PATHOGENICITY AND TRANSMISSIBILITY OF NOVEL INFLUENZA VIRUSES

by

JINGJIAO MA

M.S., China Agricultural University, 2011

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
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Abstract

Influenza A virus (IAV) is an enveloped, segmented, negative-sense RNA virus that infects avian species and mammals. Its segmented feature enables antigenic shift which can generate novel IAVs that pose a threat to animal and public health due to lack of immunity to these viruses. Pigs have been considered the "mixing vessels" of influenza A viruses to generate novel reassortant viruses that may threaten animal and public health. Therefore, it is necessary to understand the pathogenicity and transmissibility of newly emerged reassortant viruses in swine. Adding to this complexity is the newly identified bat influenza A-like viruses which have roused interest in understanding the evolutionary history and pandemic potential of bat influenza.

At least 10 different genotypes of novel reassortant H3N2 IAVs with gene(s) from 2009 pandemic H1N1 [A(H1N1)pdm09] have been identified in pigs in the United States. To date, only three genotypes of these viruses have been evaluated in animal models leaving the pathogenicity and transmissibility of the other seven genotype viruses unknown. We showed that reassortant viruses with genes from A(H1N1)pdm09 are pathogenic and transmissible in pigs. Further studies showed that avian-like glycine at position 228 of the HA receptor binding site is responsible for inefficient transmission of the reassortant H3N2 IAV with five A(H1N1)pdm09 genes.

Studying the recently discovered IAV-like sequences from bats has been hindered by the lack of live virus isolation or culturing. Using synthetic genomics, we successfully rescued modified bat influenza viruses that had the HA and NA coding regions replaced with two classical IAVs. Additional studies were performed with truncations on NS1 protein and substitution of a putative virulence mutation in bat influenza PB2. Virus reassortment

experiments demonstrated that bat influenza has limited genetic and protein compatibility with other influenza viruses; however, it readily reassorts with another divergent bat influenza virus.

Taken together, our results provide insights into the pathogenicity and transmissibility of novel reassortant H3N2 IAVs in pigs. It also indicates that the bat influenza viruses recently identified are viable viruses that pose little pandemic threat to humans. Moreover, they provide new insights into the evolution and basic biology of influenza viruses.

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Chapter 1: General Introduction

1.1 Influenza

Influenza is an acute respiratory disease caused by influenza viruses in humans and many other species of mammals such as swine, equine, canine, seal (Osterhaus et al., 2000), and whale (Hinshaw et al., 1986) as well as in avian species such as duck, chicken, and water fowl (Easterday et al., 1968; Pereira et al., 1965). Symptoms of influenza can range from mild to severe. Influenza virus infection primarily affects the upper respiratory tract including the nose and throat causing fever, runny nose, sore throat, and coughing. However, pneumonia can develop if viral infection spreads to lung.

1.2 Classification

Influenza viruses belong to the family *Orthomyxoviridae.* The nomenclature system for influenza virus includes the type of virus, host of origin, geographic origin, the successive isolate number from that location, and year of isolation. There are three types of influenza viruses: influenza A virus (IAV), influenza B virus (IBV), and influenza C virus. Aquatic birds are known to be the major reservoir of IAVs, although these animals show inapparent disease when infected. In contrast, these viruses cause epidemics of acute respiratory disease and even pandemics in humans (Webster and Govorkova, 2014). Influenza B virus is primarily a human pathogen; however, an influenza B strain was isolated from a harbor seal in the Netherlands in 2000 (Osterhaus et al., 2000). Influenza C virus infects humans and a few other species including pigs and dogs (Youzbashi et al., 1996). These different types of influenza viruses are distinguished by two internal proteins the nucleoprotein (NP) and the matrix protein 1 (M1). Influenza A and B type viruses contain eight gene segments while influenza C type virus only contains seven gene segments. The primary difference is that influenza C type viruses have only

one surface protein in contrast to influenza A and B viruses which have two surface proteins. The hemagglutinin-esterase protein (Vlasak et al., 1989) is the surface protein for influenza C virus which allows virus attachment, fusion and release (Gao et al., 2008). Another major difference between each type is their host range: IAVs infect a wide array of species while influenza B and C virus predominantly infect humans but can sporadically infect seals or pigs, respectively. Moreover, influenza A and B type viruses cause epidemics and severe disease in humans whereas influenza C principally infects children and causes common cold-like symptoms (Calvo et al., 2006).

1.3 Influenza A type viruses

IAVs infect humans and other animal species such as swine, equine, canine, and avian, including domestic poultry. These viruses cause seasonal morbidity and mortality with an annual average of 25,420 deaths in humans annually from influenza-associated respiratory and circulatory deaths over the last 23-season period in the US (Thompson et al., 2003). Additionally, IAVs are solely responsible for pandemics which have caused millions of human deaths in the past century alone.

IAV is a segmented, negative-sense, single-stranded RNA virus that contains eight gene segments. IAVs are further divided into subtypes based on the two major surface antigens, the hemagglutinin (HA) and the neuraminidase (NA) proteins. Until now, 18 HA subtype and 11 NA subtype viruses have been found. HAs are further divided into two groups based on phylogenetic analysis: H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18 belong to group 1, while H3, H4, H7, H10, H14, and H15 belong to group 2 (Russell et al., 2008). There comparison of the structure of group-specific basis indicates the prominent different in regions in changes required for membrane fusion. The virus subtypes can be further divided to three tiers based on

the epidemic or pandemic potential of virus segments from aquatic bird reservoir to currently circulating human influenza viruses. Tier 1 includes H1, H2, and H3 which have caused pandemics in humans; Tier 2 includes H5, H7, and H9 which have sporadically infected humans; Tier 3 encompasses the remaining subtypes (Koelle et al., 2010). The H17N10 and H18N11 viral sequences were recently detected in fruit bats from Guatemala (Tong et al., 2012) and Peru (Tong et al., 2013), respectively, while the H1-H16 and N1-N9 are currently circulating in aquatic birds (Table 1, Table 2).

Table 1 Influenza A Hemagglutinin Subtypes: Species Affected

Subtype	People	Poultry	Pigs	Bats / Other
H1				
H2				
H3				Other Animals
H4				Other Animals
H5				
H6				
H7				Other Animals
H8				
H9				
H10				
H11				
H12				
H13				
H14				
H15				
H16				
H17				
H18				

Table 2 Influenza A Neuaminidase Subtypes: Species Affected

Subtype	People	Poultry	Pigs	Bats / Other
N1				
N2				
N3				
N4				
N5				
N6				
N7				Other Animals
N8				Other Animals
N9				
N10				
N11				

1.4 Antigenic drift and antigenic shift

Human influenza epidemics are caused by both IAVs and IBVs, while the pandemics are only caused by IAVs. Antigenic drift and antigenic shift are two mechanisms responsible for fast changes of influenza viruses, resulting in novel viruses which cause seasonal epidemics or pandemics. After the outbreak, progeny viruses have the opportunity to establish new lineages or replace currently circulating strains.

Antigenic drift (Smith et al., 1951) refers to point mutations in the HA and NA proteins that cause gradual changes in IAVs and IBVs. These changes allow the viruses to escape immune responses already established in populations through previous viral infections or vaccinations. Initially, four antigenic sites (epitopes) on the globular head of the HA were defined by antigenic mapping and sequence analysis of the escape mutants selected by using monoclonal antibodies against the HA and NA surface proteins (Laver et al., 1979). Later studies increased the antigenic sites in globular head of HA from four to five including site A, site B, site C, site D, and site E (Wilson and Cox, 1990).

Antigenic shift (Kilbourne, 1969) refers to the reassortment of gene segments and occurs mainly in IAVs. This causes a sudden change of the HA or both the HA and NA resulting in epidemics and pandemics, due to limited or no immunity to the HA antigen in a population.

1.5 History of influenza A pandemic

The first IAV in humans was first isolated in 1933, and until now, only H1N1, H2N2, and H3N2 subtypes have caused human pandemics. Although some avian influenza viruses such as H5, H6, H7, and H9 have been reported transmit to humans occasionally causing mild to severe disease and even death, these subtypes have not established lineages in human populations and cannot maintain sustained human-to-human transmission.

In the past century, several pandemics have occurred. The 1918 H1N1 Spanish Flu was the most severe pandemic in flu history and killed upwards of 40 million people worldwide (Beveridge, 1991). Next, the H2N2 pandemic broke out in Asia in 1957 with an estimated global mortality of 1.5 million (Simonsen et al., 1998). This virus continued to circulate in human populations for a decade and disappeared in 1968. Then, the H3N2 Hong Kong Flu occurred in 1968 with a global mortality of about 1 million of people (Harkness et al., 1972). The H1N1 subtype virus disappeared from the human population in 1957 and then reemerged in 1977 (Webster and Govorkova, 2014). Most recently, the 2009 H1N1 pandemic [A(H1N1)pdm09] occurred (Smith et al., 2009).

1.6 2009 H1N1 pandemic virus

In early 2009, the A(H1N1)pdm09 emerged in humans in Mexico and quickly spread to North America and worldwide. In June 11 2009, the world health organization (WHO) announced the start of 2009 influenza pandemic (WHO, 2009). Until August 2010, the A(H1N1)pdm09 killed 18,000 people and has since spread to more than 200 countries since the emergence of this virus (WHO, 2010). Further phylogenetic analysis indicated that the A(H1N1)pdm09 is a triple reassortant virus that contains PB2, PB1, PA, HA, NP, and NS gene segments from North American triple-reassortant swine IAVs, and the NA and M segments from Eurasian swine lineages. Interestingly, this genome combination had never been detected previously (Garten et al., 2009; Smith et al., 2009).

Soon after the emergence of the A(H1N1)pdm09, this virus has transmitted from humans to other animal species such as canine, feline, and swine (Forgie et al., 2011; Hofshagen et al., 2009; Lin et al., 2012; Sponseller et al., 2010). The first case of swine infection with A(H1N1)pdm09 virus was reported in a commercial swine farm in Alberta, Canada in May,

2009 (Howden et al., 2009). Shortly afterwards, the virus was isolated from swine herds in many other areas globally including the US (Hofshagen et al., 2009; Nelson et al., 2015; Welsh et al., 2010). However, this virus did not become endemic in the swine herds. In contrast, it reassorted with endemic swine IAVs to generate novel reassortant viruses, which have been found in many areas of the world (Bi et al., 2010; Howard et al., 2011; Moreno et al., 2011; Vijaykrishna et al., 2010). In the US, H1N1, H1N2, and H3N2 subtype reassortants containing genes from A(H1N1)pdm09 have been isolated from swine (Ali et al., 2012; Ducatez et al., 2011; Karasin et al., 2000; Liu et al., 2012a; Nelson et al., 2012; Vijaykrishna et al., 2010; Webby et al., 2000). H3N2 and H1N2 variants have caused human infections in the United States (CDC, 2011; Cox et al., 2011). To date, 10 different genotypes of novel reassortant H3N2 influenza viruses containing A(H1N1)pdm09 genes have been identified in US pigs (Kitikoon et al., 2013). However, the pathogenicity and transmissibility of most of these reassortants remain unknown.

1.7 Morphology

IAV particles are irregularly shaped spherical particles ranging from 80–120 nm in diameter. The virion envelope is derived from the membranes of host cells. There are two distinct types of surface spikes (peplomers); one is rod-shaped and corresponds to the hemagglutinin (HA), and the other is mushroom-shaped and possesses neuraminidase (NA) activity. Both the HA and the NA are viral glycoproteins that attach to the viral envelope by inserting a short hydrophobic peptide into the membrane. The viral envelope surrounds a matrix protein (M) shell, which in turn surrounds the genome of eight individual molecules of single-stranded RNA (only seven in Type C influenza viruses), along with the nucleoprotein (NP) and three RNA polymerase proteins polymerase PB2, polymerase PB1, and polymerase PA (PB1, PB2, and PA) that are responsible for RNA replication and transcription. Each of the eight

genomic RNA segments encodes for one, or two, polypeptides. This nature of the individual viral gene segments results in the phenomena of high frequency recombination (reassortment of gene segments from two or more parental viruses to generate progeny with new genotypes) during mixed infections and explains the origin of some new pandemic strains of IAVs (Smith et al., 2009). The virus particles can be observed under electron microscope with direct magnification between 64,000 to 130,000 times.

1.8 IAV protein functions

The IAVs genome contains at least 10 different open reading frames (genes) in its eight segments of negative-sense RNA, typically encoding 10 to 12 proteins (Medina and Garcia-Sastre, 2011) and up to 17 proteins (Vasin et al., 2014).

Hemagglutinin

The HA protein is encoded by segment four, which is a major surface antigen and is responsible for viral attachment and pathogenicity. IAVs have two major cellular surface glycoproteins: the principal antigen, HA, is responsible for cell attachment by binding receptors, while the enzymically active NA assists matured virus release by cutting the sialic acids. For IAV, 18 antigenically distinct HAs have been recognized. The H1-H16 subtypes can be found circulating in aquatic birds while the H17 and H18 sequences have been found in fruit bats which are believed to be their natural hosts (Tong et al., 2012; Tong et al., 2013). Variation of these molecules is primarily responsible for the emergence of new strains of the virus leading to new outbreaks of influenza, resulting in failed vaccination attempts.

The HA protein is encoded by viral gene segment four and is initially synthesized as a single polypeptide precursor (HA0). The mature form of the HA protein is a rod-shaped homotrimer, and each monomer is generated by cleavage of HA0 into HA1 and HA2 subunits by

trypsin-like or furin-like protease for the virus to be infectious (Fouchier et al., 2005). The HA protein functions in initial virus attachment to its cellular receptor, which is critical in early steps of the viral replication cycle, including receptor binding and membrane fusion. The fusion of the viral envelope with an intercellular membrane allows transferring of the uncoated virus into the cell cytoplasm (Skehel and Wiley, 2000).

IAVs display host specificity. A major determinant of this specificity is the difference in cell receptor binding requirements of the HA molecule which is further complicated by differing conformations of sialic acids on cell surfaces in different species (Naeve et al., 1984). The conserved sialic acid receptor-binding pocket, located on the HA1 subunit at the distal end of the molecule, binds to monovalent sialic acid receptor analogs with relatively low affinity. However, the high abundance of HA molecules on the virion surface permit a sufficient number of low-affinity interactions to allow virus attachment and entry into host cells (Mitnaul et al., 2000). Neutralizing antibodies are used against the HA to prevent infection of host cells.

Neuraminidase

The NA protein is encoded by segment six. There are eleven recognized NA subtypes. On the surface of the virus, the NA protein forms mushroom-shaped tetramers. The head region and the enzymatic center are separated by the NA stalk from the transmembrane and cytoplasmic domains. During both entry and release from the cells, the NA is responsible for cleavage of the sialic acid-containing receptor and the release of the viral particle from the host cell, in order to remove sialic acid residues between the virus and infected cells to facilitate the mobility of virions (Hulse et al., 2004). Antibodies against the NA do not protect against infection but does confer protection against disease and reduces transmissibility (Monto et al., 2015). Zanamivir

and oseltamivir are used as neuraminidase inhibitors and are vital in managing seasonal and pandemic influenza infections (Uyeki, 2009).

It is a type II membrane glycoprotein (Colman and Ward, 1985) with an un-cleaved amino-terminal signal/anchor domain and a cytoplasmic tail that is presumed to be intracellular. The ectodomain of NA consists of a stalk and a globular head. The stalk and the transmembrane domain sequences are highly variable among the nine NA subtypes (Colman and Ward, 1985). In contrast to the rest of the NA, however, the six-amino-acid cytoplasmic tail is highly conserved within all NA subtypes of IAVs (Bilsel et al., 1993). This feature prevents self-aggregation and promotes release of the newly synthesized virus from the infected cell.

Sequence of the NA stalk varies, depending on the virus (Blok and Air, 1982). Based on previous studies, deletions or insertions of certain amino acids will not affect the infectivity of progeny viruses (Li et al., 2014). The deletions can be up to 28 amino acids and insertions can be up to 41 amino acids in the stalk region. Interestingly, the deletion of 28 amino acids of the stalk region resulted in a virus with reduced growth kinetics in Madin-Darby canine kidney (MDCK) cells. However, the insertion of 41 amino acids did not significantly interfere with viral titers in MDCK or Madin-Darby bovine kidney (MDBK) cells (Luo et al., 1993). The cysteine at position 76 is crucial for formation of infectious virus, and deletion of the cysteine at this position results in non-infectious virus (Luo et al., 1993).

Nucleoprotein

The nucleoprotein (NP) is encoded by the RNA segment five. It is a soluble antigen and is the innermost component of the influenza virion. After synthesis, it is transported into nucleus, where it binds to viral RNA and encapsidates it. It is coiled into a double helix 50 nm to 60 nm in diameter and is intimately associated with each RNA segment as well as the three different

polymerases. The NP is a multifunctional protein and is the major component of the riboucleoprotein (RNP), which is a complex molecule consisting of viral RNA (vRNA), the NP, and the RNA polymerase complex (Eisfeld et al., 2015). It encapsidates vRNA (Shimizu et al., 2011) and maintains the structure of RNP by forming homo-oligomers (Prokudina-Kantorovich and Semenova, 1996). The NP interacts with the PB1 and PB2 subunits in the viral RNA polymerase in formation of the RNP (Biswas et al., 1998). RNPs are organized in a unique pattern in the virus for transcription and replication of the viral genome (Noda et al., 2006). It is also thought to be the major switching determinant for whether genomic vRNA will be transcribed into mRNA or used as a template to synthesize complementary RNA (cRNA) for genome replication (Skorko et al., 1991).

The NP is one of the type-specific antigens. It is the second most abundant protein of the influenza virion and can be used to distinguish between genera of influenza viruses (Dowdle et al., 1974). It can be identified by enzyme-linked immunosorbent assay (ELISA), double immunodiffusion, complement fixation, single radial diffusion, agar-gel precipitation, and the hemagglutination inhibition tests.

Matrix proteins

The matrix protein 1 (M1), encoded by segment seven, is the most abundant protein in virus particles and lies inside the lipid envelope, forming a shell of the virion capsids. This structural protein surrounds the NP to form the inner part of the viral envelope. The nonglycosylated M1 is also a type-specific antigen of influenza viruses. However, antibodies against the M1 protein provide little, if any, protection against infection.

The M1 protein is multifunctional and is thought to play a fundamental role in virus assembly and the budding processes (Avalos et al., 1997; De Vleeschauwer et al., 2009). It is believed to be able to recruit viral components at the assembly site and to initiate budding and budding completion resulting in virus release (Monto et al., 2015; Portela and Digard, 2002). The M1 protein is associated with both the RNP and the viral envelope, regulating vRNP transport between the cytoplasm and the nucleus. It also regulates vRNP transcription and replication and interacts with viral envelope proteins including the HA, NA, M2 and NEP proteins.

The matrix protein 2 (M2) is transcribed from RNA segment seven and is derived from the M1 transcript by RNA splicing. The M2 protein is an integral membrane protein which forms a proton-selective ion channel (De Vleeschauwer et al., 2009), serving as a signal transportation, and is embedded in the viral envelope. The ion channel is a homotetramer formed by four M2 units and is stabilized by disulfide bonds. The ion channel is activated by a low pH which triggers viral un-coating in the endosome. One of the main reasons for the focus on virus ion channels is that they may serve as ideal anti-viral drug targets. Amantadine was one of the most studied IAV M2 proton channel inhibitors (Wang et al., 2011) and has been used clinically as an anti-influenza drug since 1966 (Oxford and Galbraith, 1980).

The M2 cytoplasmic tail (CT) domain is highly conserved among viral strains and is the longest cytoplasmic tail. The M2 CT, specifically its amphipathic helix residues 46–62, may play an important role in virus assembly and budding (Rossman and Lamb, 2011).

Nonstructural protein 1

The NS gene is transcribed by RNA segment eight and encodes two essential viral proteins, nonstructural protein 1 (NS1) and nuclear export protein (NEP). The NS1 is transcribed

by RNA-dependent RNA-polymerase (RdRp), while the NEP transcript is produced by splicing the NS1 transcripts.

The NS1 protein is a small (25kDa) regulatory protein which is an important virulence factor and has multiple functions during viral infection (Li et al., 1998). The major role of NS1 has been described as an inhibitor of host immune responses, especially as an interferon (IFN) antagonist, which is the most important function of the NS1 protein. The IFN antagonism function was discovered by using an NS1-deleted strain in which the NS1 open reading frame (Fereidouni et al.) was deleted. The NS1-deleted strain was unable to grow in an IFN-competent system in contrast to Vero cells which has an IFN-deficient system (Garcia-Sastre et al., 1998). The studies indicate that the first 73 amino acids of NS1 contain a double-stranded RNA (dsRNA) binding domain that are critical for IFN antagonism and that amino acids 38 and 41 are especially critical for dsRNA binding (Egorov et al., 1998; Garcia-Sastre et al., 1998). The binding of viral genomic dsRNA via NS1 RNA binding domain will mask the 5' triphosphate from Pattern Recognition Receptors (PRRs) (Chien et al., 2004; Hatada and Fukuda, 1992; Qian et al., 1995) in order to block the recognition of viral dsRNA.

Despite the dsRNA binding function of NS1 in IFN antagonism, the C-terminal end of the NS1 protein known as the effecter domain also has a role in IFN antagonism (Ferko et al., 2004; Talon et al., 2000b). This domain also has a protein kinase R (PKR) binding function (Hale et al., 2008). The effector domain of NS1 binding to PKR blocks the activation of PKR, which blocks translation followed by phosphorylating elF2α (Li et al., 2006; Lu et al., 1995). The NS1 protein also blocks the 2'-5'-oligoadenylate synthetase (OAS)/RNase L pathway (Min and Krug, 2006). If activated by dsRNA, OAS polymerizes ATP into 2'-5'-oligoadenylate chains which are cleaved by RNase L. These products are then recognized by PRRs (Malathi et al.,

2007). One PRR is retinoic acid-inducible gene I (RIG-I) which recognizes 5' triphosphate of vRNA. The NS1 protein has been shown to bind to RIG-I to block the activation of RIG-I (Guo et al., 2007; Mibayashi et al., 2007; Opitz et al., 2007). These functions of influenza NS1 enable it to replicate in the infected cells and to escape the innate immune response.

NS1 also inhibits host protein synthesis by inhibiting the export of host mRNAs from the nucleus, reducing translation in the cytoplasm. Suppression of host protein synthesis and initiation of virus budding from cell membranes results in cytolytic death. Influenza-induced necrosis is characterized by disruption of cytoplasmic membranes and swelling of the cytoplasm. After that, disintegration of organelles and cell lysis occurs. Necrosis induced by cell death stimulates a strong inflammatory response (Herold et al., 2012; Janke, 2014; Lam et al., 2008; Lam et al., 2011). The NS1 protein also modulates other important aspects, such as viral RNA replication, and viral protein synthesis (Bergmann et al., 2000; Guo et al., 2007; Talon et al., 2000a).

Nuclear export protein

The nuclear export protein (NEP), previously known as nonstructural protein 2 (NS2), was renamed after discovering its nuclear export function (O'Neill et al., 1998). It is composed of 121 amino acids and has a molecular weight of 14kD. The NEP is produced by splicing of the NS1 transcript. However, due to low splicing efficiency, it is produced at low concentrations. It is essential to the influenza virus life cycle and has two major functions. First, it is involved in the nuclear export machinery of synthesized vRNPs. Secondly, it is reported involved in switching the polymerase activity from transcription to replication (O'Neill et al., 1998; Perez et al., 2010; Robb et al., 2009). Some studies have also suggested a role in budding for NEP (Gorai et al., 2012).

The nuclear export of viral nucleic acids is crucial for the virus life cycle, since influenza viruses transcribe their mRNA and cRNA inside the nucleus of infected cells. The influenza NEP protein functions as a mediator between vRNP and the nuclear export machinery of the cells (O'Neill et al., 1998). The NEP associates with the M1 protein of the virus, which then interacts with the NP which is in contact with vRNAs. After NEP binds to the M1-vRNP complex, the NEP nuclear exportation is facilitated by interaction with chromosome region maintenance 1 (Crm1), an important component of the export machinery (Neumann et al., 2000).

In addition to the crucial role in nuclear export machinery, the NEP also has a role in switching the polymerase activity from transcription to replication. In one study, researchers compared cRNA and vRNA levels from RNP reconstitution assays and compared them with those from virus infections (Robb et al., 2009). The addition of NEP influenced the cRNA and vRNA levels at the expense of viral mRNA. The assays suggested that NEP would either synthesize or stabilize the viral mRNA. In addition, deleting the nuclear export signal (NES) or NEP did not influence cRNA levels or vRNA production, suggesting that the function of adjusting the cRNA and vRNA levels are separated from nuclear export function. However, the mechanism by which NEP switches the polymerase from transcription to replication remains unclear.

In addition, several studies show that NEP is involved in the generation of 20-27 virus-derived nucleotides. These nucleotides are influenza-specific, small viral RNAs (svRNAs), which correspond to the 5’ end of vRNA. The NEP also increases cRNA production, while svRNA is produced from cRNA, indicating that NEP is required for svRNA production (Perez et al., 2010).

The NEP also contributes to viral replication by adaptation. One study showed that the polymerase of an avian influenza virus was incapable of generating usable cRNA in a human cell line. However, several point mutations in NEP allowed the virus to adapt to human cells and the avian polymerase was able to generate cRNA with high efficiency (Manz et al., 2012).

Polymerase proteins

The polymerase proteins including PB2, PB1, and PA are the largest viral proteins of IAV and are encoded by segments one, two, and three. They form the heterotrimeric RdRp of influenza viruses responsible for RNA replication and transcription activities in cell nuclei (Boivin et al., 2010). The polymerase and NP as well as viral RNA form the vRNP complex, which is the smallest unit of replication function. The vRNP is released to the cytoplasm after viral infection of the cells, and then transported to nuclei. Additionally, the influenza polymerase has no proofreading function, resulting in a high gene mutation rate (Drake, 1993).

RdRp is a primer-dependent polymerase. It requires a small piece of RNA aligning the template RNA to start synthesizing; however the viral genome lacks this primer. The PB2 protein cleaves the 3' end from host mRNA which is known as "cap snatching" as the primer for synthesis of viral genome (Krug et al., 1979), causing host mRNAs to degrade. The PB1 protein is responsible for nucleotides extension. The PA subunit is associated with endonuclease activity and protease activities (Hara et al., 2001; Sanz-Ezquerro et al., 1995; Sanz-Ezquerro et al., 1996). The PB2 and PB1 proteins are necessary for cRNA synthesis, while the PA and NP proteins are required for vRNA synthesis (Liao et al., 2010; Nakagawa et al., 1996).

The influenza polymerase proteins are found to be important virulent factors and determinants of host range for certain subtype IAVs. Introduction of amino acid substitutions in polymerase proteins alters the virus' ability to adapt to physiological constraints; for instance, the

differences between temperatures in the respiratory tracts of avian and mammalian species. It is well known that mutation of the amino acid lysine (K) to glutamic acid (E) at position 627 in PB2 allows for mammalian adaptation (Subbarao et al., 1993). Normally, avian IAVs contain an E residue at position 627 and preferentially replicate in the digestive tract of birds which has a relatively higher temperature, about 41 °C (Webster et al., 1978). In contrast, human influenza viruses contain a residue K at position 627 and replicate most efficiently in the respiratory tract which has a lower temperature, about 33-37 °C (Alford et al., 1966). In addition, this substitution has been implicated with increased virulence. One previous study showed that viruses possessing E at PB2 627 replicated less well in lungs than in nasal turbinate and replicated less efficiently in cells at the lower temperature (Aggarwal et al., 2011) while K at PB2 627 enables avian H5N1 viruses to replicate efficiently in the upper and lower respiratory tracts of mammals (Hatta et al., 2007). This substitution has also been found from a fatal human infection of H7N9 (de Wit et al., 2010). Avian IAVs can quickly acquire the E627K substitution when they are passaged in mice experimentally (Li et al., 2005; Mase et al., 2006).

A mutation from aspartate (D) to asparagine (N) at positon 701 in PB2 of H5N1 virus can increase the virus virulence even without the E627K mutation. This mutation was found to promote avian H5N1 viruses adapt to mammalian species, such as mouse and human (de Jong et al., 2006; Li et al., 2005; Mase et al., 2006). The mechanism study indicated that it is due to the increased binding affinity of PB2 to importin alpha1 of mammalian cells resulting in enhanced transportation of the PB2 protein into the nucleus (Gabriel et al., 2008). However, those substitutions do not apply to all strains. For example, the A(H1N1)pdm09 human influenza virus does not carry the mammalian residues at 627K or 701N. Moreover, introduction of these

mammalian adaptation markers into the genome does not increase its virulence (Herfst et al., 2010).

