies in 100% (18/18) of the H1N2 vaccinated turkeys, while N2-ELISA detected presence of N2 antibodies in 94% (17/18) of the vaccinated turkeys (Fig. 3a). One week after second vaccination, 100% (18/18) of turkeys vaccinated with H1N1 were positive for H1 antibodies as determined by the HI assay, 74% (14/18) of the turkeys were positive for N1 antibodies as determined with the N1-NI assay, while 22% (4/18) of the turkeys were positive for N1 antibodies by the N1-ELISA (Fig. 3b). At three weeks post-second vaccination 100% (18/18) of the turkeys were antibody positive by the HI, N1-NI and N1-ELISA (Fig. 3b). None of the sera samples collected from H1N1 vaccinated and non-vaccinated turkeys were positive when tested by the N2-NI or N2-ELISA. Similarly, none of sera samples collected from H1N2 vaccinated or non-vaccinated turkeys were positive when tested by the N1-NI or N1-ELISA (data not shown).

Real Time RT PCR post challenge. Oropharingeal and cloacal swabs were collected from each bird at 3 days PI. As expected all the samples collected from the NVx/NCh, H1N1Vx/NCh, H1N2Vx/NCh groups were negative. Figure 3 shows the percentage of samples within each group of challenge turkeys that were positive by the matrix RT real time PCR (cT < 35). For the non-vaccinated turkeys 100% (12/12) and 91% (11/12) of the samples tested from the NVx/H1N2 and NVx/H1N1 groups were positive, respectively. While 75% of the samples from the H1N1Vx/H1N2Ch and H1N2Vx/H1N2Ch groups and 50% of the samples from the H1N1Vx/H1N1Ch and H1N2Vx/H1N1Ch groups were positive (fig. 4).

Hemagglutinin and neuraminidase antibody responses in non-vaccinated, vaccinated and subsequently challenge turkeys with homologous NA viral subtypes. Serum samples from turkeys were collected at one, two, and three weeks post challenge and tested by the HI, NI, and, NA ELISAs. Figure 5a, and c shows the percentage of positive samples detected by HI, N1-NI and N1-ELISA for groups H1N1Vx/NCh, and NVx/H1N1Ch, respectively. Figure 5b, and d shows the percentage of positive samples detected by HI N2-NI and N2-ELISA for groups H1N2Vx/NCh, and NVx/H1N2Ch. As expected the percentage of turkeys with antibodies against the HA and NA within the non-challenge groups decreased from 100% (6/6) in H1N1Vx/NCh (fig. 5a) and H1N2Vx/NCh (fig. 5b) at 1 week post challenge, to 66% (4/6) for the H1N1Vx/NCh (fig. 5a) and 50% (3/6) in the H1N2Vx/NCh (fig. 5b) by 3 weeks post challenge. Similarly the NA antibody response decreased from 100, 83 to 50% in the H1N1Vx/NCh and H1N2Vx/NCh groups as detected by N1-NI and N2-NI, respectively from week 1 to 3 post-challenge (fig. 5a and 5b). The N1 antibody response by week 1, 2, and 3 post-challenge was detected by the N1-ELISA in 100, 66, and 50% of the turkeys in the H1N1Vx/NCh group (fig. 5a). The N2 antibody response, by week 1, 2, and 3 post-challenge, was detected by the N2-ELISA in 100, 66, and 33% of the turkeys in the H1N2Vx/NCh group (fig. 5b). In the non-vaccinated and challenged groups the percentage of turkeys with detectable HA antibodies increased through time, 83% and 100% of the turkeys showed HA titers 3 weeks post challenge for the NVx/H1N1Ch and NVx/H1N2Ch groups respectively (fig. 5c and 5d). Similarly the percentage of infected turkeys showing NA antibody response increased from week 1 to 3 post-challenge, 16% to 83% as detected by the N1-NI assay (fig. 5c), from 33 to 100% as detected by the NI-N2 assay (fig. 5d), and from 0 to 83% as detected by the N2-ELISA (fig. 5d). On the other hand the percentage of infected turkeys showing N1

antibodies as detected by N1-ELISA decreased from 83 to 66% from week 2 to 3 post-challenge (fig. 5c). A 100% of the homologous vaccinated and challenge groups of turkeys had HA and NA antibodies from weeks 1 to 3 post-challenge as detected by HA, NI, and NA-ELISAs (data not shown).

Indirect neuraminidase ELISAs as compared to neuraminidase inhibition assay. The overall agreement, the kappa value, and the relative sensitivity and specificity of N1-ELISA and N2-ELISA to N2-NI are shown in table 1. The overall agreement and kappa value for the N1-ELISA was of 90.74% ($\kappa = 0.802$; $P < 0.05$) and for the N2-ELISA was 93.83% ($\kappa = 0.864$ $P < 0.05$). The sensitivity of the N1-ELISA to detect N1 antibodies in true positive samples was 0.88 and the sensitivity of the N2-ELISA to detect N2-antibodies in true positive samples was 0.90, indicating that the N1 and N2 ELISAs yield some false negative results. In the case of the N1-ELISA a total of 13 samples were identified as false negative, these samples belong to the either homologous and heterologues vaccinated, non-challenge, and non-vaccinated groups. A total of 10 samples were identified as false negative with the N2-ELISA, these samples belong to either heterologous vaccinated, non-vaccinated and non-challenge groups. The specificity of the N1-ELISA and N2-ELISA to identify true negative samples was 0.94 for both assays, indicating the presence of 10 and 9 false-positive reactions, respectively.

