ROLE AND IMPORTANCE OF NS1 PROTEIN OF AVIAN INFLUENZA VIRUS TO GROW IN THE PRESENCE OF INTERFERON AND EVALUATION OF THE NS1 MUTANT VIRUSES AS POTENTIAL DIVA VACCINES

A Dissertation

by

VINAYAK R. BRAHMAKSHATRIYA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2009

Major Subject: Poultry Science

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Approved by:

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Committee Members,	John El-Attrache
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ABSTRACT

Role and Importance of NS1 Protein of Avian Influenza Virus to Grow in the Presence of Interferon and Evaluation of the NS1 Mutant Viruses as Potential DIVA Vaccines.

(August 2009)

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A proper vaccination program can play a critical role in prevention and control of avian influenza (AI) in commercial poultry. Low pathogenic avian influenza viruses (LPAIV) of H5 and H7 AI subtypes cause serious economic losses to the poultry industry and have the potential to mutate to highly pathogenic AI (HPAI) strains. Due to trade implications, differentiation of infected from vaccinated animals (DIVA) is an important issue in the control of AI. Therefore, the development and characterization of vaccine candidates with DIVA properties is critical in improving vaccination programs. Keeping these aspects in mind, we investigated the role of an NS1 mutant virus as a potential live attenuated DIVA vaccine. The NS1 protein of influenza virus plays a major role in blocking the host's antiviral response. Using an eight-plasmid reverse genetics system, we recovered the low pathogenic parental (H5N3) and NS1 mutant (H5N3/NS1/144) viruses. H5N3/NS1/144 expresses only the first 144 amino acids of the

NS1 protein compared to the 230 of the parental H5N3. The growth properties of H5N3 and H5N3/NS1/144 were compared in cell culture and in different age embryonated chicken eggs. Our results confirmed that NS1 is involved in down regulation of interferon as shown by IFN-ß mRNA expression analysis and by the inability of H5N3/NS1-144 to efficiently grow in older age, interferon competent, chicken embryos. However with regards to safety the virus reverted to virulence within five back passages in chickens and was therefore not a safe vaccine candidate. However the killed form of H5N3/NS1-144 was a safer alternative and it also induced antibody titers and protection not significantly different from the parental H5N3 as vaccine. To further understand the reversion of H5N3/NS1/144 to virulence, we carried out 3 independent serial passages of H5N3/NS1/144 in increasing age of embryonated chicken eggs and examined the NS1 gene for presence of mutations. RT-PCR and sequence analysis of the NS gene in all three lineages showed the presence of a 54 amino acid deletion resulting in the generation of a 87 amino acids long NS1 ORF with a point mutation (L80V) at the site of deletion. In addition, the NS1 ORF in lineages L2 and L3 presented two additional point mutations in the RNA binding domain (Q40R and T73M). To determine if these mutations played a role in increased virulence, recombinant viruses expressing these mutant NS1 proteins in the background of parental virus were generated by reverse genetics and their replication properties and pathogenicity was examined in vitro, in ovo and in vivo systems.

Our results showed that the 87 amino acid long NS1 protein clearly increased virus replication and virulence specifically in interferon competent systems. In addition,

the two point mutations in the RNA binding domain of NS1 ORF expressing 87 a protein slightly increased the virus virulence.

Overall this study reinforces the role of NS1 in influenza virus pathogenicity and supports the use of killed inactivated NS1 mutant virus vaccines as potential DIVA vaccines.

DEDICATION

To the never ending love and support of Reshma and my family back home

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NOMENCLATURE

AI	Avian Influenza
LPAIV	Low Pathogenic Avian Influenza Virus
HPAIV	Highly Pathogenic Avian Influenza Virus
DIVA	Differentiation of Infected from Vaccinated Animals
HA	Hemagglutinin
NA	Neuraminidase
HP	High Pathogenic
LP	Low Pathogenic
RNP	Ribonucleoproteins
NP	Nucleocapsid Protein
NS1	Non-Structural Protein 1
NEP	Nuclear Export Protein
M1	Matrix Protein
M2	Membrane bound ion channel-like Protein
cRNA	Positive Strand RNA
mRNA	messenger-RNA
PRR	Pattern Recognition Receptor
PAMP	Pathogen Associated Molecular Pattern
TLR	Toll-Like Receptor
LRR	Luminal Leucine-Rich Repeat
RIG-I	Retinoic acid-Inducible Gene I
RLR	Retinoic acid-Inducible Gene I-like Receptor
IFN	Interferon
ISG	Interferon Stimulated Genes
OAS	Oligoadenylate Synthetases
Mx	Myxovirus Resistance Gene
RnaseL	Ribonuclease L
S-IgA	Secretory Immunoglobulin A
PKR	Protein Kinase R
NS1A	Influenza A Virus NS1
dsRNA	Double Stranded RNA
RNA	Ribonucleic Acid
RBD	RNA Binding Domain
eIF4GI	Eukaryotic Translation Initiation Factor 4GI
rFP-AIV-H5	Recombinant Fowlpox Virus
rFP	Recombinant Fowlpox
FBS	Fetal Bovine Serum
CEF	Chicken Embryo Fibroblasts
A-CEC	Aged Chicken Embryo Cells
DIMEM	Dulbecco’s Modified Eagle’s medium

EID_{50}	Embryo Infectious Dose 50
SPF	Specific Pathogen Free
PFU	Plaque Forming Units
BPL	Beta-Propiolactone
PFU	Plaque Forming Units
CPSF	Cleavage and Polyadenylation Specificity Factor
PABII	Poly A-Binding Protein II
MDCK	Mardin-Darby Canine Kidney
VP-SFM	Virus Production-Serum Free Media
MDT	Mean Death Time
EDP	Embryo Death Percent
PBS	Phosphate-Buffered Saline

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Classification of Influenza Viruses

Influenza viruses are members of the family Orthomyxoviridae and are important to both, veterinary as well as human health. The orthomyxoviridae family of viruses is divided into five different genera, including influenza virus A, B, C, Isavirus and Thogotovirus (Krug, 2001). Type A Influenza viruses can infect and cause disease in avian and mammalian species. Types B and C are limited to human infections, however in rare cases type C viruses have been isolated from other species (Shaw *et al.*, 2008). Isaviruses infect fish species and include infectious salmon anemia virus (Kibenge *et al.*, 2004). Thogotovirus are tick borne viruses that can infect humans as well as animals (Kuno *et al.*, 2001).

1.2 Ecology and Pathobiology of Avian Influenza Viruses

Avian influenza (AI) is a major respiratory disease of poultry caused by type A influenza viruses. AIV genome consists of eight linear negative-sense single stranded RNA segments, which code for eleven proteins, nine of which are structural and two are non-structural. The hemagglutinin (HA) and neuraminidase (NA) proteins are classified into 16 (HA) and 9 (NA) subtypes based on antigenic differences.

This dissertation follows the style of *Journal of General Virology.*

Influenza virus genome being segmented, in situations of co-infection of a single cell with two influenza viruses belonging to different subtypes, viral gene segments can reassort. This can result in 144 possible HA and NA combinations. Wild aquatic birds (water fowl and sea gulls) are the natural host for AIVs and are considered reservoir for all possible subtypes of avian influenza viruses in nature (Halvorson *et al.*, 1983). In wild birds, AIVs usually replicate in the intestinal tract without causing disease, and spread by fecal contamination in water habitat. Often, AIVs infect non-natural hosts such as land-based poultry (chickens, turkeys and quail), pigs and humans without producing any clinical signs of infection. However, occasionally, these AIVs can evolve to increased virulence causing significant morbidity and mortality.

AIVs that infect poultry are further classified based on their pathogenicity as highly pathogenic (HP) and low pathogenic (LP) avian influenza. HPAIV include viruses, which may cause mortality as high as 100%. Until now, only viruses from the H5 and H7 subtypes have been classified as HPAIV although not all H5 and H7 viruses cause HPAI. The rest of the viruses cause LPAI (Thiermann, 2007) which can be manifested by mild respiratory disease, reduction in egg production and can be exacerbated by other pathogens and environmental conditions causing a much more serious disease. It is important to note that all LPAIV of the H5 and H7 subtypes are considered notifiable (LPNAI) since they can mutate to HPAIV (Garcia *et al.*, 1996; Hall, 2004; Rohm *et al.*, 1995; Suarez *et al.*, 2004).

It is well documented that AI pathogenesis is a polygenic trait (Ito *et al.*, 2001; Rott *et al.*, 1979; Stephenson *et al.*, 2004). In this regard, it has been shown that the

cleavability of the HA protein plays an important role since it restricts tissue tropism. HA is synthesized as a precursor (HA_0), which must be cleaved, post-translationally, by host proteases to create functional HA protein and produce infectious virus particles. While HA_0 of HPAIVs are cleaved by ubiquitous furine-like proteases(Rott *et al.*, 1995), HA_0 of LPAIVs are cleaved by trypsin-like proteases present only in the respiratory and enteric tracts (Klenk & Rott, 1988). As a consequence, infection with HPAIV cause systemic infections that result in numerous vital organs being affected while LPAIV cause localized respiratory or intestinal infections.

1.3 Morphology and Composition

Morphologically influenza viruses vary from spherical to filamentous shapes with a diameter of 80-120 nm and up to several microns in length. Influenza viruses are composed of viral structural proteins and a lipid membrane from host origin (Krug, 2001). Type A influenza virus genome consists of eight negative single-stranded RNA segments, which code for 11 proteins. The three largest segments encode for the three viral polymerase subunits, PB1, PB2, and PA, and an alternate reading frame in PB1 encodes the non-structural pro-apoptotic protein PB1-F2. Two medium sized segments code for the structural glycoproteins; hemagglutinin (HA) and neuraminidase (NA), which form projections on the surface of the virus particle and are important antigenic determinants. The viral nucleoprotein together with the viral RNA and the polymerase complex forms 8 ribonucleoproteins (RNP). RNP has a helical structure and consists of the viral RNA wrapped around the nucleocapsid protein (NP), encoded by the medium

sized viral gene segment. NP makes the viral RNA accessible to the replication machinery. The two smallest segments encode two proteins each. The NS segment encodes the non-structural protein 1 (NS1) and nuclear export protein (NEP). The M segment encodes for the matrix protein (M1), which covers the inside of the viral envelope, and the membrane bound ion channel-like protein (M2). The lack of proofreading mechanisms of the viral RNA polymerase complex makes the viral genome highly variable, with viable mutations occurring in the HA and NA genes resulting in several different subtypes.

1.4 Replication of Avian Influenza Viruses

Hemagglutinins present on the surface of AIV binds to sialic acid sugars on target cells. Virus then enters the cells via receptor-mediated endocytosis and, upon acidification of the endocytic vesicle the viral membrane fuses with membrane of the vesicle releasing the viral nucleocapsid into the cytoplasm (Krug, 2001). The viral nucleocapsid is then transported to the nucleus where the negative stranded viral RNA is transcribed by the viral polymerase complex into mRNA, using capped 5' ends of host pre mRNAs as primers to initiate synthesis (Plotch *et al.*, 1981). These viral mRNAs are then transported to cytoplasm where they are translated into viral proteins. A small number of NS and M viral segments mRNAs are spliced in the nucleus prior to being transported to the cytoplasm. mRNAs specifying for viral membrane proteins (HA, NA, M2) are translated in the rough endoplasmic reticulum where they enter the secretory pathway in which they undergo glycosylation (Krug, 2001). Protein of the polymerase

complex (PA, PB1, PB2) and NP are imported into the nucleus where they catalyze synthesis of full-length positive strand RNA (cRNA) followed by negative sense virion RNA. M1 and NS1 viral proteins are also transported into the nucleus, where M1 shuts down viral mRNA synthesis and in conjunction with NS2 aids in the export of RNPs to the cytoplasm (Krug, 2001). HA, NA and M2 proteins are transported to the cell surface and become incorporated in the plasma membrane while M1 migrates to the inner part of the cell membrane making contact with the cytoplasmic tails of HA and NA and the RNPs, linking the inner core components and the membrane proteins. Assembly of the virion is then completed at this location by budding from the plasma membrane.

1.5 Immune Protection in Avian Influenza Virus Infection

The goal of AI vaccination is to induce an immune response that not only protects against disease but also prevents infection and shedding. The "gold standard" for assessing protective immunity is the use of LPAI or HPAI virus challenge models (Swayne & Kapczynski, 2008b). The criteria for protection for HPAIV challenge is in terms of morbidity and mortality (Brugh *et al.*, 1979; Stone, 1987; 1988; Wood *et al.*, 1985). On the other hand, experimental LPAIV challenges typically do not produce clinical signs or death and as a result, such criteria cannot be used in assessing vaccine protection against LPAIV. Instead, quantitative virus reduction in respiratory/digestive tract is the main criteria to evaluate protection (Capua *et al.*, 2004; Swayne, 2003; Swayne *et al.*, 1997).

1.5.1 Innate Immune Response and Avian Influenza

Innate immunity is non-specific, depends on factors that exist prior to microbe invasion and is capable of a rapid response to pathogens. The innate immune system detects pathogens through pattern recognition receptors (PRRs) (Koyama *et al.*, 2007) which recognize molecular markers of microbes known as pathogen associated molecular patterns (PAMPs). To detect and immidiately induce an innate immune response, different host species use a variety of sensors which follow in two main classes:

a. Toll like receptors (TLRs): TLRs are transmembrane proteins containing luminal leucine-rich repeats (LRRs) that sense pathogen-associated molecular patterns (Kawai & Akira, 2007). TLRs, involved in the detection of viral nucleic acids are located on the cell surface (TLR3) or in endosomal compartments (TLR3, TLR7, TLR8, and TLR9) (Iwasaki & Medzhitov, 2004). TLRs involved in detection of influenza viruses are TLR3 and TLR7/8.

b. Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs): RLR family member, RIG-I, is a cytoplasmic protein that detects single stranded viral RNA products; therefore, it can detect influenza viruses within the cytosol (Kato *et al.*, 2006).

Influenza virus infection induces TLR and RIG-I pathways leading to the activation of inflammatory cytokine production, mainly interferon (IFN) α/β (McCartney & Colonna, 2009).

Interferon produced upon stimulation of the innate immune response, binds to homologous receptor complex IFNAR and induces transcription of more than 100 IFN-stimulated genes (ISGs), whose combined action leads to the generation of an "antiviral state" in non-infected cells (Bluyssen *et al.*, 1996; Haque & Williams, 1994; Stark *et al.*, 1998). The antiviral molecules stimulated by IFN are: protein kinase stimulated by dsRNA (PKR), 2'-5' oligoadenylate synthetases (OAS) and myxovirus resistance gene (Mx) (Biron *et al.*, 2001). While Mx sequesters viral ribonucleoproteins to specific sub-cellular compartments, PKR phosphorylates downstream substrates upon recognition of dsRNA, including the elongation initiation factor eIF2-α, resulting in the inhibition of protein translation. The OAS proteins are also activated by dsRNA, leading to the generation of 2'-5' oligoadenylates, which activate ribonuclease L (Rnase L) that degrades cellular and viral RNA. Both PKR and the OAS/RNaseL systems have profound inhibitory effects on basal cellular processes that eliminate virus-infected cells by suicide (Samuel, 2001). IFN can also induce antiviral effect through other multiple pathways (Zhou *et al.*, 1999) suggesting that hosts have evolved redundant pathways to resist virus infection by using multiple mechanisms to counteract viral resistance to one particular pathway; also different factors of the IFN system mediate inhibition of specific virus families (Garcia-Sastre & Biron, 2006).

1.5.2 Adaptive Immune Response and Avian Influenza

Cytokines secreted during the induction of innate immune response stimulate and influence the nature of the adaptive immune response. A CD4+ T-helper type 1 response profile includes gamma interferon, IL-2, 15, 18 and is associated with a strong CD8+ T-

cell-specific response (Swayne & Kapczynski, 2008b). Previous studies have shown that CD8+ CTL directed against viral HA and NP epitopes conserved among influenza A viruses contribute to protection (Altstein *et al.*, 2006; Anderson *et al.*, 1992). CD4+ T helper type-2 response profile includes IL-4, 5,10 and stimulate antibody production (Swayne & Kapczynski, 2008b). In poultry, during natural infection, the humoral immune response includes systemic as well as mucosal antibody production. The humoral immune response plays a principal role in protection against AIV (Chambers *et al.*, 1988). Antibodies against HA block viral attachment to host cells, preventing infection. As a result, the immune protection offered, is strongest against the specific or closely related strains, depending on antigenic relatedness. On the other hand, antibodies to NA provide only partial protection against HPAIV challenge (Sylte *et al.*, 2007). Similarly, internal proteins have been shown to provide insufficient protection, although they induce a good antibody response (Brown *et al.*, 1992; Webster *et al.*, 1991). The strong antibody response to NP has, however important diagnostic applications as it allows the monitoring of flocks by screening for antibodies to this highly immunogenic protein. Secretory antibodies probably play an important role in the recovery of infected birds and by provides protection from further infections, particularly in the case of LPAI, which is primarily a mucosal infection. Mucosal infection by viruses like AIV also results in the induction of cell-mediated immunity, as manifested by CD4+ T helper-type 1 cells, as well as CD8+ cytotoxic T-lymphocytes. These responses result in synthesis of secretory immunoglobulin A (S-IgA) antibodies, which provide an important first line of defense against invasion of deeper tissues by

these pathogens (van Ginkel *et al.*, 2000)(ref). Resistance of S-IgA to proteolysis in external secretions and greater ability to prevent attachment of influenza virus as compared to IgY and monomeric IgA (Taylor & Dimmock, 1985) emphasizes the role and importance of mucosal immunity in controlling avian influenza.

1.6 The Influenza A Virus NS1 (NS1A) Protein

The NS gene of influenza A virus encodes two different proteins, NS1 and nuclear export protein (NEP) (Esposito *et al.*, 2006). NS1 of influenza A viruses contains around 230/237 amino acid residues depending on strain of the virus. NEP is a product of the spliced NS mRNA and shares the first 10 amino acids with NS1. NS1 is the most abundant nonstructural protein of influenza A virus expressed in infected cells. The NS1 protein of influenza viruses has been shown to block the cellular interferon pathway, which plays a crucial role in the innate antiviral defense mechanism of eukaryotic cells. (Bergmann *et al.*, 2000; Garcia-Sastre *et al.*, 1998; Hale *et al.*, 2008b; Kochs *et al.*, 2007). The amino-terminal domain of the NS1 protein of influenza A virus has a double stranded (ds) RNA binding domain, which inhibits the synthesis of IFN α/β by preventing the activation of double-stranded RNA mediated activation of protein kinase R (Bergmann *et al.*, 2000). The carboxy-terminal domain of NS1 contributes to its IFN-antagonistic properties, possibly by enhancing NS1 stability (Wang *et al.*, 2002) and by binding cellular proteins involved in mRNA synthesis. There has been increasing interest in using NS1 mutant viruses as a "modified live vaccine" since it is attenuated in growth in immunocompetent host and could also induce rapid release of several

inflammatory cytokines. However, replication of influenza virus lacking the entire NS1 coding region, is severely inhibited resulting in weak induction of protection. Therefore, influenza viruses expressing truncated NS1 proteins could have reduced virulence causing attenuation of the virus without compromising immune protection against a challenge.