The absence of mammalian adaptation markers in PB2 can be compensated by a polymerase polymorphism 590 serine (S) and 591 (R), which may affect virus replication in mammalian cells by enhancing protein interactions with cellular factors (Mehle and Doudna, 2009; Yamada et al., 2010). The adaptive studies found that the combination of 627K, 701N, with a substitution near cap-binding region T271A also enhanced polymerase activity in mammalian cells (Bussey et al., 2010). Another study suggested that in A(H1N1)pdm09 and triple-reassortant swine IAVs, the combination of 271A with the 590/591 SR is critical for virus replication and adaptation in mammalian cells (Liu et al., 2012b).

Several amino acid substitutions in PA have also been suggested as a potential mechanism that can increase polymerase activity and virulence in mouse studies (Rolling et al., 2009; Song et al., 2009). The amino acid substitutions in the avian-origin H1N1 PA gene lead to cross the species barrier and increase viral replication (Mehle et al., 2012). Additional substitutions in the PB1 and NP genes have also been suggested as virulence determinants (Gabriel et al., 2008; Salomon et al., 2006).

PB1-F2 protein

The PB1-F2 protein, a short polypeptide expressed from an alternative +1 reading frame of PB1, is important for the virulence of some influenza strains (Chen et al., 2001; McAuley et al., 2007). It contributes to virulence by inducing apoptosis of infected cells (Chen et al., 2001). A recent study also showed that the existence of a full-length PB1-F2 may contribute to pathogenesis in mice (Zamarin et al., 2006). One study demonstrated that the PB1-F2 protein promoted and increased the severity of influenza infection causing secondary bacterial

pneumonia (McAuley et al., 2007). This protein has been shown to contribute to the virulence of different influenza strains including the 1918 H1N1, 1957 H2N2, 1968 H3N2 pandemic viruses and the HPAI H5N1 strains (Conenello et al., 2007; McAuley et al., 2010; McAuley et al., 2007; Zamarin et al., 2006). One specific amino acid, N to S substitution at position 66 in PB1-F2 of 1918 pandemic influenza and HPAI H5N1 viruses, increased the virulence (Conenello et al., 2007), and showed reduced production of IFN (Varga et al., 2011). While the A(H1N1)pdm09 virus encodes a truncated PB1-F2 protein of 11 amino acids (Garten et al., 2009; Neumann et al., 2009), a restored PB1-F2 in this virus only affects the virulence modestly in mice and ferrets (Hai et al., 2010; Ozawa et al., 2011).

PA-X protein

The PA-X protein is a newly identified protein encoded by the PA gene via a shifted open frame (Jagger et al., 2012). It is a multi-functional protein that modulates the host response by depressing expression of cellular gene. Influenza viruses deficient of PA-X caused more severe disease in the mouse model due to an accelerated host response (Jagger et al., 2012). Observed influenza PA-X truncations in particular hosts is likely due to species specificity in the evolution of PA-X (Shi et al., 2012).

1.9 Influenza virus life cycle

The life cycle of the influenza virus is different from other RNA viruses in that it replicates in the nucleus. The first step of the infection is viral attachment to host cell receptors via the HA protein. After attachment, the cell membrane forms an early endosome. During the course of infection, the viral membrane must fuse to the host-cell membrane in order to deliver the viral genome into the cytoplasm. In the early endosome, the low pH triggers the cleavage of the HA protein into the HA1 and HA2 subunits, revealing the fusion protein (Medina and

Garcia-Sastre, 2011). The fusion protein then inserts hydrophobic anchors into cell membrane. Adjacent membranes do not fuse spontaneously. Envelope proteins anchored in the viral membrane generate a conformational rearrangement that bends the apposed membranes towards each other (Chernomordik et al., 1999). This close juxtaposition of the viral and cellular membrane enables fusion of the lipid bilayers, and the uncoated virus is thus delivered into the cytoplasm.

Next, the vRNPs are transported into the nucleus for replication. The vRNP complex starts transcription of negative stranded vRNAs into viral mRNAs. The PB2 protein steals the 5' cap from a host mRNA and then uses it as a transcription primer (Krug et al., 1979). The U residues at the 5' end of the vRNAs are used for polyadenylation by the viral polymerase. After transcription, the viral mRNAs are transported out of nucleus and into the cytoplasm for translation.

To produce viral genomes, the polymerase switches from production of viral mRNAs to production of cRNA which is an intermediate positive stranded RNA. The produced cRNA is then used as a template for the production of vRNA. The newly synthesized vRNA then associates with the NP protein forming a double helix and subsequent binding to polymerase proteins forms a vRNP. The new vRNPs are exported out of the nucleus by interacting with the NEP protein which has the export machinery function. The M1 protein acts as a scaffold protein, interacting between the NEP protein and the vRNPs (Shimizu et al., 2011; Ye et al., 1999). The vRNPs are transported to the cell membrane for assembly and budding, mediated by the M1 protein (Zhang et al., 2000). The vRNPs are selectively packaged resulting in the production of virus genomes containing eight viral segments, although the exact mechanisms remain unclear (Chou et al., 2012; Noda et al., 2006).

1.10 Virus receptors

Sialic acids are considered to be the receptors for influenza viruses (Schauer, 2000), which display different conformations in different species, although the receptor for H17 and H18 subtype viruses remain unclear (Sun et al., 2013; Tong et al., 2013; Wu et al., 2014). In order to attach to cells, the influenza viruses bind to the sialylglyco-conjugated *N*-acetylneuraminic acid via their HAs. The 3-D structure of a human H3 subtype HA complex with the receptor analog sialyllactose shows the receptor-binding site (RBS) of the N-acetylneuraminic acid residue (Neu5Ac) (Weis et al., 1988). Comparison of HA amino acid sequences of 13 different subtypes suggests the amino acids bind Neu5Ac moiety, indicating that IAV may bind the essential part of the receptor using a similar manner (Nobusawa et al., 1991).

Each virus subtype predominantly infects certain hosts. For example, normally avian IAVs do not infect humans, although the avian IAVs can infect humans sporadically (H9 and H5 subtypes). Similarly, swine IAVs (swine IAVs) normally only circulate in swine herds. The difference of HA molecule binding affinity is the major determinants of host specificity. Previous studies have shown that antigenically similar viruses isolated from avian and equine species preferentially bind to α 2,3 receptors, while human influenza viruses preferentially bind to α 2,6 receptors (Connor et al., 1994; Rogers and D'Souza, 1989; Rogers and Paulson, 1983). The analysis suggests that residues 226 and 228 of the H2 and H3 subtype of avian and human HAs are responsible for virus binding preference (Connor et al., 1994; Webster et al., 1992). It is evidenced that a single amino acid mutation at the HA receptor binding site will change the receptor binding preference (Matrosovich et al., 1997). In addition, the distribution of sialic acid receptors varies between species. Interestingly, domestic pigs are known to carry both α2,3 and α2,6 receptors in their respiratory tracts as shown in Figure 1.

Figure 1 Receptor distribution of α2,3 and α2,6 in the respiratory tract

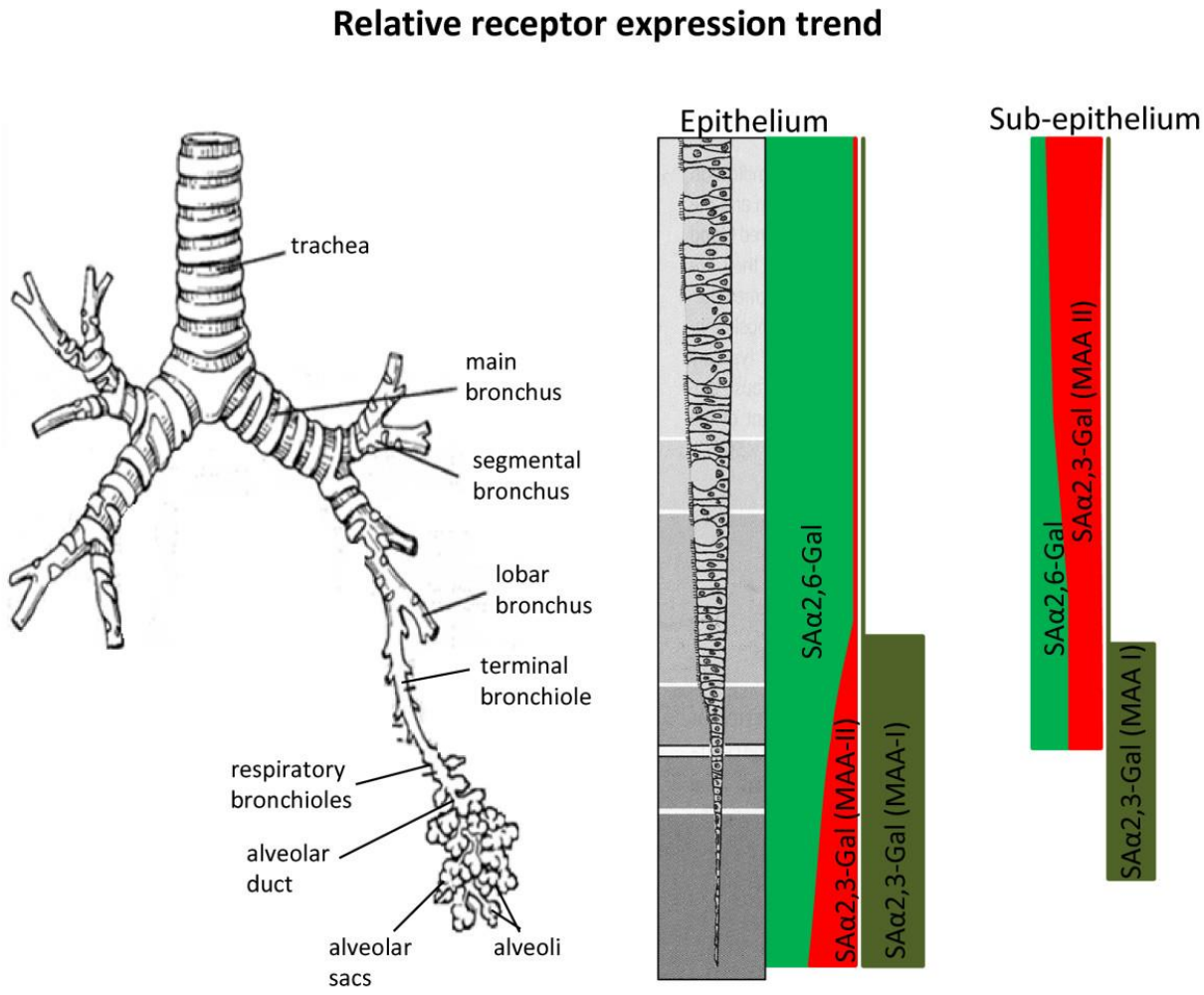

Figure 1. A qualitative assessment of receptor presence along the porcine respiratory tract. In the epithelial tract, the dominant receptor is SAα2,6-Gal, with increasing MAA II along the alveolar region. Along the sub-epithelial region, MAA II lectin binding is dominant. MAA I lectin binding is localized in the lower respiratory tract. SAα2,6-Gal (SNA); SAα2,3-Galβ(1-4) GlcNAc (MAA I) and SAα2,3-Galβ(1-3) GalNAc (MAA II). (Adapted from Nelli et al. BMC Veterinary Research 2010 6:4 doi:10.1186/1746-6148-6-4) (Nelli et al., 2010).

1.11 Swine influenza

Swine influenza is a zoonotic disease that caused by swine IAVs that results in fever, nasal discharge, and decreased appetite. Swine IAV is a common cause of respiratory disease in swine and has been isolated from pigs throughout the world. The dominant genus of influenza virus that infects swine is type A virus, although influenza B and C viruses have been isolated from swine (Kimura et al., 1997; Ran et al., 2015; Takatsy et al., 1967). There are three subtypes of swine IAVs circulating in the world, including the H1N1, H1N2, and H3N2 subtypes. However, subtype and genotype of the viruses are different in different regions of the world (Janke, 2014).

Swine influenza has received much attention due to the economic loss to swine production industry caused by swine IAVs and the potential threat to humans. Pigs also serve as an intermediate host for IAVs because they possess both α-2,3 and α-2,6 sialic acids which can be bound by both avian and human influenza viruses. As a result, swine-origin viruses might be able to transmit between pigs and other species, which was especially highlighted by the 2009 pandemic and with H3N2 variant IAVs that have infected humans (Greenbaum et al., 2015; Nelson et al., 2015).

Evolution of swine IAV in US

Influenza virus was first isolated from swine in 1930 (Shope, 1931). This virus was of the subtype H1N1 and was later termed classical swine IAV. The classical H1N1 swine IAVs continuously circulated in swine herds in the US until 1998. Then, in 1998, a severe respiratory disease emerged in a pig farm in North Carolina, US, which then spread to Texas (Zhou et al., 1999). It was a human H3N2 virus that was introduced into swine herds and reassorted with endemic viruses, resulting in novel double- and triple- reassortant swine IAVs. The double-

reassortant viruses contained HA, NA and PB1 from human IAVs and other internal genes from classical swine IAVs. It was later confirmed that the viruses that caused these outbreaks were genetically derived from avian, human, and swine IAV (Zhou et al., 1999). These viruses were later termed "triple reassortant" viruses due to the constellation of the avian (PB2 and PA), the classical H1N1 swine (M, NP, and NS), and human (PB1) genes (Webby et al., 2000; Zhou et al., 1999). This constellation of internal genes from avian, swine, and human IAV is known as the triple-reassortant internal gene cassette, or "TRIG," for short. Since then these triple-reassortant viruses have become prevalent and dominant in US pig populations (Webby et al., 2000).

Currently, there are three viral subtypes circulating in the US: the H1N1, H3N2, and H1N2 viruses. These viruses continue to circulate and generate new lineages through reassorting with seasonal human influenza virus; for example, the reassortant H1N1, human-like H1N1, and human-like H1N2 viruses (Anderson et al., 2013). The A(H1N1)pdm09 virus emerged in human in Mexico and quickly spread to other species. A(H1N1)pdm09 virus was found in swine in many areas of the world. Introduction of the A(H1N1)pdm09 virus into swine herds led to reassortment with the endemic swine IAVs, expanding the viral lineages in US swine (Liu et al., 2012a). Later, the reassortants with different reassortant patterns were reported globally (Ducatez et al., 2011). The circulating swine IAVs genotypes were changed after the emerging of A(H1N1)pdm09 as shown in Figure 2

Figure 2 Reassortant Pattern of Swine IAVs After A(H1N1)pdm09

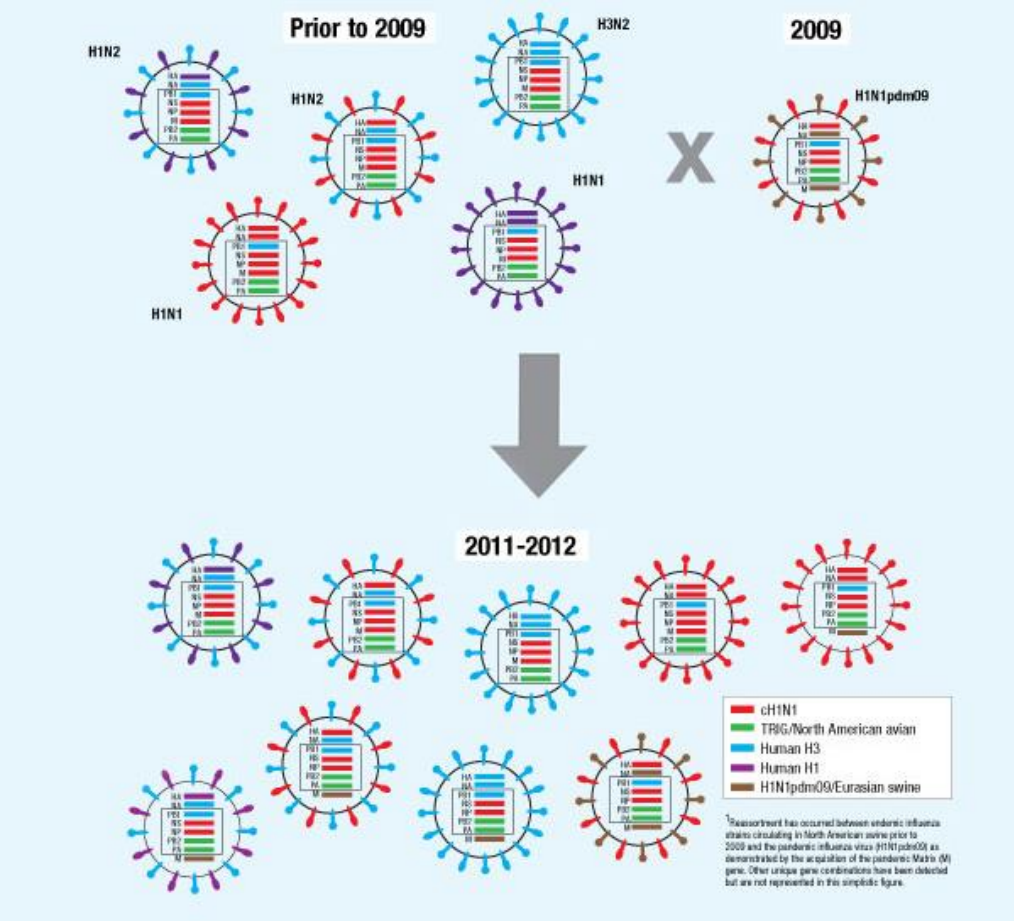

Figure 2 The swine IAV ecology in North American swine after A(H1N1)pdm09. Adapted from Phil Gauger June 19, 2012 Flu Virus Continues to Evolve in Swine

Human infections with swine IAVs

Normally, swine IAVs (swine IAVs) do not cause disease in humans. However, cases of human infection with swine IAVs have been reported in many countries of the world including the US (Myers et al., 2007). Since the first confirmed case of human infection with swine IAVs in a 16-year-old boy occurred in 1974 (Smith et al., 1976), around 50 cases of human infections have been reported (to 2005), 15 of which occurred in the US (Myers et al., 2007). In the Fort Dix case, a H1N1 subtype outbreak resulted in one mortality and 12 additional soldier infections (Gaydos et al., 1977). Since 2005, around 400 human infections with variant viruses [with or without M gene from A(H1N1)pdm09] were reported on May 11, 2015, resulting in 18 hospitalizations and one death (CDC, 2015). After the emergence of novel reassortant swine IAVs with A(H1N1)pdm09 genes, these viruses have jumped to human sporadically (Bowman et al., 2014; Bowman et al., 2012). It has been reported that during July-December 2011, 12 humans were infected with H3N2 variant viruses in the US (Lindstrom et al., 2012). Pigs are known as "mixing vessels" for avian and mammalian influenza viruses due to the presence of α-2,3 and α-2,6 (Ito et al., 1998). As the threat of influenza outbreaks continues, better understanding of the human-swine interface is needed.

Pathogenicity

The typical clinical signs and symptoms of pigs with swine IAV infection includes fever, sneezing, coughing (barking), depression, nasal discharge, breathing difficulties, eye redness and diarrhea. Although the genetic origin of swine IAVs varies, the clinical signs and lesions induced by different linages are essentially the same. The incubation period for influenza infection is 1-4 days before the onset of symptoms. Milder infection results in mild respiratory illness while few acute infections are followed shortly by death; however, swine IAVs very rarely cause mortality.

In the field, secondary bacterial infections contribute to the severity of swine influenza infection and results in severe pneumonia and higher mortality rates; however, it is hard to reveal the pathogenesis of the isolates in the *in vivo* studies (Van Reeth et al., 2002). Although pigs are susceptible to swine IAVs, they usually show less clinical signs of illness and are largely asymptomatic after infection. Pig-adapted or endemic influenza viruses usually only develop into mild or moderate clinical disease (Vincent et al., 2012). The virus infects the lungs via the airway and can be found throughout the respiratory epithelium, from nasal mucosa to alveoli. The virus has also been detected in respiratory submucosal glands. IAV replication is almost entirely limited to epithelial cells in the respiratory tract of infected pigs.

Gross lung lesions induced by swine IAV infection include multifocal to coalescing cranioventral atelectasis and varying red to purple areas of lung lobule consolidation. Adjacent lung lobes may be emphysematous as well. In milder infections, small aggregates of affected lobules are typically located in the cranial and middle lobes bilaterally. In severe infections, most of the cranial and middle lung lobes may be involved and sometimes this change extends to the cranioventral aspects of the caudal lobes. In the most severe cases, the infection may cause diffuse pulmonary consolidation with generalized pulmonary edema. Extensive amounts of foam may also be observed in bronchi and trachea along with enlarged and edematous bronchial lymph nodes. Secondary infection with bacteria may result in bacterial bronchopneumonia obscuring the underlying influenza lesions (Kennedy, 2007).

The hallmark histologic lesions of swine influenza infection are consistent with bronchointersitial pneumonia. There is prominent airway epithelium necrosis, characterized by bronchial and bronchiolar epithelial cell necrosis and accumulation of necrotic debris and neutrophils in bronchiolar lumens. Additional lesions include atelectasis, peribronchiolar,

perivascular and interstitial lymphoplasmacytic infliltrates, edema at multiple levels, and usually lesser alveolar involvement. The latter includes alveolar atelectasis or thickening of alveolar separate with inflammatory cells and scant fibrin. Occasionally, alveoli are filled with necrotic debris and neutrophils. At later infection time points, hyperplasia of airway epithelium consistent with regeneration can be seen. Previous studies have revealed that necrosis, apoptosis, and cytokines all contribute to the damage of infected cells (Henningson et al., 2015).

Vaccination in US

There are some procedures that can facilitate controlling swine IAVs and vaccination is still one of the most efficient methods. In the US, vaccination has been used by approximately 70% of large sow farms in the US (USDA. 2014). The typical influenza vaccines are adjuvanted trivalent inactivated vaccines that are efficacious and commercially available (Thacker and Janke, 2008). In addition, autogenous vaccines have also been used in about 20% of swine breeding females in the US (USDA. 2007). Alghough both inactivated and autogenous vaccins are widely used in US swine herds. The swine influenza is still not efficiently controlled and has become an endemic disease.

The inactivated and autogenous vaccines do not provide enough cross-protection against multiple antigenic strains in the field. Since the emergence of H3N2 triple-reassortant virus in 1998 and A(H1N1)pdm09 virus into swine herds, there are several different subtypes and genotypes of IAVs circulating in US pig populations (Ducatez et al., 2011; Nelson et al., 2012), and more new viruses are emerging. Commercial vaccines have to go through complicated procedures for licensing, often resulting in vaccine products that cannot match with the circulating strains. Passive acquired immunity also interfere the inactivated vaccine efficacy in the piglets. Although inactivated vaccines are immunogenic and able to stimulate high levels of

IgG in serum and lungs, they are protective only when HA antigen is antigenically close to the challenge virus.

Live attenuated vaccines stimulate stronger immune responses and provide more efficint cross-protection compared to the inactivited vaccines (Ambrose et al., 2008). A modified live attanuated vaccine such as the NS1 truncation live attenuated vaccine has been shown to be a good candidate for swine IAVs. A single dose of H3N2 Tx/98 NS1Δ126 given intranasally can fully protect challenge from a homologus virus and almost completely protect against a heterovariant virus challenge (Vincent et al., 2007). This live attenated vaccine has also been shown to be safe and efficicous in the presence of materially-dervied influenza antibody (ref), indicating that the modified live attenuated vaccine is able to overcome some of the difficulies associated with inactivated vaccines. However, no live attenuated vaccing has bee licenced yet, most likely due to safety concerns since a live attenuated vaccine might reassort with circulating viruses to generate a more virulent virus that might pose a risk to animal and human health.

1.12 Bat influenza

Bats are the second largest order among mammals and are distributed throughout most areas of the world. They are natural reservoirs for many zoonotic viruses including rabies virus, SARS coronavirus, Ebola virus and, Henipaviruses (Calisher et al., 2006; Tong et al., 2013). Given the global distribution, population density, and diversity of bats, people began to investigate potential reservoirs for influenza viruses (Turmelle and Olival, 2009). Two influenza A-like bat influenza viruses (bat influenza viruses) were identified from fruit bats in South America, which indicated that bats could also harbor a new group of influenza virus that are phylogenetically very distantly related to other IAVs (Tong et al., 2012; Tong et al., 2013).

The H17N10 subtype bat influenza virus was found from three of 316 bat rectal swabs by using pan influenza RT-PCR assay. Of one of the influenza positive bat, its liver, intestine, lung, and kidney tissues were positive but the oral swab was negative (Tong et al., 2012). The H18N11 subtype bat influenza was detected from Peru. Rectal samples were positive for influenza virus, while other specimens were negative with a pan Flu RT-PCT assay except intestine tissue was positive (Tong et al., 2013).

Based on initial sequencing of the H17 and H18 nucleotides, both viruses are clustered into the IAV family and can be grouped into group 1 depicted in Figure 3 (Wu et al., 2014). To date, neither of the two subtype bat influenza viruses have been isolated or cultured, leading to speculation that the putative viral sequences may be from DNA relics harbored in bats and not from real or viable viruses (Sun et al., 2013; Tong et al., 2012; Tong et al., 2013; Zhu et al., 2013).

Studies showed that both HA and NA lack canonical IAVs functions (Sun et al., 2013; Tong et al., 2013; Wu et al., 2014). Normally, the HA protein of IAVs is responsible for virus attachment to host cell receptors. However, the HA proteins of bat influenza viruses do not bind to sialic acid receptors as well as their counterparts. Additionally, the NAs do not have the neuraminidase functions. Thus, there is much debate as to whether or not these viruses are "real" or viable influenza viruses that exist in nature and some believe that they should be named "influenza-like" viruses. Currently, there are many vigorous tests being conducted regarding the receptors for bat influenza viruses.

Structural analysis has demonstrated that there are extensive conformational changes in the HA protein, including the conformation of rigid body orientation of the globular domain and the interhelix loop (Gamblin and Skehel). Other studies including glycan microarray analysis,

MDCK cell binding assays, and surface plasmon resonance (SPR) experiments have shown that the bat-derived HA proteins (H17/H18) cannot bind to canonical avian or human receptors (Sun et al., 2013; Tong et al., 2013; Zhu et al., 2013). One possible explanation is that the conformational change affects the structure of the HA receptor binding cavity, which reduces the strength of interaction between host cell receptors and the bat influenza HA proteins. It is likely that bat influenza viruses have acquired a different receptor.

The NA protein of IAVs is a sialidase and is responsible for cleavage of SA from the host cell surface in order to release the progeny viruses. It helps virus migration and prevents virus aggregation. If bat-derived HA proteins do not bind to SA, then there is no need for a sialidase to help with viral release. The structure of the NA-like proteins N10 and N11 have been solved. Similar to canonical IAVs, it maintains a tetrameric NA structure but other features differ. It is deficient of the 150-cavity and enzymatic active site (Tong et al., 2013; Zhu et al., 2013) and the structures of the bat-derived NA molecule do not support a neuraminidase function. Additionally, sialidase enzymatic activities were not detected for either the N10 or N11 proteins *in vitro* (Tong et al., 2013; Zhu et al., 2013).

Although efforts have been taken to isolate and culture an infectious virus from bat samples, no one has succeeded (Fereidouni et al., 2015; Tong et al., 2012; Tong et al., 2013). Reverse genetics systems have also been employed to produce an infectious bat virus. No infectious virus has been rescued (Juozapaitis et al., 2014; Zhou et al., 2014b). The unavailability of a replicative virus is the major hurdle in confirming the actual existence of such a new group of viruses and in answering questions regarding their pathogenicity in animal models and their ability to reassort with other influenza viruses.