Neuraminidase antibody responses in vaccinated/challenge turkeys with heterologous NA vaccine subtypes. To evaluate the ability of NA ELISAs to serological differentiate vaccinated and subsequently infected turkeys, sera samples collected at one, two, and three weeks post challenge were tested to determine the neuraminidase antibody response in individual turkeys, H1N1Vx/ H1N2Ch and H1N2Vx/H1N1Ch groups, were tested by N1-NI, N2-NI, N1-ELISA

and N2-ELISA. N1-ELISA Sp values and NI titers from individual positive turkeys are shown in figure 6, and results from same turkeys tested by N2-NI and N2-ELISA are shown in figure 7. The N1 antibody response in the H1N2Vx/H1N1Ch was lower than the response observed in the H1N1Vx/H1N2Ch group. By three weeks post challenge two turkeys were positive by N1-ELISA and three by N1-NI, while in the H1N1Vx/H1N2Ch group three turkeys were positive by N1-ELISA and four by N1-NI. Although more turkeys were positive for N2 antibodies in the H1N1Vx/H1N2Ch group, overall the N2 antibody response, detected by N2-NI and N2-ELISA, in individual turkeys within the H1N2Vx/H1N1Ch group was higher (fig. 7). By three weeks post-challenge a 83% and 66% of turkeys within the NVx/H1N1Ch group were identify as infected by N1-ELISA and N1-NI, respectively (fig. 8a), while a 33% and 50% of the turkeys within the H1N2Vx/H1N1Ch group were identified as infected by N1-ELISA and NI-NI, respectively (fig. 8c). For NVx/ H1N2Ch turkeys, by three weeks post-challenge 83% and 100% were identified as infected by N2-ELISA and N2-NI, respectively (fig. 8b). And 50% and 83% of the H1N1Vx/H1N2Ch group of turkeys were identified as infected by N2-ELISA and N2-NI, respectively (fig 8d).

Discussion

The development of a serological assay that allows to differentiated between vaccinated/infected and vaccinate/non-infected; and has a high-throughput capability for routine screening of vaccinated birds, is extremely important for control of AIV in a vaccinated population. The use of heterologous neuraminidase ELISA systems to monitor infected flocks during a DIVA vaccination has being evaluated before (Kim et al. 2010; Kwon et al. 2009). The first objective of this study was to develop an N2-ELISA using purified, recombinant N2 protein

and to evaluate the sensitivity and specificity of the assay. The N2-ELISA showed a high selectivity for detection of N2 antibodies with a low cross-reactivity with other neuraminidase subtypes and no cross-reactivity with others pathogens. The N2-ELISA showed a broad spectrum of reactivity with sera from N2 subtype isolates, A/turkey/Ohio/313053/04(H3N2), A/turkey/Missouri/24093/1999 (H1N2), A/Ck/CA/431/00(H6N2), A/Mallard/MN/355779/00(H5N2), A/GF/MA/14081-11/02(H7N2). In addition, data from the experimental study revealed the ability of the N2-ELISA to detect N2 antibodies in vaccinated birds, at 1 week post-second vaccination, 100% of H1N2 vaccinated turkeys were positive in the HI assay, 94% of turkeys were also positive in the N2-ELISA and 100% of H1N1 vaccinated turkeys were negative in the N2-ELISA demonstrating that the assay is able to specifically detect the presence of N2 antibodies in the sera.

The ability of the N1-ELISA and the N2-ELISA to detect infection in a non-vaccinated group was demonstrated experimentally. Turkeys infected with H1N1 virus started showing antibody against HA and NA at one week PI, similarly turkeys infected with H1N2 also starting showing presence of antibody against HA and NA at 1 week PI. Other studies demonstrated the presence of HA antibodies at the same period, 6-7 days PI (Cattoli et al. 2006; Kwon et al. 2009), however some differences in the time of detection of NA antibodies occurred. Cattoli et al (Cattoli et al. 2006) demonstrated that NA antibodies started to be detected before the HA antibodies, however the data from this research suggests that the NA antibodies starts to show at the same time or later than the HA antibodies. The percentage of turkeys positive in the HI and N1-ELISA or HI and N2-ELISA increased at 2 and 3 weeks PI reaching pikes of 66% and 83% accordance respectively at 3 weeks PI. The difference in the ability of the N1-ELISA to detect infected turkeys may not reflect the sensitivity of the assay, but be attributed to virulence of the

H1N1 isolate, since signs if replication of the H1N1 isolate could be found only in 91% of the inoculated turkeys.

The ability of N1-ELISA and N2-ELISA to detect vaccinated and infected birds was also experimentally evaluated. At one week post-challenge, the HA antibodies showed an increase in HI titers as expected, however antibodies against heterologous NA was not detected by the NA-ELISA. Even though NA antibodies to the challenge virus was not found by the ELISA, there was evidence of virus replication in the Vx/Ch turkeys, at 3 days PI, 50% of the H1N2Vx/H1N1ch turkeys and 75% of the H1N1Vx/H1N2Ch turkeys had positive results in the real time RT-PCR, confirming virus infection/replication. However, vaccinated turkeys showed ct values higher than non-vaccinated and challenge turkeys, confirming that inactivated vaccine reduce virus replication and shedding (Capua et al. 2003b, 2007; Capua et al. 2003d; Cattoli et al. 2006; Swayne 2008a; Tumpey et al. 2004). At two weeks PI, N1-ELISA and N2-ELISA started to detect presence of vaccinated and infected birds with 50% of turkeys in the H1N1Vx/H1N2Ch group being positive by the N1-ELISA and N2-ELISA. In the H1N2Vx/H1N1Ch group presence of vaccinated and infected birds was also seen at two weeks pi, however only 16% of turkeys were positive by N1-ELISA. The difference in the ability to detect vaccinated and infected birds in this particular group (H1N2Vx/H1N1ch) may be attributed to the virulence of the H1N1 isolate. Even though the N1-ELISA and N2-ELISA were able to demonstrate the presence of vaccinated and infected birds, the NI assay is more sensitive than the ELISA, however the NI assay is not suitable to high throughput analysis.