1.6.1 Structure and Function of NS1

NS1 protein was first identified more than 35 years ago. However only in the last decade, the function of NS1 during influenza A virus infection has begun to be understood. NS1 protein is multi-functional with a major function being in post-transcriptional regulation of cellular gene expression (Hale *et al.*, 2008a).

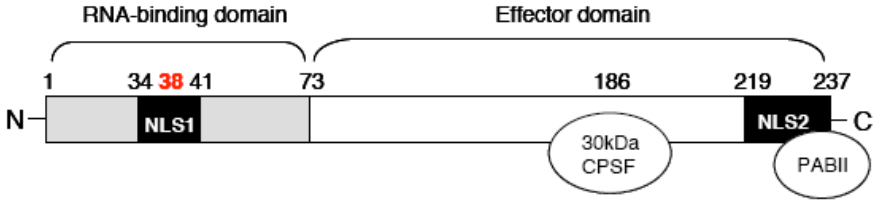

Fig.1. Schematic representation of the functional domains of the NS1 protein of type A influenza virus. The location of binding domains of known cellular proteins are indicated.

1.6.1.1 Structure and Function of the N-terminus

The RNA-binding domain (RBD) of NS1 is located at its N-terminal end. An N-terminal structural domain, which comprises the first 73 amino acids of the intact protein

NS1 (1-73), possesses all of the dsRNA binding activities of the full-length protein (Bornholdt & Prasad, 2008) as shown in Fig 1.

The NS1 RBD is almost totally α-helical and forms a symmetrical homodimer with a unique six-helical chain fold. Each polypeptide chain of the RBD consists of three α-helices (Bornholdt & Prasad, 2008): α -helix 1: Asn4-Asp24; α -helix 2: Pro31-Leu50 and α -helix 3: Ile54- Lys70 X-ray crystal structure of NS1 RBD has shown that it forms a symmetric homodimer in solution. Interestingly, the RBD of NS1 does not share any sequence homology with any known RNA binding protein (Liu *et al.*, 1997). NS1 with a unique six-helical chain fold. Each polypeptide chain of the RBD consists of three α-helices (Bornholdt & Prasad, 2008): α -helix 1: Asn4-Asp24; α -helix 2: Pro31-Leu50 and α -helix 3: Ile54- Lys70 X-ray crystal structure of NS1 RBD has shown that it forms a symmetric homodimer in solution. Interestingly, the RBD of NS1 does not share any sequence homology with any known RNA binding protein (Liu *et al.*, 1997). NS1 possesses a positive electrostatic charge due to a high content in basic amino acid residues (Chien *et al.*, 1997), which play an important role in binding sites to both double-stranded and single-stranded nucleic acids.

1.6.1.2 Structure and Function of C Terminus

The C-terminus of the NS1A protein mainly contains binding domains to three cellular proteins: eIF4GI, 30 kDa subunit of CPSF (CPSF30) and PAB II.
Like the RBD, the effector domain also forms dimers (Bornholdt & Prasad, 2006). Each monomer consists of seven β strands and three α-helices. NS1 binds two CPSF subunits

per dimmer which result in the efficient shut down of cellular mRNA maturation and export.

The NS1 effector domain inhibits host mRNA maturation by interfering with mRNA polyadenylation (Nemeroff *et al.*, 1998), mRNA nuclear export (Fortes *et al.*, 1994) and pre-mRNA splicing (Fortes *et al.*, 1994; Lu *et al.*, 1994; Qiu *et al.*, 1995). The effector domain of NS1 also enhances viral replication by interacting with the eukaryotic translation initiation factor 4GI (eIF4GI), resulting in a preferential translation of viral mRNAs over host mRNAs (Burgui *et al.*, 2003) and modulates viral RNA transcription and replication (Shimizu *et al.*, 1994). NS1 has also been shown to interact with the retinoic acid-inducible gene I product (RIG-I) inhibiting downstream activation of IRF-3 and therefore preventing transcriptional induction of IFN-β (Mibayashi *et al.*, 2007). Recently, the residue at position 92 of H5N1 virus was shown to be crucial for virulence of the virus (Seo *et al.*, 2002). Most viruses present a Asp at position 92, and its mutation to Glu is linked to increased virulence and cytokine resistance in certain H5N1 strains (Seo *et al.*, 2002). Asp interacts with Ser195 and Thr197 of NS1 protein, which is phosphorylated by the nucleocapsid protein and destabilizes NS1, leading to the induction of apoptosis. The mutation Asp92Glu lowers the efficiency of NS1 phosphorylation, resulting in a virulent phenotype by prolonging the viral life cycle (Bornholdt & Prasad, 2006).

Recently, residues 80–84 in H5N1 strains have been implicated in cytokine resistance but not virulence (Long *et al.*, 2008). These residues are part of a flexible linker between the RBD and the effector domain, and thus their deletion may greatly

alter the orientation or stability of the RBD, or both. This deletion could compact NS1 and confer more stability to the RBD, possibly by increasing dsRNA-binding affinity (Li *et al.*, 2004).

Despite the importance of the above-mentioned functions of NS1, it has been shown that NS1 is not absolutely required for virus replication. Influenza viruses lacking NS1 or containing truncated forms of the gene are able to replicate well in hosts which are defective in IFN production (Vero cells, $STAT^{-/-}$ mice and 7-day-old embryonated eggs) (Egorov *et al.*, 1998; Garcia-Sastre *et al.*, 1998; Quinlivan *et al.*, 2005), while their replication in IFN competent hosts is significantly reduced (MDCK cells, normal mice, 10-day-old embryonated chicken eggs and pigs) (Egorov *et al.*, 1998; Garcia-Sastre *et al.*, 1998; Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005).

Although the work cited above provides convincing evidence that NS1 is a major IFN antagonist, the mechanisms by which this process takes place continue to emerge. Further to this point, most experiments directed to identify the role of NS1 in counteracting the host IFN response have been conducted with human attenuated or avian viruses infecting humans in vitro, in mammalian animal models (Garcia-Sastre *et al.*, 1998; Talon *et al.*, 2000), and only more recently, with swine (Solorzano *et al.*, 2005) and chicken viruses (Cauthen *et al.*, 2007) in their natural host. In addition, because the functional properties of wild type and mutant viral proteins could be very different in their natural host, it is important to undertake a molecular and pathogenesis integrated approach to study the mechanisms of NS1 induced pathogenesis in chickens.

1.7 Prophylaxis for Avian Influenza Virus Infection

Avian influenza prevention strategies in commercial poultry are designed to achieve the following three goals (Swayne & Kapczynski, 2008a): (a) Prevention of infection; (b) Management and preventing the spread of an ongoing outbreak, to minimize economic losses; and (c) Eradication.

Strategies employed to achieve the above objectives include (Swayne & Kapczynski, 2008a):

1. Biosecurity measures: Quarantine and movement restrictions at regional, national and international levels, to prevent the spread of AI.
2. Surveillance and diagnostic programs: Aimed at early detection of potential pathogenic AIVs and to be informed on circulating AIV strains in nature.
3. Elimination of infected chickens: Policy of stamping out of infected chickens or controlled marketing of recovered or vaccinated chickens.
4. Decrease host susceptibility and increased resistance to AI: Through vaccinations and improvement in host genetics.

Criteria for vaccine licensing by the Animal and Plant Health Inspection Service (APHIS) of the USDA (Myers et al., 2003):

   a. Purity: The vaccine contents should exclusively contain the desired compounds and should be consistent in production.
   b. Safety : Vaccine should not cause any harmful effects on the host or environment.

c. Efficacy: Quantified standards of protection including decreased mortality, decreased virus shedding against homologous and heterologous challenge.
d. Potency: Maximum protection in least possible dosages.

1.8 Current and Experimental Poultry AI Vaccines

The currently licensed vaccines used in the United States are inactivated whole AIV vaccines and a recombinant fowlpox virus (rFP-AIV-H5) vaccine, which expresses the HA from A/turkey/Ireland/83/H5N8 (Swayne, 2006). A variety of HA subtype vaccines have been licensed under autogenous, conditional and full licensure categories as inactivated AI vaccines. In the US, field application of any H5 and H7 vaccines requires the approval of the state and federal government, but other subtypes require only state government approval. The licensing procedures in other countries of AI vaccines depends on the specific requirements of national veterinary biologics authority of that particular country in areas of safety, purity, potency and label approval for species, age and route of administration (Swayne & Kapczynski, 2008b). Globally, the majority of licensed vaccines are inactivated whole AI virus vaccines, principally of H5, H7, and H9 subtypes. In the current H5N1 HPAI epidemic, LPAIV seed strains from previous outbreaks have been used with the exception of A/chicken/Legok-Indonesia/03/H5N1, which is the only HPAIV used as a killed vaccine seed strain(Swayne & Kapczynski, 2008b). In recent years reverse genetic derived infectious clone seed strains have been developed using 6 internal genes from PR8 influenza A

vaccine strain and HA and NA from various HPAIV strains. In Asia, fowl pox-vectored and Newcastle disease virus- vectored vaccines incorporating HA gene, where HA gene was altered from HPAIV to an LPAIV.(Ge *et al.*, 2007).

1.8.1 Inactivated AI Virus Vaccines

Preparation of Inactivated vaccines for avian influenza for chickens require three important steps:

a. Propagation of avian influenza virus in embryonated chicken eggs
b. Chemical inactivation of the virus using formalin, beta-propiolactone or binary ethyleneimine.
c. Preparation of oil-emulsion of the inactivated virus

Preparation and administration cost for killed vaccine on a commercial scale are much higher than that for live vaccines. However, killed vaccines are much safer than live attenuated vaccines, especially in case of influenza, since AIVs have the potential to mutate and recombine raising the risk of the vaccine virus to regain virulence. To reduce costs, poultry AI vaccines are not purfied and therefore structural (HA, NA, NP, M1, M2) and non-structural (NS1) proteins are present in the vaccine preparation (Suarez, 2005). Inactivated vaccines induce a strong antibody response but a weaker cell mediated immune response (Subbarao *et al.*, 2006). In recent years, in addition to the oil phase, different adjuvants have been added to inactivated vaccine formulations to increase immunogenicity (Fatunmbi *et al.*, 1992; Stephenson *et al.*, 2005). Such adjuvants include innate immune response stimulators such as toll like receptor ligands,

microbial components, or cytokines that independently induce a cell mediated immune response (Vogel, 2000).

1.8.2 AI Protein Subunit Vaccines

Studies have been conducted, where chickens are vaccinated with only one particular recombinant protein instead of including the entire virus. In case of AI since HA is the major neutralizing antigen, HA proteins from different strains of AI have been expressed in different systems such as animal or plant cells, bacteria, viruses and yeast (Chambers *et al.*, 1988; Crawford *et al.*, 1999; Davis *et al.*, 1983; De *et al.*, 1988; Saelens *et al.*, 1999; Schultz-Cherry *et al.*, 2000). The HA protein is then purified, quantified, oil emulsified, and parenterally injected. These vaccines are very safe, because no live AI virus is involved, however vaccine production costs may be too high to replace the traditional efficacious inactivated AI vaccines. Baculovirus vectors have also been used to express the HA of both H5 and H7 subtypes in culture supernatants of insect cells, which when used to immunize poultry have provided protection from H5 and H7 HPAI challenge (Crawford *et al.*, 1999).

1.8.3 Vectored Vaccines Expressing AI Genes

Several viral vectors such as infectious laryngotracheitis virus, vaccinia virus, human adenovirus 5, Venezuelan equine encephalitis virus, and retrovirus have been studied for expression of AIV proteins (Brown *et al.*, 1992; Chambers *et al.*, 1988; De *et al.*, 1988; Gao *et al.*, 2006; Hunt *et al.*, 1988; Luschow *et al.*, 2001; Toro *et al.*, 2007; Veits *et al.*, 2006). However, the most well known example in poultry is the recombinant fowl pox (rFP) vector containing the HA gene from either H5 or H7 subtype AIV (Beard

et al., 1992; Boyle *et al.*, 2000; Bublot *et al.*, 2007; Bublot *et al.*, 2006; Jia *et al.*, 2003; Qiao *et al.*, 2003; Swayne *et al.*, 2000; Webster *et al.*, 1991). rFP vaccines expressing H5 HA have been commercialized and used extensively in Mexico, El Salvador and Guatemala with over 2 billion doses used from 1997 to 2006, and a rFP expressing both H5 and N1 genes has been used in China (Swayne, 2008; Swayne *et al.*, 1997; Villarreal, 2006). Though these vaccines need to be administered parenterally, they can be applied early on in the hatchery (1 day of age), compared to 1-2 weeks of age in case of a killed vaccine, thus saving the labor of handling chickens.

1.8.4 DNA Vaccines

Plasmid DNA-based vaccines expressing the AI HA gene, under control of a eukaryotic promoter, have been shown to elicit protective immunity in chickens following H5 and H7 HPAI challenge (Fynan *et al.*, 1993; Jiang *et al.*, 2007; Kodihalli *et al.*, 1997; Le Gall-Recule *et al.*, 2007; Robinson *et al.*, 1993; Suarez & Schultz-Cherry, 2000b). In poultry, DNA vaccines can be administered by intramuscular, subcutaneous and in ovo routes of inoculation. DNA vaccination results in protein expression eliciting both cytotoxic and humoral mediated immunity and closely mimics live virus vaccines. However, current limitations for DNA vaccines include the high cost for manufacturing and the requirement of multiple vaccinations to achieve protective immunity.

1.8.5 Live Virus AI Vaccines

Live LPAI virus vaccines can provide protection against HPAI virus challenge and may be mass applied to commercial poultry. Since live virus vaccines induce humoral, cellular, and mucosal immunity, they provide superior protection compared to

inactivated vaccines (Beard & Easterday, 1973). However, the potential for live viruses to mutate and reassortant, especially for H5 and H7 subtypes, which have been shown to mutate from LPAI to HPAI viruses, precludes their use to control AI by the poultry industry. In recent years there has been increased interest in using truncated NS1 mutant viruses as a live attenuated vaccine since the NS1 gene impacts virulence and evasion of the host immune response, making them ideal candidates for live AI virus vaccines (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005).

1.9 Evaluation of Vaccine Efficacy

1.9.1 Direct Evaluation

The assessment of poultry AI vaccines for their ability to protect against LPAIV and HPAIV is best accomplished using a challenge model using a current circulating virus strain as a challenge virus. The following measures of protection can be used:

a. Prevention of clinical signs and death (Stone, 1987).
b. Prevention of egg production drops (Brugh et al., 1979; Stone, 1987).
c. Quantitative reduction in challenge virus shed from respiratory and gastrointestinal tracts (Swayne et al., 1997). This can be achieved by virus isolation in embryonating chicken eggs or tissue culture systems. Alternatively, quantitative realtime PCR has been used in recent years (Wang et al., 2008) to quantitate virus shedding.
d. Prevention of contact transmission is a desirable goal. Contact transmission studies have been carried out by introducing uninfected chickens as contact-

control chickens into cages with infected chickens one day after infection (Wang et al., 2008). However the assessment is difficult due to multiple variables such as bird density, sanitation and ventilation standards, which have to be taken into consideration during transmission in field situation.

e. Additional Attributes: Besides reducing virus shed and preventing clinical signs other important attributes include: (1) protection against high dose challenge (2) early and long periods of protection spanning 6-12 months (Swayne et al., 1997). (3) Minimum number of vaccinations to achieve protection. (4) Protection in multiple species of birds

1.9.2 Indirect Evaluation

Direct evaluation of vaccine is important for initial demonstration of vaccine efficacy. However once the efficacy of the vaccine is established, parameters such as serological response can be used to monitor vaccine efficacy of the vaccine in field condition (Swayne, 2008). Serological assays such as neutralization or hemagglutination inhibition titers are good and reliable tests for monitoring vaccine efficacy (Swayne, 2008). Decreased immune response could be caused due to vaccine failure or due to changes in circulating viruses.

1.10 Drawbacks of Current Avian Influenza Vaccines

A major disadvantage of vaccination against avian influenza, using traditional vaccines is the need to vaccinate against different HA and NA subtypes since the heterosubtypic imunne response is not protective (Suarez & Schultz-Cherry, 2000a) and

the circulating virus subtypes keep changing. Therefore, the current influenza research focus has been to develop a vaccine that will be protective for all influenza type A viruses. There are several highly conserved influenza proteins such as NP, M1 and M2 which are potential candidates for providing broad cross protection against heterosubtypic influenza viruses (Suarez & Schultz-Cherry, 2000a). However, vaccines eliciting primarily neutralizing antibody response are effective, the antibodies against NP and M1 internal proteins are not useful, since antibody to these proteins are not neutralizing and therefore not protective. The M2 protein, an influenza surface protein, provides a broad but limited protection in mice, and it has not yet been tested in poultry (Frace et al., 1999; Zebedee & Lamb, 1988). Induction of cell-mediated immune response has been considered to play an important role in protection against virus infections (Suarez & Schultz-Cherry, 2000a). Some research has been conducted to stimulate a cell-mediated immune response to the NP, since it is a well-conserved influenza A viral protein. The use of DNA vaccines containing the NP gene has shown limited protection against a heterotypic challenge in mice and ferrets (Bot et al., 1996; Donnelly et al., 1997; Ulmer et al., 1994; Ulmer et al., 1993). Moreover, fowlpox and retrovirus vectored vaccines expressing the NP gene were unable to generate a protective response in poultry. This is surprising since the use of live virus should have stimulated a cellular immune response (Brown et al., 1992; Webster et al., 1991). Another issue with cell mediated immunity is that a detectable response is not seen, until 4 days after challenge (Kodihalli et al., 1994). This delayed cell mediated immune response, appears

unlikely to protect from HPAI that can cause death as early as 1 day after experimental challenge (Suarez et al., 1998).

In summary the current vaccines, inducing antibody response to neutralizing epitopes of avian influenza confer an effective protection against homologous but not heterologous challenge. On the other hand, induction of cell-mediated immune response to conserved structural epitopes is believed to confer protection against different subtypes. However with current understanding such vaccines have been able to demonstrate only limited protection.against avian influenza challenge.

1.11 Potential of NS1 Mutant Virus as DIVA Vaccine

Due to trade implications, vaccination of chickens is not routinely practiced because of the inability to serologically differentiate infected from vaccinated animals (DIVA). Earlier studies have shown that it is possible to differentiate vaccinated from infected chickens based on NS1 protein (Zhao *et al.*, 2005). Using runcated NS1 mutant viruses as vaccines the vaccinated chickens can be differentiated from infected chickens based on lack of antibodies against complete NS1 protein. The absence of antibodies against NS1 could be used to differentiate between vaccinated and infected chickens (Lee *et al.*, 2004; Suarez, 2005). However, commercial poultry vaccine manufacturing incorporates some non-structural proteins (Suarez, 2005) as these vaccines are not purified, which can in turn interfere in serosurvielence of AIV. In such circumstances an NS1 mutant virus could be used as a vaccine seed stock, which would enable differentiation between vaccinated and infected chickens. Because the NS1 gene impacts

virulence and evasion of the host immune response, this virus exhibited decreased replication and attenuation of infectivity, desirable traits for a live AI virus vaccine, thus providing an example for attenuated live virus vaccines.