Figure 3 Phylogenetic analysis of the hemagglutinin (HA) and neuraminidase (NA) genes of H17/H18 and NA-like N10/N11

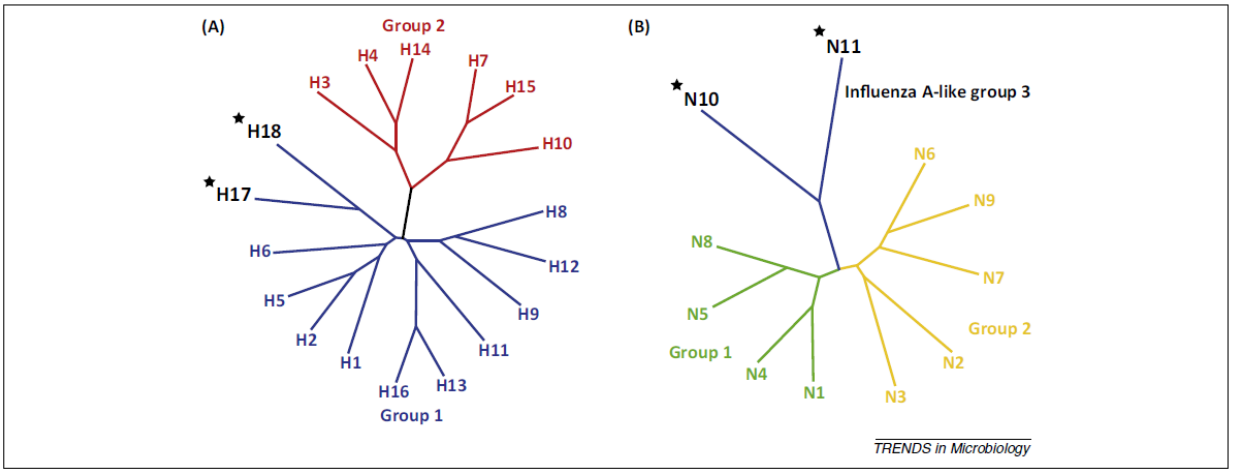

Figure 3 Phylogenetic analysis of the hemagglutinin and neuraminidase genes of H17/H18 and NA-like N10/N11. Adapted from Wu Y et al. Trends Microbiol 2014 Apr;22(4):183-91.

Chapter 2: Emergence of novel reassortant H3N2 swine IAVs with the 2009 pandemic H1N1 genes in the United States

The data in this section has been published in Arch Virol (2012) 157:555–562 by Qinfang Liu, Jingjiao Ma, et al., Emergence of novel reassortant H3N2 swine IAVs with the A(H1N1)pdm09 genes in the United States.

2.1 Introduction

Since the first report on infection of pigs with the A(H1N1)pdm09 in Canada in 2009 (Howden et al., 2009), A(H1N1)pdm09 has been isolated from pigs throughout the world including the US (UDSA, 2010). Introduction of A(H1N1)pdm09 into swine has raised concerns that novel reassortant viruses might be generated in pigs. As indeed, several reassortments of swine IAVs with A(H1N1)pdm09 in swine have been reported in Asian and European countries. The first reassortant H1N1virus was found in Hong Kong, China in 2009, which contains NA from A(H1N1)pdm09, HA from the Eurasian avian-like H1, and six internal genes from triple reassortant swine IAVs (Vijaykrishna et al., 2010). Subsequently, a reassortant H1N1 virus consisting of 7 genes from A(H1N1)pdm09 and NA from endemic swine IAVs was isolated in pigs in Germany (Starick et al., 2011). In early 2010, 3 reassortant H1N1 viruses were isolated from pigs in Thailand, which have NA from endemic H1N1 swine IAV and the remaining 7 genes from A(H1N1)pdm09 (Kitikoon et al., 2011). Recently, two reassortant H1N2 viruses were isolated from pigs in UK and Italy; one contains six internal genes of A(H1N1)pdm09 and another isolate has HA and six internal genes of A(H1N1)pdm09 and another isolate has HA and six intermal genes of A(H1N1)pdm09 and the remaining genes from endemic swine IAVs (Howard et al., 2011; Moreno et al., 2011). In the US, nine H1N2 swine IAVs and one H1N1 reassortant swine IAV have been detected in swine herds from Indiana, Minnesota and North

Carolina. All of these reassortant viruses contain the M gene and additional one to four internal genes from A(H1N1)pdm09 and the remaining genes from endemic triple reassortant swine IAVs (Ducatez et al., 2011; Sun D, 2011).

Here we report the characterization of seven reassortant H3N2 swine IAVs containing internal genes from A(H1N1)pdm09. These novel reassortant H3N2 swine IAVs were isolated between winter of 2010 and spring of 2011 from five swine farms in the Midwestern USA which outbreaks of respiratory disease had occurred.

2.2 Materials and Methods

Sample collection and virus isolation

Samples were collected between winter of 2010 and spring of 2011 from five swine farms in the Midwestern USA in which outbreaks of respiratory disease had occurred, and by the attending veterinarians who observed gross lesions of pneumonia suggestive of influenza at necropsy. Lung tissues and nasal swab samples from diseased pigs were submitted to the Kansas State Veterinary Diagnostic Laboratory (KSVDL) for further testing. First, 10% lung homogenates were centrifuged at 640×g for 10 minutes. The supernantant was then filtered by passing through 0.45 μm filters (Thermo, US). The bacteria free supernatants were then incubated in the monolayer Madin-Darby canine kidney (MDCK) cells prepared one day prior in 24-well-plates. The MDCK cells were incubated in infection medium of Eagle's Minimum Essential Medium (MEM) containing 0.3% bocine albumin (Sigma, USA) and 1 μg/ml TPCK-trypsin (Sigma) at 37°C with 5% CO2. The cells were observed daily for cytopathic effects (CPE). After observation of CPE, the cells supernatants were harvested for further tests.

Hemagglutinin inhibition assay (HI)

The virus isolates were first determined by Hemagglutination Assay. Briefly, 50 µl of 1×PBS was added to each well of a V-bottom microtiter plate. Then 50 µl of virus stock was added to the first well of each column and subsequently serially diluted twofold down the column. The last 50 µl from last row were discarded after dilution was completed. Then, 50 µl of 0.5% RBC was added to each well. Titers were then read after incubation at room temperature for 30-45 minutes. Four unit antigen solutions were prepared before Hemagglutinination inhibition assay. Briefly, 25 µl of 1×PBS was added to each well of V-bottom microtiter plate. Treated sera ws added to the first well of each column and serially diluted twofold. The prepared antigen solution of 25 µl was added each well and incubated at room temperature for 30 minutes. The 50 µl of 0.5% RBC was added to each well and incubate at room temperature for another 30 minutes. Then plates were analyzed.

Reverse transcription-PCR and Real-time PCR

The cell cultural supernatants containing the viruses were centrifuged at 1,200 rpm for 5 minutes to remove cellular debris. Virus RNAs were extracted by QIAamp Viral RNA Mini Kit (250) (Qiagen, US) following the manufacture's instruction. Real-time PCR targeting M genes of A(H1N1)pdm09 virus were performed as previously described (Ma et al., 2010). The probe is Pan-M-Probe: 5'- TTG CAT GGG CCT CAT ATA CAA C-3' and the A(H1N1)pdm09 specific primers are Pan-M-F: 5'- GGT GTC ACT AAG CTA TTC AA-3' and Pan-M-R: 5'- CAA AAG CAG CTT CTG TGG TC-3'. The RNA of all isolates was then extracted. The RNAs were then reverse transcribed into DNA complement (cDNA) using SSII RNA transcriptase (Life technology, CA) with uni-12 (5'-AGCAAAAGCAGG-3') primer. If the M segment is positive of A(H1N1)pdm09, the full genome sequences of all seven isolates were obtained by sequencing

all eight gene segments using Hoffman universal primers; if not, only HA, NA, and M gene will be amplified and sequenced.

Genome analysis

The nucleotide sequences from the isolates were aligned by DNASTAR Lasergene 9.0 SEQMAN. The aligned sequences were sent to BLAST (http://blast.ncbi.nlm.nih.gov) to determine the source of the individual genes. Phylogenetic tree analyses were generated by MegAlign software version 4.1.

2.3 Results

In December 2010, an outbreak of respiratory disease occurred in nursery pigs in a commercial swine farm (farm #1) in the Midwestern USA. In mid-January 2011, 50% of the sows that provided piglets to farm #1 (6000 sows in sow farm #1) were sick with acute respiratory signs, and more than 100 sows were suddenly dead within 24 hours after the occurrence of clinical signs. Subsequently, in February 2011, one independent farm (farm #2) located in the same area and farm #1 had an outbreak of respiratory disease in nursery pigs; both received piglets from sow farm #1. In mid-March 2011, an outbreak of respiratory disease occurred in nursery pigs in another independent farm (farm #3) that does not purchase piglets from sow farm #1. At the beginning of April 2011, farm #2 and another independent farm (farm #4) had an outbreak of respiratory disease in nursery pigs, and both farms had purchased piglets from the sow farm #1. During the outbreak, pigs showed respiratory signs, such as coughing, sneezing and nasal discharge. The morbidity was high (>60%) and the mortality was rather low (<3%) in the affected herds. The infection persisted in the swine herds throughout the winter in all affected farms. At necropsy, the attending veterinarian observed gross lesions of pneumonia suggestive of influenza. Lung tissues and nasal swab samples from diseased pigs were submitted

to the Kansas State Veterinary Diagnostic Laboratory (KSVDL). Swine IAVs were detected and isolated from samples collected from diseased pigs of all five farms (1 sow farm and 4 nursery farms) by standard real-time RT-PCR and virus isolation in the KSVDL. Lung tissues from these pigs were also found to be positive for porcine circovirus type 2 and Streptococcus suis. Porcine reproductive and respiratory syndrome virus was detected in lung tissues of pigs from two affected nursery farms (farm #1 and #2). All seven swine IAVs were isolated in cell culture using MDCK cells and identified to be of the H3N2 subtype by hemagglutinin inhibition and gel-based RT-PCR assays using standard methods. All isolates were positive by A(H1N1)pdm09 M-gene specific real-time RT-PCR (Ma et al., 2010). The full genome sequences of all seven isolates were obtained by sequencing all eight gene segments (sequence primers are available upon request). BLAST (http://blast.ncbi.nlm.nih.gov) and phylogenetic tree (MegAlign software version 4.1) analyses were conducted to determine the source of the individual genes from the isolates.

Based on sequence analysis, seven H3N2 isolates were identified to be reassortants of A(H1N1)pdm09 and endemic H3N2 swine IAVs. Phylogenetic analysis revealed that the NP, M and NS genes of all seven novel H3N2 reassortant viruses grouped within the A(H1N1)pdm09 cluster. The PB2 and PA genes of A/swine/Kansas/11-107824/2011 isolated from farm #3 also clustered into the A(H1N1)pdm09 group (Table 3). The HA genes of these seven viruses belonged to the North American triple reassortant H3N2 virus lineage. The NA gene of A/swine/Kansas/11-110529/2011 isolated from farm #4 grouped within the human-like lineage, whereas the NA genes of the other 6 H3N2 isolates clustered into the North American triple reassortant H3N2 lineage (Figure 8). In addition, all of the other internal genes that are not A(H1N1)pdm09-like were grouped within the triple reassortant swine IAVs cluster (Figure 4,

Figure 5, Figure 6, Figure 7, Figure 9, Figure 10, and Figure 11). Based on the HA phylogenetic tree, the H3N2 triple reassortant viruses in the field are genetically diverse (Figure 7), because four genetic clusters of H3N2 viruses are circulating presently in US swine herds. These genetically different H3N2 viruses were generated by reassortment events due to at least three introductions of different seasonal human H3N2 viruses into the swine herds in the late 1990s (Richt et al., 2003; Vincent et al., 2008; Webby et al., 2004).

Molecular analysis showed that M2 protein of all seven novel reassortant H3N2 viruses had an S31N amantadine-resistance mutation, similar to A(H1N1)pdm09 viruses; the NS1 gene of these seven viruses encoded a truncated 220-amino-acid protein that is identical to A(H1N1)pdm09 NS1. There were no specific mutations for adaptation to mammalian host (627E and 701D) (Gabriel et al., 2005; Li et al., 2005; Subbarao et al., 1993) in the PB2 of any of the seven novel reassortant H3N2 viruses, but they all had a 271A and SR polymorphism at positions 590/591, which is believed to compensate for the lack of 627K (Bussey et al., 2010; Mehle and Doudna, 2009). The HA proteins of all seven H3N2 had 226V and 228S at the receptor-binding sites except for the HA of the isolate A/swine/Kansas/11-107824/2011,which contained 226V and 228G is different from those of most of avian (226Q/228G) and human (226L/228S) influenza virus HAs(Matrosovich et al., 1997). But it is present in the majority of HAs (>90%) of North American triple reassortant H3N2 swine IAVs. The 226V/228G combination in the HA receptor-binding site is rarely found. The receptor specificity of the 226V/228S and 226V/228G combinations remains unknown and needs to be investigated in the future. In addition, the NA protein had 119E, 292R and 274H, suggesting susceptibility to oseltamivir.

2.4 Discussion

H1 subtype (i.e., H1N1 and H1N2) reassortants of A(H1N1)pdm09 and endemic swine IAVs have been isolated from pigs worldwide (Ducatez et al., 2011; Howard et al., 2011; Kitikoon et al., 2011; Moreno et al., 2011; Starick et al., 2011; Sun D, 2011; Vijaykrishna et al., 2010). A reassortant H3N2 swine IAV was only recently isolated from Minnesotan pigs and had PA, NP and M genes from the H1N1 (Ducatez et al., 2011). In this report, seven novel H3N2 reassortants were isolated from diseased pigs from five different farms. Six of the H3N2 viruses had a similar genetic constellation, i.e., NP, M and NS were derived from A(H1N1)pdm09, and the remaining genes were from endemic H3N2 swine IAVs; one isolate had PB2, PA, NP, M and NS from A(H1N1)pdm09 and the remaining genes from endemic H3N2 triple reassortant swine IAVs. Three reassortant H1N2 swine IAVs containing a similar genetic constellation, carrying the A(H1N1)pdm09 NP, M and NS genes, were recently detected in pigs (Ducatez et al., 2011), indicating that this genotype of novel reassortant swine IAVs seems to be preferred in different subtypes and seem to be stable. The novel reassortant H3N2 viruses having A(H1N1)pdm09 NP, M and NS genes were isolated from diseased sows (sow farm #1) and nursery pigs from three farms (farms #1, #2 and #4) that obtained piglets from sow farm #1, indicating that the piglets may have been infected at the sow farm before transportation to the nursery. Importantly, similar H3N2 reassortant viruses were also isolated from farms #1 and #2 at later time points (two months after the first isolations occurred), suggesting that the virus had been established and continued to circulate within the affected production systems. An H3N2 isolate containing all internal genes from A(H1N1)pdm09 except PB1 was isolated from another farm (farm #3); this farm does not receive piglets from sow farm #1. Whether novel H3N2 reassortant viruses have increased pathogenicity and are transmitted among pigs more efficiently than A(H1N1)pdm09 or

parental H3N2 swine IAVs remains unknown; the role of internal genes from A(H1N1)pdm09 in pathogenesis and transmissibility of these novel viruses need to be investigated.

Concurrent epidemiological surveillance has revealed that these novel reassortant H3N2 swine IAVs are circulating in Midwest swine herds although triple reassortant H1N1, H1N2 and H3N2 swine IAVs have also been isolated from other swine farms in the same area. A Kansas boy who was in contact with healthy pigs while attending a county fair in 2009 was reported to have been infected with a triple reassortant H3N2 swine IAV. This H3N2 virus does not seem to be transmitted efficiently among humans because his three household contacts did not show signs of illness (Cox et al., 2011). Recently, two younger children from Indiana and Pennsylvania were infected by reassortant H3N2 swine IAVs that contained only the M gene from A(H1N1)pdm09 (CDC, 2011) Two other children in Pennsylvania who were directly exposed to swine at an agricultural fair had confirmed infection with a similar A(H1N1)pdm09 reassortant H3N2 influenza virus (Health, 2011). Although no reported human illness due to influenza infection was associated with the Kansas farms where the novel reassortant H3N2 viruses were isolated, it remains unclear whether these novel reassortant H3N2 swine IAVs can be transmitted to and then infect humans. If so, they most likely will pose a threat, especially to children born after 1998, due to the lack of immunity to these viruses. Notably, the isolate A/swine/Kansas/11-110529/2011 has an NA gene that is similar to those of human H3N2 viruses, including the novel reassortant H3N2 viruses that recently infected children in Indiana and Pennsylvania (CDC, 2011; Health, 2011). Although the NA is grouped within the human-like H3N2 influenza lineage, North American triple reassortant swine IAVs containing a similar NA gene have been circulating in US swine herds for more than 5 years. Nevertheless, continuous circulation of A(H1N1)pdm09 in swine will increase the chance of further reassortment with

human, avian or swine IAVs (Kimble et al., 2011; Schrauwen et al., 2011; Vijaykrishna et al., 2010) and could result in a novel virus with the potential to cause infection and efficient transmission among humans.

2.5 Conclusion

In conclusion, the emergence of novel reassortant H3N2 swine IAVs in US swine is further evidence of reassortment between A(H1N1)pdm09 and endemic swine IAVs. The occurrence of human infection with novel reassortant H3N2 viruses warrants continuous surveillance in swine and human populations.

Table 3 Reassortant patterns of novel H3N2 swine IAVs in the USA

Virus name	Subtype	Gene segment							
		PB2	PB1	PA	HA	NP	NA	M	NS
A/swine/Kansas/10-91088/2010	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-101926/2011	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-104465/2011	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-104467/2011	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-107824/2011	H3N2	P	T	P	T	P	T	P	P
A/swine/Kansas/11-109700/2011	H3N2	T	T	T	T	P	T	P	P
A/swine/Kansas/11-110529/2011	H3N2	T	T	T	T	P	T	P	P

PB, polymerase basic protein; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleo protein; NA, neuraminidase; M, matrix; NS, nonstructural; T: gene has closest homology with triple reassortant swine IAV; P: gene has closest homology with 2009 pandemic H1N1 virus

Figure 4 Phylogenetic Tree of the PB2 gene of the seven reassortant H3N2 swine IAVs