Overall, the ELISAs were capable to detect vaccinated and subsequently infected turkeys. Although the ELISA showed lower sensitivity than the NI assay, it was demonstrated that screening for NA antibodies by ELISAs were an effective, rapid, and easy to interpret assay

capable to identify exposure to challenge virus within a vaccinated population of birds during a DIVA heterologous NA vaccination strategy. During field application ELISAs should be utilized and interpreted as a flock assay and should be complemented by other rapid diagnostic assays.

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Figure 5.1. Expression of recombinant N2 protein. a, b, c and d indirect imunofluorescence (iIFA); e. western blot analysis of N2 secreted protein. a and c, mock infected Sf-9 cells; b and d Sf-9 cells infected with recombinant Baculovirus (N2TKsigLsBacV). a and b Sf-9 cells incubated with anti-RGS-6xHis mab and visualized using FITC-coupled anti-mouse IgG. c and d Sf-9 cells incubated with H3N2 chicken antisera visualized using FITC-coupled anti-chicken IgY antibodies. e western blot analysis of N2 (H3N2) protein secreted in the culture supernatant, purified by metal affinity chromatography (IMAC) and concentrated by centrifugation in an Amicon filter (Millipore). Western blot was incubated lane 1, anti-RGS-6xHis mab, lane 2, H3N2 chicken antiserum, lane 3, H7N2 chicken antiserum and visualized by chemiluminescence.

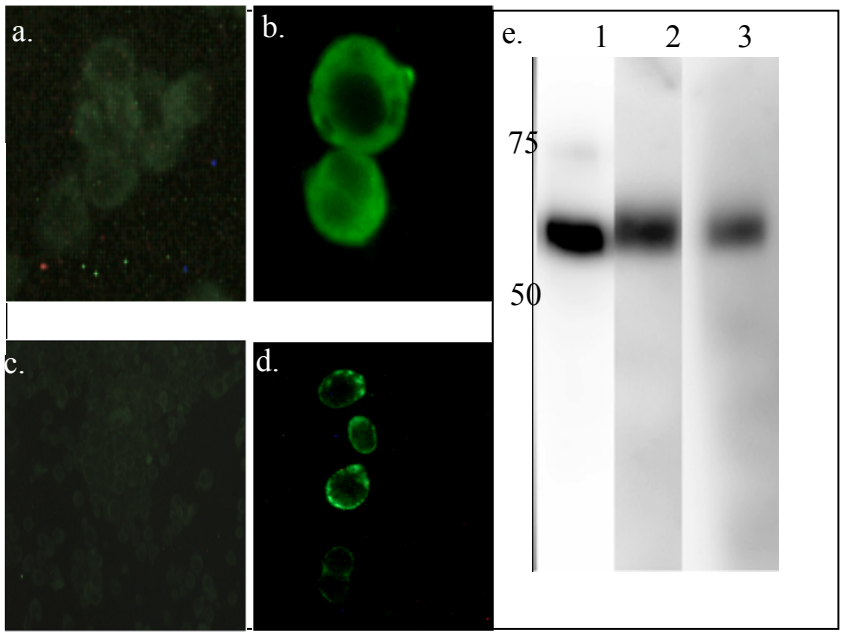


Figure 5.2. Determination of the N2-ELISA specificity using polyclonal chicken sera raised against AIV neuraminidase subtypes N1 to N9. N1: A/CK/NY/94 (H1N1), A/EQ/Tenn/76 (H7N1), A/TK/KS/80 (H1N1); N2: A/MA/NY/78, (H2N2), A/TK/MA/65 (H6N2), A/TK/DE (H7N2); N3: A/TK/OR/71 (H7N3), A/TK/England (H0N3), TK/OR/71 (H7N3); N4:A/TK/ONT/67, A/TK/ONT/67; N5: A/DK/ALB/76 (H12N5), A/Nws/England (H0N5), MA/Gurjev/82 (H14N5); N6: A/GU/MD/77 (H13N6), A/DK/England (H0N6), A/SH/WA/79 (H15N6); N7: A/CK/Germ/49 (H10N7), A/CK/Germ/49 (H10N7), A/CK/Germ/49 (H10N7); N8: A/CK/ALB/78 (H4N8), A/EQ/England (H0N8), Mallard/OH/421/87 (H7N8); N9: TK/WI/68 (H5N9), A/DK/Nws/546(H0N9), A/DK/Memphis/74 (H11N9).