Recently, an AI virus with natural truncation in NS1 ORF was passaged for several rounds in 14-day-old ECE causing further truncations in the NS1 gene. This virus was shown to be attenuated in chickens and was shown to induce protection against a subsequent virulent challenge (Steel *et al.*, 2009; Wang *et al.*, 2008). On similar lines in a different study besides truncations in NS1 ORF point mutation was introduced in PB2, changing K at position 627 to E. This change was shown to cause further attenuation. These viruses were shown to be attenuated and protective, however their safety in terms of reversion to virulence was not tested in chickens.

CHAPTER II

CHARACTERIZATION AND EVALUATION OF AVIAN INFLUENZA NS1 MUTANT VIRUS AS A POTENTIAL LIVE AND KILLED DIVA (DIFFERENTIATING BETWEEN INFECTED AND VACCINATED ANIMALS) VACCINE FOR CHICKENS

2.1 Introduction

Avian influenza is a major respiratory disease of poultry caused by type A influenza viruses. Type A influenza viruses are members of the *Orthomyxoviridae* family and their genome consists of 8 RNA segments of negative polarity. The 8 segments code for a total of 11 proteins: 3 polymerase components (PA, PB1 and PB2), 3 membrane proteins (hemagglutinin or HA, neuraminidase or NA and ion channel protein or M2), a matrix protein (M1), a nucleocapsid protein (NP), a nuclear export protein (NEP) and two non-structural proteins (NS1 and PB1-F2).

Type A influenza viruses are also classified into subtypes based on the antigenic reactivity of the HA (16 subtypes) and NA (9 subtypes) proteins present on the viral envelope (Fouchier *et al.*, 2005; Webster *et al.*, 1992). Wild aquatic birds (water fowl and sea gulls) are the natural reservoirs for all subtypes of type A influenza viruses (Fouchier *et al.*, 2005; Webster *et al.*, 1992). On occasions, these viruses can infect non-natural hosts such as land-based poultry (chickens, turkeys and quail) without producing any clinical signs of infection. However, sporadically, these influenza viruses can evolve to increased virulence causing extensive economic losses (Alexander, 2000;

2007; Garcia *et al.*, 1996; Rohm *et al.*, 1995). Avian influenza viruses (AIV) are also classified as highly pathogenic (HP) or low pathogenic (LP) based on their virulence. HPAIV produce systemic infections and mortality may be as high as 100%. On the other hand, LPAIV cause a mild, primarily respiratory disease in poultry, which may be exacerbated by other infections or environmental conditions. HPAI originates from LPAI viruses, which are present in many wild bird species throughout the world. Thus all countries are potentially at risk of being infected with HPAI, this phenomenon has been well demonstrated in recent years with the global spread of H5N1 (Swayne & Kapczynski, 2008a).

Pathogenesis of AIV is a polygenic trait and HA, PB1, PB2, PA, N and NS1 have been implicated in host range and pathogenicity of influenza viruses (Asplin, 1970Cheung, 2002 #5356; Easterday *et al.*, 1968; Hatta *et al.*, 2001; Hinshaw *et al.*, 1986; Li *et al.*, 2005; Lipkind *et al.*, 1981; Perkins & Swayne, 2002; 2003; Seo *et al.*, 2002; Shinya *et al.*, 2004) The non-structural protein, NS1 is considered a virulence factor due to its ability to block the cellular interferon pathway (Garcia-Sastre, 2001; Wang *et al.*, 2000). The amino-terminal end of NS1 has a double stranded (ds) RNA binding domain, which inhibits the synthesis of IFN α/β by preventing the activation of double-stranded RNA mediated activation of protein kinase R (Wang *et al.*, 2000). The carboxy-terminal end of NS1 contributes to its IFN-antagonistic properties, possibly by enhancing NS1 stability (Nemeroff *et al.*, 1995; Wang *et al.*, 2002) and by binding cellular proteins involved in mRNA synthesis. There has been increased interest in the use of NS1 mutant influenza viruses as “modified live vaccines” because of their

attenuated growth in immunocompetent hosts and also their induction of several inflammatory cytokines such as IL1β and IL6 (Ferko *et al.*, 2004). Besides inhibiting the innate immune response, NS1 can also affect the adaptive immune response by inhibiting dendritic cell maturation and their capacity to induce T cell responses (Fernandez-Sesma *et al.*, 2006). However, replication of influenza virus lacking the entire NS1 coding region, is inhibited to a great extent resulting in weak induction of immune response. Therefore, attenuated truncated NS1 mutant viruses are potential candidates for live attenuated vaccines.

Due to trade implications, vaccination of chickens is not routinely practiced because of the inability to serologically differentiate infected from vaccinated animals (DIVA) (Suarez, 2005). Different approaches have been sought, for developing AIV vaccines with DIVA properties. It has also been shown that it is possible to differentiate vaccinated from infected chickens based on antibodies to NS1 protein using killed vaccine (Zhao *et al.*, 2005). NS1 being a non-structural protein, is absent when purified virions are used as a killed vaccine. However, poultry vaccine manufacturing on a commercial scale includes non-structural proteins (Suarez, 2005) and in such a situation an NS1 mutant AIV in which the more immunogenic domain (carboxy end) (Birch-Machin *et al.*, 1997) has been deleted could be used as a seed virus for making vaccines and DIVA strategy could be feasible option. A similar DIVA approach was demonstrated for swine influenza viruses, where the vaccinated animals could be serologically differentiated based on the presence or absence of antibodies to NS1 while still inducing protection against challenge (Richt *et al.*, 2006).

A DIVA approach would have a great impact for the poultry industry where AIV vaccines are used. In this study we assessed the biological properties and potential use and safety of an NS1 mutant virus with DIVA potential as live attenuated as well as killed vaccine.

2.2 Materials and Methods

2.2.1 Cells and Viruses

DF1, a chicken embryo fibroblast cell line, was cultured in Leibovitz and McCoy's growth medium containing 5% fetal bovine serum (FBS). Chicken embryo fibroblasts (CEF), aged chicken embryo cells (A-CEC) and 293-T human embryonic kidney cells were maintained in growth medium containing Dulbecco's modified Eagle's medium (DMEM) and 5% fetal bovine serum (FBS). Recombinant parental (H5N3) and NS1 mutant (H5N3/NS1/144) viruses were generated from A/Ck/TX/02 (H5N3) virus by the eight plasmid reverse genetics technique (8). H5N3/NS1/144 expresses the first 144 amino acids of the NS1 protein compared to the full length (230 amino acids) of the parental H5N3 virus. Viral stocks for H5N3 and H5N3/NS1/144 viruses were generated in 10 and 7 day-old embryonated chicken eggs, respectively. Viruses were titrated and the embryo infectious dose 50 (EID_{50}) was determined by the Reed and Muench method (Reed & Muench, 1938).

2.2.2 Cloning of A/Chicken/TX/02 H5N3 Genes for Reverse Genetics

To clone full-length genes of A/Ck/TX/02 (H5N3), viral RNA was isolated from 200 µl allantoic fluid with the RNeasy-Kit (Qiagen, Valencia, CA), according to the

manufacturer's instructions. First strand cDNA of all eight vRNA segments was synthesized using Uni12 primer (Hoffmann *et al.*, 2001) and OmniProII RT (Promega, Madison, WI) reverse transcriptase as per manufacturer's instructions. Individual viral segments were PCR amplified using *PFU-Ultra* polymerase (Stratagene, La Jolla, CA) and segment specific primers as previously described (Hoffmann *et al.*, 2001). PCR products were cloned into pCRBlunt (Invitrogen, Carlsbad, CA) and sequenced. A plasmid (pDualPol) containing a RNA polymerase I promoter and terminator sequence flanked by a RNA polymerase II promoter and polyadenylation signal was generated as previously described (Chin *et al.*, 2002). Once transferred to the pDualPol vector, the correct orientation of the viral cDNAs was confirmed by sequencing. H5N3/NS1/144 was generated by inserting an oligonucleotide containing an *Asc*I restriction site and stop codons in all three open reading frames (TAGCTAGGCGCGCCTAGCTA) at the *Psh*AI restriction site (nt 430) of the NS segment cDNA (Figure 1). This insertion resulted in the disruption of the carboxy terminal half (145-230 amino acids) of the NS1 protein maintaining intact the coding region of NEP.

2.2.3 Recovery of Viruses Using Reverse Genetics

Infectious H5N3 and mutant H5N3/NS1/144 viruses were generated as previously described (Hoffmann *et al.*, 2000) with some modifications. Briefly, 293-T cells and CEF were co-cultured at 1:1 ratio in a six well plate. After 24 hours, the cell culture media was replaced with 2 ml of OptiMEM supplemented with 2% FBS. Cells were transfected with 333 ng of each of the 8 plasmids mixed with 9 µl of Trans IT (Mirrus, Madison, WI) transfection reagent. Twenty four hours post transfection, the

media was changed to OptiMEM supplemented with 0.15% FBS and 1% BSA followed 24 hours later with OptiMEM supplemented with 0.15% FBS 1% BSA and 0.0005 µg/ml of TPCK trypsin. Seventy-two hours post transfection, 100 µl supernatant of H5N3 and H5N3/NS1/144 transfected cells was used to inoculate 10 and 7-day old specific pathogen free (SPF) embryonated chicken eggs, respectively. Viral stocks for samples positive for virus, as determined by HA, were further generated in SPF embryonated chicken eggs and titers determined as EID_{50}/ml.

2.2.4 Viral Growth Kinetics

Comparison of H5N3 and H5N3/NS1/144 viral growth kinetics was carried out in interferon competent A-CEC and in embryonated chicken eggs at different age in development. A-CECs were generated by aging freshly prepared CEF for 10 days as previously described (Sekellick & Marcus, 1986). A-CEC plated in 60 mm dishes were infected with either H5N3 or H5N3/NS1/144 at a multiplicity of infection (MOI) of 0.001 in DMEM media supplemented with 0.0005 µg/ml of TPCK trypsin, and cell culture supernatants were collected every 24 hours for five days. Virus titrations were carried out in DF1 cells and expressed as plaque forming units (PFU/ml). To study growth kinetics in embryonated chicken eggs, 100, 10, and 1 PFUs were inoculated into 6 and 13 day old embryonated chicken eggs, via the chorioallantoic sac route. Embryos were observed daily for mortality, up to 5 days post-inoculation, and allantoic fluid from dead embryos was assessed for HA activity.

2.2.5 Plaque Assay

Confluent monolayers of DF-1 cells in 6 wells were infected with 10-fold serial dilutions of infected A-CEC supernantants. After adsorbing the virus at 37°C for 1 h, the infected cells were overlaid with 1% agarose in VP-SFM supplemented with 0.0005 µg/ml of TPCK trypsin and were incubated at 37°C for 48 hours. Plates were then fixed overnight with 10% buffered formalin, stained with 1% crystal violet, plaques counted and virus titer expressed as PFU/ml.

2.2.6 Quantification of IFN-ß mRNA by Real-Time RT-PCR

The relative levels of IFN-ß m-RNA produced by chicken cells in response to H5N3 and H5N3/NS1/144 virus infection was evaluated. Briefly, A-CEC were infected with H5N3 and H5N3/NS1/144 viruses at an MOI of 0.001 and 24 hours post infection, cells were trypsinized and total RNA isolated using the Ambion 4PCR RNA extraction kit (Ambion, Austin, TX) as per manufacturer's instructions. cDNA was synthesized from purified mRNA with poly(dT) primer (Ambion, Austin, TX) and Superscript II reverse transcriptase (Ambion, Austin, TX) and measured by real-time PCR analysis using primers specific for chicken IFN-ß (Forward 5' AGCTCTCACCACCACCTTCTCCT 3' and Reverse 5' TGGCTGCTTGCTTCTTGTCCTT 3') in an iCycler (BioRad Laboratories, Hercules, CA) for 40 cycles. Expression of chicken ß-actin mRNA was used to normalize cellular RNA levels between samples (Primers: Forward 5' TATTGTGATGGACTCTGGTGATG 3' and Reverse 5' TCGGCTGTGGTGGTGAAG 3') using standard procedures (Quinlivan *et al.*, 2005).

2.2.7 Experimental Design for Challenge Study Using Live H5N3/NS1/144 *Vaccine*

Two groups of 15 six-week-old Hyline chickens were inoculated by the intra-tracheal and intranasal routes with 0.2 ml of 10^6 EID_{50}/ml of H5N3 or H5N3/NS1/144 at 6-weeks of age. The immunized chickens were then challenged 4-weeks post immunization with 0.2 ml of 10^6 EID_{50}/ml H5N3 virus via the intra-choanal and intranasal route. Four days post immunization and post challenge five chickens from each group were euthanized and tracheal swabs collected for virus re-isolation and estimation of virus load. Virus re-isolation was performed in 8-day-old chicken embryos. Virus replication was determined by real-time RT-PCR for influenza matrix gene using AgPath-ID™ AIV- M kit (Ambion, Austin, TX) as per manufacturers instructions. Trachea and lung samples were collected for histopathological studies. All chickens were bled at 1,2 3, and 4 weeks post-immunization and sera was tested for presence of antibodies against NP using a commercial ELISA kit (Synbiotics Inc., San Diego, CA), HA using hemagglutinin inhibition test and NS1 using Western blot analysis.

2.2.8 Assessing Stability and Safety of H5N3/NS1/144 Vaccine as a Live Attenuated Vaccine

The stability and safety of the live H5N3/NS1/144 virus as a live vaccine was assessed by five back passages in 6-8 week old chickens. Three different lineages with one chicken per back passage were used to determine the reproducibility of the results. Briefly, three 6-week old chickens were inoculated intra-tracheally with $10^{6.0}$ EID_{50} and four days post inoculation tracheal swabs were collected in 2 ml DMEM and chickens euthanized and examined for lesions. One hundred µl of tracheal swab sample was used

to infect one chicken and this process was repeated five times. Two hundred µl of tracheal swabs fluid was used to extract viral RNA using the RNA Mag Max kit (Ambion Inc, Austin, TX) as per manufacturer's instructions. Viral cDNA was prepared using Uni-12 primer (reference) and MLV-RT (Ambion, Austin, TX) and full length NS viral gene was PCR amplified using *PFU-Ultra* polymerase (Stratagene, La Jolla CA) and segment specific primers as previously described (Hoffmann *et al.*, 2002). PCR products were separated on a 1% agarose gel in TAE, gel purified using PurelLink™ Quick Gel Extraction Kit (Invitrogen, Carlsbad CA), cloned into pCRBlunt™ vector (Invitrogen, Carlsbad CA) and sequenced.

2.2.9 Preparation of Killed Vaccine

Seed stocks of H5N3 and H5N3/NS1/144 viruses were propagated in thirty 10- and 7-day-old embryonated chicken eggs, respectively. Allantoic fluid was collected from embryos that died between 48-120 hours post inoculation, and tested for HA activity and bacterial contamination. Based on HA titers, allantoic fluids from embryos infected with a particular virus were pooled together and virus stock was inactivated by adding beta-propiolactone (BPL) at a ratio of 1:2000 (vol/vol) and allowing the fluid to remain at room temperature for 4 h followed by 24 h at 4°C. Inactivated virus was then concentrated by ultracentrifugation through a 30% sucrose cushion at 4°C for 1 h at 27,000 xg. Viral pellets were then resuspended and sonicated in appropriate volumes of STE buffer (10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA). Inactivation of the virus stocks was confirmed by the absence of detectable infectious virus upon passage of the treated and concentrated allantoic fluid in 7 day-old embryonated chicken eggs. HA

titers of the vaccine stocks were standardized to 128 HA units. Inactivated viruses were then emulsified in mineral oil as described by Stone et.al, (Stone, 1987; Stone *et al.*, 1983) prior to vaccination.

2.2.10 Experimental Design for Challenge Study Using Killed H5N3/NS1/144 Vaccine

Groups of 10 chickens were vaccinated, at three weeks of age, subcutaneously in the nape of the neck with 0.5 ml (128 HA units) of either H5N3 or H5N3/NS1/144 killed viruses. Vaccinated chickens were boosted with second dose two weeks later by the same method and route. Two weeks later (seven weeks of age), chickens were challenged with 0.2 ml of 10^6 EID_{50}/ml parental H5N3 virus via the intrachoanal/intranasal route and observed for clinical signs of disease up to 7 days post challenge. Four days post challenge all chickens were euthanized and tracheal swabs collected for virus re-isolation in 8-day-old embryonated chicken eggs. In addition, virus replication at four days post challenge was determined by real-time RT-PCR for influenza matrix gene using AgPath-ID™ AIV- M kit (Ambion, Austin, TX) as per manufacturers instructions. Chickens were bled at 1, 2, 3 and 4 weeks post vaccination and sera tested for presence of antibodies against NP using a commercial ELISA kit (Synbiotics Inc.) and HA using hemagglutinin inhibition test .

2.2.11 SDS-PAGE and Western Blot Analysis

The immune response to NP and NS1 proteins in chickens vaccinated with H5N3 and H5N3/NS1/144 viruses was examined by Western blot analysis. Briefly, baculovirus expressed and purified NP and NS1 proteins (10.5 ng of /lane) (Watson *et al.*, 2008) were separated in 12% SDS-PAGE gels and transferred to an Immobilon-P membrane

(Millipore, Billerica, MA). Serum samples were diluted 1:35 in 5% non-fat dry milk in PBS and incubated on the membrane at room temperature for 1 h. Membranes were washed 3 times/5 min each with PBST (PBS. 0.05% Tween 20) and then incubated with 1:1,000 dilution of rabbit anti-chicken HRP labeled secondary antibody (Bethyl Laboratories, Montgomery, TX). Membranes were incubated 1 h at room temperature and washed three times before adding TMB membrane substrate (KPL, Gaithersburg, MD).

2.3. Results

2.3.1 Viral Growth in Embryonated Chicken Eggs

To assess viral growth in interferon incompetent and competent systems, ten, 6- and 13-day-old eggs respectively, were inoculated with 1, 10 and 100 PFU of H5N3 or H5N3/NS1/144 viruses. Viral growth was measured in terms of embryo mortality and was confirmed by HA activity in the allantoic fluid of dead embryos. Interestingly, in 6-day-old embryonated chicken eggs, H5N3/NS1/144 grew better than parental H5N3 virus. However, in 13-day-old embryonated chicken eggs, H5N3/NS1/144 virus replication was significantly impaired when compared to H5N3 as shown in Fig. 2.