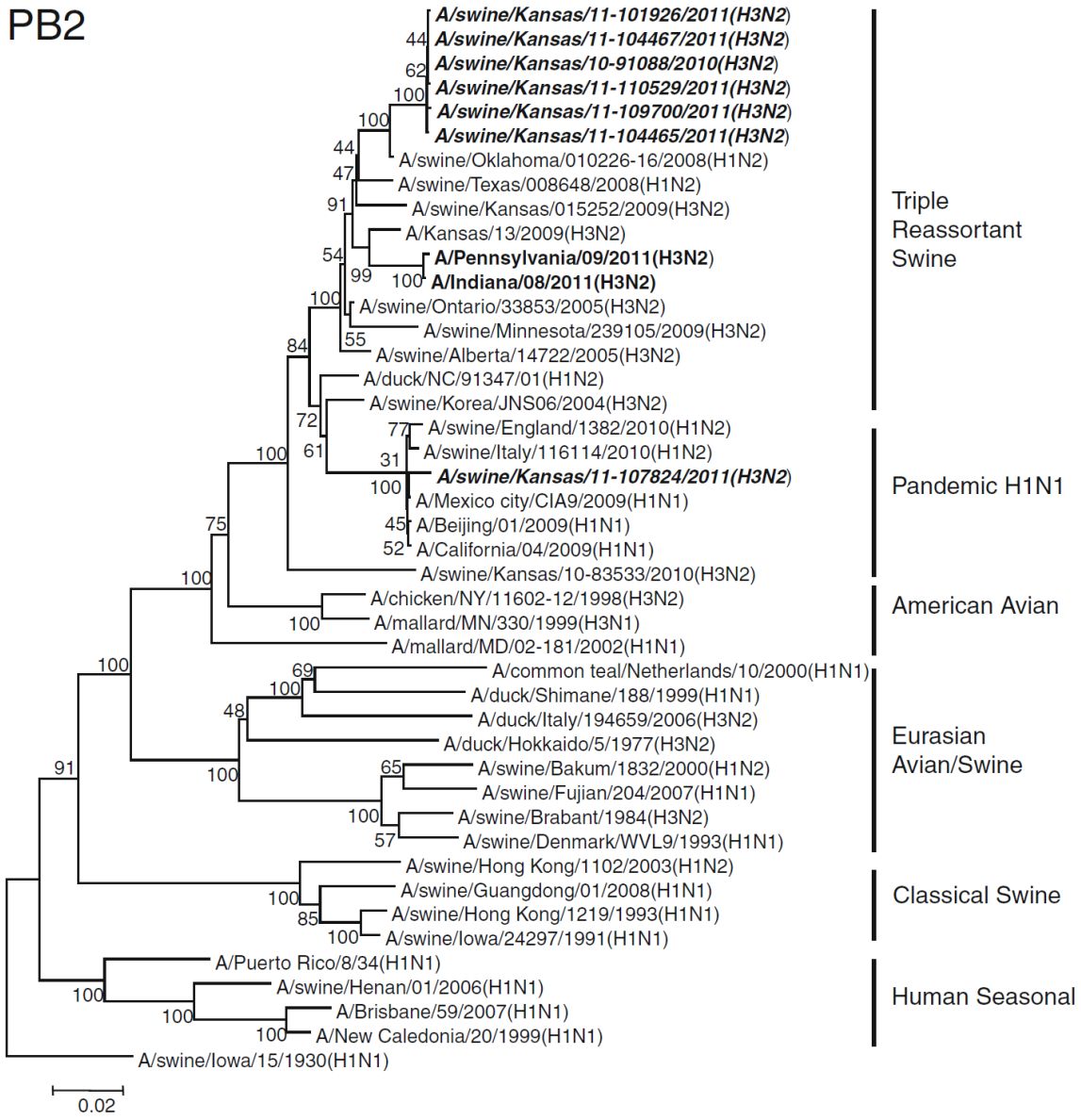

Figure 5 Phylogenetic tree of the PB1 gene of seven reassortant H3N2 swine IAVs

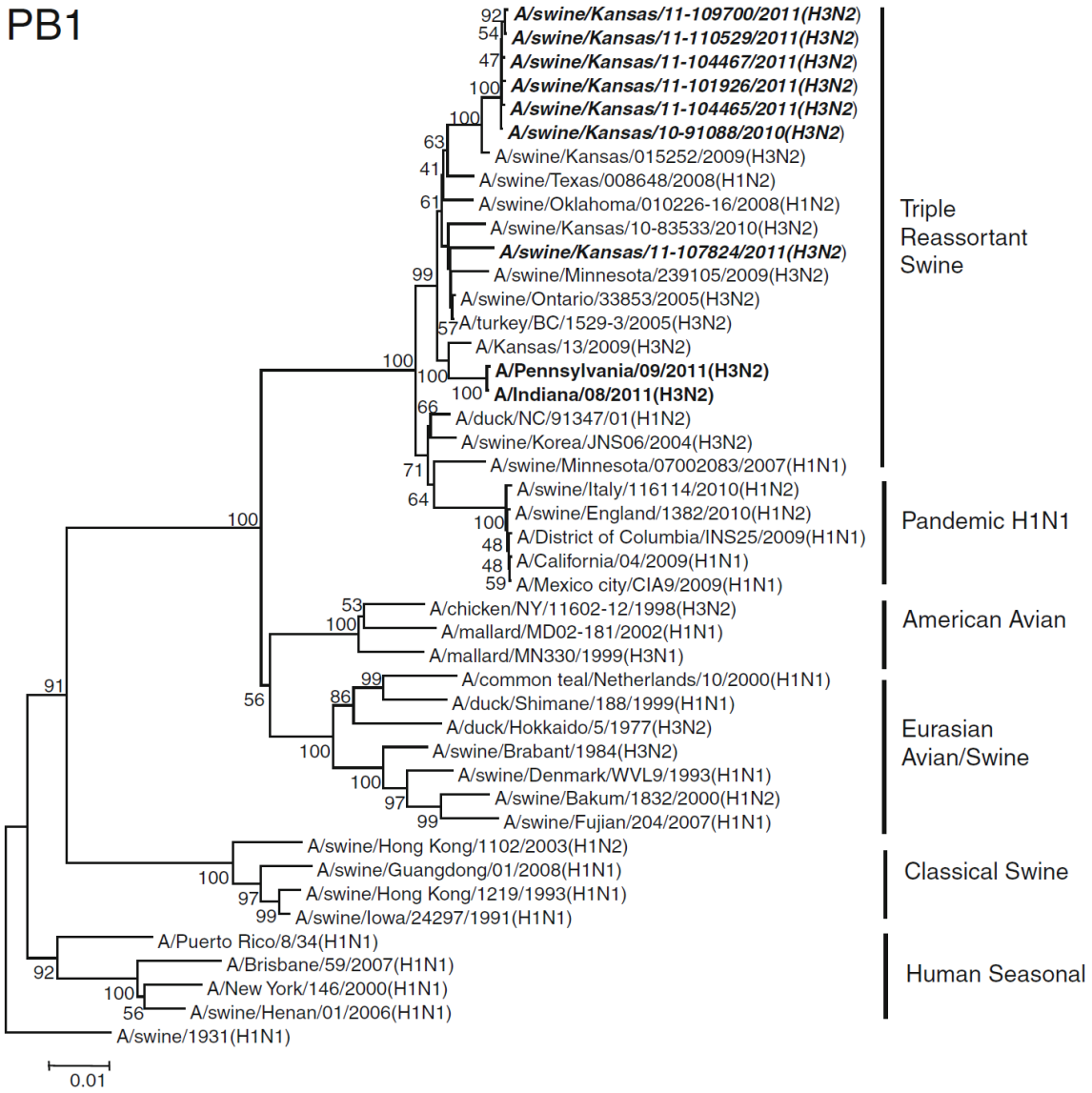

Figure 6 Phylogenetic tree of the PA gene of seven reassortant H3N2 swine IAVs

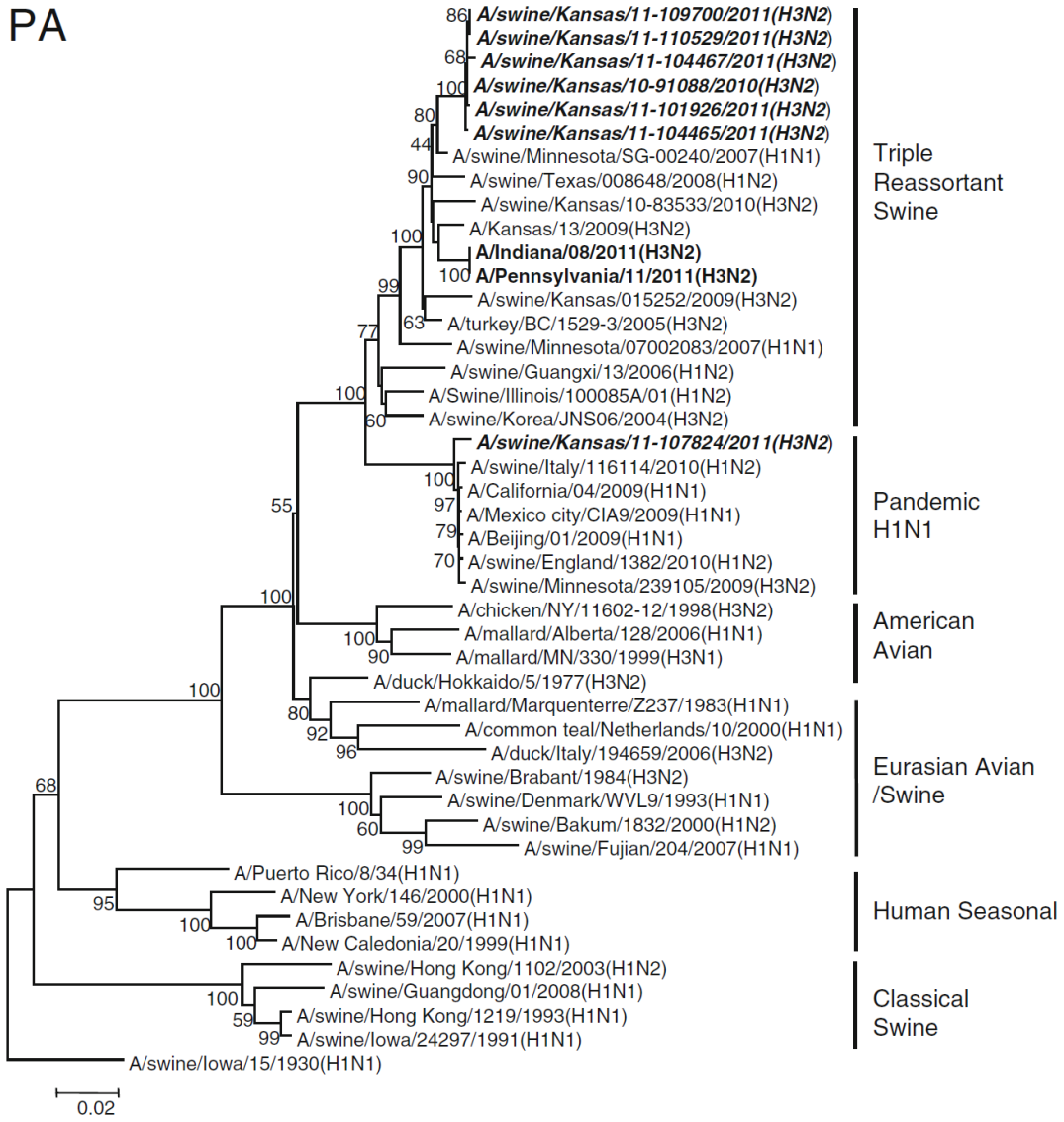

Figure 7 Phylogenetic tree of the HA gene of seven reassortant H3N2 swine IAVs

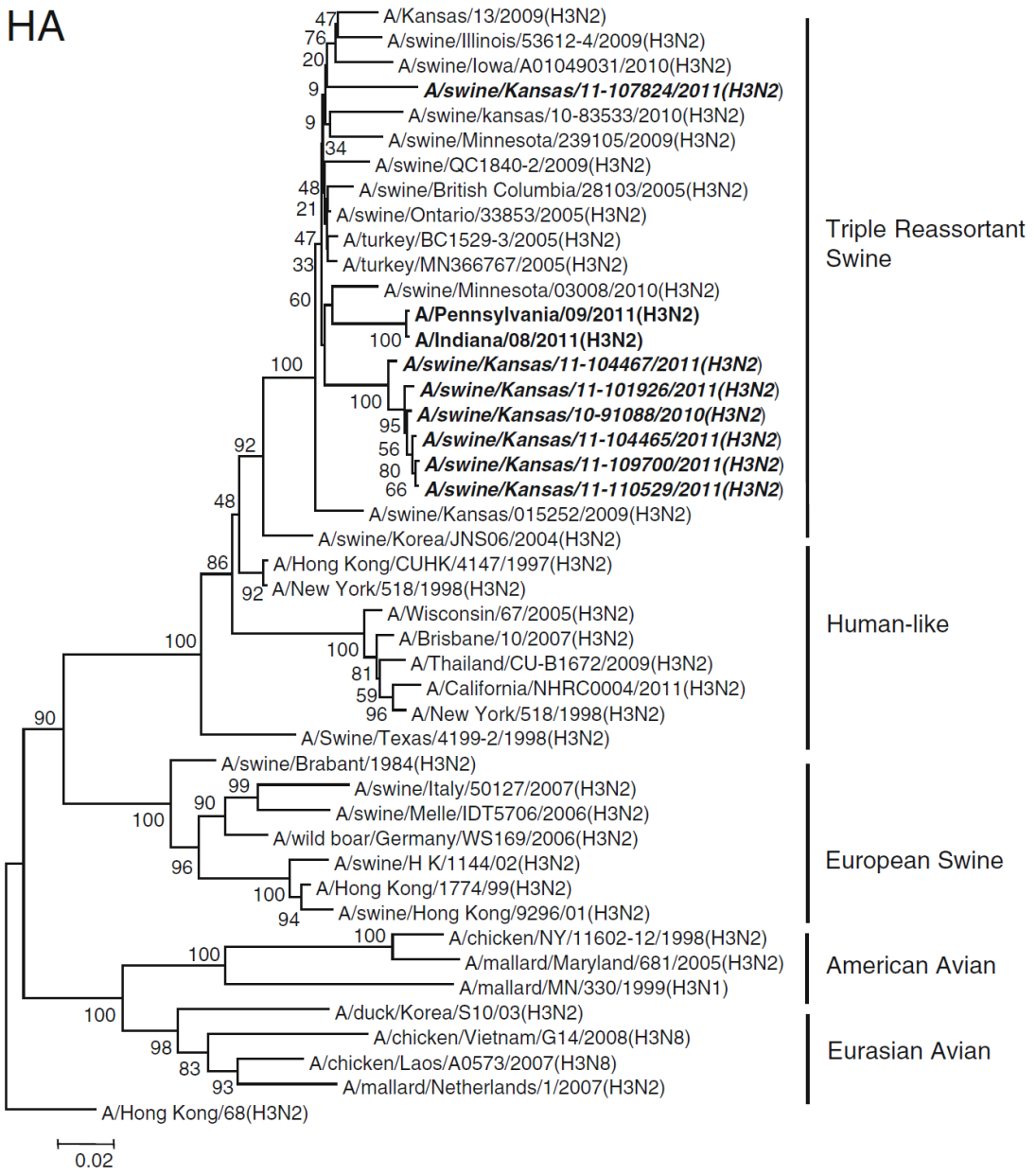

Figure 8 Phylogenetic tree of the NA gene of seven reassortant H3N2 swine IAVs

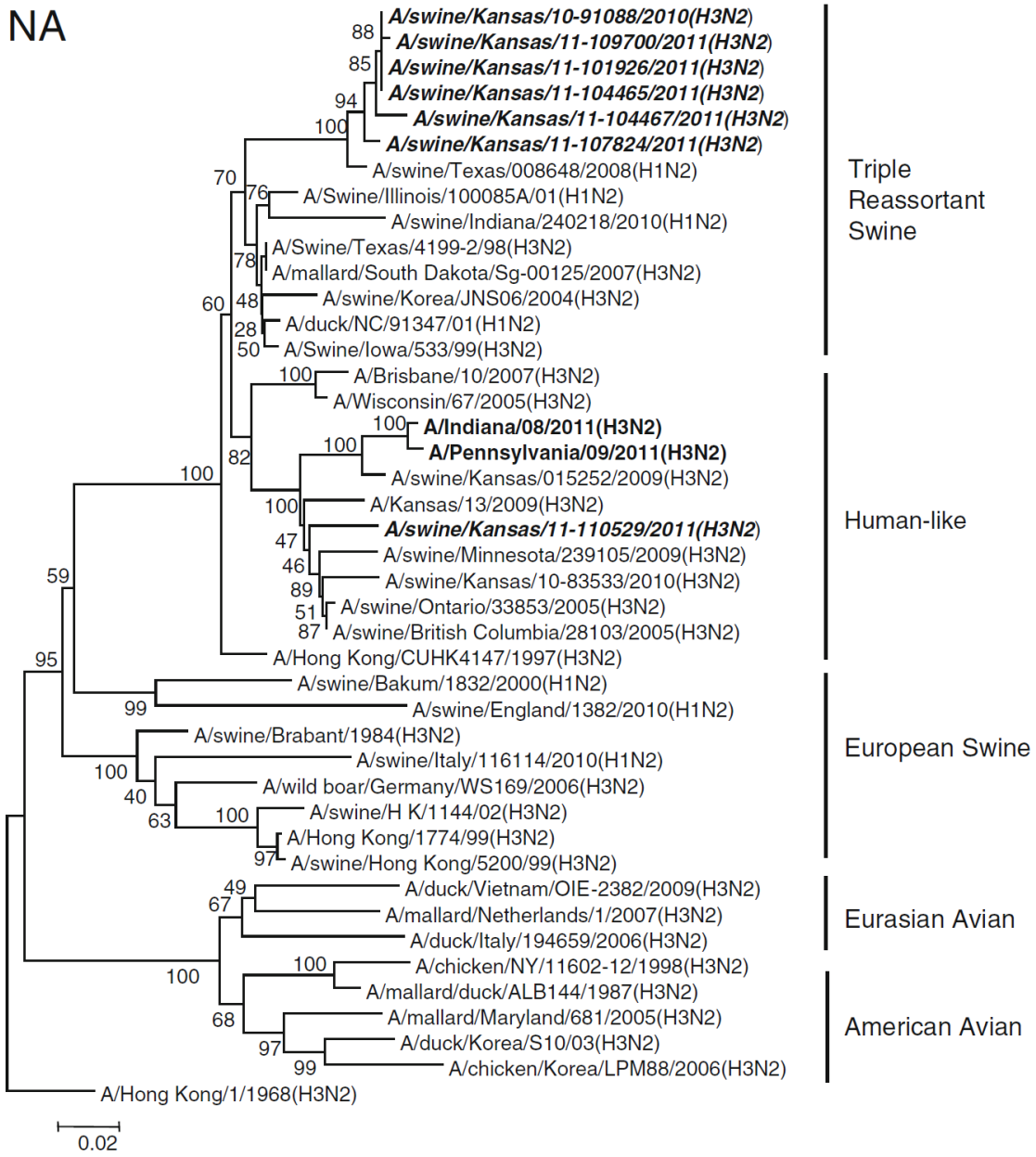

Figure 9 Phylogenetic tree of the NP gene of seven reassortant H3N2 swine IAVs

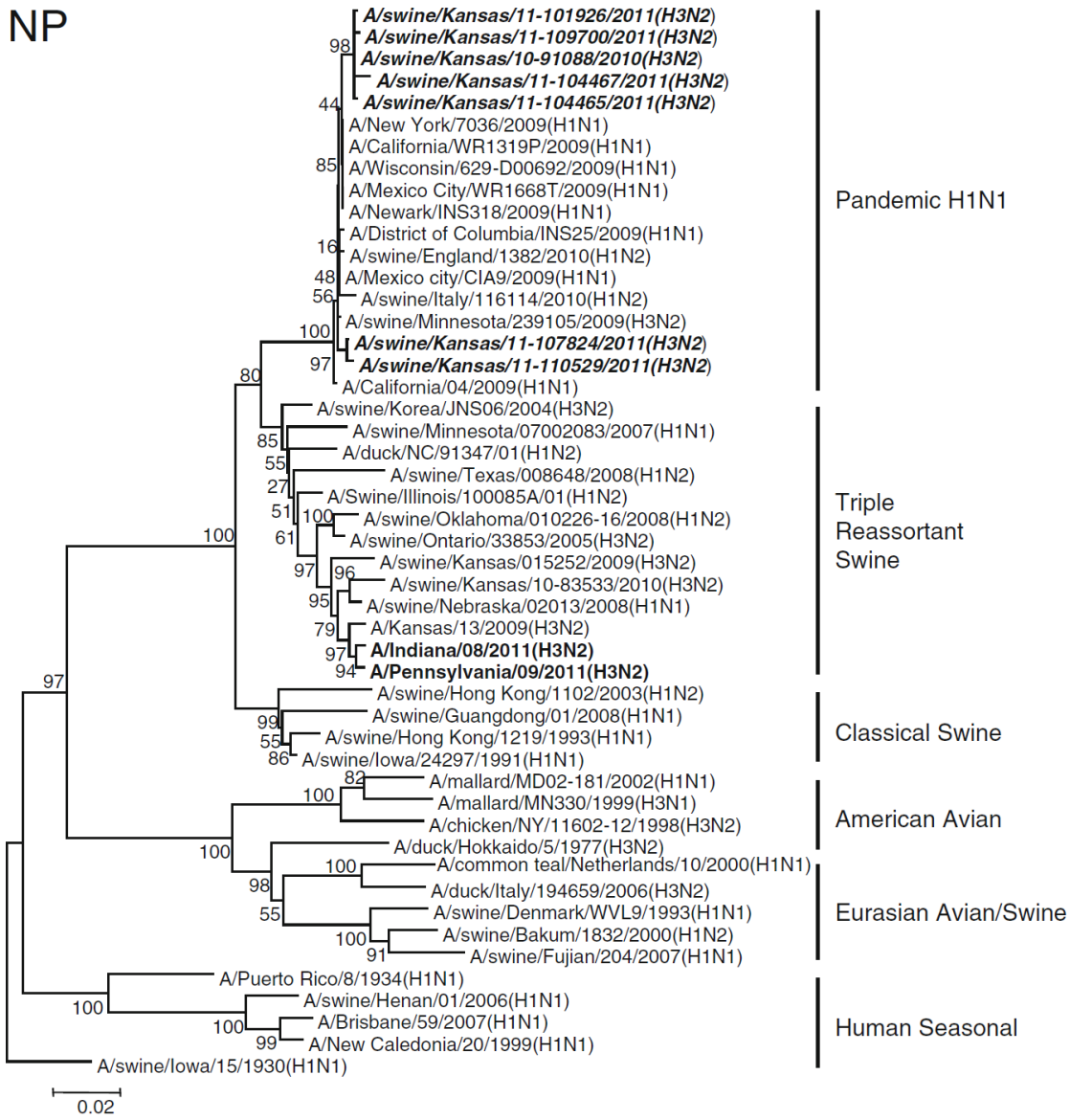

Figure 10 Phylogenetic tree of the M gene of seven reassortant H3N2 swine IAVs

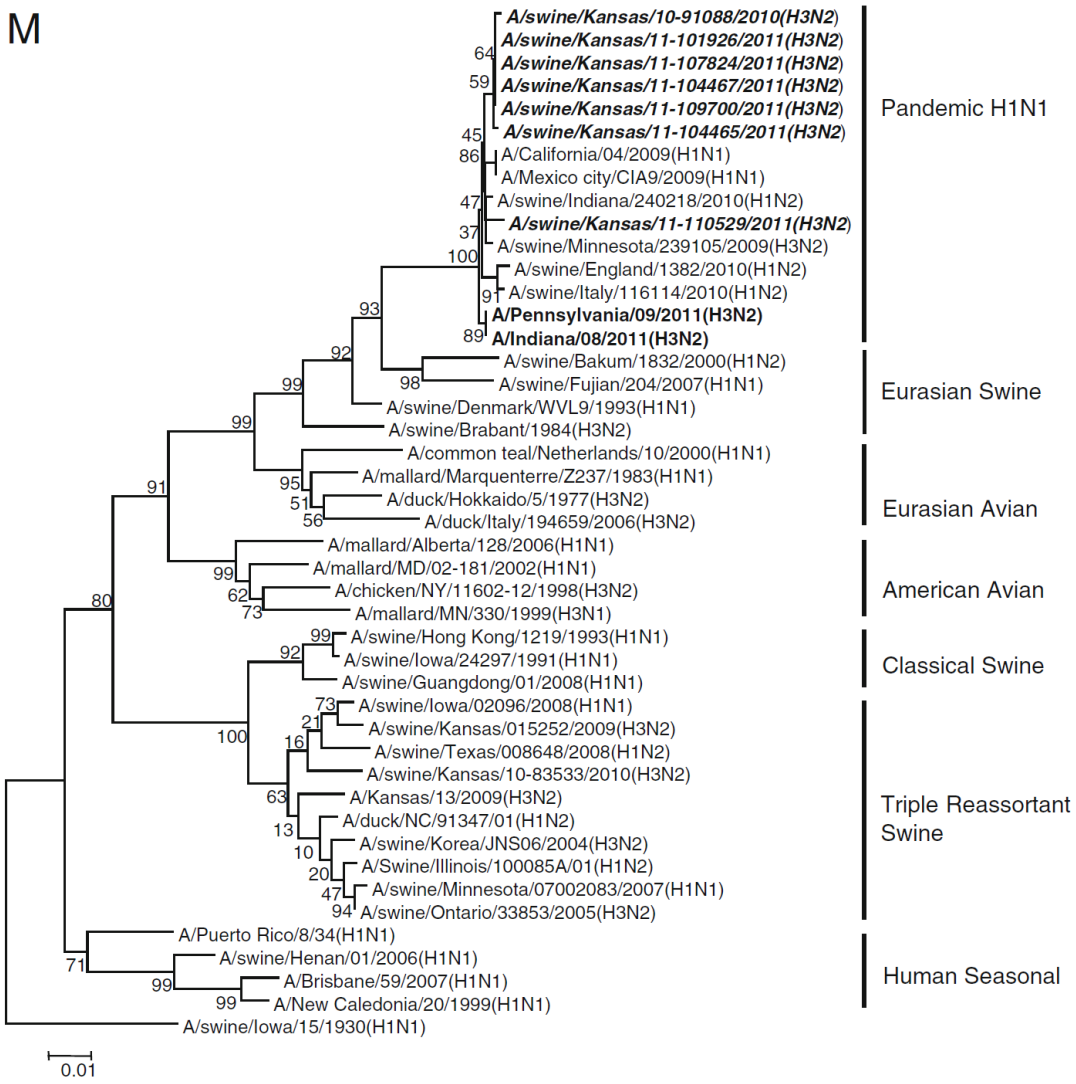

Figure 11 Phylogenetic tree of the NS gene of seven reassortant H3N2 swine IAVs

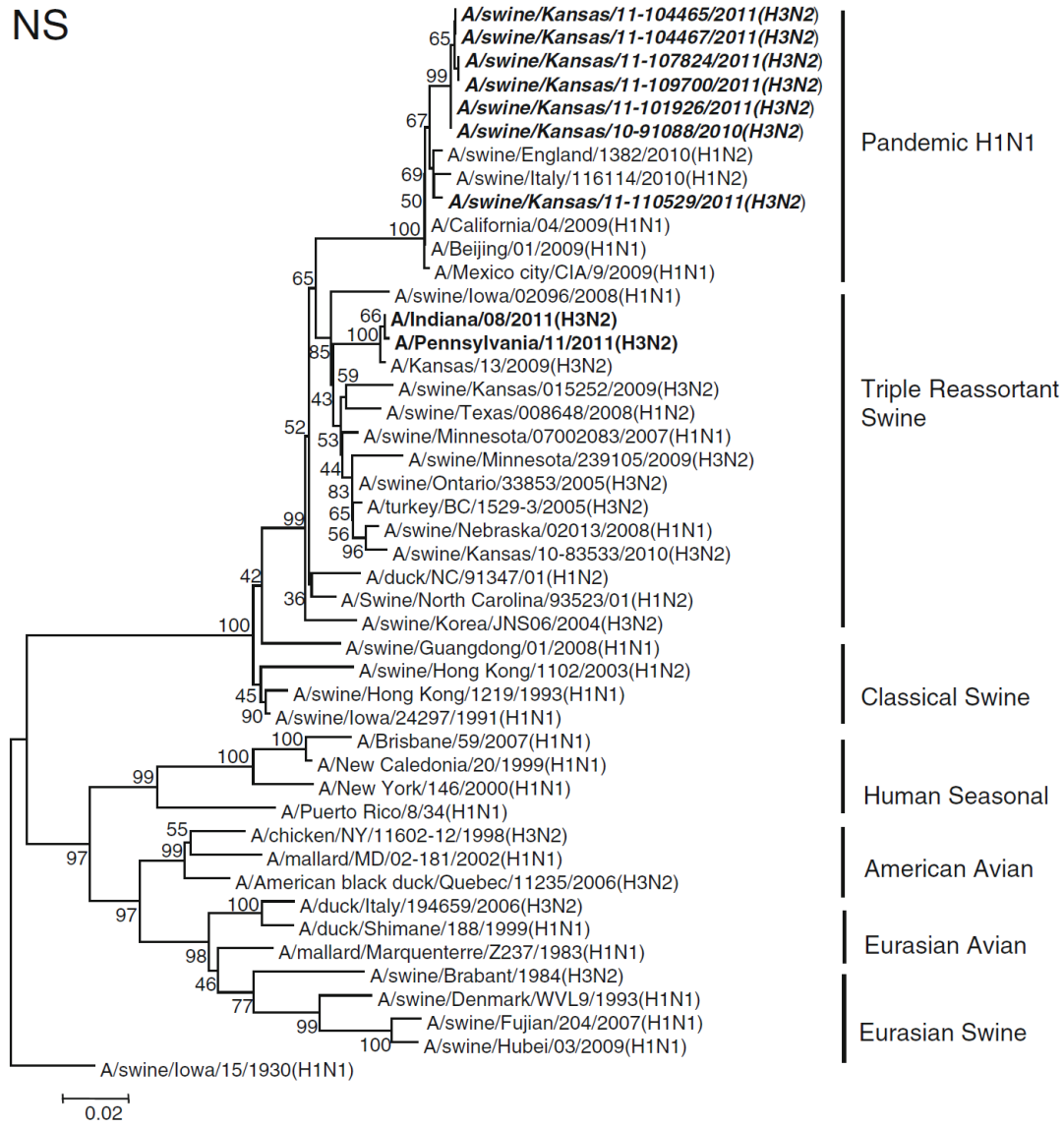

Figure 11 The tree was generated by the distance-based neighbor-joining method in the software MEGA 4.1. The reliability of the tree was assessed by bootstrap analysis with 1,000 replications. Horizontal distances are proportional to genetic distance. The viruses isolated in this study are in italic and bold. The viruses (H3N2) that infected two children in Pennsylvania and Indiana are in bold.

Chapter 3: Pathogenicity and transmissibility of novel reassortant H3N2 swine IAVs with 2009 pandemic H1N1 genes in pigs

The data in this section has been published in J Virol 2015 Mar 1;89(5):2831-41 Jingjiao Ma, Huigang Shen, Qinfang Liu, Bhupinder Bawa, Wenbao Qi, Michael Duff, Yuekun Lang, Jinhwa Lee, Hai Yu, Jianfa Bai, Guangzhi Tong, Richard A. Hesse, Jürgen A. Richt, Wenjun Ma. Pathogenicity and Transmissibility of Novel Reassortant H3N2 Influenza Viruses with 2009 Pandemic H1N1 Genes in Pigs.

3.1 Introduction

Swine influenza is a zoonotic disease that threatens animal and public health. Currently, there are three subtypes of influenza A viruses that predominantly infect pigs worldwide: H1N1, H1N2, and H3N2 (Ducatez et al., 2008). Since the emergence of triple-reassortant influenza A viruses containing genes of human, swine, and avian influenza viruses in swine in North America in 1998 (Karasin et al., 2000; Zhou et al., 1999), triplereassortant H1N1, H1N2, and H3N2 subtype viruses have been endemic in North American swine herds. In particular, triplereassortant H3N2 viruses have become a major cause of swine influenza in North America (Olsen, 2002; Vincent et al., 2008) and also sporadically cause human infections (Cox et al., 2011; Lindstrom et al., 2012). The 2009 pandemic was caused by a reassortant H1N1 virus whose genes are from North American triple-reassortant (PB2, PB1, PA, HA, NP, and NS) and Eurasian avian-like (NA and M) H1N1 swine viruses (Smith et al., 2009). The A(H1N1)pdm09 virus circulated in humans and crossed the species barrier to infect other animals, including swine, dogs, cats, and wild mammals (Fiorentini et al., 2011; Howden et al., 2009; Lin et al., 2012; Schrenzel et al., 2011; Weingartl et al., 2010). Importantly, the virus has been isolated from pigs worldwide, including Europe, Asia, South America, and North America (Pereda et al.,

2010; Sreta et al., 2010; Weingartl et al., 2010; Welsh et al., 2010). Human influenza viruses tend to bind to α-2,6-linked sialic acids on the host cell surface, which are present in the upper respiratory tract in humans and other mammals (Shinya et al., 2006). In contrast, avian influenza viruses preferentially bind to α-2,3-linked sialic acids. They are reported to be abundant in the avian intestinal tract, as well as in the human lower respiratory tract (Nicholls et al., 2007). Swine have been considered "mixing vessels" for avian, human, and swine IAVs because the swine respiratory tract has receptors for both avian and mammalian influenza viruses. Thus, if two influenza viruses infect one pig concurrently, they may randomly exchange gene segments, resulting in novel reassortant viruses through an event called reassortment. Since the first reassortant influenza virus containing A(H1N1)pdm09 genes was found in pigs in 2009 in Hong Kong (Vijaykrishna et al., 2010), similar reassortant viruses containing genes from influenza viruses endemic in pigs and A(H1N1)pdm09 have been reported from other countries, including novel reassortant H1N2 viruses in the United Kingdom and Italy (Howard et al., 2011; Moreno et al., 2011); reassortant H1N1 viruses in Germany and Thailand (Sreta et al., 2010; Starick et al., 2011); and reassortant H1N1, H1N2, and H3N2 viruses in the United States (Ali et al., 2012; Ducatez et al., 2011; Liu et al., 2012a). In addition, reassortant H3N2 viruses were isolated from mink and swine in Canada (Tremblay et al., 2011). This has raised concerns that these novel reassortant influenza viruses in swine may pose a threat to humans and gain the ability for human-to human transmission. Indeed, novel reassortant H3N2 viruses containing the matrix gene from A(H1N1)pdm09 (H3N2 variants [H3N2v]) that emerged in swine have been reported to infect humans in the United States, and most of the infected patients had been either directly or indirectly exposed to pigs (Bowman et al., 2014; Jhung et al., 2013). Furthermore, limited human-to-human transmission has been found (Jhung et al., 2013).

Previously, we reported the isolation of 7 reassortant H3N2 influenza viruses with 3 or 5 genes derived from A(H1N1)pdm09 from diseased pigs from Midwestern swine farms with outbreaks of respiratory disease (Liu et al., 2012a). To date, the pathogenicity and transmissibility of these novel reassortant H3N2 viruses in pigs remain unknown. Additionally, whether these viruses could be maintained and circulate in swine herds needs to be investigated. To evaluate the pathogenicity and transmissibility of these novel reassortant H3N2 viruses, we selected three novel reassortant viruses carrying either 3 (NP, M, and NS) or 5 (PB2, PA, NP, M, and NS) genes from A(H1N1)pdm09 for the pig study, using a recently isolated endemic triple-reassortant H3N2 influenza virus from diseased pigs as a control.

3.2 Materials and Methods

Ethics statement

The pig study was conducted at the Large Animal Research Center (a biosafety level 2+ facility) at Kansas State University in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching of the U.S. Department of Agriculture. The protocol was approved by the Institutional Animal Care and Use Committee of Kansas State University (IACUC no. 3146).

Cells

Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) with 5% fetal bovine serum (FBS) (HyClone, Logan, UT), 1×L-glutamine (Invitrogen, Carlsbad, CA), 1×MEM vitamins (Invitrogen, Carlsbad, CA), and 1% antibiotics (Invitrogen, Carlsbad, CA). Human lung adenocarcinoma epithelial cells (A549) and porcine kidney cells (PK-15) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 1×MEM vitamins, 1×L-glutamine, and 1% antibiotics. The cells were

infected with viruses using MEM infecting medium that contained 0.3% bovine albumin (Sigma, St. Louis, MO), 1% antibiotics (Invitrogen, Carlsbad, CA), and 1 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma, St. Louis, MO).

Viruses

Three novel reassortant H3N2 influenza viruses isolated from swine and containing either 3 or 5 genes from A(H1N1)pdm09 were used in this study: A/swine/Kansas/10-91088/2010 (KS-91088) [NP, M, and NS genes from A(H1N1)pdm09 and its eight-gene segment, GenBank accession number JN409388-95], A/swine/Kansas/11-107824/2011 (KS- 107824) [PA, PB2, NP, M, and NS genes from A(H1N1)pdm09 and its eight-gene segment, GenBank accession number JN409420-27], and A/swine/Kansas/11-110529/2011 (KS-110529) [NP, M, and NS genes from A(H1N1)pdm09 and its eight-gene segment, GenBank accession number JN409436-43]. The KS-110529 virus contains an NA gene derived from recent human influenza viruses that has genetically diverged from the NAs of the above-mentioned 2 reassortant viruses (their NAs are from early human influenza viruses). One endemic triple-reassortant A/swine/Kansas/10-83533/2010 virus (KS-83533N) and its eight-gene segment, GenBank accession number KP270886-93, which was isolated in the same area from diseased pigs and also contains an NA gene from the recent human influenza viruses, was used as a control in this study.

Growth kinetics

To study the growth kinetics of viruses in different cells, including A549 (multiplicity of infection [MOI] = 0.01), MDCK (MOI = 0.1), and PK-15 (MOI = 0.01) cells, confluent cells were infected with each virus at the indicated MOI. The supernatants of the infected cells were collected at 12, 24, 36, and 48 h postinoculation (p.i.). The virus titers of the collected

supernatants were determined by inoculating confluent monolayers of MDCK cells in 96-well plates, and the 50% tissue culture infective dose ($TCID_{50}$)/ml was calculated by the method of Reed and Muench. A plaque assay was conducted to compare the sizes of plaques formed by each virus on MDCK and PK-15 cells.