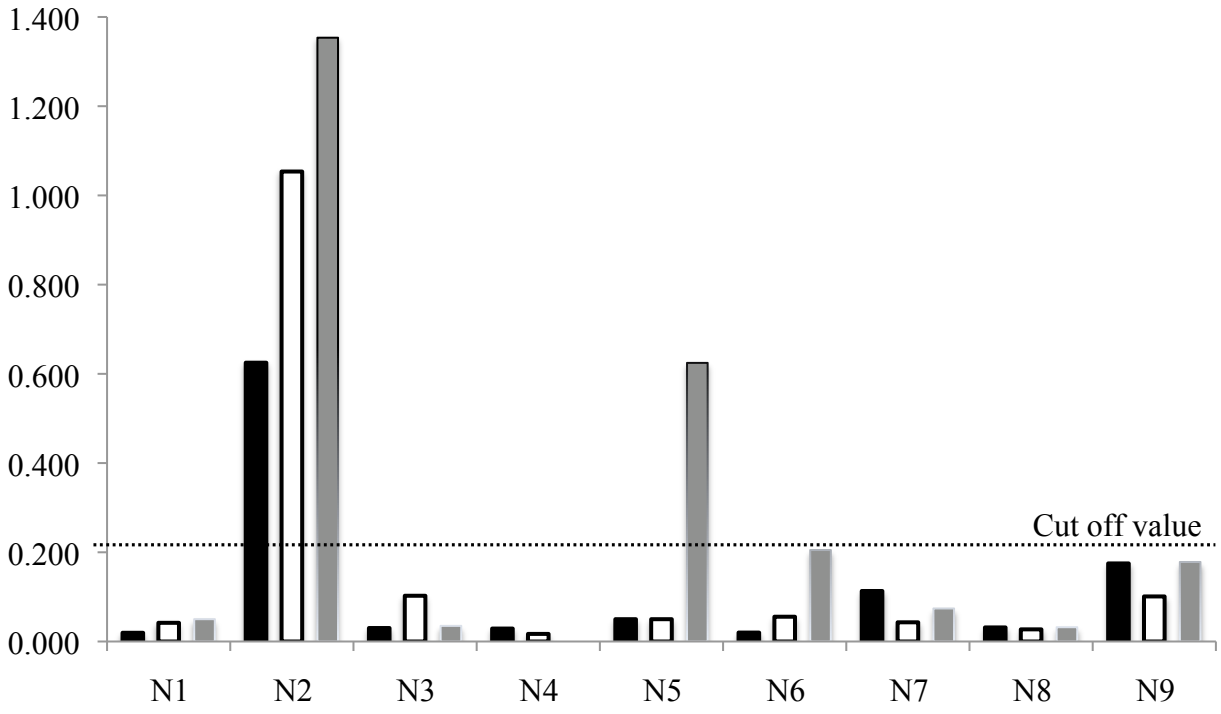


Figure 5.3 Evaluation of hemagglutinin and neuraminidase antibodies in turkeys vaccinated with A/turkey/Missouri/24093/1999 (H1N2) and NC/TK/88 (H1N1). a. Percent of positive samples as determined by HI, NI (N2) and ELISA (N2) out of 18 turkeys vaccinated with inactivated H1N2. b. Percent of positive samples as determined by HI, N1 (NI) and ELISA (N1) out of 18 turkeys vaccinated with inactivated H1N1vaccine.

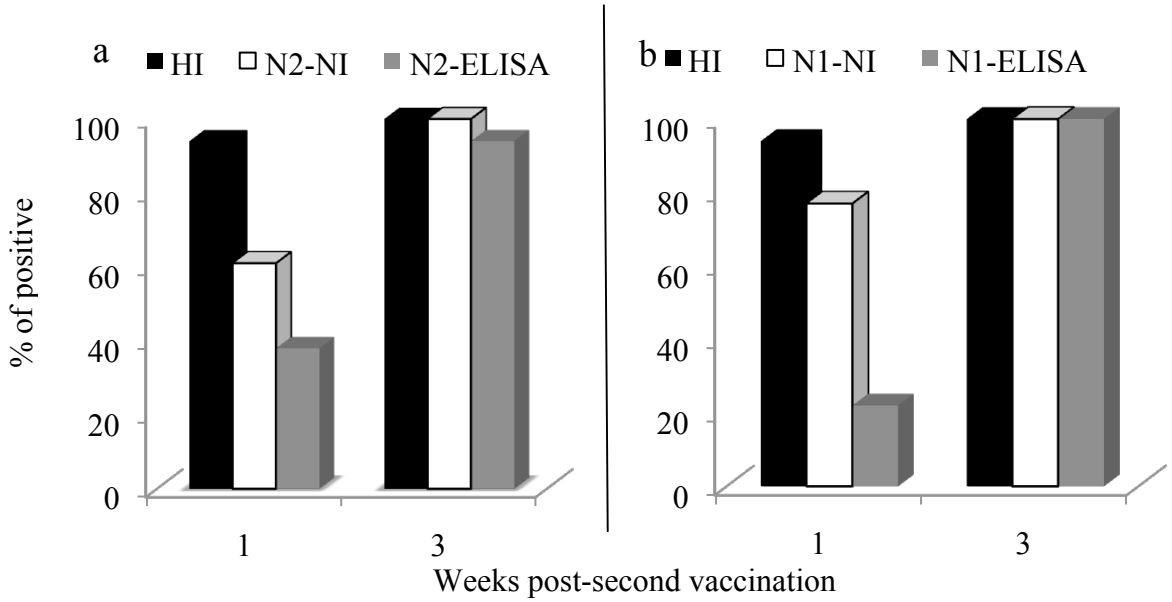


Figure 5.4. Reverse Transcriptase (RT) real time-PCR. Percentage of positive samples (out of 12 oropharingeal and cloacal swabs) in the different groups.