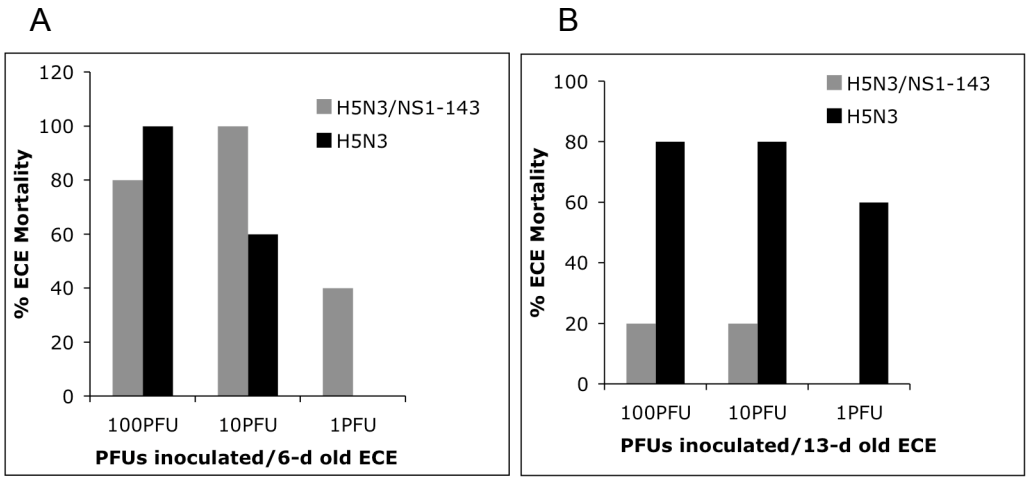

Fig. 2. Characterization of viral growth in embryonated chicken eggs (ECE). (a) The allantoic cavities of 6-day-old (A)and 13-day-old (B) embryonated eggs were infected with 100, 10 and 1 plaque forming units (PFUs) of the recombinant viruses. The infected ECE were examined for mortality upto 5 days post inoculation and represented as percent dead.

2.3.2 H5N3/NS1/144 Replication is Impaired in A-CEC

In order to investigate the multi-step growth properties of H5N3/NS1/144 and H5N3 viruses in interferon competent A-CEC, confluent monolayers of A-CEC were infected at a low MOI (MOI = 0.001) in media supplemented with trypsin (0.0005 μg/ml). Supernatants from infected cells were collected at different time points post-infection (24, 48, 72 and 96 h) and virus titers determined by plaque assay on DF1 cells. Like in interferon competent 13-day-old ECE, replication of H5N3/NS1/144 virus in A-CEC was significantly reduced compared to H5N3 as shown in Fig. 3.

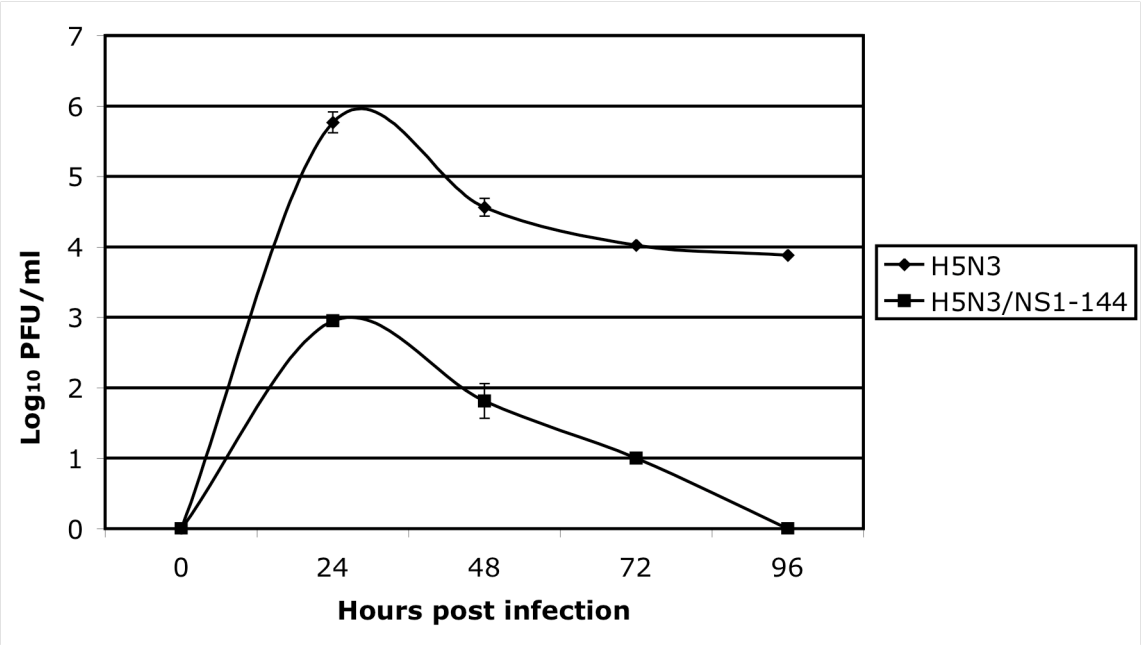

Fig. 3. Characterization of viral growth in aged chicken embryo cells (A-CEC). A comparison of viral growth was done for the recombinant viruses in A-CEC cells. A-CEC cells were infected with each of the viruses at an MOI of 0.001. Viral titers in infected cell culture supernatants were determined at the indicated times post-inoculation by plaque assay on DF1 cells.

2.3.3 Interferon Induction by H5N3/NS1/144

The NS1 protein of influenza virus has previously been shown to act as an interferon-α/ß antagonist (Cauthen *et al.*, 2007; Egorov *et al.*, 1998; Garcia-Sastre *et al.*, 1998). To determine whether the induction of antiviral state in A-CEC infected with H5N3/NS1/144 and H5N3 correlates with the level of IFN-ß induction, relative levels of IFN-ß mRNA in the infected cells was quantitated by real-time RT-PCR at 24 hours post infection. As shown in Fig. 4, H5N3/NS1/144 appears to induce significantly higher

levels of interferon than parental H5N3 virus. These results reflect the inability of H5N3/NS1/144 to induce anti-interferon activity in interferon competent systems.

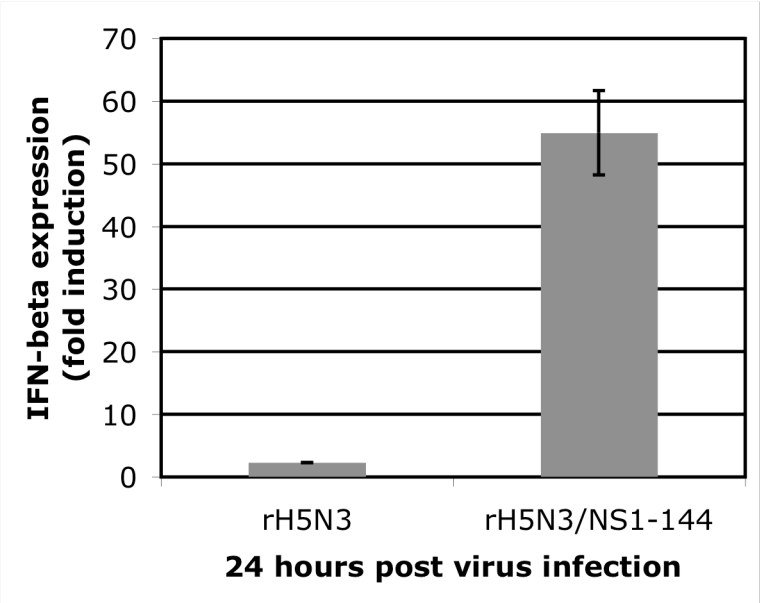

Fig. 4. Quantification of IFN-ß mRNA synthesis by A-CEC infected with H5N3, H5N3/NS1/144, at MOI of infection 0.001. Twenty-four hours post infection, cells were collected, RNA extracted and IFN-β mRNA expression determined by real-time RT-PCR. The results represent relative IFN-β mRNA levels normalized with levels of β-actin mRNA.

2.3.4 Efficacy of H5N3/NS1/144 *Virus as a Live Attenuated Vaccine*

Attenuation of H5N3/NS1/144 was based on lesions and virus isolation from immunized chickens. On the other hand, efficacy of H5N3/NS1/144 virus as a live vaccine was evaluated based on protection against homologous, H5N3 challenge. Chickens immunized with H5N3 but not with H5N3/NS1/144 virus showed gross lesions 4 days post inoculation. Lesions observed with H5N3 were tracheal plugs and

inflammation of the trachea. Histologically, severe lymphocytic and mononuclear infiltration was observed in chickens immunized with H5N3 but not with H5N3/NS1/144. Virus replication was demonstrated by virus re-isolation from tracheal swabs of chickens immunized with both H5N3 and H5N3/NS1/144 viruses as shown in (Table 1). In addition, virus quantification, in terms of Ct values from real-time RT-PCR of viral RNA isolated from swab samples, indicated that H5N3/NS1/144 was significantly attenuated in its replication.

Table 1. Lesions and virus shedding in chickens vaccinated with live virus vaccines and challenged with 10^5 EID_{50} of H5N3 virus. Tracheal swabs were collected for virus re-isolation and lung and tracheal sections were collected for histopathology 4 days post immunization and 4 days post challenge.

Vaccine Group	4 dpi*			4 dpc**		
	Gross Lesions	Virus Re-isolation	Viral RNA Levels***	Gross Lesions	Virus Re-isolation	Viral RNA Levels***
H5N3/NS1/144	0/5	4/5	35.2 (±5.0)	0/5	3/5	35.6 (±3.2)
H5N3	5/5	5/5	25.4 (±0.5)	0/5	0/5	> 40
Challenge Control				5/5	5/5	23.7 (±0.9)
Negative Control	0/5	0/5	> 40	0/5	0/5	> 40

*dpi = days post immunization
**dpc = days post challenge
*** RNA levels represented by Ct values. Numbers in parentheses represent standard deviation of mean

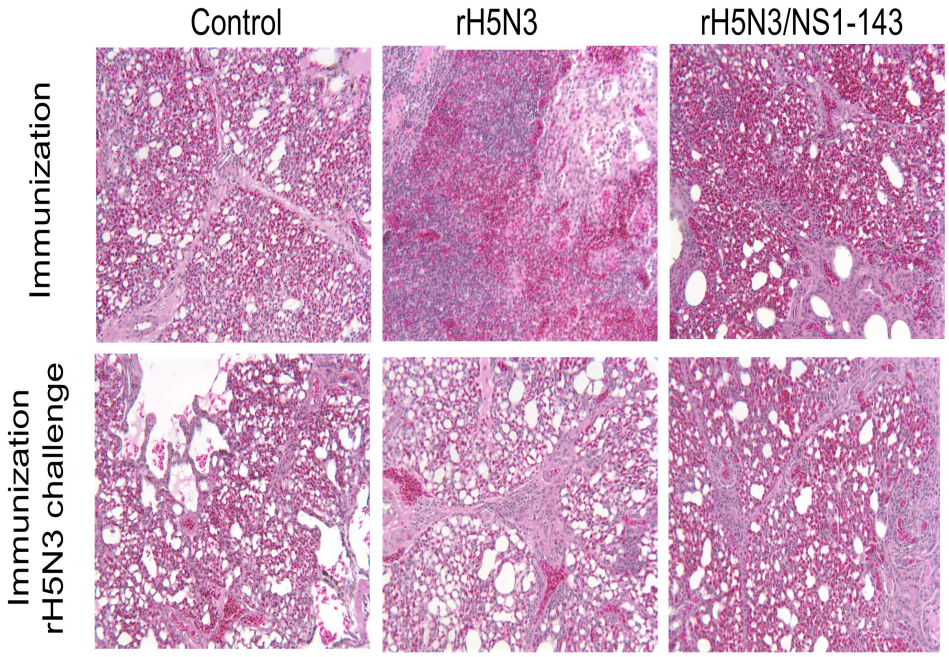

Fig. 5. H&E staining of lung sections of, rH5N3 and rH5N3/NS1/144 immunized (upper panel) and immunized and challenged (lower panel) chickens. Lungs of rH5N3 immunized chickens show severe lymphocytic and mononuclear infiltration with heterophilic inflammation 4 days PI. Similar lymphocytic and mononuclear infiltration is observed in lungs of rH5N3/NS1-144 immunized chickens 4 days PI but to a much lesser degree while the heterophilic infiltration appears to be absent. On the other hand, only slight lymphocytic infiltration was observed in chickens immunized with rH5N3 and H5N3/NS1-144 and challenged with H5N3.

Protection conferred by H5N3/NS1/144 and H5N3 viruses at four weeks post immunization, was compared and measured based on the amount of virus shedding and protection from gross and histo-pathological lesions at 4 days post challenge with H5N3 virus. No gross lesions were observed in the H5N3 and H5N3/NS1/144 immunized chickens when challenged with H5N3 virus; however, minor histo-pathological lesions were observed only in the lungs of H5N3/NS1/144 vaccinated chickens as seen in Fig. 5.

We were unable to re-isolate any challenge virus from chickens previously immunized with H5N3. However, 3 out of 5 chickens that received the H5N3/NS1/144 live vaccine were positive for virus re-isolation when tested in embryonated chicken eggs. These results were confirmed by real time RT-PCR of viral RNA isolated from tracheal swabs collected at 4 days post inoculation in H5N3 and H5N3/NS1/144 vaccinated chickens. Additionally, to assess protection, serum samples were collected every week for 4 weeks post vaccination to monitor antibody response. Significantly stronger immune response was observed in chickens vaccinated with H5N3 compared to H5N3/NS1/144 at one week post-vaccination based on HI and NP antibody titers. However, at 2, 3, and 4 weeks, post-vaccination the antibody response induced by H5N3/NS1/144 was not significantly different from H5N3 as shown in Fig 6.

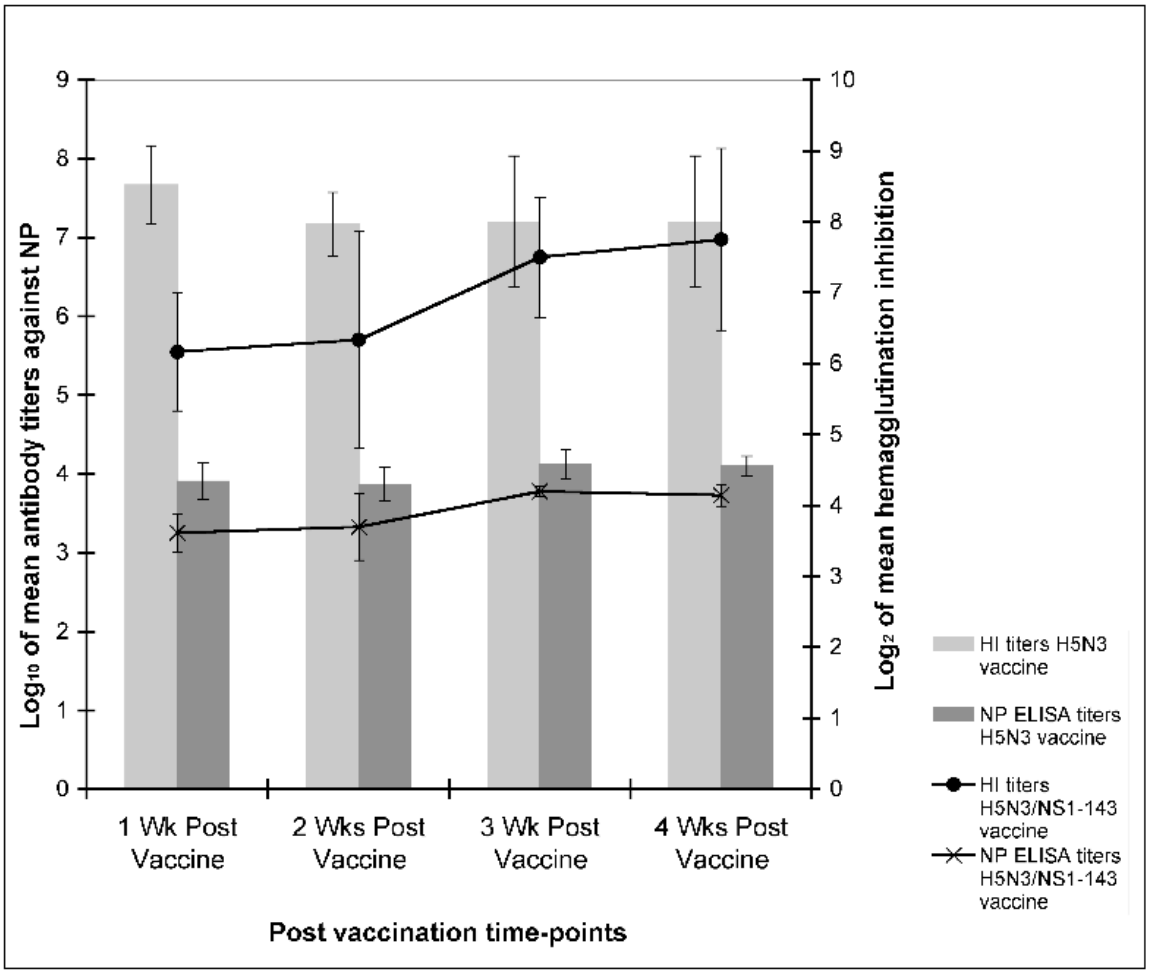

Fig. 6. Antibody levels (NP and HA) in live-vaccinated chickens. Serum samples were collected weekly after vaccination. Serological response was estimated based on $\log_{10}$ of average value of the HI titers and antibody titers against NP protein.

2.3.5 Safety of H5N3/NS1/144 Virus as a Live Attenuated Vaccine

To ensure the safety of H5N3/NS1/144 as a live attenuated vaccine, the virus was back passaged for 5 rounds in three independent lineages of 6-8 week old chickens. Interestingly, after two rounds of passage, lesions similar to those induced by H5N3 virus were noticed in the trachea and lungs of infected chickens. After five rounds of

passage, the stability of the NS gene was determined by RT-PCR amplification of genome segment 8 RNA extracted from tracheal swabs. It was interesting to see that various truncated forms of genome segment 8, were isolated from each of the 3 chickens tested. Upon sequencing it was evident that in lineages 1 and 2, a region containing three amino acids before and one amino acid after the stop codon were deleted (amino acid 142-145), resulting in an NS1 ORF protein with an internal deletion of only four amino acids compared to the parental H5N3 NS1 ORF. In lineage 3, a mixed population of different size gene segment 8 was detected Fig. 7; in one of the NS genes sequenced (L3a) only the stop codon was deleted, resulting in an ORF encoding the entire 230 amino acids NS1 protein. However, in another sequenced gene (L3b), 57 amino acids, from position 80-136, were deleted resulting in a truncated NS1 protein expressing only 87 amino acids. Since viruses from all three lineages showed increased pathogenicity, which correlated with the loss of the stop codon and/or the generation of deletions, we conclude that the H5N3/NS1/144 virus is unstable and reverted to virulence in chickens.