Plasmid construction and minigenome replication assay

To determine the polymerase activity of each H3N2 virus, a minigenome assay was performed as described previously (Bortz et al.). pPol1-NS-Luciferase carries an influenza A virus reporter minigenome in which the firefly luciferase gene is flanked by the influenzaAvirus NS gene noncoding regions, a truncated PolI promoter, and the hepatitis delta virus ribozyme. The polymerase (PB1, PB2, and PA) and NP genes of each virus were cloned into the pGEM-T vector (Promega, Madison, WI) and then subcloned into the pCAGGS/MCS vector. All the plasmids were confirmed by sequencing. Confluent 293T cells were cotransfected with pPol1-NS-Luciferase (100 ng); pSV-Renilla (50 ng) carrying the *Renilla* luciferase gene under simian virus 40 (SV40) RNA polymerase II promoter as a control; and four pCAGGS plasmids expressing viral PB2, PB1, PA, and NP from each strain (the amounts of PB2, PB1, PA, and NP plasmids used were 50 ng, 100 ng, 100 ng, and 500 ng (Bortz et al.)). Twenty-four hours after transfection, cells were collected and lysed using passive lysis buffer, and then the cell lysates were used to conduct a dual-luciferase reporter assay according to the manufacturer's protocol (Promega). The influenza virus polymerase activity derived from the firefly luciferase plasmid (pPol1-NS-Luciferase) was calculated and normalized based on transfection efficiency using the *Renilla* luciferase activity values from pSV-Renilla. Each cotransfection experiment was repeated three times.

Neuraminidase activity assay

The NA activities of endemic and reassortant H3N2 viruses (KS-83533N, KS-91088, KS-107824, and KS-110529) were determined with an NA-XTD Influenza Neuraminidase Assay Kit (Life Technologies). The assay was performed following the manufacturer's instructions. Briefly, all the viruses were diluted in the same titer of 10^5 $TCID_{50}$ in 50 μl NA-XTD assay buffer as the virus stock. Serial 1:2 dilutions of virus stocks were made using the NA-XTD assay buffer. The diluted viruses (50 μl) were mixed with 25 μl of NA-XTD chemiluminescent substrate [5 μM; sodium (3-chloro-5-(4-methoxyspiro<1,2-dioxetane-3,2=-(5-chloro)tricycle[3.3.1.13,7]decan>-4-yl-phenyl5-acetamido-3,5-dideoxy-_-D-glycero-D-galacto-2-nonulopyranoside)onate] in a 96-well plate and incubated at 37°C for 30 min. Then, 60 _l of NA-XTD accelerator was added to each well, and the plate was read with Fluostar Omega (BMG Labtech) using a 1-s/well reading time as recommended by the manufacturer. Three independent replicate assays were conducted for each virus.

Generation of wild-type KS-107824 and its single-amino-acid-mutated virus (HA G228S) by reverse genetics

Eight-gene segments of the H3N2 KS-107824 virus were amplified using universal primers and cloned into the pHW2000 vector as described previously (Hoffmann et al., 2000) to establish a reverse-genetic system for KS-107824, resulting in plasmids pHW2000-PB1, -PB2, -PA, -HA, -NP, -NA, -M, and -NS. All the cloned genes were confirmed by sequencing. A single substitution at position 228 in HA (glycine to serine) was introduced with a site-directed mutagenesis kit (Invitrogen) according to the manufacturer's recommendations, resulting in plasmid pHW2000-HA-G228S, which was confirmed by sequencing. Both the wild-type (rgKS-107824) virus and a mutated virus with a single substitution in HA, G228S (rgKS-107824-

G228S), were rescued as described previously (Hoffmann et al., 2000) by reverse genetics and propagated in MDCK cells for *in vitro* and pig studies. Both wild-type and singly mutated viruses were confirmed by sequencing prior to the animal study.

HA receptor binding preferences of endemic, reassortant, and reverse-genetics-derived H3N2 viruses using hemagglutinating receptor-specific red blood cells

The receptor binding preferences of endemic, reassortant, and reverse-genetics-derived H3N2 viruses were analyzed using hemagglutinating receptor-specific red blood cells (RBCs). For this experiment, normal turkey red blood cells (containing both α-2,6 and α-2,3 receptors), α-2,3-specific neuraminidase-treated turkey red blood cells (containing only α-2,6 receptor after treatment), and sheep red blood cells (mainly expressing α-2,3 receptor) were used (Medeiros et al., 2001). To remove α-2,3-linked *N*-acetyl-neuraminic acid residues from oligosaccharides of turkey red blood cells, they were treated with α-2,3 neuraminidase (Matrosovich et al.). Briefly, 10% RBCs in 1 ml of 1X G4 reaction buffer and 1X BSA was incubated at 37°C in the presence of 1,000 IU α-2,3-specific neuraminidase for 1 h. The treated red blood cells were washed three times with phosphate-buffered saline (PBS) before use. The final working solution was 0.5% RBCs in PBS for the hemagglutination assay. The Hemagglutination assay was performed with 0.5% different red blood cells with specific hemagglutinating receptors in 96-well V-bottom microtiter plates by incubating equal volumes (50 μl) of 2-fold serially diluted viruses. The Hemagglutination titer was defined as the reciprocal of the highest virus dilution that hemagglutinated red blood cells. To determine the specific receptors on the treated and untreated red blood cells, avian influenza A/chicken/Jena/4836/1983 (H2N2) and human influenza A/Brisbane/59/2007(H1N1) viruses were included in the receptor binding assay as controls.

Solid-phase HA receptor binding assay

Viruses amplified in chicken embryos were collected and centrifuged at 1,200 rpm for 5 min to remove debris. One hundred microliters of sialyl-glycopolymer 3'-sialyl-*N*acetyllactosamine (3'-SLN) and 6'-sialyl-*N*-acetyllactosamine (6'-SLN) (V-Lab; 10 μg/ml or 2.5 μg/ml in carbonate) was added to each well of the microplates. The microplates were coated at 4°C overnight. After washing the plates with cold PBS five times, the wells were blocked with 100 μl of PBST (PBS with 0.05% Tween 20) containing 4% lipid-free bovine serum albumin (BSA) at 4°C for 6 h. The plates were washed with 200 μl of cold PBST five times, and 50 μl virus supernatant containing 64 HA units was added to each well and incubated at 4°C overnight. The solution was discarded, and the plates were washed with 200 μl of cold PBST five times. Fifty microliters of solution containing anti-influenza virus NP monoclonal antibody (Thermo Scientific) was added to each well for 2 h at 4°C. After washing, the plates were incubated with 50 μl of anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (DakoCytomation, Denmark; 1:1,000) diluted with PBST containing 1% lipid-free BSA for 2 h at 4°C. The plates were washed with 200 μl of cold PBST five times. The color reaction was developed by incubating with 100 μl of *o*-phenylenediamine dihydrochloride (OPD) (Sigma) solution (in 100 mM phosphate-citrate buffer, pH 5.0) at 37°C for 15 min. The reactions were stopped by adding 50 μl of 1 N H_2SO_4 solution (Sigma). The absorbance was measured at 490 nm using an enzymelinked immunosorbent assay (ELISA) reader.

Pig study

In the first pig study, 73 5-week-old influenza H1 and H3 subtype virus- and porcine reproductive and respiratory syndrome virus-seronegative crossbred pigs were randomly allocated into 5 groups (4 infected and 1 control groups). Each infected group contained 16 pigs,

while the control group had 9 pigs. Twelve pigs from each infected group and the 9 control pigs were intratracheally inoculated with 10^6 $TCID_{50}$ of each virus (KS-91088, KS-107824, KS-110529, or KS-83533N) or virus free MEM as described previously (Ma et al., 2007). The remaining 4 naive pigs from each infected group were commingled with inoculated pigs at 2 days p.i. to investigate viral transmission. Body temperature and clinical symptoms for all experimental pigs were monitored throughout the experiment. Four infected pigs from each inoculated group and 3 control animals were euthanized at 3, 5, and 7 days p.i., and 4 contact pigs were necropsied at 5 days post contact (p.c.). Blood samples were collected before challenge or contact and on necropsy days. Nasal swabs were collected at 0, 3, 5, and 7 days p.i. for inoculated pigs and at 2, 4, and 5 days p.c. for contact animals. During necropsy, the percentage of gross lesions on each lung lobe was scored by a single experienced veterinarian. Bronchoalveolar lavage fluid (BALF) samples were collected by flushing a lung with 50 ml of MEM. The virus titers of BALF and nasal-swab samples were determined on MDCK cells in 96-well plates. Tissue samples from the nasal turbinate, the trachea, and the right cardiac lung lobe were collected and fixed in 10% buffered formalin for the pathological examination. The lung sections were examined by a veterinary pathologist in a blinded fashion and given a score of 0 to 3 to reflect the severity of bronchial epithelial injury, as described previously (Ma et al., 2007; Richt et al., 2003).

In the second pig study, 25 3-week-old influenza H1 and H3 subtype virus- and porcine reproductive and respiratory syndrome virus-seronegative crossbred pigs were purchased and randomly allocated into 3 groups (2 infected and 1 control groups). There were 10 pigs in each infection group and 5 pigs in the control group. Since younger pigs were used in this study (in contrast to 5-week-old pigs in the first study), a lower dose of 10^4 $TCID_{50}$ of each virus was used

for infection. Six pigs from each infected group and the 5 pigs from the control group were intratracheally inoculated with each virus (rgKS-107824 or rgKS-107824-G228S) or virus-free MEM. The remaining 4 naive pigs from each infected group were commingled with inoculated pigs at 2 days p.i. to investigate viral transmission. Three infected pigs and 3 (or 2) control pigs from each group were necropsied at 5 and 7 days p.i., and 4 contact pigs were necropsied at 5 days p.c. The other procedures were conducted in the same manner as in the first pig study.

Statistical analysis

Macroscopic and microscopic lung lesion scores and virus titers were analyzed by using analysis of variance (ANOVA) in GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA); a *P* value of 0.05 or less was considered significant. The response variables shown to have a significant effect by treatment group were subjected to comparisons for all pairs by using the Tukey-Kramer test. Pairwise mean comparisons between inoculated and control groups were made using the Student *t* test.

3.3 Results

Novel reassortant H3N2 viruses replicate more efficiently than non-reassortant endemic H3N2 virus in cell cultures

Plaque assays showed that the three novel reassortant H3N2 viruses (KS-91088, KS-107824, and KS-110529) formed plaque sizes similar to those of the nonreassortant endemic control KS-83533N virus in MDCK cells; endemic and reassortant H3N2 viruses with 5 A(H1N1)pdm09 genes formed similar-size plaques in PK-15 cells and in MDCK cells, whereas novel reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes formed smaller plaques in PK-15 cells than in MDCK cells. The novel reassortant viruses grew more efficiently than the control nonreassortant KS-83533N virus in cultured cells, including MDCK, PK-15, and A549 cells. In

MDCK cells, the three novel reassortant viruses grew to significantly higher titers than the nonreassortant endemic virus (KS-83533N) at 24, 36, and 48 h p.i. (Figure 12A). The KS-107824 virus containing 5 (PA, PB2, NP, M, and NS) genes from A(H1N1)pdm09 grew to significantly higher titers than the control nonreassortant endemic KS-83533N virus at all the tested time points on both PK-15 and A549 cells (Figure 12 B and C), whereas the KS-110529 virus with 3 (NP, M, and NS) genes from A(H1N1)pdm09 had significantly higher titers only at 24, 36, and 48 h p.i. on these cells (Figure 12 B and C). For the KS-91088 virus, significant differences were observed only at 36 and 48 h p.i. compared to the control endemic KS- 83533N virus on both PK-15 and A549 cells (Figure 12 B and C). No significant difference was observed in terms of growth kinetics in canine, human, and swine cells among the three novel reassortant H3N2 viruses.

Novel reassortant H3N2 viruses are pathogenic in pigs

Five-week-old pigs infected with a high dose (10^6 $TCID_{50}$ /pig) of either of the three novel reassortant H3N2 viruses displayed fever (around 25-100% of pigs from 1-7 dpi), which were similar to those infected with the endemic control H3N2 virus. No fever was seen in the mock-infected group. All 3 reassortant viruses as well as the endemic H3N2 virus caused significantly more macroscopic lung lesions in infected pigs when compared to the control group (Table 4). However, no significant differences in macroscopic lung lesions were observed among the infected groups.

All 3 novel reassortant H3N2 viruses as well as the endemic H3N2 KS-83533N viruses replicated efficiently in pigs' lungs. Virus was detected in lungs of pigs infected with either reassortant or endemic H3N2 viruses on 3 and 5 dpi, with the exception of one pig from the KS-107824 [5 genes from A(H1N1)pdm09] infection group on 5 dpi. No virus was found in the

lungs of pigs infected with the different viruses on 7 dpi (Figure 13). Although virus titers were variable between the different groups on the indicated necropsy days, no significant differences in virus titers were observed among infected groups (Figure 13). The microscopic pathology score (0-3) was between 1.50 to 2.50 on 3, 5 and 7 dpi in all 4 inoculated groups compared with a score of 0.00 in control pigs. No significant difference was observed among the infected groups (Table 4). All infected pigs had variable degrees of lung damage, ranging from mild to moderate bronchointerstitial pneumonia, atelectasis, acute to subacute bronchiolitis with epithelial necrosis, and variable lymphocytic cuffing of bronchioles on 3, 5 and 7 dpi (Table 4, Figure 14).

Novel reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes transmit more efficiently than the virus with 5 A(H1N1)pdm09 genes in pigs

All three reassortant and the endemic H3N2 viruses transmitted to sentinel pigs from primary infected animals. Most of pigs infected with the novel reassortant H3N2 virus with 3 genes from A(H1N1)pdm09 (KS110529 or KS-91088) shed virus via the nasal cavity on 3 (92%-100%) and 5 (100%) dpi with high titers ($10^{3.23}$-$10^{4.26}$ $TCID_{50}$/ml), similar to the endemic KS-83533N infected pigs (80-100%). However, only 42% (5/12) and 25% (2/8) of pigs infected with the KS-107824 with 5 genes from A(H1N1)pdm09 shed virus via the nasal cavity on 3 and 5 dpi, respectively, with lower titers ($10^{1.60}$-$10^{2.62}$ $TCID_{50}$/ml) than the reassortant viruses with 3 A(H1N1)pdm09 genes and the control endemic virus (Figure 13 B).

All contact pigs (100%, 4/4) from groups infected with either endemic KS-83533N or reassortant viruses (KS-91088 or KS-110529) with 3 genes from A(H1N1)pdm09 had fever on 2 and 3 days p.c., and 50% of contact pigs from these 3 groups still displayed fever on 4 and 5 days p.c.. In contrast, only 50% of contact pigs in the group infected with KS-107824 with 5 A(H1N1)pdm09 genes showed fever on 2 and 3 days p.c. and only 25% of contact animals had

fever on 4 and 5 days p.c.. Only minimal gross lung lesions were observed in the latter contact group of pigs, whereas moderate lung lesions were found in all contact pigs from the other contact groups. Notably, more severe lung lesions were observed in contact pigs of the KS-110529 infection group, in contrast to the other 3 infection groups (Table 4 Macroscopic and microscopic lung lesion scores of infected and contact pigs). Virus was detected on 5 days p.c. in lungs of all contact pigs of each group with the exception of 2 contact pigs of the KS-107824 [5 A(H1N1)pdm09 genes]-infected group (Figure 13A). Nasal virus shedding was detected from contact pigs of all infected groups. The reassortant KS-91088 with 3 A(H1N1)pdm09 genes displayed kinetics in virus nasal shedding and transmission in contact pigs similar to those of the endemic H3N2 virus. The reassortant KS-107824 with 5 A(H1N1)pdm09 genes showed delayed and inefficient transmission and shedding kinetics since nasal shedding was only detected on 4 days p.c., but not on 2 and 5 days p.c. (Figure 13C). The reassortant KS-110529 virus with 3 A(H1N1)pdm09 genes shed most efficiently in contact pigs (Figure 13C) and also caused microscopic lung lesions in contact pigs significantly more severe than the endemic and the other 2 reassortant viruses (Table 4).

Polymerase and Neuraminidase activities of the reassortant KS-107824 virus with 5 A(H1N1)pdm09 genes

Compared to endemic and reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes, the reassortant KS-107824 additionally obtained the polymerase PB2 and PA genes from A(H1N1)pdm09. Therefore, we performed a minigenome assay to investigate the effect of the different polymerase complexes (PB1, PB2, PA and NP) from each H3N2 virus on polymerase activity. In this assay the amount of luciferase expression is correlated with the polymerase activity of each virus. The polymerase complex from KS-107824 showed significantly (10-fold)

greater polymerase activity than the other 3 combinations from either endemic KS-83533N or reassortant H3N2 (KS-110529 and KS-91088) viruses with 3 A(H1N1)pdm09 genes (Figure 15A). The polymerase complex from either endemic KS-83533N or reassortant H3N2 (KS-110529 and KS-91088) viruses displayed variable polymerase activity, but no significant difference was observed.

The KS-107824 and KS-83533N viruses, as well as the KS-91088 virus had an NA from early human influenza viruses in contrast to the NA of the KS110529 derived from recent seasonal human influenza viruses. To investigate whether the different NA has influence on virus features, we performed a neuraminidase assay. The results showed that the early NA from the endemic KS-83533N displayed a higher enzyme activity than those from reassortant H3N2 viruses with 3 or 5 A(H1N1)pdm09 genes and from the A(H1N1)pdm09 virus, independent whether the NA was derived from an early or recent human influenza virus. The NA from both KS-107824 (an early human influenza N2) and KS-110529 viruses (a recent human influenza N2) as well as from KS-91088 (an early human influenza N2) had similar enzyme activities as that of the A(H1N1)pdm09 virus (Figure 15B).

HA receptor binding preference of novel reassortant H3N2 and single mutated viruses

Our previous studies showed that the KS-107824 HA contains 226V and 228G at the receptor-binding sites, whereas the HA proteins of endemic and reassorant H3N2 swine viruses used in this study have 226V and 228S at their receptor-binding sites (commonly found in triple reassortant H3N2 swine IAVs). The 226V-228G combination in the HA receptor binding site is rarely found, and its role in virus receptor binding, replication or transmission in pigs remains unknown. Therefore, we first determined its role in receptor binding specificity. The three reassortant and endemic H3N2 viruses were able to bind rooster red blood cells that contain both

α-2,3 and α-2,6 receptors. The KS-107824 bound to both α-2,6 and α-2,3 receptors with a low affinity to α-2,3 receptors. In contrast, the two reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes and the endemic H3N2 virus only bound to α-2,6 receptors. The single substitution (G to S) at position 228 in HA resulted in greatly enhanced affinity to α-2,6 receptors, and reduced its binding affinity to α-2,3 receptors (Table 4). Although the rescued wild type rgKS-107824 and the single mutated rgKS-107824-G228S virus displayed different HA receptor binding preference, they showed similar growth kinetics on MDCK cells. To confirm the receptor binding properties of each novel H3N2 virus, we examined the receptor binding affinities of the virus with different glycans using a solid-phase binding assay. As shown in Figure 15, results similar to those with the Hemagglutination assay using resialylated red blood cells were obtained. The endemic virus (KS-83533N) and the reassortant H3N2 virus with 3 A(H1N1)pdm09 genes preferentially bound to α-2,6 sialic acid glycans, whereas the KS-107824 preferentially bound to α-2,3 sialic acid glycans. A single substitution (G to S) at position 228 in HA noticeably switched the receptor binding specificity of the KS-107824 virus from α-2,3 sialic acid to α-2,6 sialic acid glycans.

Glycine at position 228 in HA is responsible for inefficient transmission of the KS-107824

No obvious respiratory signs were observed from 3-week-old pigs infected with a low dose (10^4 $TCID_{50}$/pig) of reverse-genetic-derived wild-type rgKS-107824 and singly mutated rgKS-107824-G228S viruses. Fever was observed in one out of six pigs infected with the rgKS-107824-G228S virus on 3 dpi and lasted for three days. Similarly, only one pig (1/6) infected with the rgKS-107824 virus displayed fever on 6 dpi, which lasted for 1 day. In contrast to the control group, both the rgKS-107824 and rgKS-107824-G228S viruses caused gross lung lesions

at both 5 and 7 dpi. Furthermore, the rgKS-107824-G228S induced more severe lung lesions than wild type rgKS-107824 virus at 5 dpi (Table 6). Both viruses were detected in lungs of all the infected pigs at 5 dpi, but also in 2 out of 3 pigs at 7 dpi. Noticeably, the titers of rgKS-107824-G228S were higher than those of wild-type rgKS-107824 at the tested time points, but no significant difference was observed (Figure 16 A).

Although both viruses were transmitted to contact pigs, the efficiencies of nasal shedding of the two viruses were very different. The rgKS-107824-G228S could be detected in 3 out of 6 nasal swab samples collected from infected pigs at early as 3 days p.i., whereas virus was found in nasal-swab samples from only one pig infected with rgKS-107824. At later time points (5 and 7 dpi) virus was detected in nasal swabs collected from both infected groups; more infected pigs with higher titers were found in the rgKS-107824-G228S infected group than in the wild type rgKS-107824-infected group (Figure 16 B). Virus was detected in lungs of all contact animals (4/4) in each group, but the titers detected in the rgKS-107824-G228S group were higher than those detected in the rgKS-107824 group (Figure 16 A). The wild type rgKS-107824 exhibited delayed and inefficient nasal shedding in sentinel animals, as no contact pigs in the rgKS-107824 group shed virus at 2 days p.c. and only one and two pigs shed virus at 4 and 5 days p.c.. In contrast, all contact pigs (4/4) in the rgKS-107824-G228S group shed virus with significant higher titers at both 4 and 5 days p.c. (Figure 16 C).

3.4 Discussion

At least 10 different genotypes of reassortant H3N2 viruses with 1, 2, 3, 4, 5 or 6 genes from A(H1N1)pdm09 have been detected in U.S. swine (Ducatez et al., 2011; Kitikoon et al., 2013; Kitikoon et al., ; Liu et al., 2012a). Viruses with an A(H1N1)pdm09 M gene(s), called H3N2 variants, or H3N2v, have been transmitted to and infected humans, mainly during state

fair events (Bowman et al., 2014; Lindstrom et al., 2012). From August 2011 to October 2014, 343 cases of human infections with the H3N2 variant have been reported, including 18 hospitalizations and one death (Prevention, 2014). To our knowledge, the H3N2 variant with a single M gene from A(H1N1)pdm09 is responsible for most human infections, although other genotypes of reassortant H3N2 viruses containing A(H1N1)pdm09 PA and M genes; A(H1N1) pdm09 NP and M genes; or A(H1N1)pdm09 PA, NP, and M genes have been reported to infect humans. This has raised the question of whether other genotypes of novel reassortant H3N2 viruses not yet detected in humans will be maintained or even become the predominant viruses in swine herds and whether they might cross the species barrier to infect humans.

To date, three genotypes of novel reassortant H3N2 viruses with an A(H1N1)pdm09 gene(s) have been evaluated in ferret and pig models (Ducatez et al., 2011; Kitikoon et al., ; Pearce et al., 2012). Ducatez et al. showed that an H3N2 reassortant virus with A(H1N1)pdm09 PA, NP, and M genes caused only mild clinical signs and replicated in ferrets to the same extent as the A(H1N1)pdm09 virus and an early triple-reassortant H3N2 swine virus (Ducatez et al., 2011), indicating that no enhancement of virulence occurred in ferrets through reassortment. Another study performed in pigs compared the pathogenicity and transmissibility of a human H3N2 variant with those of a reassortant H3N2 swine isolate with 5 A(H1N1)pdm09 (PA, PB1, NP, M, and NS) genes using an endemic H3N2 swine virus as a control. The study showed that no increased virulence and transmissibility were detected in either the human variant or the swine reassortant H3N2 isolate compared to the endemic H3N2 virus (Kitikoon et al., 2012). A recent study showed that H3N2 variant human isolates could be transmitted efficiently by direct contact and respiratory droplets to naive ferrets and that they replicated in human Calu-3 cells to significantly higher titers than seasonal H3N2 viruses (Pearce et al., 2012).

In this study, we evaluated three novel reassortant H3N2 influenza viruses with 3 (NP, M, and NS) or 5 (PA, PB2, NP, M, and NS) genes from A(H1N1)pdm09 virus isolated from diseased pigs *in vitro* and *in vivo*. These reassortant H3N2 viruses have different genetic constellations and belong to different genotypes than the reassortant H3N2 viruses used in previously published studies. Our results revealed that introduction of 3 or 5 genes from A(H1N1)pdm09 virus conferred efficient virus replication in canine, swine, and human cells compared to a contemporary endemic H3N2 virus. Both reassortant H3N2 viruses (KS-110529 and KS-91088) with 3 genes from A(H1N1)pdm09 displayed properties similar to those of the endemic H3N2 virus in infected pigs in terms of virus replication and pathogenicity. However, the KS-110529 virus was more transmissible in pigs than the endemic and KS-91088 viruses, as evidenced by the presence of severe microscopic and macroscopic lung lesions in sentinel pigs and the fact that more sentinels shed viruses via the nasal cavity. The KS-110529 virus has a similar recent human influenza N2 gene, as well as 4 other (PB1, PB2, PA, and HA) genes, but differs from the endemic virus in having NP, M, and NS genes derived from the A(H1N1)pdm09 virus (Liu et al., 2012a); this suggests that introduction of 3 internal genes from A(H1N1)pdm09 enhances viral transmission in pigs. KS-110529 and KS-91088 viruses with 3 genes from A(H1N1)pdm09 have similar genetic constellations but differ in the N2 gene (Liu et al., 2012a), suggesting that the recent human influenza virus N2 plays a critical role in enhancing virus transmission in pigs. Taken together, both the 3 A(H1N1)pdm09 virus internal genes (NP, M, and NS genes) and the recent human influenza virus N2 gene are important for efficient viral transmission. The KS-107824 virus with 5 genes from A(H1N1)pdm09 exhibited virus replication and pathogenesis in infected pigs similar to those of the endemic and reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes. However, it showed delayed and inefficient pig-to-

pig transmission compared to the endemic and reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes. This is supported by the facts that (i) fewer originally infected pigs shed virus and at a lower titer (virus was detected in the lungs of all infected animals), (ii) virus was detected in the lungs of only 50% of the contact pigs, and (iii) delayed nasal shedding was observed in contact pigs.

Multiple viral factors, including PB2-specific amino acid residues (e.g., 627K/701N) and the balance between the activities of HA and NA have been shown to influence influenza virus transmission and pathogenicity in mammals, including humans (Gao et al., 2009; Pascua et al., 2013; Subbarao et al., 1993; Van Hoeven et al., 2009; Yen et al., 2011). The cause of inefficient transmission of KS-107824 with 5 A(H1N1)pdm09 genes might reside in its different polymerase complex and NA proteins compared to the endemic and the two reassortant H3N2 viruses. The KS-107824 virus has the avian origin PB2 containing 627E and 701D residues, as well as SR polymorphism (590S/591R) that was demonstrated to partly compensate for the absence of 627K in its polymerase activity and virus pathogenicity (Liu et al., 2012b; Mehle and Doudna, 2009). However, the polymerase complex of the KS-107824 virus showed significantly higher polymerase activity than those from the endemic and the two reassortant H3N2 viruses with 3 A(H1N1)pdm09 genes. Furthermore, the NA from both KS-107824 and KS-110529 viruses (derived from early and recent human influenza viruses) had enzyme activities similar to that of the 2009 A(H1N1)pdm09 virus. Whether the presence of an early or recent NA (N2) protein is beneficial for the balance with its H3, resulting in different transmission efficacies needs to be investigated in future. Taken together, these data indicate that the different polymerase and NA genes present in the reassortant H3N2 viruses might not be responsible for inefficient transmission of the KS-107824 virus.

HA receptor specificity is another important factor that has been known to play a major role in influenza virus cross-species transmission (Ito, 2000; Liu et al., 2014; Tumpey et al., 2007). The swine H3 HA receptor binding site with the combination of 226V-228S is different from the HAs of most avian (226Q-228G) and human (226L-228S) influenza viruses and is present in 90% of North American triple-reassortant H3N2 influenza viruses in swine (Matrosovich et al., 1997). The combination 226V-228S at the swine H3 HA receptor binding site binds only to α-2,6 receptors, whereas the combination 226V-228G found in the KS-107824 HA binds to both α-2,3 and α-2,6 receptors in our receptor binding assay. The single substitution G228S in the HA of the KS-107824 virus resulted in a receptor binding preference change. Importantly, in subsequent studies, it could be found that the avian-like 228G within the HA of KS-107824 is at least partially responsible for inefficient viral replication and transmission in pigs. This is supported by the facts that the singly mutated virus (rgKS-107824-G228S) replicated more efficiently than wild-type virus in infected and contact pigs and that more animals in this group shed virus at the tested time points with higher titers.