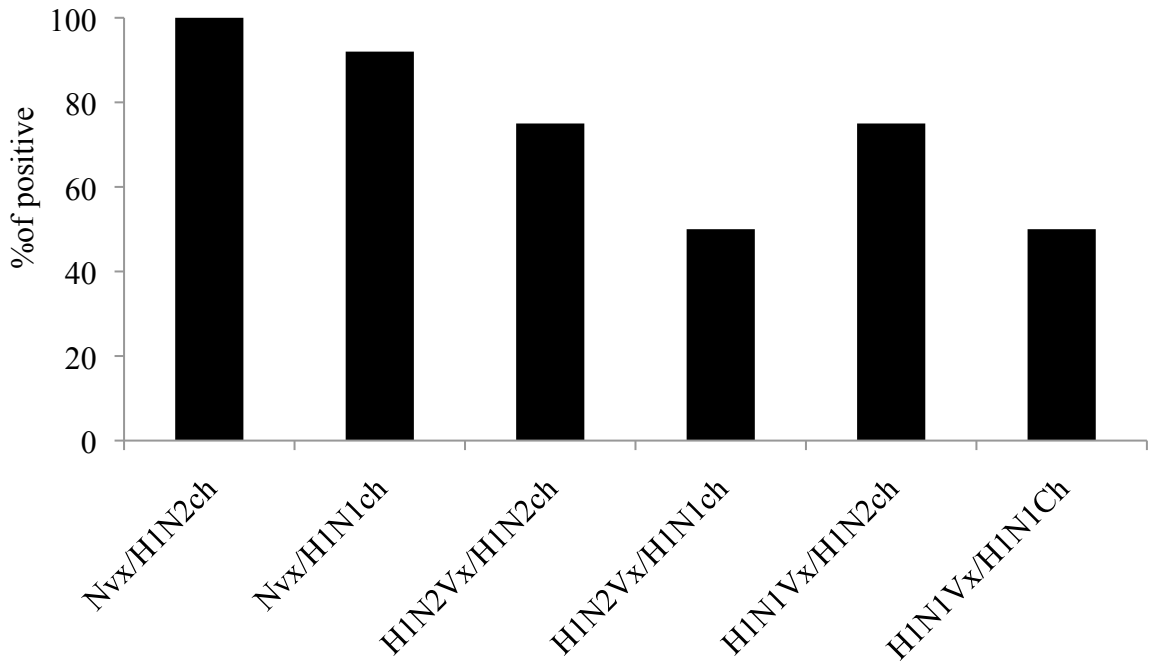


Figure 5.5. Evaluation of hemagglutinin and neuraminidase antibody responses in vaccinated and challenge turkeys with homologous NA subtype H1 viruses as determined by HI, N1 and NA ELISAs. Figures 5a, 5c tested by HI, NI (N1), ELISA (N1) and figures 5b, 5d tested by HI, NI (N2) and ELISA (N2). Group of birds per graph: (5a) NVx/H1N1Ch; (5b) Nvx/H1N2Ch; (5c) H1N1Vx/NCh, (5d) H1N2Vx/NCh. Legend NA-ELISA, NI, and HI and y axis number of positives samples per group of birds, x axis weeks post challenge.

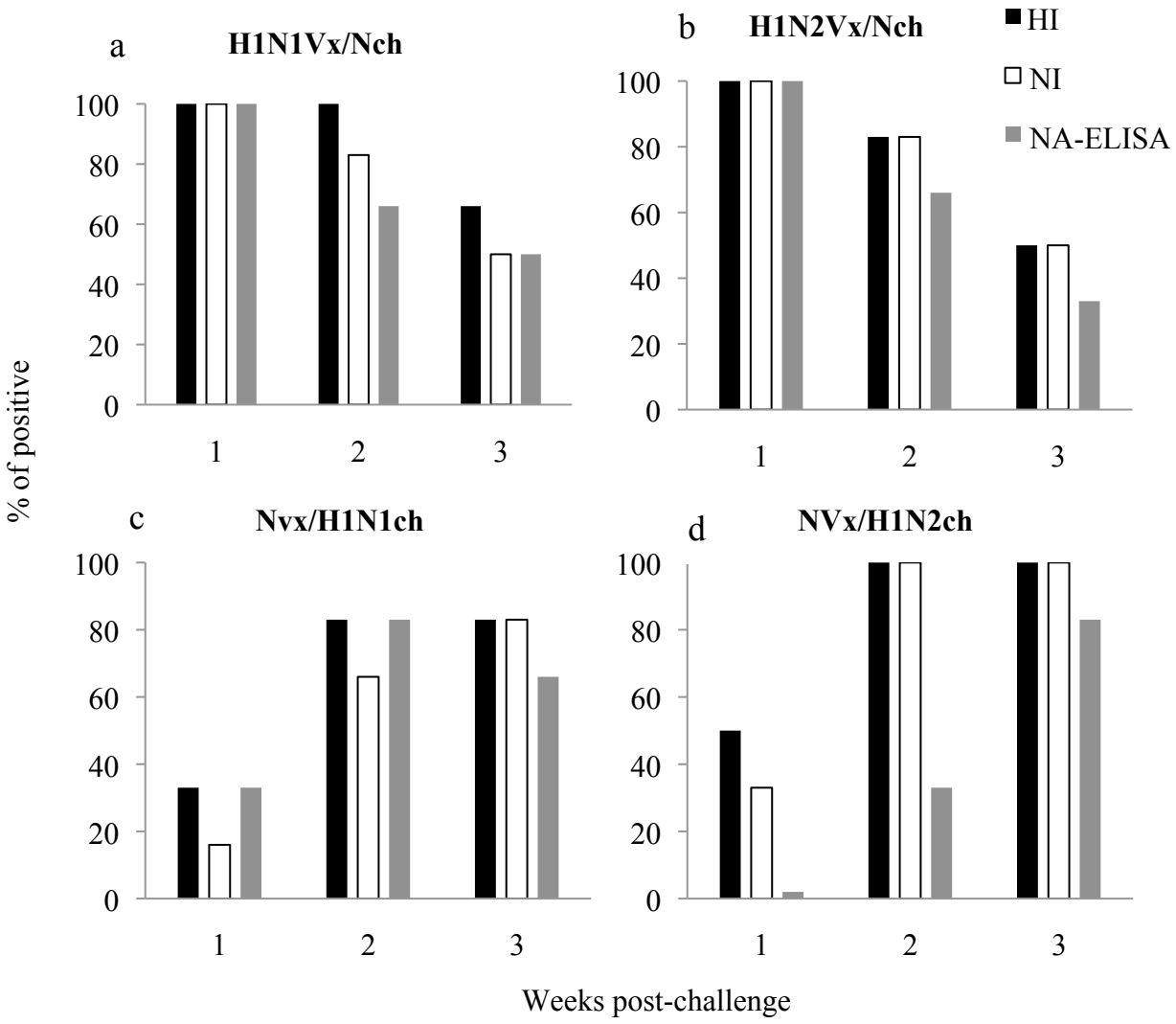


Table 5.1. Evaluation of N1 and N2- ELISAs sensitivity and specificity as compared to subtype specific neuraminidase inhibition assays (NI).