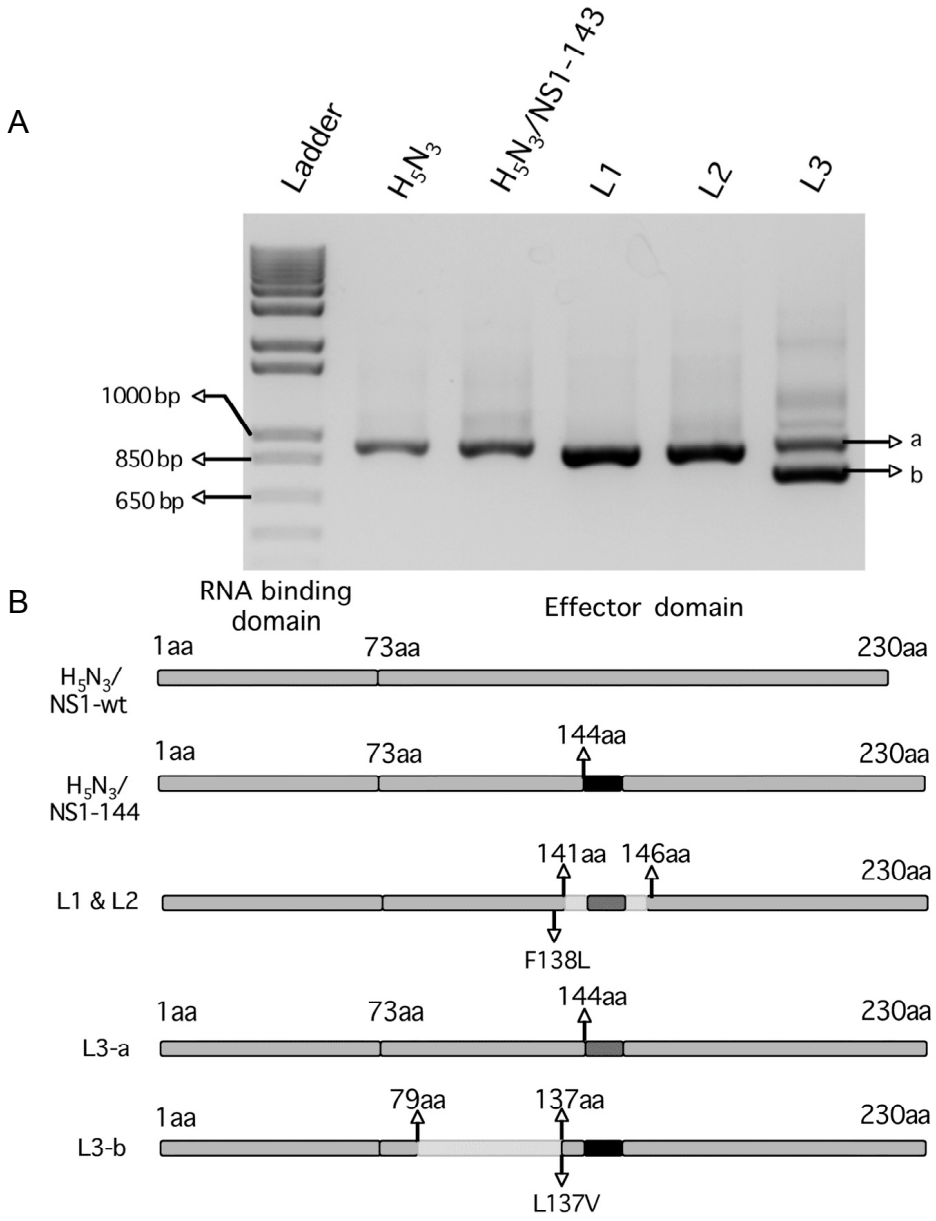

Fig.7. NS1 ORF after back passage of H5N3/NS1/144 in 6-8 week old chickens. A. Agarose gel electrophoresis of RT-PCR products of the NS gene segment of H5N3/NS1/144 after 5 rounds of back passage in chickens. PCR products for parental H5N3, NS1 mutant H5N3/NS1/144 and passage viruses from three different lineages (L1, L2 and L3) are shown. B. Schematic representation of the deletion (light colored regions) and point mutations in each of the three passage lineages (L1, L2 and L3) of H5N3/NS1/144 in chickens.

2.3.6 Efficacy of H5N3/NS1/144 as a Killed Vaccine

Killed vaccines were prepared from H5N3 and H5N3/NS1/144 virus stocks as described in Material and Methods and the level of protection was compared based on HI antibody titers and re-isolation of challenge virus from vaccinated chickens. Four days post challenge tracheal swab samples were taken from all chickens for virus re-isolation and viral RNA detection. Virus was re-isolated from tracheal swabs from 2/10 chickens immunized with H5N3/NS1/144 vaccinated while no virus was re-isolated from H5N3 vaccinated chickens as shown in table 2.

Table 2. Virus re-isolation and viral RNA detection in tracheal swabs of chickens vaccinated with killed H5N3 and H5N3/NS1/144 viruses and challenged with H5N3 1 week post boost.

Vaccine Group	**4 dpc***		
	Gross Lesions	**Virus Re-isolation**	**Viral RNA Levels****
H5N3/NS1/144	0/10	2/10	32.3 (±3.75)
H5N3	0/10	0/10	33.3 (±3.45)
Challenge Control	6/10	10/10	22.6 (±3.66)
Negative Control	0/10	0/10	> 40

* dpc = days post challenge

** expressed as Ct values as determined by Real-time RT-PCR using the AgPath-ID™ AIV-M Ambion kit

However, when viral RNA levels were compared by real time RT-PCR, no significant difference between the two vaccinated groups was noticed. Antibody titers, determined by HI and NP specific ELISA (Synbiotics Inc, San Diego, CA), were not detected at 1 week post vaccination with either vaccine, but were detected at 2 weeks post vaccination and steadily increased after booster vaccination with no significant differences observed between H5N3/NS1/144 and H5N3 vaccinated groups as shown in Fig. 8.

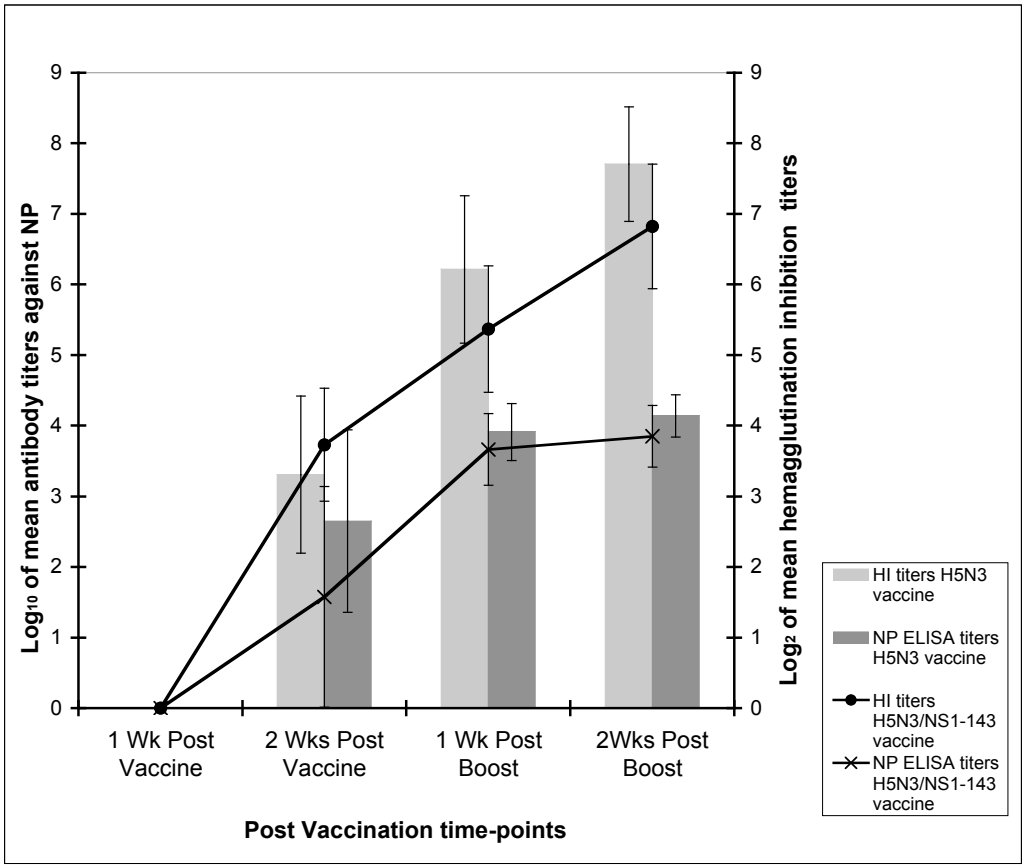

Fig 8. Antibody levels (NP and HA) in killed-vaccinated chickens. Chickens were vaccinated at 2 weeks of age and 2 weeks post vaccination chicken were boosted. Serum samples were collected weekly after vaccination. Serological response was estimated based on $\log_{10}$ of average value of the HI titers and antibody titers against NP protein.

2.3.7 Differentiation between Vaccinated and Infected Chickens

Presence of antibodies to NS1 protein in chickens vaccinated with live H5N3 and H5N3/NS1/144 was determined by Western blot analysis. As shown in Fig.9, chickens vaccinated with live H5N3 virus produced antibodies against NS1 protein while antibodies were absent or detected at very low levels, in chickens vaccinated with live H5N3/NS1/144. By comparison, antibody levels to NP were detected to similar levels in chickens vaccinated with live H5N3 and H5N3/NS1/144 viruses.

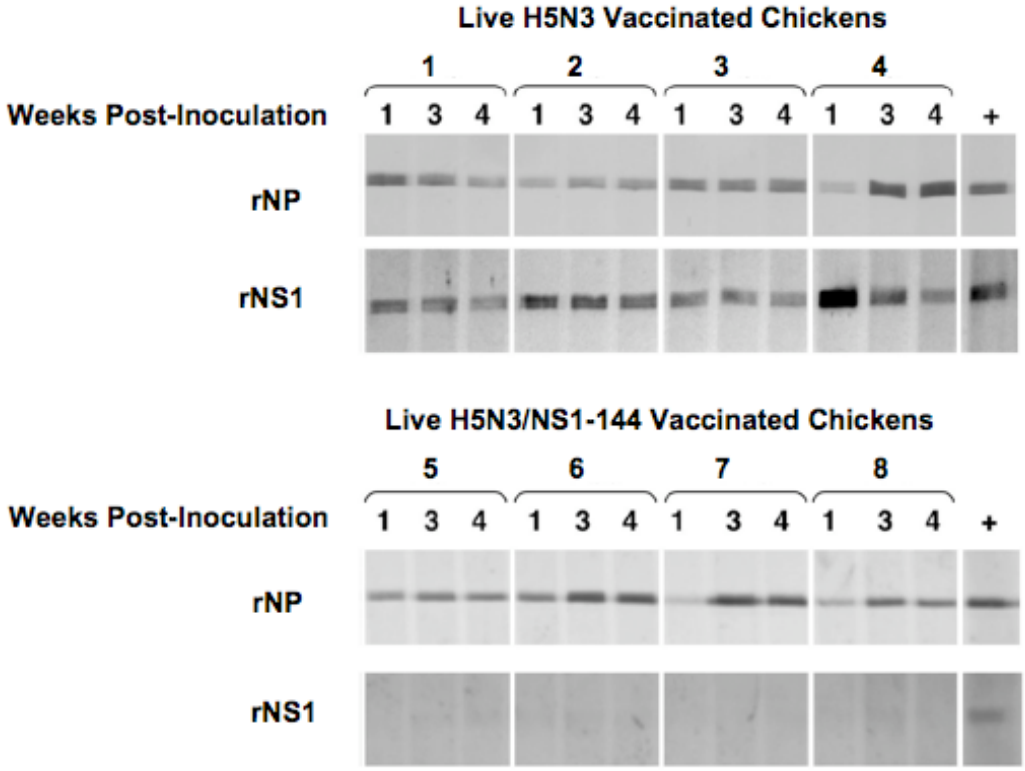

Fig. 9. Antibody response to rH5N3 and rH5N3/NS1-144 viruses. Western blot analysis of purified rNP, rNS1 and rM1 proteins using sera obtained from chickens inoculated with H5N3 (1, 2, 3 and 4) or rH5N3/NS1-144 (5, 6, 7 and 8) at 1, 3 and 4 weeks post vaccination. 10.5 ng of purified protein was loaded per lane. Chicken sera samples were diluted at 1:35.

2.4 Discussion

The increasing number of AI outbreaks worldwide highlights the difficulties encountered in controlling this disease. As a result, vaccination, which has rarely been used in the past, has become a recommended tool to support eradication efforts and to limit increasing economic losses due to AI. However, due to trade implications and the common use of serology in AI surveillance programs in commercial poultry, traditional vaccine strategies are not feasible and vaccines with DIVA properties have been recommended by international health organizations. Vaccines with DIVA properties have successfully been used to control AIV outbreaks (Capua *et al.*, 2002; Cattoli *et al.*, 2003; Lee *et al.*, 2004). To date the DIVA system that has been effectively used is based on heterologous vaccination, where the vaccine is made with a virus possessing the same hemagglutinin as the field virus and a neuraminidase of a different subtype. Although both HA and NA contain neutralizing epitopes, antibodies against HA are more important in protection (Suarez, 2005). As a result, vaccinating with a homologous HA subtype but a NA different to that of a circulating AI strain provides protection and at the same time makes it possible to differentiate between vaccinated and infected chickens. This strategy has been proven to be very effective in AIV outbreaks in chickens (Capua *et al.*, 2002), however it limits the number of potential effective vaccine candidates that can be selected since inclusion of the NA of the circulating viruses have shown to be more protective compared to a heterologous NA vaccine. A DIVA system based on the NS1 protein has been proposed for AI (Tumpey *et al.*, 2005). Targeting the NS1 protein enables the generation of attenuated vaccine viruses since NS1 is a

virulence factor due to its anti-interferon activity. This makes NS1 mutant viruses ideal candidates for a live attenuated vaccines with DIVA properties. In this study we evaluated the pathogenesis, protection and safety of such a LPAIV live vaccine candidate, which expresses a truncated NS1 protein (144 amino acids).

To generate the NS1 mutant virus, we established a reverse genetics system for the LPAIV A/Ck/Texas/2002 H5N3 virus. The NS mutant gene segment was generated by insertion of an oligonucleotide, with stop codons in all 3 open reading frames, at amino acid position 144 of the NS1 gene. The recovered NS1 mutant virus, H5N3/NS1/144, was attenuated compared to parental H5N3 virus in terms of growth and pathogenicity in interferon competent system *in vitro*, *in ovo* and *in vivo*. It was expected that that older age embryos would provide more of a challenge for H5N3/NS1/144 replication because the IFN-α/β system in older eggs is more mature (Sekellick *et al.*, 1990; Sekellick & Marcus, 1985). Consistent with previous studies, it was found that H5N3/NS1/144 virus was severely attenuated in comparison to the parental virus in 13-day-old embryonated chicken eggs (Fig. 1). In contrast, embryo mortality caused by H5N3/NS1/144 was, higher in 6-day-old embryonated chicken eggs compared to parental H5N3 virus (Fig. 1). Attenuation was also observed, in terms of significantly lower titers, for H5N3/NS1/144 in A-CEC when compared to parental H5N3. The lower virus titers in A-CEC correlated with increased type I interferon induction as shown by significantly increased mRNA expression of IFN-β (Fig. 3). These results are in accord with the observations of studies carried out in mice, swine, equine and chickens in which the IFN-β inducing capacity of influenza viruses expressing truncated NS1 was elevated

and corresponded with decrease ability of the virus to replicate in interferon competent systems (Cauthen *et al.*, 2007; Egorov *et al.*, 1998; Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005). H5N3/NS1/144, was also attenuated in chickens based on decreased virus re-isolation and pathogenicity while still induced an antibody response to NP similar to parental H5N3 virus. HI titers were only slightly lower for H5N3/NS1/144 at 1, 3 and 4 weeks post-vaccination when compared to parental H5N3 virus (Fig. 4). However, both groups of chickens were protected against lesions caused by homologous parental H5N3 challenge. In addition, there was a significant reduction of challenge virus in the tracheal swabs of H5N3 and H5N3/NS1/144 vaccinated and challenge groups compared to the non-vaccinated challenge control group. This demonstrates, the ability of H5N3/NS1/144 to induce a good immune response, conferring protection against a homologous challenge. However, significantly lower levels of challenge virus were detected in chickens pre-immunized with H5N3 virus compared to H5N3/NS1/144. This difference in protection could be due to attenuation of H5N3/NS1/144, which results in reduced replication in target organs inducing a weaker overall (cell and antibody mediated) immune response compared to parental H5N3 virus.

Immune sera of chickens inoculated with H5N3/NS1/144 displayed a high level antibody response against NP and HA proteins but below detectable levels of antibodies to NS1 (Fig. 8) making it possible to differentiate H5N3/NS1/144 vaccinated from H5N3 infected chickens.

We also evaluated the safety and stability of H5N3/NS1/144 virus as a live vaccine by carrying out 5 back passages in 6-8 week old chickens, in triplicate. After 5

back passages in chickens, H5N3/NS1/144 underwent immune selection and the virus reverted to virulence causing lung lesions similar to those produced by parental H5N3 virus. The NS gene segment of H5N3/NS1/144 was not stable and the engineered stop codon was deleted in all three, lineages, resulting in an almost full-length NS1 proteins. These results emphasize the importance of NS1 protein in pathogenesis of influenza viruses in interferon competent systems. Interestingly, passage lineage 3 also contained a truncated population of NS1 expressing only 87 amino acids. It will be interesting to study the replication properties and pathogenesis of a recombinant H5N3 virus expressing only 87 amino acids of NS1. Previous reports on the role of truncated NS1 mutants in equine and swine influenza viruses (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005) indicate that shorter forms of NS1 result in more stable proteins, ensuing in a more functional NS1 protein. In these studies, influenza viruses expressing shorter NS1 proteins were attenuated compared to parental virus; however, these viruses were more virulent when compared to other NS1 mutant viruses expressing longer proteins. Interestingly, when a naturally truncated NS1 mutant LPAIV was serially passaged in older age embryonated chicken eggs, further truncations in the NS1 gene segment resulted in further attenuation (Wang *et al.*, 2008). The differences observed in pathogenicity for viruses from the above mention study and the current study could be strain specific. The presence of an AIV population with a truncated NS1 ORF expressing 87 amino acids, after serially passage in chickens suggests that AIVs with shorter NS1 could have a selective advantage to help regain its virulence. However, the reversion of H5N3/NS1/144 to virulence in chickens cannot be fully attributed to the presence of an

87 amino acid protein since a population of viruses expressing a near full length NS1 protein (with 4 amino acid deletion at position142-145) was also present. Further studies are needed to address the significance of different size NS1 mutant viruses in virulence in chickens.