In addition, our results revealed that host factors are also critical for viral virulence and transmissibility. The KS-107824 virus could not be detected in the lungs of 5-week-old pigs at 7 days p.i.,although a high infection dose of virus (10^6 $TCID_{50}$/pig) was used. However, both the wild-type rgKS-107824 virus and the singly mutated rgKS-107824-G228S virus given at a low infection dose (10^4 $TCID_{50}$/pig) were able to replicate in 3-week-old pigs until 7 days p.i. These data indicate that both virus and host factors, such as age and immune status, might influence viral replication, transmission, and evolution (Ma et al., 2014). It would be very interesting to compare the immune responses of the animals at different ages upon infection with influenza virus in future studies. In conclusion, we demonstrate that the reassortant H3N2 virus with 3

A(H1N1) pdm09 genes (NP, M, and NS) and a recent human N2 gene replicates and is transmitted in pigs more efficiently than three other H3N2viruses (2 reassortant and 1 endemic) and that theHA228S, in contrast to HA 228G, is critical for the transmissibility of these reassortant H3N2 viruses in pigs. These results are in agreement with our concurrent surveillance data in the Kansas area, where more than 50% of H3N2 swine isolates are KS-110529-like viruses. This is also true for the majority of influenza virus N2 sequences in swine isolates that are circulating in North American swine herds based on national surveillance data (Kitikoon et al., 2013; Nelson et al., 2012).

3.5 Conclusion

Our study suggested that this kind of reassortant H3N2 virus continually circulates in Midwestern swine herds and could become the dominant H3N2 virus in swine populations. Our results provide insights into the pathogenesis and transmission of novel reassortant H3N2 viruses that are circulating in U.S. swine herds and warrant future surveillance and mitigation strategies.

Figure 12 Growth kinetics of reassortant and endemic swine H3N2 influenza viruses in cell cultures

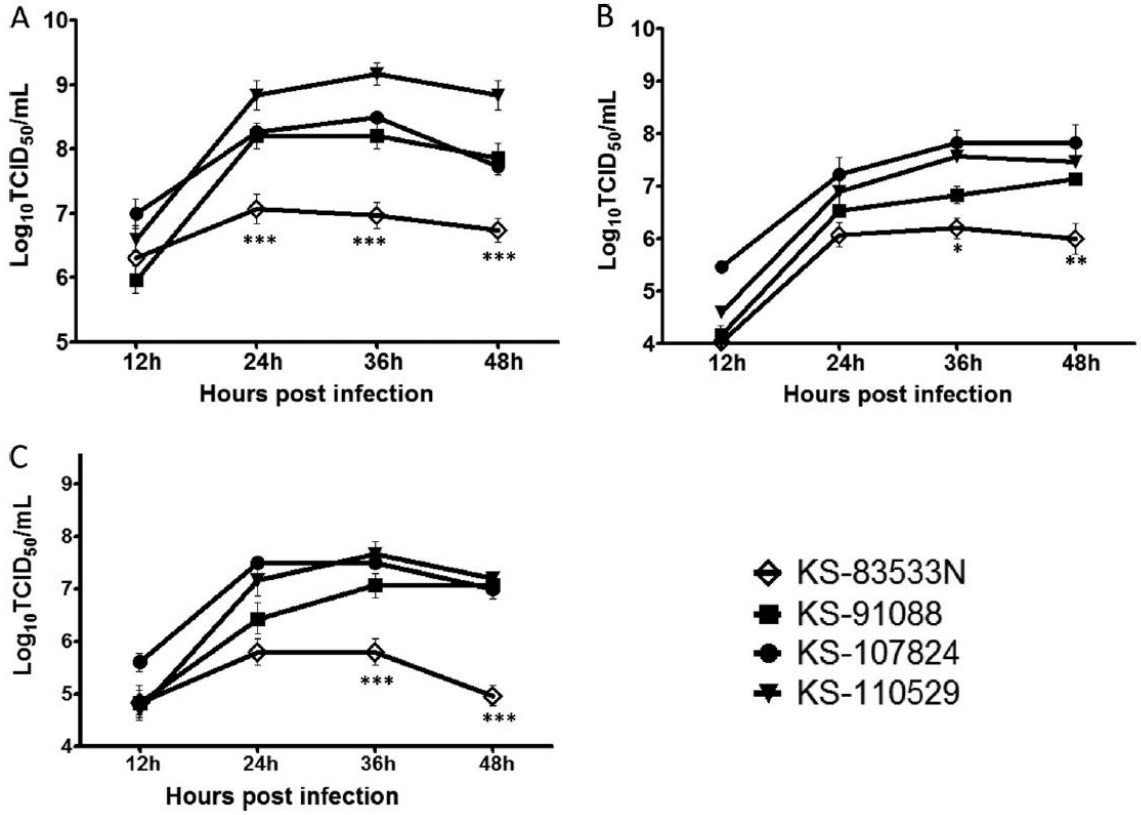

Figure 12 (A) MDCK cells were infected with different viruses at an MOI of 0.1. (B) PK-15 cells were infected with different viruses at an MOI of 0.01. (C) A549 cells were infected with different viruses at an MOI of 0.01. The data points of the curves indicate the means of the results of 3 independent experiments, and the error bars indicate standard errors of the mean (SEM). *, $P<0.05$;**, $P< 0.01$;***, $P< 0.001$.

Table 4 Macroscopic and microscopic lung lesion scores of infected and contact pigs

TABLE 1 Macroscopic and microscopic lung lesion scores of infected and contact pigs

		Lung lesion score[a]			
		Infected pigs			Contact pigs
Lesion size	Virus	3 days p.i.	5 days p.i.	7 days p.i.	(5 days p.c.)
Macroscopic	KS-83533N	13.71 ± 4.95	6.00 ± 1.55	4.71 ± 1.13	3.00 ± 0.61
	KS-91088	10.68 ± 2.75	4.54 ± 1.97	2.43 ± 0.72	4.18 ± 0.87
	KS-110529	11.71 ± 0.61	9.71 ± 0.87	5.86 ± 0.35	7.68 ± 2.73
	KS-107824	15.93 ± 7.66	5.89 ± 1.19	4.11 ± 1.15	1.32 ± 1.96
	Control	0.00	0.00	0.00	NA
Microscopic	KS-83533N	2.50 ± 0.20	2.38 ± 0.24	1.75 ± 0.14	1.50 ± 0.20[b]
	KS-91088	1.88 ± 0.13	1.88 ± 0.31	1.75 ± 0.32	1.38 ± 0.24[c]
	KS-110529	2.25 ± 0.25	2.27 ± 0.24	2.50 ± 0.20	2.38 ± 0.24[d]
	KS-107824	1.75 ± 0.48	2.13 ± 0.38	1.50 ± 0.20	0.75 ± 0.14[b,c,d]
	Control	0.00	0.00	0.00	NA

[a] The macroscopic lung lesion scores were determined as percentages of the lung. The microscopic lung lesion scores were determined by the following criteria: 0, no lesion; 1, mild; 2, moderate; 3, severe. The data are means ± standard errors of the mean (SEM). NA, not applicable: there were no control pigs in the contact group.
[b] Significant differences were observed between the 2 groups ($p<0.05$).
[c] Significant differences were observed between the 2 groups ($p<0.05$).
[d] Significant differences were observed between the 2 groups ($p<0.001$).

Figure 13 Virus loads in nasal swabs and BALF samples of contact and principal pigs infected with endemic and different reassortant H3N2 viruses

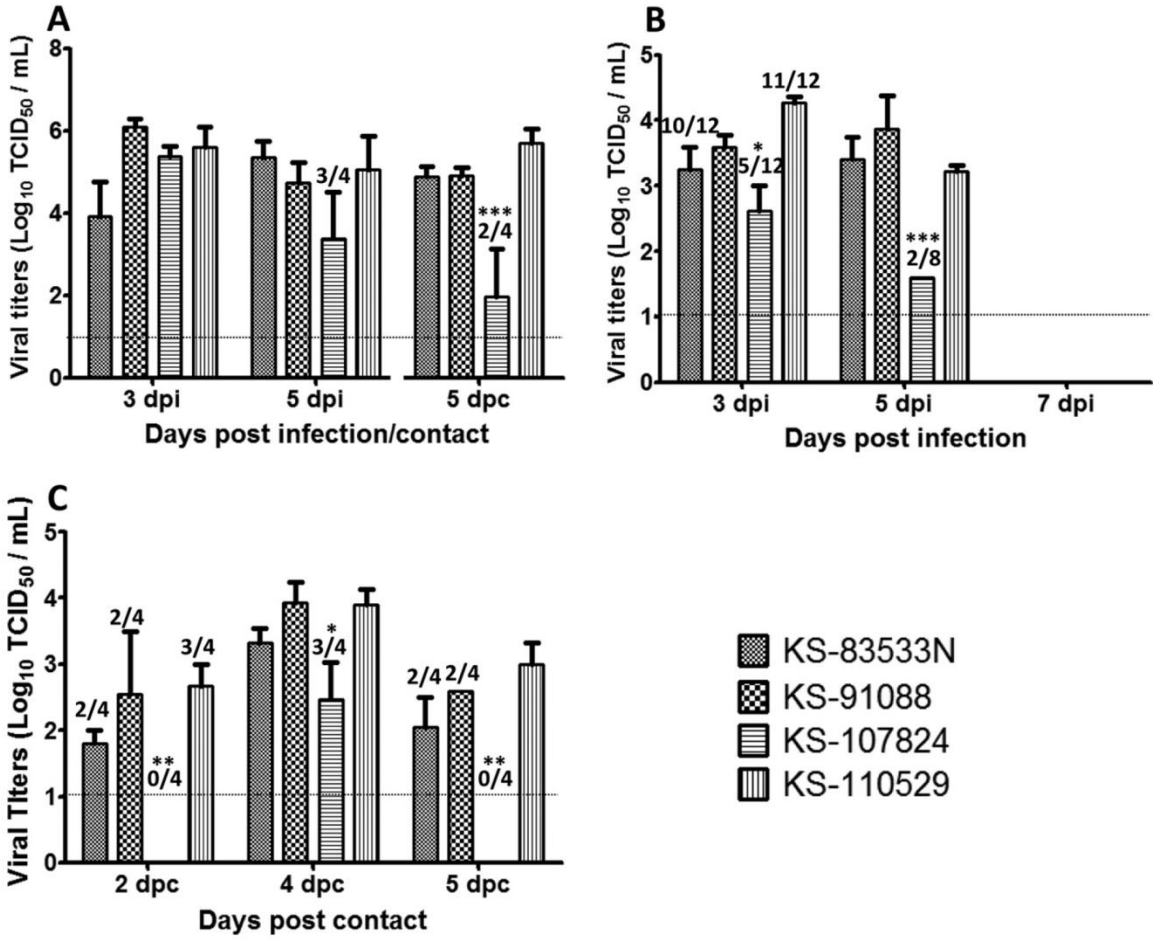

Figure 13 (A) Virus titers in BALF samples of infected and contact pigs. (B) Virus titers in nasal swabs of infected pigs. (C) Virus titers in nasal swabs of contact pigs. All animals were positive for virus isolation at the time points shown unless otherwise indicated (e.g., 2/4 means 2 of 4 animals were positive). The dotted lines represent the limits of detection. The error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 14 Microscopic lung sections from pigs infected with various H3N2 viruses at 5 days post-infection

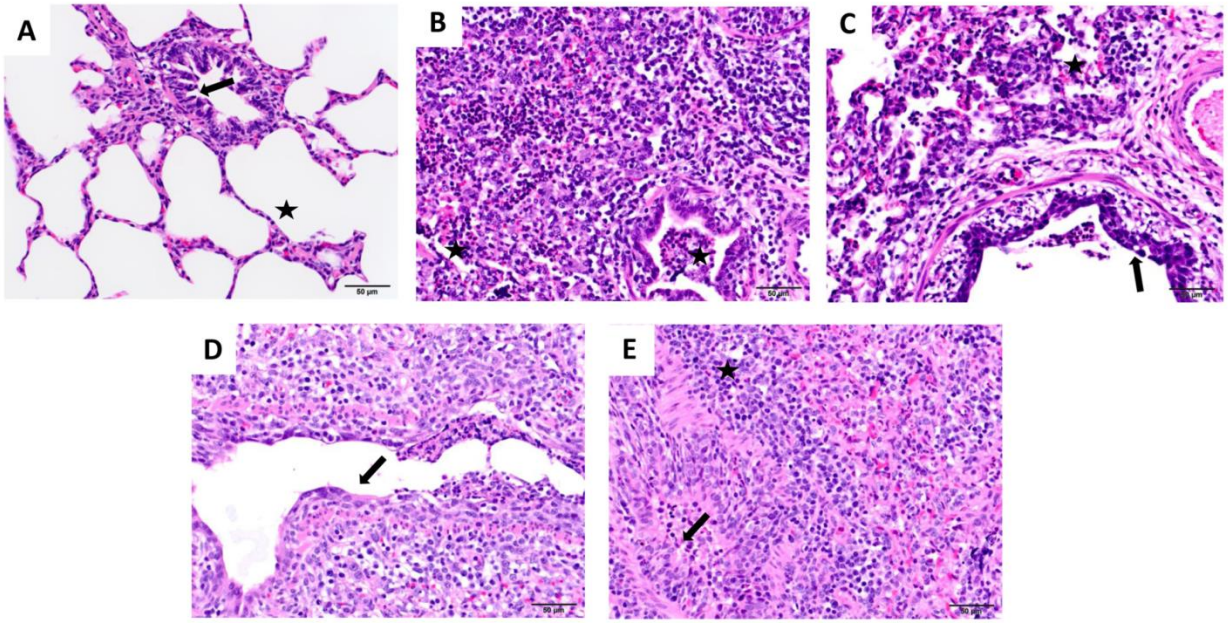

Figure 14 (A) The bronchioles are lined by normal cuboidal epithelium (arrow) and the alveoli are clear (asterisk) in the control group. (B) The bronchiolar and alveolar lumen is filled with large numbers of degenerate and intact neutrophils (asterisks), and moderate interstitial, peribronchiolar, and perivascular lymphocytic infiltration is also seen in the KS-83533N group. (C) Moderate bronchiolar epithelial loss and early regeneration are seen (arrow), and the alveolar and bronchiolar lumen contains small numbers of neutrophils (asterisk) in the KS-91088 group. (D) Moderate to severe bronchiolar epithelial degeneration and necrosis with early regeneration are observed (arrow) in the KS-107824 group. (E) Bronchiolar epithelial necrosis with sloughing of necrotic cells in the lumen was noticed (arrow), and the peribronchiolar and interstitial areas contained moderate numbers of lymphocytes and fewer neutrophils (asterisk) in the KS-110529 group. Scale bars, 50 μm.

Table 5 HA receptor binding preferences of wild-type and rescued viruses

Virus	Residue at HA 226/228	Titer for receptor(s)[a]: α-2,3 + α-2,6	α-2,6	α-2,3
KS-83533N	V/S	64	64	0
KS-91088	V/S	128	128	0
KS-110529	V/S	128	64	0
KS-107824	V/G	64	32	4
rgKS-107824	V/G	64	32	8
rgKS-107824-G228S	V/S	256	256	0
A/chicken/Jena/4836/1983 (H2N2)	Q/G	256	0	128
A/Brisbane/59/2007 (H1N1)	–[b]	512	512	0

[a] The titer shown is the reciprocal of the highest virus dilution that hemagglutinated RBCs bearing various sialic acid receptors.
[b] -, not applicable.

Figure 15 Polymerase and neuraminidase activities of endemic and different reassortant H3N2 viruses

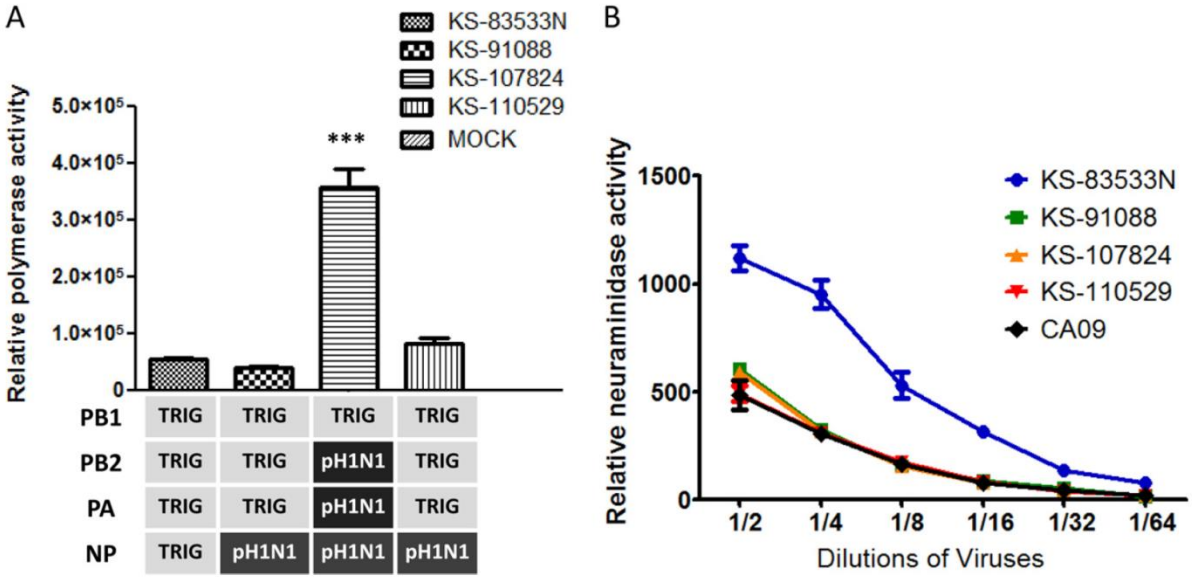

Figure 15 (A) Comparison of polymerase activities of endemic and different reassortant H3N2 viruses containing 3 or 5 genes from A(H1N1)pdm09 virus in 293T cells at 37°C. (B) Comparison of neuraminidase activities of different reassortant and endemic H3N2 swine viruses with that of the A(H1N1)pdm09 virus. For each virus, three independent replicate experiments were conducted. The error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. TRIG, North American triple-reassortant internal gene; pH1N1, A(H1N1)pdm09 virus-like gene; CA09, A/California/04/2009.

Table 6 Macroscopic lung lesions of contact and principal pigs infected with the rgKS-107824 or singly mutated rgKS-107824-G228S virus

	Lung lesion score[a]		
	Infected pigs		Contact pigs
Virus	5 days p.i.	7 days p.i.	(5 days p.c.)
rgKS-107824	15.33 ± 4.84	7.66 ± 3.84	7.25 ± 4.13
rgKS-107824-G228S	35.33 ± 11.92	8.66 ± 2.72	5.50 ± 2.06
Control	0.00	0.00	NA

[a] The lung lesion scores were determined as percentages of the lung; the data are means ± SEM. NA, not applicable; there were no control pigs in the contact group.

Figure 16 Virus titers in nasal swabs and BALF samples of contact and principal pigs infected with the rgKS-107824 and/or singly mutated virus

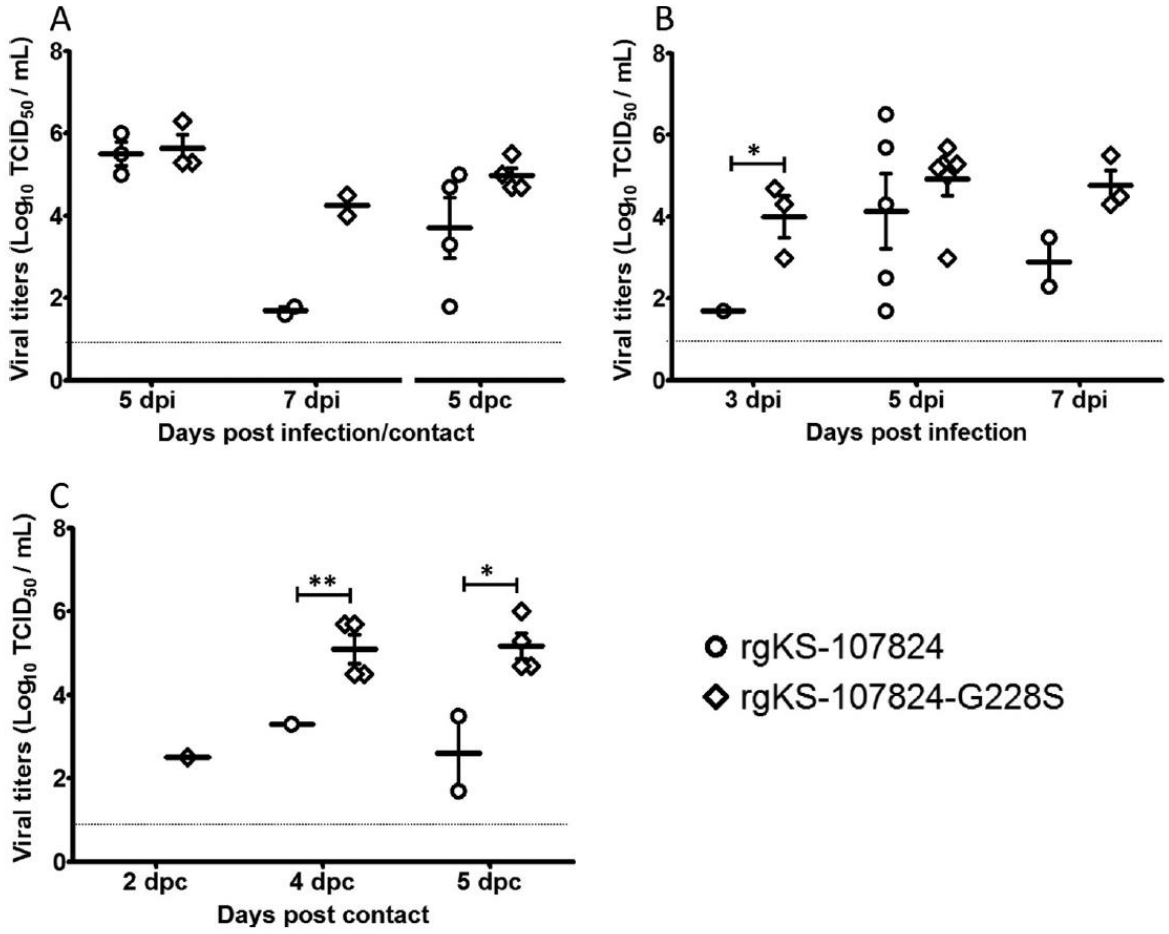

Figure 16 Virus titers in nasal swabs and BALF samples of contact and principal pigs infected with the rgKS-107824 and/or singly mutated virus. (A) Virus titers in BALF samples of principal infected and contact pigs. (B) Virus titers in nasal swabs of infected pigs. (C) Virus titers in nasal swabs of contact pigs. The error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$. The dotted line indicates the detection limit of the assay.

Chapter 4: Characterization of uncultivable bat influenza virus using a replicative synthetic virus

The data in this section has been published in PLoS Pathog 10(10): e1004420. Zhou B, Ma J, Liu Q, Bawa B, Wang W, et al. (2014) Characterization of Uncultivable Bat Influenza Virus Using a Replicative Synthetic Virus.

4.1 Introduction

Bats are present throughout most of the world and account for more than fifth of mammalian species. They are natural reservoirs of some of the most deadly zoonotic viruses; including, rabies virus, Ebola virus, Henipaviruses, and SARS coronavirus (Calisher et al., 2006; Tong et al., 2013). Recently, nucleic acids obtained from bat samples indicated bats may be a reservoir of a new group of influenza viruses, bat influenza that are phylogenetically very distantly related to other influenza viruses (Tong et al., 2012; Tong et al., 2013). Type A, B, and C influenza viruses belong to the *Orthomyxoviridae* family and their genomes are composed of 7-8 negative sense RNA segments (vRNAs). While influenza B (IBV) and C viruses mainly infect human hosts, influenza A virus (IAV) has a broad host range; including humans, marine mammals, horses, pigs, waterfowl, and poultry. New subtypes of IAV, which have novel hemagglutinin (HA) and/or neuraminidase (NA) surface glycoproteins, are introduced into the human population by zoonosis and this periodically leads to devastating pandemics. Past pandemics include the "Spanish flu" (H1N1) in 1918, "Asian flu" (H2N2) in 1957, "Hong Kong flu" (H3N2) in 1968, "Russian flu" (H1N1) in 1977, and the recent "swine origin" [A(H1N1)pdm09] in 2009. Pandemic viruses are often reassortant viruses composed of vRNAs that are derived from multiple IAV lineages that previously circulated in swine and/or avian reservoirs (e.g., 1957 avian-human reassortant, 1968 avian-human reassortant, and 2009 avian-

swine-human reassortant). The discovery of putative bat influenza viruses expands the known host species reservoirs that may serve as a source of novel viruses, which is major concern for public and animal health (Wu et al., 2014).

Infectious bat influenza viruses couldn't be isolated (Tong et al., 2012; Tong et al., 2013) and although several structural and biochemical characterization studies have been conducted with the putative bat influenza HA, NA, and part of PA, none of the vRNAs have been shown to be functional in a replicative virus (Chotpitayasunondh et al., 2005; Liu et al., 2012b; Tong et al., 2012; Tong et al., 2013; Wu et al., 2014; Zhu et al., 2012). This has led to questions such as: (1) are the putative bat influenza vRNA sequences identified derived from infectious viruses or are they merely nucleic acid relics harbored in bats (Wu et al., 2014), (2) are the vRNA segments sequenced from a single bat influenza virus or are they from multiple potentially incompatible viruses, and (Watanabe et al., 2011) were the sequences of the complete gene segments, which is a significant technical challenge, determined accurately. The inability to culture infectious viruses is the major hurdle to confirm the existence of these novel influenza viruses, and to answer important questions, such as pathogenicity in animal models, ability to reassort with other influenza viruses, and their potential risk to public health (Garcia-Sastre, 2012; Wu et al., 2014). The goals of this study were to synthesize the complete viral genome, characterize the bat influenza virus using non-infectious approaches, then generate a replicative virus, and use it as a model to better understand bat influenza viruses.

4.2 Materials and Methods

Biosafety and ethics statement

The study was reviewed and approved by the Institutional Biosafety Committee at Kansas State University (protocol #903), and by the institutional biosafety committee at the J.

Craig Venter Institute (protocol # 3414). We conducted the initial studies using PR8 gene fragments to generate the modified bat influenza viruses and to test the reassortment potential because PR8 is a widely used lab/mouse adapted BSL2 virus that poses very low risk to humans or livestock. Subsequently, TX98 H3N2 genes were used in a few experiments because this is a BSL2 swine virus, which we have used previously and the viruses generated were considered low risk.

The animal studies were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol (protocol #3339) was reviewed and approved by the Institutional Animal Care and Use Committee at Kansas State University. All animal studies were performed in a Biosafety Level 3 facility located at the Biosecurity Research Institute at Kansas State University under the approved protocol #3339 following the American Veterinary Medicine Association guidelines on euthanasia. For virus inoculation, each mouse was anesthetized by inhaling 4% isoflurane. Mice were euthanized if more than 25% of weight lost after virus inoculation. Euthanasia of mice was conducted by inhaling 4% isoflurane followed by cardiac puncture and cervical dislocation. No survival surgery was performed, and all efforts were made to minimize suffering.

Cells

Human embryonic kidney 293T (HEK-293T) cells, mouse rectum epithelial carcinoma (CMT-93) cells, and African green monkey kidney (Vero) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) supplemented with 5% FBS. Human lung epithelial (A549) cells, bat lung epithelial (Tb1Lu) cells, mink lung epithelial (Mv1Lu) cells and swine testis (ST) cells were maintained in MEM

supplemented with 10% FBS. Human lung epithelial (Calu-3) cells were maintained in MEM supplemented with 10% FBS, 1% nonessential amino acids, and 1 mM sodium pyruvate.