	%Agreement	Kappa value (P)[a]	Sensitivity[b]	Specificity[c]
N1-Elisa	90.74	0.802 (0.034) [0.735-0.870][d]	0.88	0.94
N2- Elisa	93.83	0.864(0.003) [0.807-0.922]	0.90	0.94

[a] probability < 0.05 indicating agreement is not due to chance.[b] sensitivity: number of samples positive by ELISA and NI/number of samples positive by.
[d] 95% confidence interval

Figure 5.6. N1 antibody responses in NA heterologous vaccinated turkeys. N1 antibody responses measure by N1-ELISA (a and c) and N1-NI (b and d) in turkeys vaccinated with NA heterologous vaccines (H1N2Vx/H1N1Ch and H1N1Vx/H1N2Ch). ELISA S/p rations or NI titers of individual turkeys in the y-axis; weeks post challenge in the x-axis.

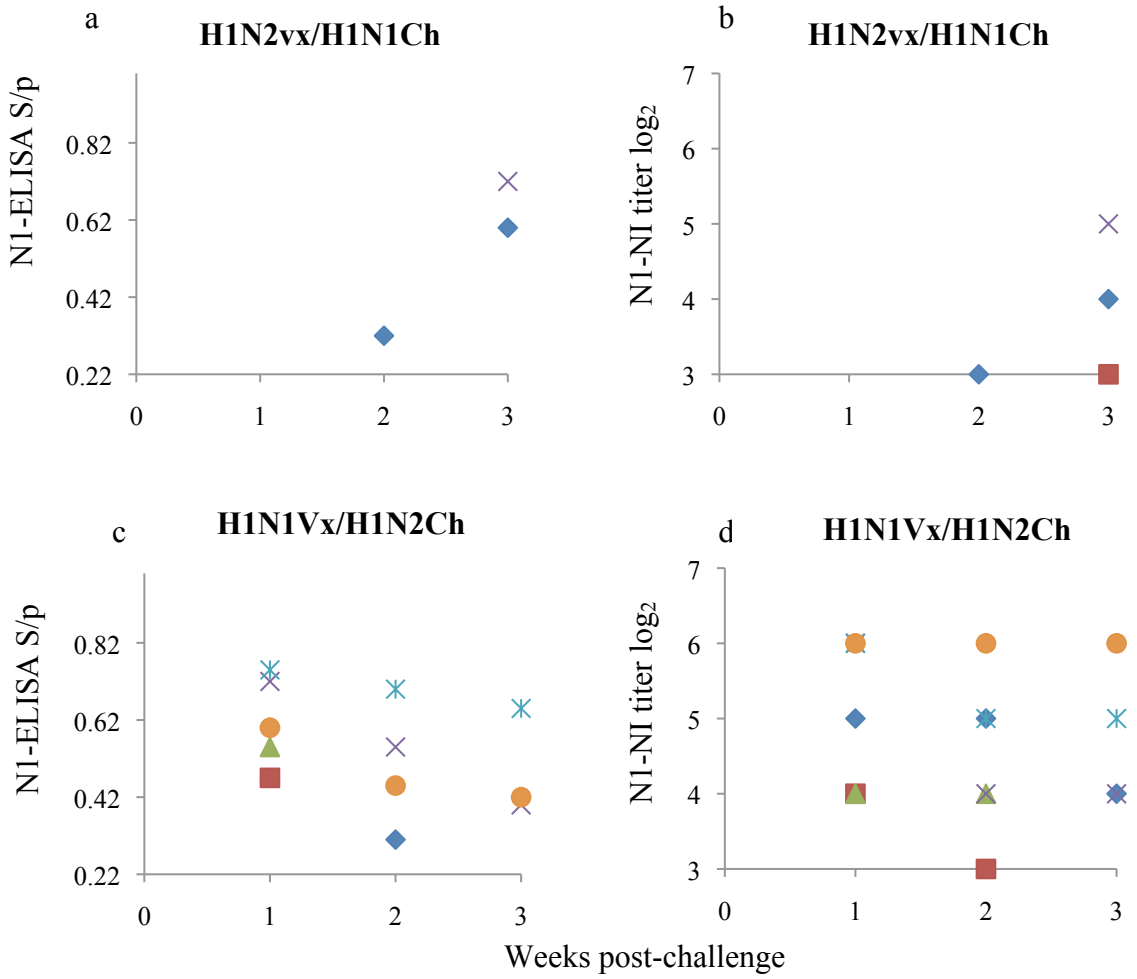


Figure 5.7. N2 antibody responses in NA heterologous vaccinated turkeys. N2 antibody responses measure by N2-ELISA (a and c) and by N1-NI (b and d) in turkeys vaccinated with NA heterologous vaccine (H1N2Vx/H1N1ch and H1N1Vx/H1N2ch). ELISA S/p or NI titers of individual turkeys in the y-axis; weeks post challenge in the x-axis.