Though H5N3/NS1/144, used in this study was not stable and reverted to virulence during back passage in chickens, in a separate study (unpublished results) H5N3/NS1/144 infected chickens did not transmit the virus to uninfected cage-mates when compared to parental H5N3 infected chickens. The lack of natural transmission of H5N3/NS1/144 could restrict its circulation in chickens, precluding the possibility of reversion to virulence. It is possible that by deleting the carboxy-end of NS1 instead of inserting a stop codon we could avoid the reversion of virus to virulence. However, this is unlikely, since shorter NS1 proteins seem to be more stable and more functional, as demonstrated in other studies (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005). Consequently, more comprehensive studies are needed to characterize mutant viruses with shorter NS1 proteins. Alternatively, to ensure no reversion to virulence, a killed vaccine made out of mutant viruses with shorter NS1 protein as seed virus, as shown in this study, would be a much safer and feasible option.

The efficacy of parental H5N3 and H5N3/NS1/144 inactivated vaccines was tested against a homologous, H5N3 virus challenge. Virus shed was significantly reduced in chickens vaccinated with either killed vaccines and no significant difference in terms of antibody titers was observed between the two vaccinated groups (Table 2).

In summary, AIV vaccines with a truncated NS1 protein could be used to differentiate between vaccinated and infected chickens making DIVA strategy a feasible option. The NS1 mutant AIV generated in this study was highly effective as killed vaccine. However, its use as live virus raises safety concern, because back passage in chickens, resulted in reversion back to virulent phenotype. The future development of live NS1 mutants can only be feasible by identifying mutants that are unable to revert back and by developing mutant viruses unable to regain the wild type gene segment through re-assortment.

CHAPTER III

CHARACTERIZATION OF NS1 MUTANT AVIAN INFLUENZA VIRUSES TO GROW IN INTERFERON COMPETENT SYSTEMS

3.1 Introduction

Influenza A viruses are members of the Orthomyxoviridae family and their genome consists of eight single-stranded, negative sense RNA segments which encode 11 different proteins (PB2, PB1, PB1-F2, PA, HA, NP, NA, M1, M2, NS1, and NS2) (Chen *et al.*, 2001). Influenza A viruses are classified into subtypes based on their envelope proteins, hemagglutinin (HA) and neuraminidase (NA). Currently, 16 HA and 9 NA subtypes have been identified. Wild birds serve as reservoirs to all subtypes of influenza A viruses and transmission of these viruses to domestic poultry and their adaptation to the new host is well established (Swayne, 2007). Some of the avian influenza virus (AIV) subtypes, such as H5 and H7, which spread into domestic chickens, have shown to turn into highly pathogenic viruses (Swayne, 2007). Better understanding of AIV virulence factors is likely to help, generate safe and efficacious vaccines and design better intervention strategies. Besides HA, NA and proteins of the polymerase complex, the non-structural protein NS1 is also an important virulence factor (Ito *et al.*, 2001; Stephenson *et al.*, 2004). NS1 inhibits and resist innate antiviral immune response through several different mechanisms and at different stages of induction. These mechanisms have been elucidated using both artificial and natural

truncations as well as point mutations in the NS1 gene segment as reviewed by Hale *et.al.* (Hale *et al.*, 2008b).

NS1 protein consist of two domains an RNA binding domain and an effector domain. The RNA binding domain of the NS1 protein has been shown to play a crucial role in inhibiting host interferon response through its ability to bind to the replicative forms of viral RNA. Arginine at position 38 and lysine at position 41 within the RNA binding domain are thought to directly interact with RNA, mediating binding. The main function of the carboxy-terminal 157 amino acids of the NS1 protein, which contain the effector domain (amino acids 134 to 161) (Nemeroff *et al.*, 1995), is to stabilize and/or facilitate NS1 dimerization (Wang *et al.*, 2002) and has binding sites for several cellular proteins such as cleavage and polyadenylation specificity factor (CPSF), and poly (A)-binding protein II (PABII). The carboxy-terminal region of NS1 also contains a PDZ-binding motif (Chen *et al.*, 1999; Nemeroff *et al.*, 1998; Obenauer *et al.*, 2006), which directly or indirectly affects interferon induction. The presence of amino acid Glutamine (Q) at position 92 of NS1 in the H5N1/97 influenza viruses has been implicated in its ability to modulate the cytokine response and has been associated with the high virulence of these viruses in pigs. Recently, it was shown that the amino acid at position 149 of NS1 in highly pathogenic avian influenza (HPAI)-H5N1 affected the ability of the virus to antagonize the induction of IFN α/β (Li *et al.*, 2006). The valine to alanine mutation at position 149 caused increased expression of NS1 protein, which is believed to increase its ability to inhibit interferon, thereby making the virus more virulent in

chickens. This increase in virulence was demonstrated in terms of increased lethality in embryonated chicken eggs (ECE) and chickens.

Phylogenetically, NS1 proteins of influenza viruses are divided into alleles A and B (Ludwig *et al.*, 1991). Allele B includes exclusively NS1 proteins from avian viruses while allele A includes influenza viruses isolated from mammalian species. As little as 62% identity has been observed between alleles A and B (Hale *et al.*, 2008b). Most of the functional studies of NS1 have been limited to laboratory strains and highly pathogenic H5N1 strain of AIV, both of which belong to allele A. Recent studies have also shown that NS1 of allele B presents anti-interferon activity in chickens (Cauthen *et al.*, 2007). However, studies addressing the role of NS1 in the host-pathogen interaction between AIVs with allele B NS1 viruses and chickens are lacking.

We have recently shown, that a recombinant H5N3 LPAIV expressing first 144 amino acids of NS1 (H5N3/NS1/144) is attenuated with regards to growth and virulence when compared to parental virus (Brahmakshatriya VR, 2009). However, when H5N3/NS1/144 was propagated under innate immune pressure in older age ECE, further truncation of the NS1 gene segment occurred resulting in the expression of a NS1 protein with only 87 amino acids. Upon sequencing, we detected two additional point mutations in the RNA binding domain (position, 40 and 73) of two of the lineage passages. Since the RNA binding domain is critical in the anti-interferon activity of NS1, we sought to determine the significance of the truncation and point mutations on the virulence of the virus. Using reverse genetics, two NS1 mutant viruses (H5N3/NS1/87 and H5N3/NS1/87P) were generated and their biological characteristics determined *in*

ovo, *in vitro* and *in vivo* systems. Our studies show that the H5N3/NS1/87 and H5N3/NS1/87P viruses, showed increased virulence compared to H5N3/NS1/144 and this gain in virulence correlated with increased ability of the virus to inhibit interferon.

3.2 Materials and Methods

3.2.1 Cells and Viruses

DF1, a chicken embryo fibroblast cell line, was cultured in Leibovitz and McCoy's growth medium containing 5% fetal bovine serum (FBS). Chicken embryo fibroblasts (CEF), aged chicken embryo cells (A-CEC) and 293-T human embryonic kidney cells were propagated in growth medium containing Dulbecco's modified Eagle's medium (DMEM) and 5% FBS. Mardin-Darby Canine Kidney (MDCK) cells were propagated and maintained in Virus Production-Serum Free Media (VP-SFM) (Gibco, New York, USA). Recombinant parental (H5N3) and NS1 mutant viruses (H5N3/NS1/144, H5N3/NS1/87and H5N3/NS1/87P) (see description of viruses Sections 3.2.2 and 3.2.3) were generated by the eight plasmid reverse genetics technique (Hoffmann *et al.*, 2000).

3.2.2 Cloning of A/Chicken/TX/02 H5N3 Genes for Reverse Genetics

To clone full-length genes of A/Ck/TX/02/H5N3, viral RNA was isolated from 200 µl allantoic fluid using the RNeasy-Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. First strand cDNA of all eight vRNA segments was synthesized using Uni12 primer (Hoffmann *et al.*, 2001) and OmniProII RT (Promega, Madison, WI) reverse transcriptase as per manufacturer's instructions. Individual viral

segments were PCR amplified using *PFU-Ultra* polymerase (Stratagene, La Jolla, CA) and segment specific primers as previously described (Hoffmann *et al.*, 2001). PCR products were cloned into pCRBlunt (Invitrogen, Carlsbad, CA) and sequenced. A plasmid (pDualPol) containing a RNA polymerase I promoter and terminator sequence flanked by a RNA polymerase II promoter and polyadenylation signal was generated as previously described (Hoffmann *et al.*, 2000; Reddy, 2006). Once transferred to the pDualPol vector, the correct orientation of the viral cDNAs was confirmed by PCR. H5N3/NS1/144, an NS1 mutant virus, which expresses the first 144 amino acids of the NS1 protein (Figure 1), maintaining intact coding region of NEP, has already been described in earlier chapter.

3.2.3 Serial Passage of H5N3/NS1/144 in Older-Age-ECE

Allantoic fluid of H5N3/NS1/144 virus was serially passaged in increasing age embryonated chicken eggs (ECE) as shown in Fig.10. Virus propagation in the chorio-allantoic cavity of ECE was done as previously described (Swayne, 1998). Three independent lineages (L1, L2 and L3) were carried for 15 passages and allantoic fluid was collected 72-96 hours post-inoculation for every passage.

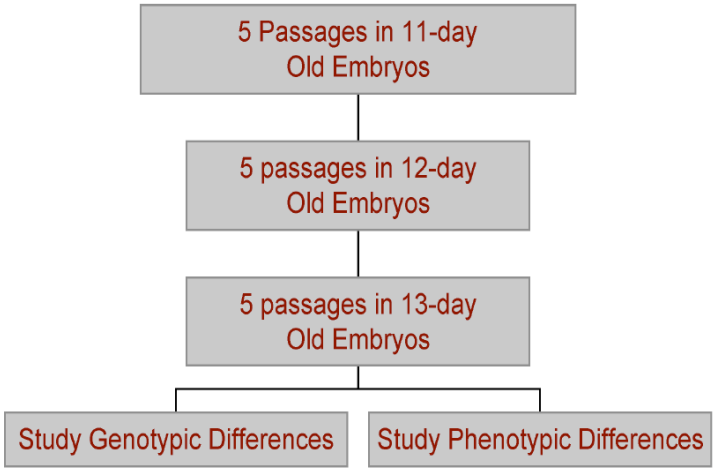

Fig.10. Schematic for serial passage of H5N3/NS1/144 in older age ECE.

The allantoic fluid collected was tested for HA activity and positive samples were diluted 10^{-3} or 10^{-4}, before the next round of propagation in ECE. After the first passage, the diluted virus was passaged into 3-4 ECE however, allantoic fluid from only one embryo was passaged, and allantoic fluid from the other infected embryos of the same lineage were kept as a backup. At no point during the serial passage were the viruses from two different eggs or lineages pooled. After 15 rounds of serial passages in older age ECE, each lineage was subjected to three rounds of plaque purification and plaque-purified viruses were propagated in 13-day-old ECE.

3.2.4 Viral RNA Extraction and NS1 Gene Specific Cloning and Sequencing

Viral RNA was isolated from plaque-purified viruses propagated in 13-d-old-ECE using the RNA Mag Max kit (Ambion Inc, Austin, TX) as per manufacturer's instructions. Viral cDNA was prepared using Uni-12 primer (Hoffmann *et al.*, 2001) and MLV-RT (Ambion, Austin, TX) and full length NS viral gene was PCR amplified using

PFU-Ultra polymerase (Stratagene, La Jolla CA) and segment specific primers as previously described (Hoffmann *et al.*, 2001). PCR products were separated on a 1.5% agarose gel in TAE, gel purified using PurelLink™ Quick Gel Extraction Kit (Invitrogen, Carlsbad CA), cloned into pCRBlunt™ vector (Invitrogen, Carlsbad CA) and sequenced.

3.2.5 Recovery of Viruses Using Reverse Genetics

Infectious parental (H5N3) and NS1 mutant viruses (H5N3/NS1/144, H5N3/NS1/87 and H5N3/NS1/87P) were generated as described by Hoffman et.al. (Hoffmann *et al.*, 2000). Briefly, 293-T cells and chicken embryo fibroblasts were co-cultured (1:1 ratio) in a six well plate. After 24 hours, the cell culture media was replaced with 2 ml of OptiMEM supplemented with 2% FBS. Cells were transfected with 333 ng of each plasmid mixed with 9 µl of Trans IT (Mirrus, Madison, WI) transfection reagent. Twenty-four hours post transfection, the cell culture media was changed to OptiMEM supplemented with 0.15% FBS and 1% BSA followed 24 hours later with OptiMEM supplemented with 0.15% FBS 1% BSA and 0.0005 µg/ml of TPCK trypsin. Seventy-two hours post transfection, 100 µl supernatant of H5N3, H5N3/NS1/87 and H5N3/NS1/87P transfected cells were inoculated into 10- day-old specific pathogen free (SPF) ECE while H5N3/NS1/144 was propagated in 7-day-old ECE. Viral stocks for recombinant viruses, were further generated in 10 or 7-day-old SPF ECE and virus titers were determined by plaque assay in DF1 cells and expressed as plaque forming units per ml (PFU/ml).

3.2.6 Viral Growth Kinetics

Comparison of the growth kinetics of H5N3, H5N3/NS1/87 and H5N3/NS1/87P viruses was carried out in DF1 and A-CEC. A-CECs were generated by aging primary chicken cells, obtained from 10-day old embryos, for 10 days as described earlier (Sekellick & Marcus, 1985). A-CEC plated, in 60 mm dishes were infected with either H5N3, H5N3/NS1/144, H5N3/NS1/87 or H5N3/NS1/87P at a multiplicity of infection (MOI) of 0.001 in DMEM media supplemented with 0.0005 µg/ml of TPCK trypsin. Cell culture supernatants were collected every 24 hours for five days in case of A-CEC and for 3 days in case of DF1 cells. Virus titers in the collected supernatants was determined by plaque assay in DF1 cells and determined as PFU/ml.

3.2.7 Plaque Assay

Confluent monolayers of DF-1 or MDCK cells in 6 well plates were infected with 10-fold serial dilution of infected A-CEC supernatants or viral stocks generated in ECE. After adsorbing the virus at 37°C for 1 h, the inoculum was removed and the infected cells were overlaid with 1% agarose in VP-SFM supplemented with 0.0005 µg/ml of TPCK trypsin and incubated at 37°C for 48 hours. Plates fixed over night with 10% buffered formalin, stained with 1% crystal violet, plaques counted and virus titer determined as PFU/ml.

3.2.8 Mean Death Time (MDT) and Embryo Death Percent (EDP) in Different Ages of ECE

The pathogenicity of H5N3, H5N3/NS1/87 and H5N3/NS1/87P viruses was examined by determining mean death time (MDT) and embryo death percent (EDP) in 7,

10 and 13-day-old ECE. To determine MDT and EDP, groups of twenty, 7- and 10-days-old ECE and twenty-five 13-day-old ECE, were inoculated via the chorioallantoic sac route with 10^2 PFUs of each virus or sham inoculated with phosphate-buffered saline (PBS). Embryos were then candled at 8-hour intervals for 6 days, embryo mortality recorded and presence of virus in dead embryos was confirmed by HA. Based on the number of ECE dead and time of death post inoculation, MDT was calculated as follows: the total sum of the product of, number of dead embryos at each time point and the time point in hours divided by the total number of dead embryos in that group (Perdue *et al.*, 1990).

3.2.9 Quantification of IFN-ß mRNA by Real-Time RT-PCR

The relative levels of IFN-ß m-RNA produced by chicken cells in response to replication of H5N3, H5N3/NS1/144, H5N3/NS1/87 and H5N3/NS1/87P viruses were evaluated. Briefly, A-CECs were infected with H5N3, H5N3/NS1/144, H5N3/NS1/87 or H5N3/NS1/87P at an MOI of 0.01, 0.001 and 0.0001. Six hours post infection, cells were trypsinized and total RNA was isolated using the Ambion 4PCR RNA extraction kit (Ambion, Austin, TX) as per manufacturer's instructions. cDNA was synthesized from mRNA with poly(dT) primer (Ambion, Austin, TX) and Superscript II reverse transcriptase (Ambion, Austin, TX) and levels of IFN-ß mRNA expression determined by real-time PCR analysis using primers specific for chicken IFN-ß (Forward 5' AGCTCTCACCAC CACCTTCTCCT 3' and Reverse 5' TGGCTGCTTGCTTCTTG TCCTT 3') in an iCycler (BioRad Laboratories, Hercules, CA) for 40 cycles. Expression of chicken ß-actin mRNA was used to normalize cellular RNA levels between samples

(Primers: Forward 5' TATTGTGATGGACTCTGGTGATG 3' and Reverse 5' TCGGCTGTGGTGGTGAAG 3'), using a standard procedure (Jaini *et al.*, 2006). Relative quantitation of mRNA expression was calculated as fold increase in inoculated versus mock-inoculated control A-CEC.

3.2.10 In Vivo Characterization of NS1 Mutant Viruses

The virulence of the NS1 mutant viruses was further examined by comparing virus growth of H5N3, H5N3/NS1/144, H5N3/NS1/87 and H5N3/NS1/87P viruses in 1-day-old chickens. Five groups of twenty, 1-day-old commercial layer-type Hyline chickens were inoculated by the intranasal/intrachoanal route with 0.1 ml containing 10^4 PFU of parental or NS1 mutant viruses. Five contact control chickens were introduced into the same cage one day post-infection. The chickens were observed daily for clinical signs of disease and four days post-inoculation all inoculated chickens were euthanized and observed for gross lesions. Lung samples were collected in tubes containing 1 ml tryptose phosphate broth to determine viral titers in terms of PFU/ml in DF1 cells and EID_{50}/ml in ECE. To assess the ability of the viruses to transmit, contact chickens were bled two weeks after exposure to virus-inoculated chickens and were checked for sero-conversion using a commercially available NP ELISA kit for avian influenza (Synbiotics, San Diego, CA).

3.3 Results

3.3.1 Immune Pressure Result in Genotypic Differences in NS1

After serial passage of H5N3/NS1/144 in older age ECE, the virus was adapted to grow in 13-day-old ECE. This serial passage caused selection of NS gene segments with a truncation between amino acid position 79 and 137 resulting in and ORF of only 87 amino acids in all three lineages (L1, L2 and L3). This deletion also caused a point mutation, changing leucine at position 137 of the parental H5N3/NS1/144 to valine at amino acid position 80 in the new, 87 amino acid long ORF. This virus is referred as H5N3/NS1/87, throughout this study. (Fig. 2). Interestingly, viruses in L2 and L3 had two additional point mutations in the RNA binding domain at position 40 where glutamine was replaced by arginine (Q40R) and at position 73 where a threonine was replaced by a methionine (T70M) (Fig. 11), and is referred throughout this study as H5N3/NS1/87P.