Complete genome synthesis and plasmid construction

Nucleotide sequences of the eight gene segments of A/little yellow-shouldered bat/Guatemala/164/2009 (H10N17) (Bat09) were retrieved from the GenBank database. A total of 472 oligonucleotides of 56-60 bases in length were designed for enzymatic assembly of the eight segments. The assembly and error correction processes were performed as recently described (Dormitzer et al., 2013; Li et al., 2014), modified with increased time at all extension steps (from 72^{o}C for 1 min to 72^{o}C for 2 min) for efficient assembly of the polymerase segments. The synthesized segments (Figure 17) were cloned into the modified bidirectional influenza reverse genetics vectors pBZ66A12 (Zhou et al., 1999) using the recombination-based method (Zhou et al., 1999) and transformed into Stella competent *E. coli* cells (Clontech). Colonies were selected and sequenced. The appropriate clones for each segment were propagated for plasmid preparation and verified by sequencing. The resulting plasmids are pBZ146A1 (PB2), pBZ147A11 (PB1), pBZ148A20 (PA), pBZ149A30 (HA), pBZ150A31 (NP), pBZ151A36 (NA), pBZ152A42 (M) and pBZ153A45 (NS). The whole process only took seven days to complete. The plasmids containing Bat09 PB2 mutations were constructed by site-directed mutagenesis using the pBZ146A1 as template. The NS1 truncation constructs were generated by Gibson Assembly and details of the truncations are diagramed in Figure 18C. The modified (m) Bat09 HA and NA (mH1, mN1, mH1ss, and mN1ss, see Figure 18A, B for diagrams, and Figure 20 for sequence alignment) were synthetized by Gibson assembly from oligonucleotides. Silent substitutions (ss) were introduced to disrupt the putative packaging signals in the PR8 HA and NA terminal coding regions. The mH1ss and mN1ss are thus more appropriate than the mH1 and

mN1 to assess the HA and NA packaging signal compatibility between Bat09 and PR8. The Batps-PR8-NP, PR8ps-Bat-NP, Batps-PR8-NS, and PR8ps-Bat-NP constructs were constructed similarly and diagramed in Figure 18D. As a comparison of the speed of different synthesis strategies, the eight gene segments of A/flat-faced bat/Peru/033/2010 (H18N11) (Bat10) were synthesized by Genewiz (NJ, USA) in the vector plasmid of pUC57 based on the GenBank database and subcloned into pHW2000 vector. The resulting plasmids (pHW-H18-PB1, pHW-H18-PB2, pHW-H18-PA, pHW-H18-NP, pHW-H18-HA, pHW-H18-NA, pHW-H18-M and pHW-H18-NS) were confirmed by sequencing. The whole process took more than one month. The PB2, PB1, PA and NP gene were also subcloned into the pDZ vector to be used in the mini-genome assay. Diagrams of the mutant or modified genes of Bat09 and Bat10 are described in Figure 18. The pPol1-NS-Luc reporters used in the mini-genome polymerase activity assay were described in Figure 18E. Sequences of all constructs used in this study were confirmed to ensure absence of unwanted mutations and the GenBank accession numbers are KM203345-KM203356.

Virus Rescue

Briefly, 0.6 µg of plasmid for each gene segment was mixed and incubated with 15 µl of Mirus TranIT-LT1 (Mirus Bio, Madison, WI) at 20°C for 20 min. The transfection mixture was transferred to 90% confluent 293T/MDCK cell monolayers in a 35-mm tissue culture dish and incubated at 37°C with 5% CO_2 for 8 h. The transfection supernatant was replaced with 3 ml of Opti-Mem I medium (Life Technologies) supplemented with 0.3% bovine serum albumin (BSA) fraction V (Life Technologies), 3 µg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington, Lakewood, NJ), and 1% antibiotic-antimycotic (Life Technologies). Three days post-transfection, culture supernatant (passage 0, P0) was collected and 0.5 ml of that was inoculated into MDCK cells in 6-well plates at 37°C. Supernatant (P1)

was collected at 4 days post-inoculation (dpi), or when severe cytopathic effect (CPE) was observed. The P1 supernatant was further passaged blindly for two passage before determined to be negative for rescue. Titers of the viruses used in this study were determined by $TCID_{50}$ assay in MDCK cells.

Rescue efficiency/easiness definition. Very easy (++++): P0 viral titer 106-108 TCID50/ml, or severe CPE observed in P1 within 1 dpi; Moderate (+++): P0 titer 104-106 TCID50/ml, or obvious CPE observed in P1 within 2 dpi; Difficult (++): P0 titer 102-104 TCID50/ml, or weak CPE observed in P1 within 4 dpi; Very difficult (+): P0 titer lower than 102 TCID50/ml, or CPE not observed until P2/P3; Negative (Neg): rescue failed, no CPE observed through passage 3.

Various transfection conditions including different transfection reagents, temperatures, and incubation time before supernatant collection were attempted to rescue the wild type Bat09 virus and the reassortants between Bat09 and PR8. However, none of them generated any positive rescue results if they were negative under standard rescue condition described above. Bat09 transfection supernatants were also transferred to various cells (MDCK, mink lung Mv1-Lu, swine testis, Vero, A549 cells, Calu-3, bat lung epithelial Tb1Lu) and embryonated chicken eggs and passaged at least three times. The real-time RT-PCR assays targeting Bat09 and PR8 M genes were used to confirm negative results (primers and probes are possible upon request).

Electron microscopy

To determine whether virus particles of Bat09 and other viruses can be produced by reverse genetics system, a total of thirty-five ml of transfected 293T cell supernatants for each virus were collected at 48 hours post transfection and centrifuged at 8000 rpm for 20 minutes to remove the cell debris. Then the clear supernatant was loaded on 30% (w/v) sucrose in centrifuge

tubes and was concentrated at 27,000 rpm (OptimaTM LE-80K ultracentrifuge, Beckman Clouter) for 2 hours. The virus pellets was dissolved in 100 µl of water and the viral particles were fixed by incubating with 0.2% paraformaldehyde at 37°C for 48 hours. The fixed particles were dipped on a 200 mesh copper grid and the grid was dried and stained with negative staining before observation under an electron microscope.

Virus replication in vitro and in ovo

MDCK monolayers in 12-well plates were washed twice with PBS, and then 2 ml of virus growth medium (VGM) was added to each well. The cells were inoculated at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell with the Bat09:mH1mN1 virus or PR8 virus and incubated at 37 ^{o}C. Supernatants were collected at 1, 2, and 3 days post inoculation (dpi). Inoculations of Calu-3 cells were performed similarly, except that an MOI of 0.02 $TCID_{50}$/cell was used for the following viruses: Bat09:mH1mN1ss, Bat09:mH1mN1ss-NS1-73, Bat09:mH1mN1ss-NS1-128, PR8, PR8-NS1-73, and PR8-NS1-126. The VGM used for MDCK cells was EMEM supplemented with 0.15% BSA fraction V, 2 µg/ml TPCK-trypsin, and 1% antibiotic-antimycotic, and the VGM used for Calu-3 cells was EMEM supplemented with 0.3% BSA fraction V, 1 µg/ml TPCK-trypsin, and 1% antibiotic-antimycotic. All virus titers were determined by $TCID_{50}$ assay using MDCK cells.

Six of 10-day-old embryonated chicken eggs were inoculated with Bat09:mH1mN1 or PR8 at 10^3 $TCID_{50}$/egg. After 2 days incubation at 35 ^{o}C, allantoic fluid was collected from each egg and titrated individually. The 4 eggs with the highest titers in each virus group was used to calculate the average titer and generate the graph in Figure 21 E.

Next generation sequencing and analysis

A modified Multi-segment RT-PCR (Zhou et al., 2009; Zhou et al., 2014a) was used to amplify influenza-specific segments. The only modification to the procedure was the primers used for amplification were changed to match Bat influenza termini. The oligonucleotide primers used were Uni12-Inf-1G5 (5'-GGGGGGAGCAGAAGCAGG-3') and Uni13/Inf-1 (5'-CGGGTTATTAGTAGAAACAAGG-3'). The M-RTPCR amplicons were used for Illumina Miseq librarly construction via Next era DNA sample prep kit (Illumina, Inc.) and sequenced using the Illumina MiSeq (Illumina, Inc.) according to manufacturer's instructions. SNP variations were identified using custom software that applies statistical tests to minimize false positive SNP calls that could be caused by the types of sequence-specific errors that may occur in Illumina reads identified and described in Nakamura, et al. (Kimura et al., 1997). To overcome this problem, the protocol requires observing the same SNP, at a statistically significant level, in both sequencing directions. Once a minimum minor allele frequency threshold and significance level are established by the user, the number of minor allele observations and major allele observations in each direction and the minimum minor allele frequency threshold are used to calculate a p-value based on the binomial distribution cumulative probability, and if the p-values calculated in each of the two sequencing directions are both less than the Bonferroni-corrected significance level, then the SNP call is accepted. For our analyses, we used a significance level of 0.05 (Bonferroni-corrected for tests in each direction to 0.025), and a minimum minor allele frequency threshold of 10% of the read population.

Interferon-β reporter assay

To measure the IFN-antagonist function of NS1, a luciferase-based, Sendai virus-mediated IFN-β promoter activation assay was conducted as previously described (Leung et al.,

2010). Briefly, 293T cells in 24-well plates were transfected with empty vector (200 ng) or increasing amounts of wild type (WT) or carboxyl terminal truncated NS1 from Bat09 and PR8 (2 ng, 10 ng, and 50 ng of NS1 expression plasmids supplemented with 198 ng, 190 ng, and 150 ng of empty vector, respectively). Also co-transfected were 200 ng of an IFN-β-promoter-luciferase reporter plasmid (pIFNβ-Luc) and 20ng of a plasmid constitutively expressing Renilla luciferase (pRL-TK from Promega). At 18 hours post transfection, cells were infected with Sendai virus to induce the IFN-β promoter. A dual-luciferase assay was performed at 18 hour post virus inoculation, and firefly luciferase was normalized to Renilla luciferase activity. The relative luciferase activity of the group with empty vector was set as 100%, and the other groups were presented relative to that.

Interferon bioassay with VSV-luciferase virus

As previously described for the VSV-GFP virus mediated interferon bioassay (Zhou et al., 2010), in the VSV-Luciferase virus mediated bioassay, A549 cells were inoculated with one of the wild type or NS1 truncated viruses at an MOI of 4 $TCID_{50}$/cell, or were mock-inoculated; supernatants were then collected at 24 hpi. Supernatants were treated with UV irradiation to inactivate viruses and were then transferred to naïve A549 cells. Following 24h of incubation at 37^{o}C, supernatants were removed, and the cells were inoculated with VSV-Luciferase virus (Chandran et al., 2005), at an MOI of 2 $TCID_{50}$/cell. The firefly luciferase expression in the cells was measured using the Luciferase Assay System (Promega) at 4 hpi with VSV-Luciferase.

Mini-genome polymerase activity assay

The luciferase-mediated mini-genome polymerase activity assay was performed as previously described, using a PolI-driven reporter plasmid and pDZ-based PB2, PB1, PA, and

NP bidirectional expression plasmids (Quinlivan et al., 2005; Zhou et al., 2009). To determine the effects of PB2 mutations on polymerase activity (Figure 27) 293T cells were co-transfected with 0.2 µg each of the PB2 (WT or mutant), PB1, PA, NP, and a pPol1-FluA-NS-Luc (firefly luciferase flanked by A/New York/1682/2009 (Zhou et al., 2011)). As a control for transfection efficiency, 0.02 µg of the Renilla luciferase plasmid pRL-TK (Promega) was also co-transfected. After 18 hours of incubation at 33^{o}C, 37^{o}C, and 39^{o}C, luciferase production was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. Firefly luciferase expression was normalized to Renilla luciferase expression (relative activity). The relative activity of the PB2-WT was set as 1 fold, and the relative activities of the PB2 mutants were presented relative to that (Figure 27).

To test the compatibility between RNPs (PB2, PB1, PA, and NP) and viral RNA promoters from bat influenza virus (Bat09) (Figure 23), IAV (A/PR/8/1934), and IBV (B/Russia/1969), 293T cells were co-transfected with 0.2 µg each of the PB2, PB1, PA, NP, and a pPol1-NS-Luc reporter plasmid, followed by incubation at 37^{o}C for 18 hours. Three reporters were used in this study, including pPolI-Bat-NS-Luc (firefly luciferase flanked by Bat09 NS non-coding regions), pPol1-FluA-NS-Luc, and pPolI-FluB-NS-Luc (firefly luciferase flanked by B/Russia/1969 NS non-coding regions) (Figure 18 D). For each combination of RNP and pPolI-NS-Luc reporter (from Bat09, A, or B Type), three independent replicates were conducted. For each RNP, the luciferase activity with the reporter from the same virus (e.g., Bat-RNP and pPol1-Bat-NS-Luc) was set at 100%, and the activities with the other two reporters (e.g., pPol1-FluA-NS-Luc and pPol1-FluB-NS-Luc) were presented relative to that (Figure 19).

The PB2, PB1, PA, and NP compatibility between Bat09 and the following influenza viruses was examined in the study (Figure 28): A/PR/8/1934 (lab adapted human H1N1), A/Ann

Arbor/6/1960 (human H2N2), A/New York/238/2005 (human H3N2); A/New York/1692/2009 (human H1N1 seasonal), A/New York/1682/2009 (human H1N1 pandemic), A/canine/New York/6977983/2010 (canine H3N8), A/turkey/Ontario/7732/1966 (avian H5N9), A/Hong Kong/213/2003 (avian H5N1), A/Anhui/1/2013 (human H7N9), B/Russia/1969 (lab adapted human IBV), and A/flat-faced bat/Peru/033/2010 (bat H18N11). For the compatibility test between Bat09 and IAVs (Figure 28 A – I), 293T cells were co-transfected with 0.2 µg each of the PB2, PB1, PA, NP (from Bat09 or IAV), 0.1 µg of pPolI-Bat-NS-Luc plasmid and 0.1 µg of pPolI-FluA-NS-Luc. For compatibility test between Bat09 and IBV (Figure 28 J), 293T cells were co-transfected with 0.2 µg each of the PB2, PB1, PA, NP (from Bat09 or B/Russia/1969), 0.1 µg of pPolI-Bat-NS-Luc plasmid and 0.1 µg of pPolI-FluB-NS-Luc. For compatibility test between Bat09 and Bat10 (Figure 28), 0.2 µg each of the PB2, PB1, PA, NP (from Bat09 or Bat10), and pPolI-Bat-NS-Luc plasmids were used (The NS non-coding regions of Bat09 and Bat10 have the same sequence). Renilla luciferase was also co-transfected and dual-luciferase reporter assay system was used. For each combination of PB2, PB1, PA, and NP (from Bat09 or another influenza virus), three independent replicates were conducted at 37^{o}C, the luciferase activity of the all-Bat09-combination (Bat09-PB2/Bat09-PB1/Bat09-PA/Bat09-NP) was set at 100%, and the activities of other 15 combinations were presented relative to that.

Pathogenicity of PR8, modified bat influenza virus (Bat09:mH1mN1) and PB2 mutants

A total of 98 female BALB/c mice aged 6 to 7 weeks were randomly allocated to 7 groups (14 mice/group). Six mice were intranasally inoculated with 10^3 $TCID_{50}$ of each virus (Bat 09:mH1mN1, Bat09:mH1mN1-PB2-701D, Bat09:mH1mN1-PB2-627K701D, Bat09:mH1mN1-PB2-158G701D, Bat09:mH1mN1-PB2-158G, PR8, or MEM Mock) in 50µL

fresh MEM medium while under light anesthesia by inhalation of 4% isoflurane. To determine the virus replication in mouse lungs, three mice from each group were euthanized on both 3 and 5 day post-inoculation (dpi). Another 8 mice from each group were intranasally inoculated with 10^4 $TCID_{50}$ of viruses in 50μL MEM medium; all eight mice were kept to monitor body weights and clinical signs. Weights were recorded daily and general health status was observed twice daily. After the onset of disease, the general health status was observed three times daily. Severely affected mice (i.e., more than 25% body weight loss) were euthanized immediately, and the remaining mice were euthanized on 14 dpi. All control mice were intranasally inoculated with 50 μL fresh MEM (mock group), three control mice were necropsied at 3 and 5 dpi, the remaining mice were kept until the end of the animal study.

During necropsy, the right part of the lung was frozen at -80^{o}C for virus titration, and the left part of the lung was fixed in 10% formalin for histopathologic examination. For virus titration, the 10% lung homogenate was prepared in cold fresh MEM medium by using a Mini Bead Beater-8 (Biospec Products; 16 Bartlesville, OK). The homogenate was centrifuged at 6000 rpm for 5 minutes, and the supernatant was titrated by infecting MDCK cells in 96-well plates. For the histopathologic examination, lung tissues fixed in 10% phosphate-buffered formalin were processed routinely and stained with hematoxylin and eosin. The lungs were examined microscopically both for the percentage of the lung involved and for the histopathologic changes seen, including bronchiolar and alveolar epithelial necrosis, intraalveolar neutrophilic inflammation, peribronchiolar inflammation, and bronchiolar epithelial hyperplasia and atypia. For detection of virus NP antigens in lung sections on day 5 post infection, a rabbit anti-H1N1 (2009 flu pandemic) NP polyclonal antibody was used (Genscript, USA). A pathologist examined each slide in a blinded fashion.

Pathogenicity of modified bat influenza viruses (Bat09:mH1mN1ss) containing truncated NS1 genes

A total of 70 female BALB/c mice aged 6 to 7 weeks were randomly allocated to 5 groups (14 mice/group). To determine virus replication, six mice were intranasally inoculated with 10^4 $TCID_{50}$ of each virus (Bat09:mH1mN1ss-NS1-WT, Bat09:mH1N1ss-NS1-73, Bat09:mH1mN1ss-NS1-128, and PR8-NS1-126) in 50μL MEM medium while under light anesthesia by inhalation of 4% isoflurane. Three mice from each group were killed on both 3 and 5 day post-inoculation (dpi). Another 8 mice from each group were intranasally inoculated with 10^5 $TCID_{50}$ of each virus in 50μL MEM medium for morbidity and mortality comparison. All the other procedures are same with described previously.

Pathogenicity of TX98 and modified bat influenza (Bat09:mH3mN2) viruses

A total of 42 female BALB/c mice aged 6 to 7 weeks were randomly allocated to 8 groups (14 mice/group). To investigate virus replication in mice, six mice from each group were intranasally inoculated with 3×10^4 $TCID_{50}$ of virus or mock-inoculated with 50μL fresh MEM medium while under light anesthesia by inhalation of 4% isoflurane. Three of six inoculated mice from each group were euthanized at 3 and 5 day post-inoculation (dpi). To evaluate viral pathogenicity in mice, the remaining eight mice from each group were intranasally inoculated with 3×10^5 $TCID_{50}$ of virus (Bat09:mH3mN2, and TX98) in 50μL fresh MEM medium or mock-inoculated with 50μL fresh MEM medium. The mice were monitored body weights and general health status daily. After the onset of disease, the general health status was observed twice per day. Severely affected mice (i.e., more than 25% body weight loss) were humanly euthanized, and the remaining mice were euthanized and bloods were collected from each mouse

to isolate serum for the HI assay at 14 dpi. Sample collection and analysis, and virus titration were performed as described as above.

Co-infection study for assessment of reassortment

To study the reassortment between Bat09:mH1mN1 and PR8 or Bat10:mH1mN1, confluent monolayer of MDCK cells in 6-well-plates were co-infected with both viruses (Bat09:mH1mN1 and PR8, or Bat09:mH1mN1 and Bat10:mH1mN1). Both modified Bat09:mH1mN1 and Bat10:mH1mN1 viruses showed similar replication kinetics in MDCK cells, whereas the PR8 replicated more efficiently than both modified viruses in MDCK cells. Therefore, for the co-infection study with PR8 and Bat09:mH1mN1 viruses, the cells were infected with the PR8 at MOI of 1 and with the Bat09:mH1mN1 at MOI of 4 (a ratio of both viruses is 1:4). For the co-infection study with Bat09:mH1mN1 and Bat10:mH1mN1 viruses, the cells were infected with each virus at MOI of 1 (a ratio of both viruses is 1:1). Then, the co-infected MDCK cells were incubated in 37°C with 5% CO2 for 1 hour. After 1 hour of incubation, the supernatant was removed and the infected cells were washed with fresh MEM for 10 times. One mL of infection medium supplemented with 1 μg/mL TPCK-trypsin (Worthington, Lakewood, NJ) was added on cells. The supernatant containing progeny viruses was collected at 24 hours after inoculation. Plaque assays were performed in MDCK cells to pick up single virus from co-infected supernatants. The purified single virus was amplified for further analysis. To identify the origin of each gene of the purified single virus, specific RT-PCR was used to differentiate internal genes from Bat09:mH1mN1, Bat10:mH1mN1 and PR8 viruses (primers for specific RT-PCR are available upon request). The surface HA and NA genes were differentiated by sequencing HA and NA non-coding regions (packaging signals) since three parental viruses contain identical HA and NA ORF sequences and different sequences in non-

coding region (it is difficult to differentiate them by RT-PCR). For the RT-PCR, RNAs were extracted from each amplified single virus using a QIAamp Viral RNA Mini Kit (Qiagen). cDNA was synthesized by using an influenza A universal 12 primer (5'-AGCRAAAGCAGG-3') for the samples from the co-infection study with Bat09:mH1mN1 and Bat10:mH1mN1 viruses, and by using a mixture of an influenza universal 12 primer (5'-AGCRAAAGCAGG-3') and the universal bat primer (5'-AGCAGAAGCAGG-3') for the samples from the co-infection study with Bat09:mH1mN1 and PR8 viruses. If the origin of internal genes determined by the specific RT-PCR was not sure, sequencing was used to confirm the results from specific RT-PCR (All sequence primers are available upon request).

Statistical analysis

Luciferase activity, virus titers, and mouse weights were analyzed by using analysis of variance (ANOVA) in GraphPad Prism version 5.0 (GraphPad software Inc, CA). One-way ANOVA with Dunnett's multiple comparison test was used to determine the significance of the differences ($P < 0.05$) among different groups. For simple comparisons, Student's *t* test was used to examine the significance of differences observed. Error bars represent standard deviation (±SD).

4.3 Results

Synthetic genomics generated bat influenza virus-like particles but they were not infectious in many host cell substrates

Lack of infectious particles in the original bat specimens is a potential factor in the inability to isolate/culture bat influenza using multiple host cell substrates (Tong et al., 2012). Based on digital sequence information published by Tong et al (Tong et al., 2012), we synthesized the complete genome of A/little yellow-shouldered bat/Guatemala/164/2009

(H17N10) (Figure 17) and cloned it into reverse genetics plasmids to rescue this putative bat influenza virus (Bat09). Thousands of spherical influenza-like particles budded into the supernatants of human cells (293T) transfected with the Bat09 reverse genetics plasmids (Figure 21 **A**). The supernatants were inoculated into embryonated chicken eggs and cell lines derived from many species (canine (MDCK), mink (Mv1-Lu), swine (ST), African green monkey (Vero), human (A549, Calu-3), and free-tailed bat (Tadarida brasiliensis, Tb1Lu)); however, none of the host cell substrates tested supported productive virus infection (determined by serial passage and subsequent real-time RT-PCR).

Previous biochemical and structural studies with purified proteins of Bat09 hemagglutinin (HA) and neuraminidase (NA) indicate that that the HA doesn't bind to canonical sialic acid receptors of influenza viruses and the NA doesn't have neuraminidase activity, which is characteristic of IAV and IBV NAs (Li et al., 2012; Wu et al., 2014; Zhu et al., 2012; Zhu et al., 2013). To further examine if the HA and NA proteins are the major blocks to the propagation of the Bat09 virus, we attempted to rescue reassortant viruses that contained the 6 internal protein coding vRNAs (PB2, PB1, PA, NP, M, and NS) from Bat09 and the surface glycoprotein vRNAs (HA and/or NA) from a recombinant A/Puerto Rico/8/1934 (PR8). PR8 is a lab adapted H1N1 virus that has been used for many years in research and vaccine settings because it replicates efficiently in embryonated chicken eggs, cell lines (e.g., MDCK) and in the mice, but has low risk to humans. However, the three PR8-HA/NA reassortant genotypes containing the Bat09 internal protein vRNAs couldn't be rescued following transfection (Figure 21 **B**). While the Bat09 internal protein/vRNAs are capable of generating proteins and producing influenza-like particles, they may have critical mutations that were inhibiting infectivity, or they can't cooperate efficiently with the PR8-HA/NA proteins/vRNAs.

Modified bat influenza virus could be generated and it replicated efficiently in vitro, in ovo, and in vivo

To further address the inability to rescue Bat09 or the Bat:PR8-HA/NA reassortants, we created a modified HA vRNA (mH1) that contained the protein coding region from PR8-H1 flanked by putative cis-acting terminal packaging signals from Bat09 that we hypothesized would be similar to the regions known to be central to packaging of A/WSN/1933 and PR8 (Gao and Palese, 2009; Watanabe et al., 2003) (Figure 21 C, Figure 18). The Bat09 NA gene segment was modified using a similar strategy to replace the NA coding region with PR8-N1, while the putative bat NA packaging signals were retained (mN1) (Figure 21 C, Figure 18). Co-expression of the mH1 and mN1 vRNAs with the six Bat09 internal protein vRNAs efficiently rescued a reassortant Bat09:mH1mN1 virus (Figure 21B). The reassortant Bat09:mH1mN1 formed particles similar to that of Bat09 (Figure 21A) and replicated robustly *in vitro* and *in ovo* (Figure 21D, E). Next generation sequencing demonstrated that the consensus sequence of the virus stocks from 1 passage in MDCK cells or embryonated chicken eggs was identical to that of the reverse genetics plasmids. Furthermore, even after 3 passages in MDCK cells, we still didn't identify any nucleotide polymorphisms accounting for >10% of the genomic population that would suggest strong selective pressure on Bat09 genes or the modified HA/NA genes of PR8.

To investigate whether Bat09:mH1mN1 is able to infect and replicate in mice, a mouse study was performed using the mouse adapted PR8 IAV as a positive control. Bat09:mH1mN1 replicated efficiently in mouse lungs (Figure 22A), and caused significant weight loss as early as at 4 days post inoculation (4 dpi) (Figure 22B). The virulence of Bat09:mH1mN1 (75% mortality) was close to that of the PR8 virus (100% mortality) (Figure 22C). Histopathological analysis showed that the Bat09:mH1mN1 virus caused typical influenza-like lesions characterized by a varying degree of broncho-alveolar epithelial degeneration and necrosis, and

interstitial pneumonia. The peribronchiolar and perivascular areas were infiltrated by moderate numbers of lymphocytes and plasma cells (Figure 22D). The histopathology identified correlates with presence of virus antigen in the mouse lungs (Figure 22E).

Next generation sequencing was used to determine if the Bat09 vRNAs were genetically stable in mice. Although nucleotide polymorphisms (at the level of 12%–36%) were detected at sporadic loci throughout the Bat09 vRNAs, each lung sample only had one such polymorphism on average, and none of the mutations were found in more than one mouse. Nonetheless, serial passage of this virus in mice may identify mutations in the Bat09 backbone critical to replication/pathogenesis in mice. We did identify a low level nucleotide polymorphism in the modified PR8 HA at residue at 187 that emerged in multiple Bat09:mH1mN1 inoculated mouse lung samples collected at 3 and 5 dpi (HA-K187E, 10%–20% of the genomic population). This unanticipated result may have also occurred in PR8 inoculated mice; however the lung specimens from these mice were not sequenced.

The virulence of the Bat09:mH1mN1 in mice could partly result from the H1 and N1 of the mouse adapted PR8 virus. To further investigate pathogenicity of Bat09-like viruses we rescued another modified Bat09 virus that expresses H3N2 surface glycoproteins from A/swine/Texas/4199-2/1998 (H3N2) (TX98), which we have used in pigs previously (Solorzano et al., 2005). The HA/NA vRNAs of Bat09:mH3mN2 were modified using a similar strategy used to substitute the coding regions of PR8 H1N1 effectively replacing the coding regions Bat09 glycoproteins with TX98 H3N2, while the putative Bat09 packaging signals were retained (mH3mN2) (Figure 23A). The rescued Bat09:mH3mN2 virus replicated to peak titers close to that of TX98 (Figure 23B) and both viruses were inoculated into mice to compare the morbidity (weight loss), mortality and virus replication at various times post inoculation. All mice survived

infection and both viruses (Bat09:mH3mN2 and TX98) caused little effect on weight gain as compared to the mock inoculated animals (Figure 23C), indicating little overall disease. Titration of virus in the lung tissues showed that the Bat09:mH3mN2 virus replicated as efficiently as the TX98 control in the mice at early time points, yet it appeared to be cleared more rapidly (Figure 23D). This data suggests that some of the pathogenicity observed in the Bat09:mH1mN1 infected mice likely results from the mouse adapted HA/NA of PR8. However, it is clear that the bat influenza internal protein vRNAs do support replication of the modified viruses (Bat09:mH1mN1 and Bat09:mH3mN2) in vitro, in ovo, and in the mouse lungs. The slightly lower replication efficiency and pathogenicity of those two viruses compared to the corresponding PR8 and TX98 viruses could be ascribable to either the nature of the Bat09 internal protein vRNAs or the engineering of the modified HAs and NAs.