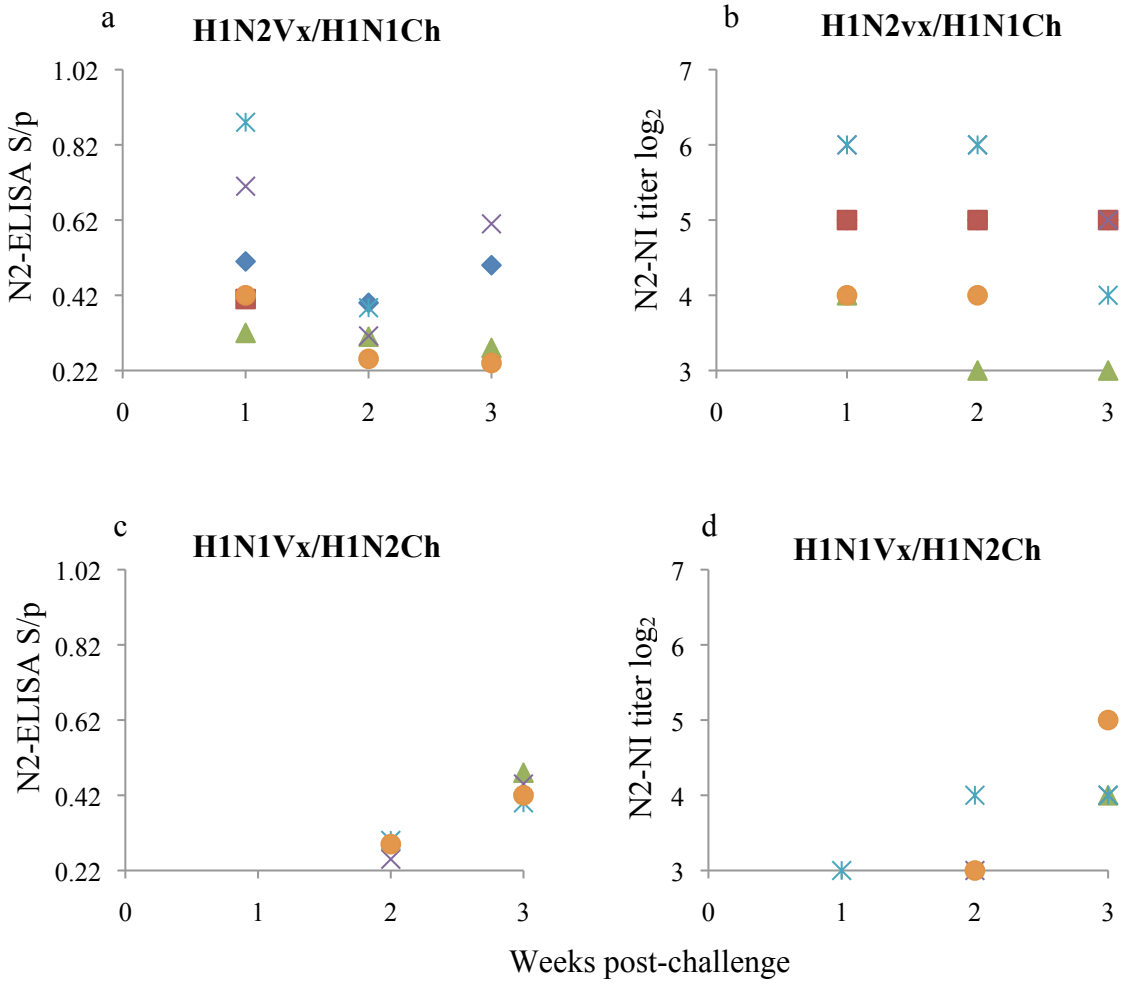
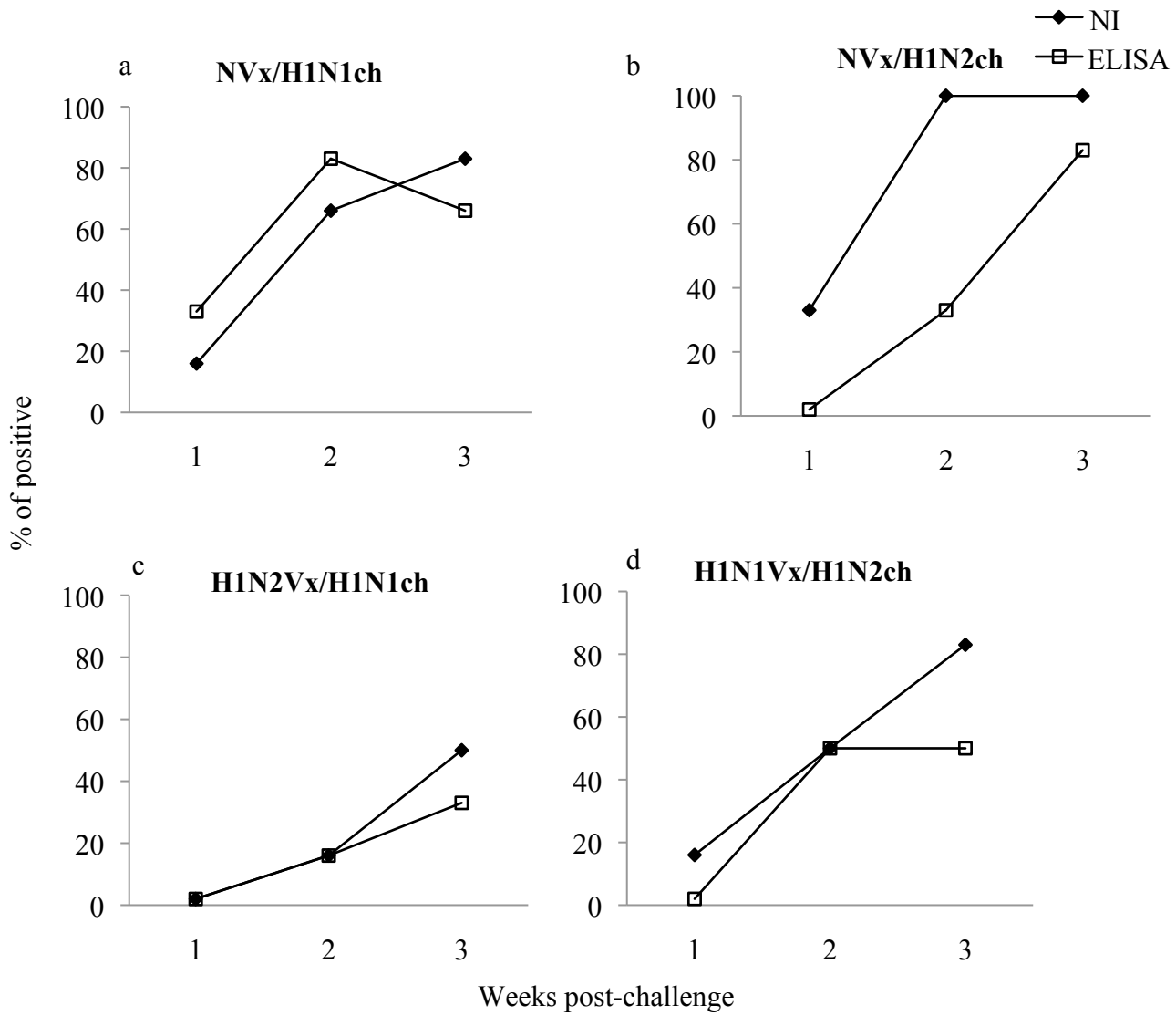