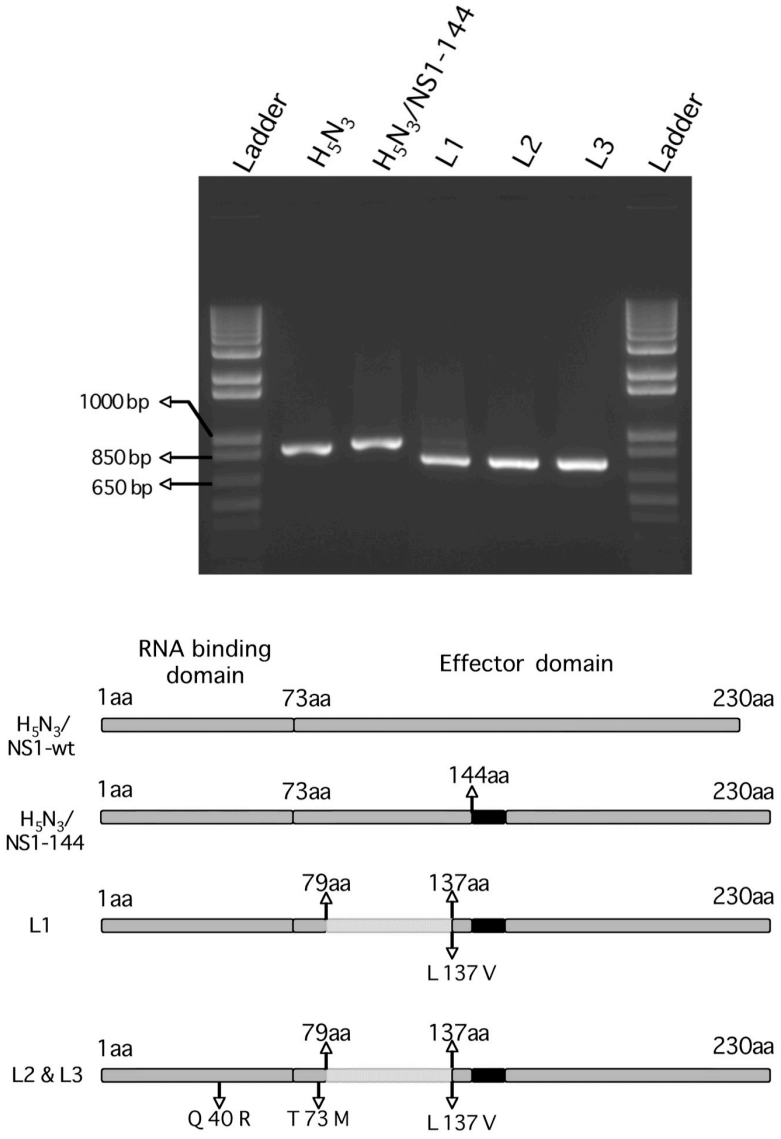

Fig.11. A. Truncated forms of NS1 ORF after serial passage of H5N3/NS1/144 in older age ECE. A. Agarose gel electrophoresis of RT-PCR products of the NS gene segment of H5N3/NS1/144 after 15 serial passages in older age ECE. PCR products for parental H5N3, NS1 mutant H5N3/NS1/144 and passage viruses from three different lineages (L1, L2 and L3) are shown. B. Schematic representation of the deletion and point mutations in each of the three passage lineages (L1, L2 and L3) of H5N3/NS1/144 in older age ECE. The NS gene of lineages 1 and 2 were incorporated into the wild-type H5N3 background using reverse genetics generating viruses H5N3/NS1/87and H5N3/NS1/87P, respectively.

To study the significance of the deletion and point mutations in virus replication and pathogenesis, these mutations were incorporated by reverse genetics into a parental virus background and phenotypic differences *in vitro*, *in ovo* and *in vivo* were examined.

3.3.2 Viral Growth in ECE

The growth properties of parental and NS1 mutant viruses was next assessed in 7, 10 and 13-day-old ECE. Viability of embryos inoculated with H5N3, H5N3/NS1/144, H5N3/NS1/87 and H5N3/NS1/87P viruses was monitored every 8 hours to study the mortality pattern. Mortality pattern was based on percent dead embryos and MDT for each virus. As expected, all viruses tested caused 100% mortality and showed a very similar MDT (38-48 h) in 7- and 10-day-old ECE (Fig.12). On the other hand, when embryos were inoculated at 13-days of age, MDT ranged from 79-144 h, with the lowest MDT for H5N3 virus followed by H5N3/NS1/87P, H5N3/NS1/87and H5N3/NS1/144. It should be noted that not all the 13-day-old ECE died, and the MDT was calculated based on the number of dead embryos. Similar trend in mortality pattern was observed in three independent experiments.

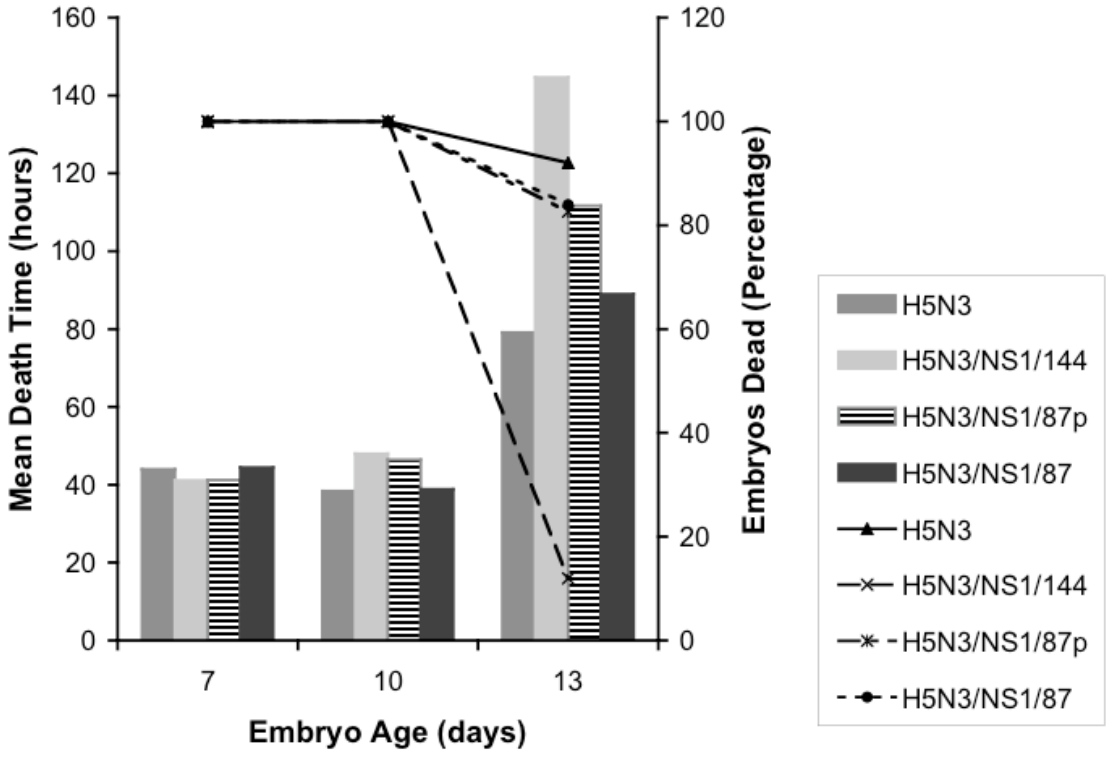

Fig.12. Comparisons of mean death time (MDT) and embryo death percent (EDP) in 7, 10 and 13-day old ECE. The bars indicate MDT and the lines indicate EDP. MDT was determined as described in materials and methods using 100 PFU/embryo.

3.3.3 Growth Characteristics in Cell Culture

Plaque phenotype has been shown to be an indicator of fitness and a correlate of virulence. Clear differences were noted in plaque size in all three lineages of H5N3/NS1/144 in older age ECE as shown in Fig. 13. While L1 formed smaller and indistinct plaques on MDCK, L2 and L3 formed plaques bigger than parental H5N3 and H5N3/NS1/144 viruses. In addition, H5N3/NS1/87 and H5N3/NS1/87P derived by reverse genetics presented plaque morphologies similar to passage lineage virus L1 and

L2 from which their respective NS gene segment was derived. This demonstrates that the plaque morphology on MDCK cells was determined by the truncation and point mutations in the NS1 gene segment.

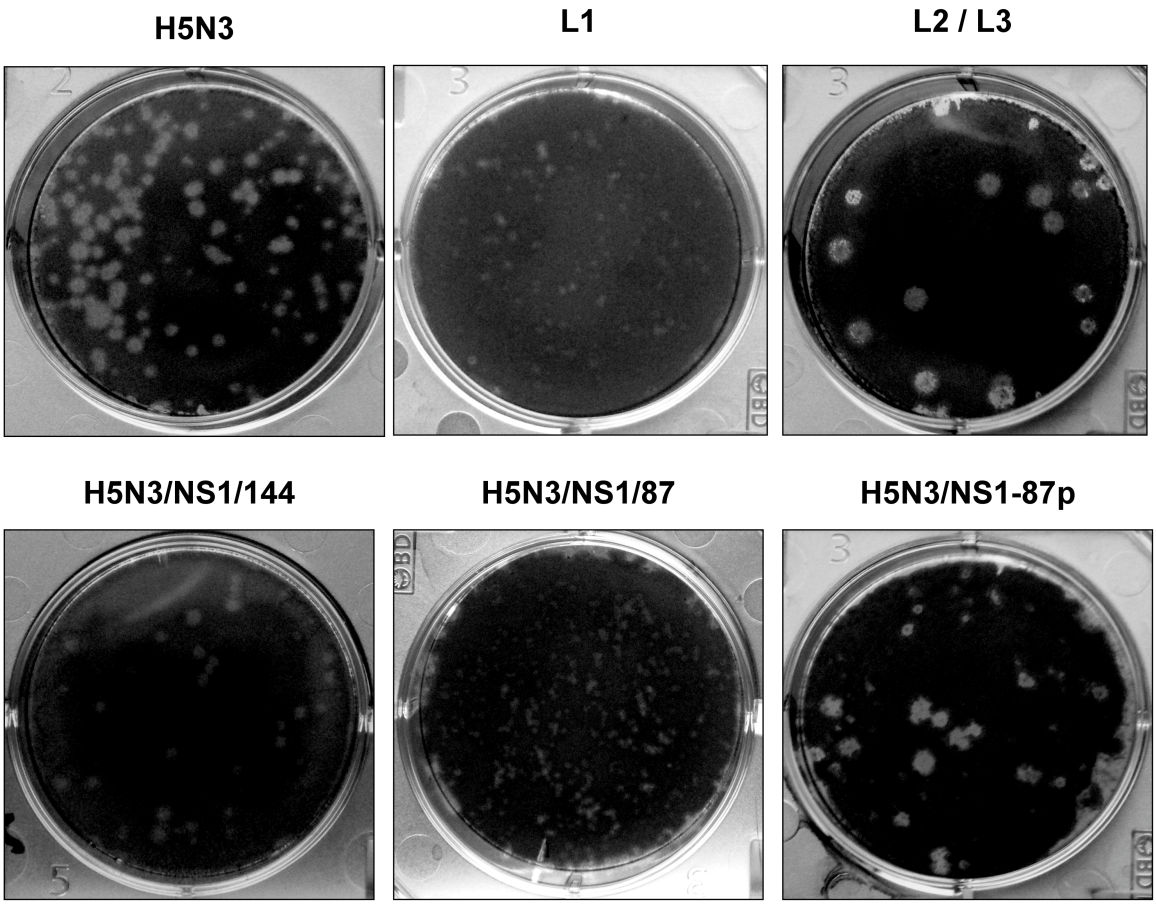

Fig.13. Plaque phenotypes of passage and reverse genetic generated viruses on MDCK cell monolayers 72 hours post infection.

Next, the growth properties of H5N3, H5N3/NS1/144, H5N3/NS1/87 and H5N3/NS1/87P viruses were assessed in A-CEC, as aging of chicken embryo cells has been shown to make chicken cells more interferon competent compared to non-aged cells (Sekellick & Marcus, 1985).

To determine multi-step growth properties of the parental and NS1 mutant viruses, cells were inoculated at a low multiplicity of infection (0.001 PFU/cell) and supernatants from infected cells were collected at different time-points and titrated by plaque assay on DF1 cells. The growth kinetics of the NS1 mutant viruses in A-CEC was clearly different from parental H5N3 virus. As expected, H5N3/NS1/144 was the most compromised in growth, followed by H5N3/NS1/87 and H5N3/NS1/87P. At earlier time points (24-72 h), H5N3 grew to significantly higher titer compared to the NS1 mutants. Titers for H5N3/NS1/144 reached a peak at 24h post-inoculation and then fell precipitously. Interestingly, an increase in viral titers was noticed for H5N3/NS1/87 and H5N3/NS1/87P at 96 hours post-inoculation and no significant differences were observed between H5N3, H5N3/NS1/87 and H5N3/NS1/87P at 120 h post inoculation (Fig.14. A). In contrast, all four viruses showed similar growth kinetics in infected DF1 cells (Fig. 14. B).

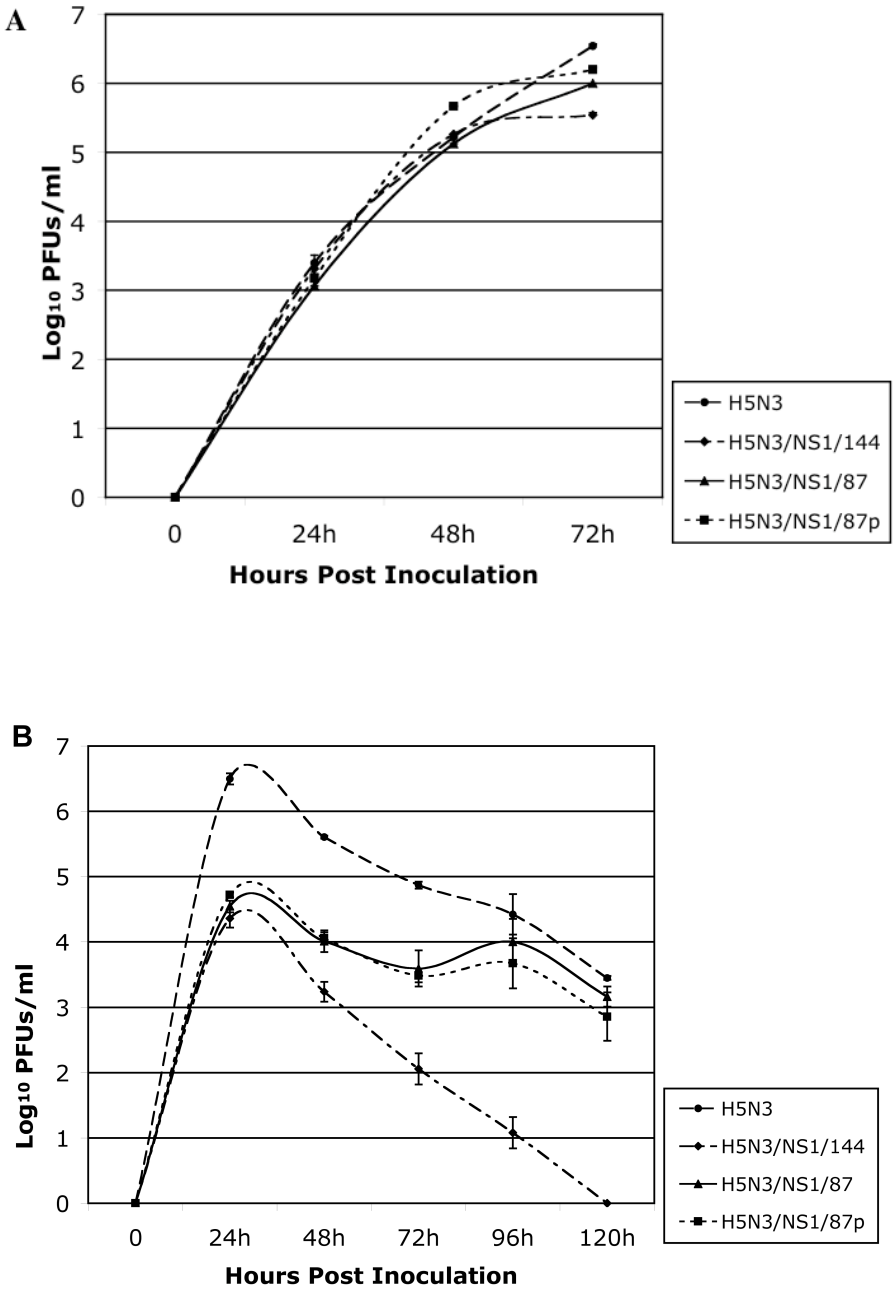

Fig.14. Growth kinetics of recovered parental and NS1 mutant viruses in (A) DF1 and (B) A-CEC. Cells were infected at an MOI of 0.001 with the indicated viruses. Viral titers in infected cell culture supernatants were determined at the indicated times post-inoculation by plaque assay on DF1 cells.

3.3.4 Interferon Induction by NS1 Mutant Viruses

To examine whether the differences in the replication and virulence of parental H5N3 and NS1 mutant viruses were directly correlated with their respective abilities to inhibit the IFN-α/ß system, the production of IFN-ß in cells infected with these viruses was assessed. The relative levels of IFN-ß mRNA in A-CEC infected with 0.0001, 0.001 and 0.01 MOI was quantified by real-time RT-PCR at 6 hours post infection. As shown in Fig. 15, H5N3/NS1/144 induced markedly higher levels of interferon mRNA than parental H5N3, H5N3/NS1/87P and H5N3/NS1/87 viruses. Within the NS1 mutant viruses expressing 87 amino acids the differences in levels of interferon induction were small, but were significantly higher for H5N3/NS1/87 compared to H5N3/NS1/87 at the two highest MOIs tested in this study. These results reflect the ability of H5N3/NS1/87P and H5N3/NS1/87 viruses to regain considerable virulence over the H5N3/NS1/144 through their increased ability to inhibit interferon induction.

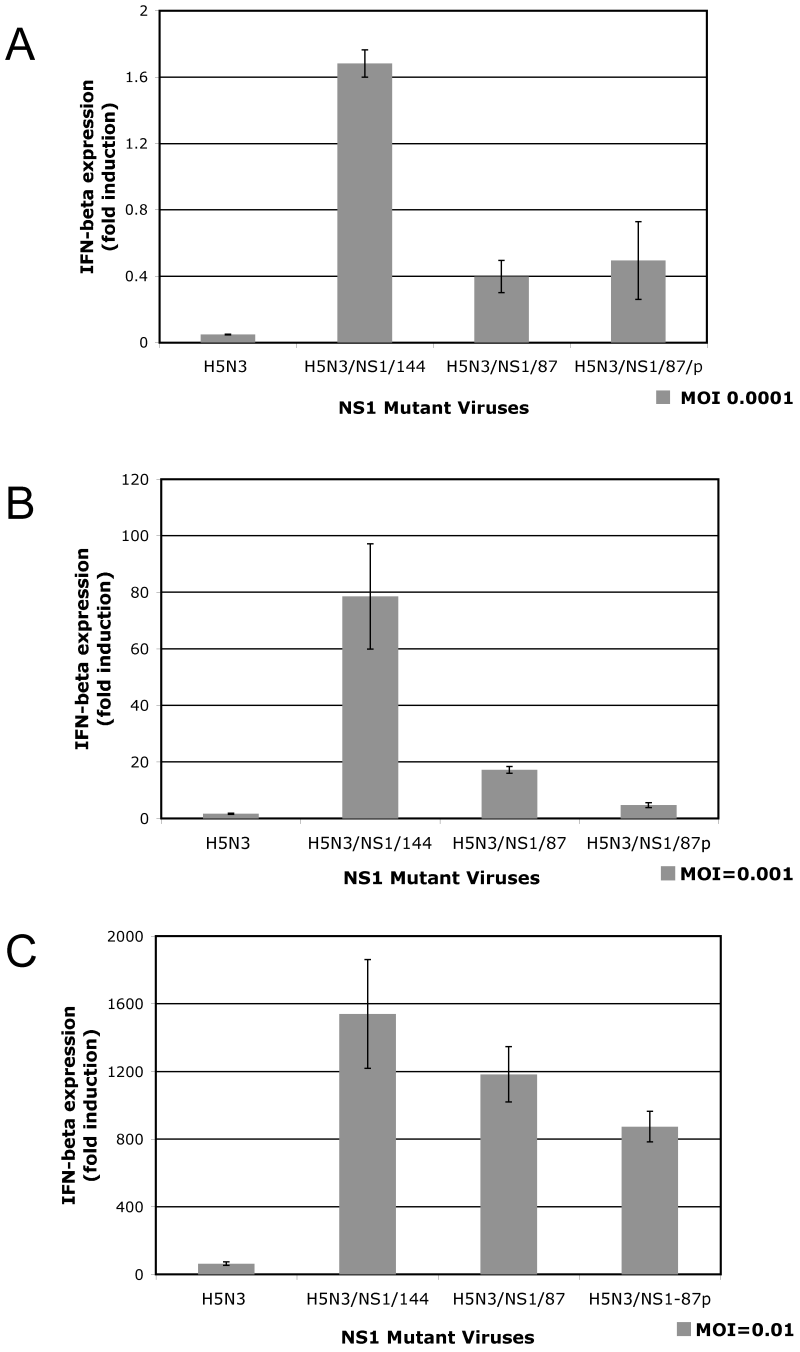

Fig. 15. Quantification of IFN-ß mRNA synthesis by A-CEC infected with H5N3, H5N3/NS1/144, H5N3/NS1/87P and H5N3/NS1/87 at increasing MOI of infection (A: 0.0001 MOI, B: 0.001 MOI, C: 0.01 MOI). Six-hours post infection, cells were collected, RNA extracted and IFN-β mRNA expression determined by real-time RT-PCR. The results represent relative IFN-β mRNA levels normalized with levels of β-actin mRNA. Levels of IFN-β induction increase with increasing MOI.

3.3.5 Replication and Virulence of NS1 Mutant Viruses in Chickens

Groups of twenty-two 1-day-old chickens were inoculated via the intranasal/intra-choanal route with 10^4 PFU of H5N3, H5N3/NS1/144, H5N3/NS1/87P and H5N3/NS1/87 viruses or mock-infected with allantoic fluid without virus. One day after inoculation, five uninfected contact chickens were included in each of the infected groups of chickens to determine the ability of the viruses to transmit. Mild clinical signs, such as depression, dyspnea and gasping were noted in chickens infected with parental H5N3 virus and in few chickens infected with H5N3/NS1/87P but no mortality was recorded in any of the treatment groups. Four days post-inoculation, all chickens in each group, except the contact chickens, were euthanized and necropsy was performed. Gross lesions in the respiratory tract (trachea and lung) were recorded, and lungs were collected to determine virus titers. Typical lesions observed included, plum-colored consolidated areas on individual lobes of the lungs and in some chickens, mucoid tracheal plugs were also present. H5N3/NS1/87P and H5N3 infected groups had equal number of chickens (10/22) showing gross lesions, followed by H5N3/NS1/87 (7/22) and H5N3/NS1/144 (2/22). We were able to isolate virus from significantly greater numbers of chickens infected with H5N3 (17/22), followed by H5N3/NS1/87P (16/22) and H5N3/NS1/87 (14/22) compared to H5N3/NS1/144 (8/21). The average titers in lungs from chickens infected with H5N3 were $10^{3.7}$ ELD_{50}/g of tissue followed by H5N3/NS1/87P, H5N3/NS1/87 and H5N3/NS1/144 with $10^{2.8}$ ELD_{50}/g $10^{1.7}$ ELD_{50}/g $10^{1.3}$ ELD_{50}/g, respectively (Table 3). Serconversion of contact chickens was observed

for one of the contact chickens infected with H5N3 virus, while none of the contact chickens in other groups seroconverted.

Table 3. Gross lesions and virus isolation with mean titers from homogenized lung tissues four days post infection.

Groups	Gross Lesions	Virus Isolation *
H5N3	10/22	17/22 (3.7)**
H5N3/NS1/144	2/22	8/21 (1.3)
H5N3/NS1-87/80V	7/22	14/22 (1.7)
H5N3/NS1-87/40R/73M/80V	10/22	16/22 (2.8)

* Virus was isolated 4 days post challenge from homogenized lung tissues
** Number of chickens positive / number tested with average titers expressed as Log_{10} ELD_{50} / g of homogenized lung tissue

3.4 Discussion

In recent years, economic losses associated with AI infections have resulted in the use of large-scale vaccination in certain countries. The objective of current vaccines is to increase resistance in chickens against field challenge virus, reduce mortality rates and more importantly, limit AIV shed into the environment (Iqbal, 2009). Reduced virus shedding not only results in decreased transmission of AIV among chickens, but also minimizes the risk of evolution of new AIV strains, which can infect humans (Iqbal, 2009).

In recent years, there has been increased interest in using NS1 mutant viruses, in different animal species, as a potential live attenuated vaccines due to the anti-interferon

function of NS1 and the ability of such vaccines to differentiate between vaccinated and infected animals (DIVA) (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005; Steel *et al.*, 2009; Wang *et al.*, 2008). Efficacy of NS1 mutant viruses as live vaccine candidates studies should not only be evaluated based on attenuation and protection offered by the vaccine, but also by the stability of the mutation and its reversion to wild-type phenotype. This is very important especially for commercial poultry, where chicken are under a variety of production stresses, and NS1 mutant viruses, though attenuated, can still replicate and reach moderate titers (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005), giving the NS1 mutant vaccine virus the opportunity to mutate and regain virulence. In a recent study, we observed such a phenomenon with H5N3/NS1/144, a carboxy-terminal truncated NS1 mutant virus. Within five back-passages in chickens,the virus underwent mutations that resulted in deletion of the inserted stop codon and was able to express both the amino and carboxy ends of NS1 protein. The current study and other reports, underscore the importance of NS1 in viral pathogenicity (Cauthen *et al.*, 2007; Quinlivan *et al.*, 2005) and prove that NS1 mutant viruses are able to revert to virulence under severe immune pressure when propagated in interferon competent host systems.

To further understand the strategies used by LPAI NS1 mutant viruses to compensate the anti-interferon function of NS1 in its host, we serially passaged H5N3/NS1/144 in increasing age ECE. Older age ECEs are considered to be more interferon competent (Morahan & Grossberg, 1970; Sekellick *et al.*, 1990; Sekellick & Marcus, 1985) and are also considered a good system to assess pathogenicity of AIVs affecting chickens (Perdue *et al.*, 1990). H5N3/NS1/144 was earlier characterized and

shown to be severely attenuated *in vitro*, *in ovo* and *in vivo* compared to the parental H5N3 virus. After fifteen serial passages of H5N3/NS1/144 in older age ECE, the NS1 gene in all three lineages was further truncated (87 amino acids) with a point mutation occurring at the point of truncation (L80V). Viruses from two of three lineages also had point mutations at positions 40 (Q40R) and 73 (T73M), of the RNA binding domain.

These naturally selected mutant viruses showed differences in plaque phenotype when plated on MDCK cells. Large plaque size has been associated with better virus replication and virulence (Pappas *et al.*, 2008). Interestingly, the same truncation was also present as a part of mixed population after five rounds of back passage of H5N3/NS1/144 in 6-8 week old chickens. The significance of the further deletion in the 144 amino acid long NS1 ORF is not known. However, it has been previously shown that shorter NS1 mutant proteins (<90 amino acids) are more stable, and are expressed at higher levels than longer mutant proteins (~140 amino acids) (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005). Recently, Lee et al., showed that serial passage in 14-day-old ECE of a virus with a natural truncation in NS1 also resulted in further truncations (Wang *et al.*, 2008). However, in that report, the authors detected various sizes of the NS1 ORF while in this study, we consistently detected a deletion of 58 amino acids between amino acids 79 and 137. Although the cause of the differences observed is not known, they could be attributed to the different strains of LPAIV used in the two studies.

To study the role and importance of the naturally selected truncated forms of NS1 along with the mutations in the RNA binding region, we used reverse genetics to construct two new NS1 mutant viruses: H5N3/NS1/87 and H5N3/NS1/87P which

differed from each other and parental virus only in the NS gene segment. Both, H5N3 and H5N3/NS1/144 viruses showed stark contrast in their ability to replicate in A-CEC as expected and observed earlier. A-CECs were chosen as they have been reported to have higher interferon production potential compared to fresh chicken embryo cells (Sekellick *et al.*, 1990). Replication of H5N3/NS1/87 and H5N3/NS1/87P in A-CECs was intermediate when compared to parental H5N3 and H5N3/NS1/144 viruses. Interestingly, at later time points (96 h post-inoculation) H5N3/NS1/87 and H5N3/NS1/87P showed a slight increase in titers, compared to a slight drop in viral titers for the parental H5N3 virus. To our knowledge, this change in growth kinetics pattern at later time points has never been reported. It is possible that the NS1 protein of H5N3/NS1/87 and H5N3/NS1/87P follow a different IFN inhibition pathway from the parent virus. A recent study demonstrated that in for some strains increased virus replication can out competed the antiviral response of the infected host (Grimm *et al.*, 2007). Therefore, further studies are required to understand the role of NS1 in the growth kinetics differences observed. On the other hand, growth kinetics in DF1 cells, which do not express interferon upon virus infection (Karpala *et al.*, 2008), were the same for all four viruses examined, suggesting that the differences observed in A-CEC were due to differences in interferon induction. In addition, this observation also rules out the possibility of NS1 mutations affecting the replication ability of the virus.

Our study showed that levels of IFN-β induction increased with increasing MOI of infection. In addition, the attenuation of virus replication in A-CEC correlated with levels of interferon mRNA induced by virus replication. These data suggest that shorter

forms of NS1 regained functionality allowing for better replication of virus in interferon competent systems as observed with swine and equine influenza viruses (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005). These data also explain, the natural selection of shorter forms of NS1 after serial passage of H5N3/NS1/144 under innate immune pressure. Of the two shorter NS1 mutants generated in this study, H5N3/NS1/87P, which contains two point mutations in the RNA binding domain, showed a very small but significantly lower level of IFN-β induction compared to H5N3/NS1/87, at the higher MOIs used in this study. Although these differences could be attributed to the point mutations in the RNA binding domain, further biochemical studies are warranted to elucidate their role in the anti-IFN activity of H5N3/NS1/87P.

We also found that the pathogenicity of the NS1 mutant viruses was different in terms of their ability to cause embryo mortality in increasing age ECE. In 13-day-old ECE a sharp decrease in embryo mortality was observed for H5N3/NS1/144 compared to just a slight drop in embryo mortality for the other viruses tested. The MDT, which is considered a good measure of pathogenicity (Perdue *et al.*, 1990), indicated that H5N3/NS1/87P was more pathogenic than H5N3/NS1/87 and slightly less pathogenic than parental H5N3 virus. A similar trend in pathogenicity of the NS1 mutant viruses was observed in terms of their replication and ability to cause lesions in chicken lungs (Table 1). Collectively, these data suggest that NS1 is a crucial virulence factor and under selective pressure larger truncations in the NS1 ORF are generated resulting in increased virulence compared to H5N3/NS1/144 virus. In addition, the point mutations detected in the RNA binding domain seem to have some added advantage *in vivo*;

however, additional functional studies are need to understand the role of these point mutations in virus replication.

In summary, in this study we have shown that the truncations detected in the NS1 protein of passaged viruses play an important role in the virus reversion to virulence. In addition, these studies support that the use of NS1 mutant viruses as live vaccines should be taken with a word of caution, and vaccine candidates should undergo several back-passages in chickens or older age ECE to ensure the stability of the NS1 gene, before they can be introduced as a live attenuated vaccines in the field.

CHAPTER IV

SUMMARY

NS1 is one of the avian influenza virus (AIV) proteins associated with virulence due to its anti-interferon activity (Garcia-Sastre *et al.*, 1998; Krug *et al.*, 2003). Interferon plays a crucial role in the innate antiviral defense mechanism of eukaryotic cells. There has been increased interest in using NS1 mutant viruses as "modified live vaccines" since deletions in NS1 result in attenuation in growth in immunocompetent hosts. Besides inhibiting the innate immune response, NS1 can also inhibit the adaptive immune response by inhibiting dendritic cell maturation and their capacity to induce T cell responses (Fernandez-Sesma *et al.*, 2006). NS1 protein of influenza A viruses utilizes several different mechanisms for its anti-interferon activity and these mechanisms differ from one strain to another (Hayman *et al.*, 2007; Hayman *et al.*, 2006). Most on the studies on the mechanisms of NS-1 anti-interferon activity have focused on human strains. However, the role and importance of NS1 in anti-interferon activity of LPAIV in chickens needs more investigation. The *rationale* behind the present work is that by gaining knowledge into the role of NS1 in AIV pathogenesis in chickens, it will be possible to evaluate the use of NS1 mutant viruses as potential DIVA vaccines for poultry.

The first part of this study focused on the development a NS1 mutant virus, which is attenuated in its growth in interferon competent systems, and the evaluation of its use as a live attenuated vaccine for chickens. The second part of this project

concentrated on the *in vitro*, *in ovo* and *in vivo* characterization of growth properties and pathogenesis of different NS1 mutant viruses to better understand the importance of NS1 in virus-host interaction.

The AIV under study is a low pathogenic H5N3 virus. Our laboratory has developed an eight-plasmid reverse genetics system to generate recombinant AIVs. Initially, the NS gene segment was manipulated to generate a NS1 mutant virus expressing only the first 144 amino acids. The H5N3/NS1/144 virus was significantly attenuated in growth in interferon competent systems *in vitro*, *in-ovo* and *in-vivo*. In addition, attenuation of H5N3/NS1/144 correlated with lack of inhibition of interferon induction by the mutant virus, which was in accordance with published reports (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005). Once its attenuation was confirmed, H5N3/NS1/144 was evaluated as a live vaccine in chickens. H5N3/NS1/144 induced a good immune response, as measured by HI, and also protected chickens from lesions after challenge with parental H5N3. However, safety studies indicated that H5N3/NS1/144 could revert to virulence. Within 5 rounds of back passage of the virus in chickens, the inserted stop codon at position 144 of the NS1 ORF was deleted and the virus was able to express an almost complete NS1 protein. As a consequence, the back-passage viruses produced lesions, in the respiratory tract, similar to parental H5N3. However, based on the absence of antibodies against the carboxy end of NS1 it was possible to clearly differentiate H5N3/NS1-144 from parental H5N3 inoculated chickens. In an attempt to make a safer vaccine, we evaluated H5N3/NS1/144 as killed virus vaccine and compared its efficacy with that of parental H5N3 virus against a

homologous H5N3 challenge. As expected, both vaccine preparations induced similar antibody titers and reduced virus shedding considerably. H5N3/NS1/144 certainly showed potential to be used as a killed vaccine for its DIVA properties.

As indicated earlier back passage of H5N3/NS1/144 in chickens caused the deletion of the stop codon we introduced in the NS1 gene of H5N3/NS1/144. Interestingly, one of the three back-passage lineages of H5N3/NS1/144 also had a virus population with an internal truncation in the NS1 coding region reducing the NS1 ORF to only 87 amino acids. This further truncation was of interest because in swine and equine influenza viruses it was shown that shorter mutant NS1 proteins were more stable and were more functional than longer ones. To study the relevance and importance of the NS1 truncation observed during serial passage in chickens, we carried out serial passages of H5N3/NS1/144 in increasing age embroyonated chicken eggs (ECE). Older age ECE were chosen for two reasons: a) They are believed to be more interferon competent; b) The ability of AIV to grow in older-age ECE is an indicator of pathogenicity of the virus. After 15 rounds of serial passages in increasing age ECE the NS1 ORF was truncated. Interestingly, the truncation observed was similar to that observed in one of the chicken back-passage lineages. This suggests that there is some relevance for natural selection of shorter NS1 ORF. In addition, two of the embryo passage lineage had two mutations at amino residues 40 (Q40R) and 73 (T73M) of the RNA binding domain. To understand the importance of the large truncation in NS1 ORF and the role of the two point mutations in the RNA binding domain, we incorporated these particular NS gene segments into the parental H5N3 background and recovered the

viruses. *In vitro*, *in ovo* and *in vivo* studies indicate that H5N3/NS1/87 and H5N3/NS1/87P were more virulent and replicated better than H5N3/NS1/144; however, they were still partially attenuated compared to parental H5N3. Interestingly, H5N3/NS1/87P was more virulent than H5N3/NS1/87 *in ovo* and *in vivo*; however, *in vitro* replication studies in aged chicken embryo cells (A-CEC) did not show any significant difference between the two viruses. Further studies are required to identify the role of the RNA binding domain mutation in pathogenesis.

Based on our results future lines of research should include the use of adjuvants such as TLR receptor ligands along with killed NS1 mutant vaccines, as this would mimic a live virus infection and boost an early immune response, which was absent in the oil adjuvanted vaccine used in this study. It would also be interesting to compare the effect of the NS1 mutant viruses on host gene regulation especially with regards to the host immune response, this could provide information on the mechanism used by the NS1 mutant viruses with shorter NS1 proteins to counteract interferon pathway and increase virus replication compared to NS1 mutant with longer NS1 proteins. Also it would be of interest to study the effect of the point mutations at positions 40 and 73 in parental H5N3 virus and study its growth properties.

In summary: (1) H5N3/NS1/144, is not a safe live vaccine candidate; however, it can be safely used as a killed DIVA vaccine; (2) Any potential NS1 mutant live virus vaccine should undergo several serial passages in either older age ECE and/or in chickens to ensure its stability and safety; (3) Shorter NS1 proteins help the virus regain its virulence considerably; (4) Mutations at position 40 and 73 confer added advantage to

the virus expressing an 87 amino acids long NS1 protein in terms of virulence and replication in older age ECE and chickens.

Further studies on the role of NS1 protein domains and specific amino acids in anti-interferon activity may lead to the development of improved live attenuated vaccines or other novel control strategies.

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