Fig 5.8. Detection of infected turkeys by NI and NA-ELISAs in vaccinated/challenge with heterologous NA subtypes (N1 and N2) as compared to inon-vaccinated/challenge groups. Figures 6a, 6c shows samples tested by NI (N1), ELISA (N1) and figures 6b, and 6d, shows serum samples tested by NI (N2) and ELISA (N2).Legend NA-ELISA, and NI, axis percent of positive samples per group and x axis weeks post challenge.



Chapter 6

Discussion

Given the rapid movement of people and animal products around the world, the emergence and spread of zoonotic diseases, particularly avian influenza (AI) is a growing concern. The emergence of highly pathogenic H5 avian influenza and subsequent human infections in Hong Kong in 1997 increased the awareness to AI and to the risk of human infection by avian influenza. Prior to 1997, the transmission of avian influenza viruses to humans was not considered to be a serious health threat; this new information increased the necessity to understand and control avian influenza outbreaks.

Although commercial poultry is not the natural host of AIV, infections occur frequently (Alexander 2000) It is believed that the ability of the LPAI viruses to transmit is closely linked to the amount of virus released from the intestinal route of infected birds. However, the duration and extent of AIV shedding, particularly of LPAIV in infected poultry, is poorly understood and can be highly variable (Alexander 2007; Jackwood et al. 2010; Morales et al. 2009; Pillai et al. 2010a). Similar to other studies (Alexander 2007; Jackwood et al. 2010; Morales et al. 2009; Pillai et al. 2010a), we detected that the level of cloaca shedding and fecal contamination was highly variable and significantly influenced by host and viral strains. In this study regardless of the viral strain, turkeys and quails shed lower titers than broilers and layers and independently of the host while fecal contamination with A/Ck/CA/431/00(H6N2) virus was significantly higher

than A/Mallard/MN/355779/00(H5N2), and A/turkey/Ohio/313053/04(H3N2). Overall this study shows that shedding of LPAIV is an extremely intricate process dependent on host, viral isolate degree of adaptation to the host, and viral tropism in that particular host; and even though in this study LPAI viruses retained infectivity in litter and manure for only 24 hours, biossecurity measures, disinfection of chicken houses, and proper handling of poultry by products, such as the composting of carcasses and windrow composting of litter during AI outbreaks should be implemented, particularly in lower temperature climates.

The current strategy to control avian influenza, in cases where low pathogenic AI is endemic such as Mexico the H5N2 (Villarreal 2009), and in the Middle East the H9N2 (Banet-Noach et al. 2007), is biosecurity together with vaccination. Vaccination has potential benefits, such as the reduction of clinical signs of diseases, reduction of viral shedding and increases resistance of flocks be re-infected (Capua et al. 2003a). However, vaccination also has drawbacks; vaccination with inactivated vaccines hinders serological surveillance to identify infected flocks, since vaccinated flocks cannot be distinguished from naturally infected birds. Subsequently the inability to identify infected flocks severely affects international trade of poultry products (Suarez 2005; Swayne 2003a). Therefore, it is important to differentiate naturally infected from vaccinated birds and also to identify vaccinated birds that become infected (Suarez 2005). We developed an N2 indirect ELISA using a recombinant purified N2 protein, and evaluated the ability of indirect N1 (Liu et al., 2010) and N2 ELISAs to identify experimentally vaccinated/infected turkeys using heterologous NA DIVA vaccination strategy. N1-ELISA and N2-ELISA were able to differentiate vaccinated from infected birds, and they are also able to show the presence of infection in vaccinated birds. Although the ELISA showed lower sensitivity than the NI assay, it was demonstrated that screening for NA antibodies by

ELISAs were an effective, rapid, and easy to interpret assay capable to identify exposure to challenge virus within a vaccinated population of birds during a DIVA heterologous NA vaccination strategy. Overall, AIV surveillance allow us to detect outbreaks before they have the opportunity to spread, and field application of N1 and N2- ELISAs should be utilized and interpreted as a flock assay and should be complemented by other rapid diagnostic assays

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