Bat influenza NS1 shows strong innate immune suppression in human cells and in mice

Bat influenza viruses appear to have diverged from IAV a very long time ago and its internal protein vRNAs have many unique features that are not seen in IAVs (Tong et al., 2012; Tong et al., 2013). Therefore, the biological roles of the various vRNA segments and their protein products are likely to have both similarities and intriguing differences. Many deadly bat viruses (e.g., filoviruses) have evolved powerful molecular mechanisms to inhibit host (e.g., human) immune responses (Leung et al., 2010; Ramanan et al., 2012; Reid et al., 2007; Valmas et al., 2010). Therefore, to gain an understanding of how Bat influenza viruses may evade the host innate immune response we analyzed the Bat09 NS1 protein using interferon induction experiments and carboxy-terminal truncation mutations known to attenuate IAVs. The NS1 protein of IAVs is critical for pathogenicity of many strains because of its ability to antagonize

the host interferon response (Hale et al., 2008). To compare the direct effect of Bat09-NS1 and PR8-NS1 on interferon-β production, we expressed the proteins ectopically in human HEK-293T and then infected them with Sendai virus to stimulate the innate immune response. Activation of interferon-β promoter was determined by a luciferase mediated reporter assay (Leung et al., 2010). Bat09-NS1 inhibited host interferon-β induction comparable to that of the PR8-NS1, and carboxy-terminal truncation of Bat-NS1 protein (NS1-128 and NS1-73, see Figure 18) decreased its ability to inhibit interferon-β production (Figure 24A). These results are consistent with the attenuating effect that these NS1 truncations have on PR8 (Figure 24A) and other IAV NS1 proteins; thereby, providing a strategy to generate live attenuated influenza vaccines (Quinlivan et al., 2005; Solorzano et al., 2005; Talon et al., 2000b; Zhou et al., 2010).

A VSV-luciferase virus mediated bioassay was also performed to compare the effect the NS1 truncations have on the Bat09 viruses' ability to inhibit host innate immune response (Zhou et al., 1999). The replication of the VSV-luciferase virus, which is sensitive to innate immune activation, is inversely correlated with type I interferon induced by influenza virus. Truncation of the Bat09-NS1 modestly reduced VSV replication, whereas truncation of the PR8-NS1 severely inhibited VSV replication (i.e., luciferase expression) (Figure 24B). These results were confirmed by analysis of influenza virus replication kinetics in a human lung epithelial cell line (Figure 24C). The Bat09-NS1 truncated viruses (Bat09:mH1mN1ss-NS1-128 and Bat09:mH1mN1ss-NS1-73) replicated to titers of 10^6 - 10^7 $TCID_{50}$/ml (near wild type NS1; Bat09:mH1mN1ss), whereas the PR8-NS1 truncation mutants had 100 – 1000 fold lower titers than PR8 (Figure 24C, Figure 18 for gene and virus diagrams).

To analyze the impact of these Bat NS1 truncation mutations in vivo we inoculated mice with the same panel modified Bat09 viruses, or the PR8-NS1-126 as a control. In contrast to the

significant attenuation conferred by the truncated NS1 in PR8 (PR8-NS1-126), recombinant bat influenza viruses with truncated NS1 genes (Bat09:mH1mN1ss-NS1-128 and Bat09:mH1mN1ss-NS1-73) replicated efficiently in the lungs (Figure 25A), caused significant morbidity (Figure 25B), and remained 100% lethal in mice (Figure 25C). Altogether the NS1 studies show that the Bat09 NS1 protein inhibits host interferon-β production and carboxy-terminal truncation mutations reduce its ability to antagonize this response, likely through mechanisms similar to IAV (Figure 24A). However, in contrast to IAV, truncation (NS1-128, NS1-73) of the Bat09 NS1 didn't dramatically impact the viruses' ability to antagonize the host innate response, or replicate and cause disease in mice (Figure 24B,C, Figure 25).

Pathogenesis of the modified Bat09 virus can be manipulated by amino acid substitutions at residues important in virulence of IAVs

We analyzed the Bat09 PB2 gene because of its central role in the species specificity of IAVs, and some of the critical residues involved are known to be virulence determinants in mice and ferrets (Gabriel et al., 2008; Gao et al., 2009; Hatta et al., 2001; Li et al., 2005; Subbarao et al., 1993; Zhou et al., 2011). Asparagine (N) 701 in the PB2 protein is a mammalian-signature in IAVs and when this residue was mutated to aspartic acid (D, an avian-signature) in the modified Bat09 (Bat-701D), it decreased virus titers in lungs, morbidity (minor weight loss), and resulted in 100% survival (Figure 26). The bat influenza PB2 also has a serine (S) residue at position 627, which is unlike either mammalian or avian IAVs. Replacing the serine 627 with the mammalian-signature residue lysine (K) (Hatta et al., 2001; Subbarao et al., 1993) in the context of 701D (Bat-627K/701D) increased virus replication in the lungs but only caused slightly more weight loss (compared to the Bat-701D virus) and it remained attenuated in mice (Figure 26). In contrast, introducing another virulence marker PB2-E158G (Zhou et al., 2011) into the PB2-

N701D virus (Bat-158G/701D) dramatically increased the pathogenicity of the Bat09 virus (100% mortality), which was higher than the Bat09 virus with wild type PB2 (Bat09:mH1mN1, Figure 26). In addition, introducing the PB2-E158G (Bat-158G) into the wild type PB2 resulted the most significant increase of virus replication, morbidity, and mortality (Figure 26), indicating there is an additive effect between the two virulence determinants (PB2-158G and PB2-701N) in the Bat09 PB2. All viruses collected from mouse lungs were deep sequenced to confirm the stability of the engineered mutations and although sporadic nucleotide polymorphisms (10% - 44%) were detected in the viral genomes (1 to 2 such polymorphisms per mouse sample on average), none of them occurred at the engineered loci. The high genetic stability of the modified Bat09 viruses in mice is consistent with the notion that the bat influenza viruses are mammalian viruses that have been evolving and adapting in the bats for a long period of time.

To determine the molecular basis for the altered pathogenicity imparted by the various mutations in the PB2 we examined their effects on the viral polymerase activity in human 293T cells using a luciferase-mediated mini-genome replication assay (Figure 27). At all temperatures tested, the PB2-N701D mutation decreased the polymerase activity and the PB2-E158G mutation enhanced the polymerase activity, consistent with the decreased and increased pathogenicity in mice, respectively (Figure 26). Interestingly, the PB2-627S showed intermediate polymerase activity compared to the PB2-627K and PB2-627E (Figure 27). In addition, the polymerase activity of the PB2-158G and PB2-627E/K mutants decreased proportionally when they were combined with the PB2-701D mutation (Figure 27). This result is consistent with the observation that Bat-158G/701D appeared to be less pathogenic than the Bat-158G virus (Figure 26). Collectively, the data collected on the Bat09 PB2 show that amino acid residues known to be

important in replication, species specificity, transmission, and/or pathogenesis of IAV are important in the replication and pathogenesis of Bat09.

Internal protein coding vRNAs of bat influenza don't efficiently reassort with IAV or IBV

Reassortment of IAVs is important in the evolution of IAVs and generation of panzootic and pandemic strains. Furthermore, efficient replication of bat influenza internal protein vRNAs in human cells and mice, as well as their pathogenicity, necessitated an assessment of reassortment potential between Bat09 and other influenza viruses. Replication of vRNAs from different parental viruses is a factor critical in the generation of reassortant progeny. Transcription/replication of mini-genome reporter constructs showed that the viral RNA dependent RNA polymerase (RdRp), which is a heterotrimer of PB1, PB2, and PA, from bat influenza, IAVs, and IBVs recognize and transcribe their cognate vRNAs more efficiently than non-cognate vRNAs (Figure 19). Additionally, most RdRp combinations (PB2, PB1, PA) between bat influenza and IAVs nearly abolished the polymerase activity in this very sensitive mini-genome reporter assay (Figure 28 A-I). Interestingly, the NP protein, which is a single-strand RNA-binding nucleoprotein, is very compatible between Bat09 and IAVs (Figure 28 A-I), but it is incompatible between the bat influenza and IBV (Figure 28 J).

Although some gene segment combinations showed limited polymerase activity in the mini-genome assays, we couldn't generate any reassortant viruses using reverse genetics between Bat09:mH1mN1 and PR8 that contain partly compatible RdRp components (e.g., Bat-PB2/PR8-PB1/PR8-PA), including the highly compatible NP vRNA/protein (Table 9**,** Table 10). Instead, the PR8-M segment could unidirectionally substitute for the Bat09-M segment (Table 10). This likely results from the highly conserved nature of the M vRNA and proteins (M1, M2).

Swapping the putative cis-acting packaging signals of the Bat-NP and known packaging signals of the PR8-NP, or between the Bat-NS and PR8-NS didn't enable rescue of viruses containing either the NP or NS vRNAs in a heterologous virus background (Table 11**,** Figure 18).

HA and NA vRNAs of bat and other influenza viruses have limited compatibility

Low efficiency of packaging at least some vRNA segments from the heterologous virus is also a major restrictive factor for reassortment. For instance, a reassortant virus containing six internal protein vRNAs from Bat09 and the HA and NA from PR8 couldn't be rescued, whereas the PR8 HA and NA coding regions flanked by Bat09 packaging regions (mH1 and mN1) can efficiently reassort with the Bat09 internal genes (Figure 21 B, Table 11). Nevertheless, PR8 HA and NA can individually reassort (7:1) with the Bat09 six internal protein vRNAs when mN1 and mH1 were provided, respectively (Table 12). The inability to rescue the 6:2 reassortant Bat09:PR8-H1N1 virus may result from compounding the low efficiency of packaging for each of the wild type PR8-HA and PR8-NA vRNAs into the bat influenza backbone.

The mN1 can also reassort with the other seven segments from PR8, even when many silent substitutions (ss) were introduced into the N1 coding regions to disrupt the remaining PR8 packaging signals (Table 12). Actually, another modified NA that contains the coding region from IBV NA flanked by the putative packaging region of the Bat09-NA (Bat-N10ps-FluB-NA) can also be rescued in the PR8 background, strongly suggesting that the bat influenza NA segment could be efficiently packaged into the PR8 virus, whereas the Bat HA packaging signal didn't mediate efficient packaging of the mH1 into the PR8 backbone (Table 12).

Interrogation of reassortment between IAV and Bat09 using a classical co-infection approach

While the generation of reassortants through plasmid-based reverse genetics is a powerful and sensitive way to rescue influenza viruses, it is difficult to generate every combination of gene constellation and accompanying minor nucleotide variations within replicating vRNAs that could give rise to progeny reassortants. Therefore, we attempted to generate reassortants between modified Bat09 and PR8 using a classical co-infection approach. However, when MDCK cells were inoculated at a high multiplicity of infection (MOI) with both PR8 and Bat09:mH1mN1 viruses, reassortment between the two parental viruses was not detected. We plaque purified 118 progeny viruses from the co-infection and 53 of them were the parental PR8 virus and 65 of them were the parental Bat09:mH1mN1 virus. Although more exhaustive classical reassortant studies using powerful selective pressures are needed to completely evaluate the generation of natural reassortants between these viruses, the data indicate that PR8 and Bat09:mH1mN1 don't efficiently reassort.

Divergent bat influenza viruses are highly compatible for reassortment

Recently, another bat influenza virus A/flat-faced bat/Peru/033/2010 (H18N11) (Bat10) was identified in Peru and phylogenetic analysis indicated this virus diverged from the bat influenza viruses identified in Guatemala (e.g., Bat09) so long ago that genetic diversity between these two bat influenza viruses is higher than that of IAVs (Tong et al., 2013). Reassortment of the PB2, PB1, PA, and NP segments in mini-genome polymerase activity assay demonstrated that the Bat09 and Bat10 viruses were fully compatible (Figure 28 K). Most importantly, successful reassortment between the two modified bat viruses (Bat09:mH1mN1ss and Bat10:mH1mN1ss) (Table 13, Figure 18) proved that these genetically divergent bat influenza

virus vRNAs were highly interchangeable, in contrast to their very low compatibility with IAV and IBV. Interestingly, classical co-infection of the Bat09:mH1mN1 and Bat10:mH1mN1 viruses in MDCK cells readily generated reassortant progeny viruses with various genotypes, and some were apparently preferably selected (e.g., Bat10:Bat09-NS reassortant, Table 8), demonstrating the merit of classic co-infection strategy in identification of gene constellations that may have certain advantages. Collectively the mini-genome replication, reverse genetics reassortment, and co-infection reassortment experiments strongly suggest that while bat influenza viruses readily reassort with another divergent bat influenza strain they won't reassort with canonical IAVs in the natural setting.

4.4 Discussion

The generation of synthetic modified bat influenza viruses (e.g., Bat09:mH1mN1) that grow to high titers in commonly used influenza virus culture substrates and mice is an important step toward understanding these novel bat influenza virus. The rescue of Bat09:mH1mN1 and Bat09:mH3mN2 viruses demonstrates that the putative vRNAs of Bat09 function efficiently together and are probably derived from either one virus, or a group of compatible viruses, whose NS, M, NP, PA, PB1, and PB2 proteins efficiently replicate and package vRNAs in host cells commonly used to culture influenza viruses (Figure 21). Importantly, the data also shows that the bat HA and NA were the sole determinants inhibiting Bat09 virus rescue, and that the terminal regions of HA and NA of bat influenza viruses selected for our constructs contain cis-acting vRNA packaging signals. Although wild type bat influenza virus (Bat09) couldn't be propagated in the human, canine, mink, avian, porcine or bat cell lines we tested, consistent with Tong et al. (Tong et al., 2012), it is likely that the bat influenza virus infects some other cell cultures from other species and/or tissues, especially cells derived from appropriate bat species.

Our Bat09:mH1mN1 studies provide other unique insights, which can't be gleaned from non-infectious assays. For instance, non-infectious assays (interferon-β reporter assay, Figure 24**A**) showed the Bat09 NS1 carboxy-terminal truncationss (NS1-128 and NS1-73) were similar to the truncated PR8 NS1 (NS1-126 and NS1-73), which largely lost the ability to inhibit the host interferon response. However, mouse experiments with the replicative bat influenza viruses revealed that the truncation of Bat09 NS1 had minimal effects on the viral pathogenesis compared to the truncation of PR8 NS1 (Figure 25). Differences in the attenuating impact observed in the PR8-NS1 and the Bat09-NS1 truncated viruses suggests that Bat09 has novel molecular mechanisms that have evolved in the amino terminal portion of NS1 and/or other internal protein vRNAs to antagonize/evade the host innate immune response.

The PB2 of IAV plays important roles in replication, species specificity, transmission, and pathogenesis (Gabriel et al., 2008; Gao et al., 2009; Hatta et al., 2001; Li et al., 2005; Subbarao et al., 1993; Zhou et al., 2011). Our analysis of Bat influenza PB2 demonstrated that it is also a virulence determinant and as anticipated conversion of mammalian-signature residues at position 701 to avian-signature (N701D) attenuated the virus, and the E158G substitution enhanced virulence. PB2-627 is one of the most studied positions differentiating avian viruses (glutamic acid) and mammalian viruses (lysine) (Hatta et al., 2001; Subbarao et al., 1993). Intriguingly, the bat influenza PB2 has a serine at position 627, which is unlike mammalian or avian IAVs. Our data show that PB2-627S has intermediate polymerase activity compared to PB2-627E and PB2-627K in mammalian cells, suggesting an alternative evolutionary pathway that avian influenza viruses may be able to take for mammalian adaptation.

Reassortment of the segmented genomes of Orthomyxoviruses is a powerful evolutionary mechanism that is central to the success of these pathogens. Viruses within a Genus readily

reassort upon co-infection of a single host cell (e.g., avian and swine IAV); whereas, viruses from a different Genus (e.g., IAV and IBV) don't reassort. The factors important for generation of reassortant progeny from two parental influenza viruses include; recognition and replication of vRNAs by parental virus RdRp, protein-protein interaction/compatibility (e.g, heterotrimeric RdRp), and vRNA-protein interactions needed for virion morphogenesis. The RNA transcription/replication promoter of each influenza vRNA segment is formed by base pairing of highly conserved nucleotides at the 5′ and 3′ termini, which form a partially double-stranded structure. The IAV Genus has specific nucleotide variations within the termini that distinguish it from IBV. The termini of bat influenza vRNAs also show conserved 5' and 3' complementarity; however, they also have distinct nucleotide variation. Therefore, we used mini-genome replication studies to analyze promoter recognition and RdRp activity of various combinations of the PB1, PB2, PA subunits in combination with various NPs from IAV, IBV, or bat influenza. The data show that the wild type RdRp most efficiently replicate their cognate vRNAs, and that both IAV and IBV RdRp have 50-60% reduction in activity with the Bat influenza mini-genome. Many PB1, PB2, PA combinations between bat influenza and IAV/IBV dramatically reduce activity, which demonstrates protein-protein incompatibility between the RdRp subunits. Interestingly, the Bat influenza NP and IAV NP were very compatible in the mini-genome assay, however NP reassortant viruses could be generated (Table 10) suggesting that the vRNAs are incompatible.

IAVs of various subtypes can infect and reassort in bat cell lines (Dlugolenski et al., 2013; Hoffmann et al., 2013), providing a permissive environment for them to reassort with bat influenza viruses. However, our reassortant analysis indicates that while two divergent Bat influenzas readily reassort, Bat influenza and IAVs don't easily reassort in co-infection

experiments. Reverse genetics reassortment studies showed the PB2, PB1, PA, NP, and NS vRNAs of bat influenza don't efficiently reassort with the IAV or IBV, and provide many additional tantalizing results. For example, reassortants were not rescued from relatively compatible RdRp combinations in the mini-genome assay (e.g. Bat-PB2/PR8-PB1/PR8-PA, Fig. 8A) and demonstrate that divergent Bat09 and Bat10 can efficiently reassort with each other (Table 13). The M segment is the most highly conserved gene among influenza A and B viruses. We found that the PR8-M segment could substitute for the Bat09-M segment (Table 10), indicating that the M vRNAs/protein(s) of PR8 and Bat09 have enough conservation in both cis-acting packaging signals and functional domains of the proteins (M1/M2) to enable the replication of the modified Bat09 virus. In contrast, putative packaging signal swapping of the NP and NS segments didn't overcome reassortment defects suggesting that protein level incompatibility is likely to be a critical factor inhibiting reassortment between the bat influenza and other influenza viruses. Alternatively, one could argue that that since the vRNA packaging signals of Bat influenza NP and NS segments have not been delineated; therefore, the putative packaging regions incorporated in the Batps-PR8 constructs may not be sufficient for packaging the modified vRNAs. However, the well-defined PR8 packaging signals incorporated in our modified gene segments should be sufficient to package the corresponding bat influenza NP and NS vRNAs (PR8ps-Bat-NP and PR8ns-Bat-NS, Figure 18 D) in the PR8 backbone. The failure to rescue the PR8ps-Bat NP or NS viruses, as well as the PR8:Bat-M reassortant virus, strongly suggests protein-vRNA level incompatibility and provides a unique opportunity to better understand the functional domains of these proteins through characterizing chimeric/mosaic proteins containing motifs/domains from both viruses.

Another caveat with our bat influenza reassortment experiments is the focus on interactions with the laboratory adapted PR8 virus, which was chosen primarily due to biosafety concerns. Reassortment between the Bat09:mH1mN1 virus and other IAVs, particularly avian viruses (e.g., H5N1, H7N9) that appear to be more compatible in the mini-genome assay (Figure 28), are needed to fully assess reassortment potential of bat influenza. However, based on our results from the NP reassortment and the Bat-PB2/PR8-PB1/PR8-PA reassortment experiments (Table 9, Table 10), the likelihood of rescuing a reassortant with RdRp components from both Bat and IAVs is very low. Finally, since the HAs and NAs of the bat influenza viruses can't be used to rescue viruses using contemporary influenza virus host substrates, we were not able to assess the ability of the HA or NA to reassort with other influenza viruses. Therefore, the known bat influenza viruses (Bat09, Bat10) could pose a pandemic threat if their HA and NA acquire mutations that impart binding to canonical influenza virus receptors and rescuing the NA for neuraminidase activity, or acquisition of binding and entry through alternative human cell surface receptors.

Collectively, our experiments suggest that the bat influenza virus is unlikely to reassort with an IAV or IBV and spread to other species even if they were to infect the same host cell. The restriction on reassortment appears to result from multiple levels of incompatibility (RNA-RNA, RNA-protein, or protein-protein) that are either additive or synergistic. Consequently, our data suggest that due to the extremely limited ability of genetic information exchange between bat influenza and IAV or IBV, the International Committee on Taxonomy of Viruses should consider classifying these two bat influenza virus lineages as a new Genus within the *Orthomyxoviridae*.

This study also demonstrated the power of synthetic genomics in rapid characterization and risk assessment of an emerging virus, even when the virus itself is not readily cultured. The synthetic genomics/reverse genetics strategy employed provides an infinite supply of wild type bat influenza particles that can be used to identify permissive cells or animals. The availability of our modified bat influenza virus, opens many other avenues of investigation and discovery. For instance, to gain a better understanding of cis-acting signals in the vRNAs that are important in bat influenza transcription, replication, packaging/particle morphogenesis, and using forward genetics to elucidate viral protein-protein and/or viral protein-host protein interactions.

4.5 Conclusion

Taken together, our study suggested that the newly discovered bat influenza viruses may have less chance reassort with IAVs and IBVs which pose less threat to public health. Continued study of bat influenza viruses and integration of data from other contemporary influenza viruses is important in the elucidation of the evolutionary history of influenza viruses.

Figure 17 Synthetic generation of the eight full-length genomic segments of A/little yellow-shouldered bat/Guatemala/164/2009 (Bat09)

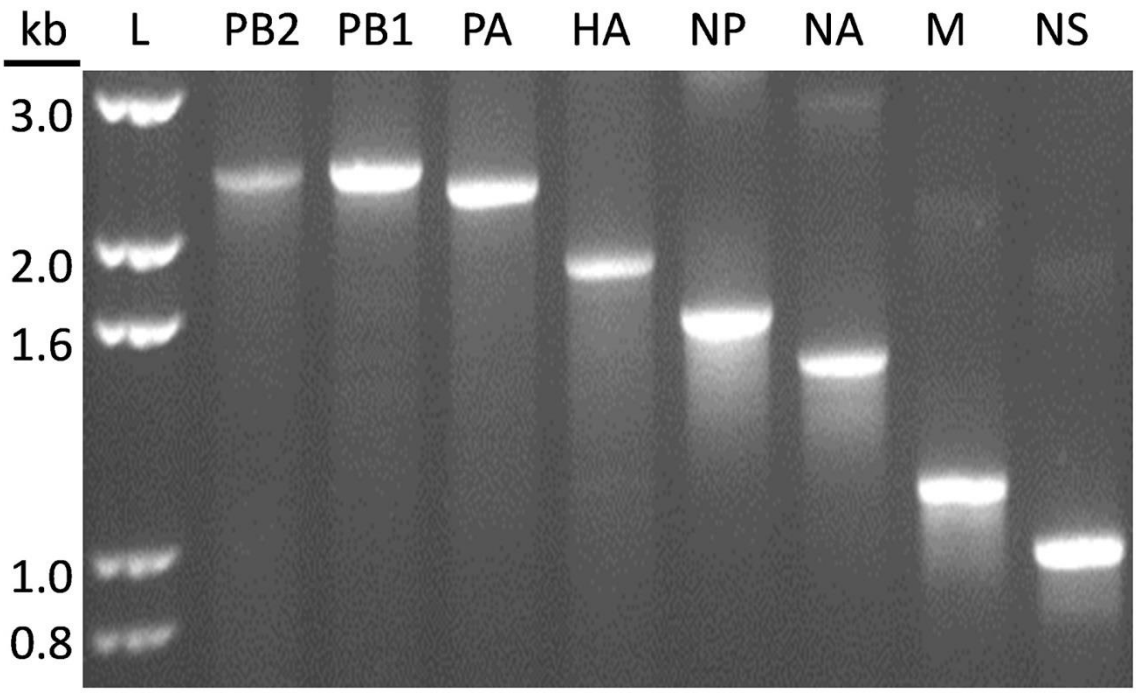

Figure 17 The products were assembled from oligonucleotides and error corrected. L: 1 Kb Plus DNA ladder from Life Technologies. doi:10.1371/journal.ppat.1004420.s001

Figure 18 Diagrams of select constructs and viruses used in this study

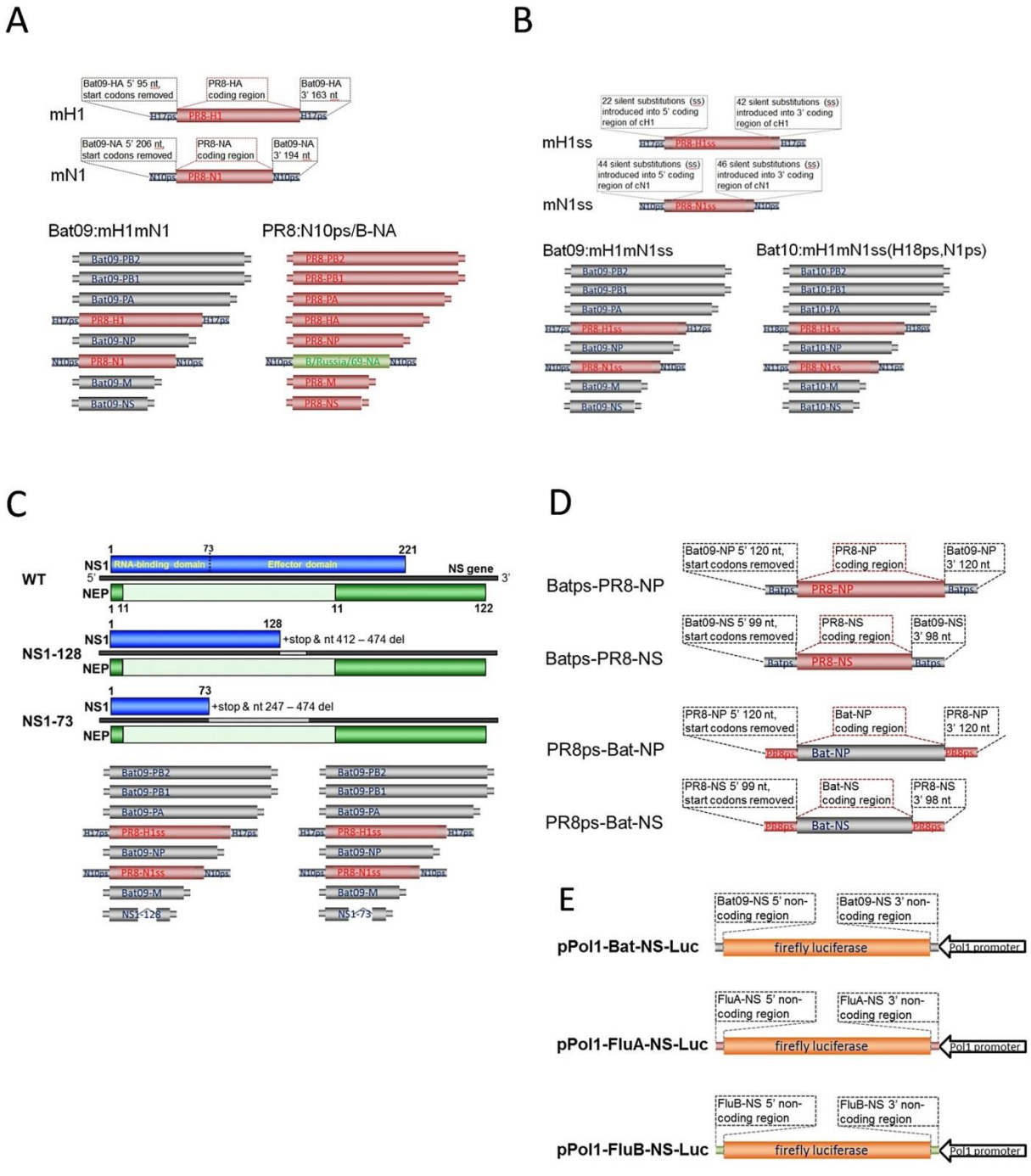

Figure 18 All constructs shown are in cDNA sense complementary to viral RNA. (**A**) Modified HA (mH1) and modified NA (mN1). To construct the mH1, PR8-HA coding region was flanked by the putative packaging regions from Bat09-HA and all ATG in the Bat09-HA 5′ packaging region were mutated. To construct the mN1, PR8-NA coding region was flanked by the putative packaging regions from Bat09-NA and all ATG in the Bat09-NA 5′ packaging region were mutated. Batps-B/NA was constructed similarly with the packaging regions from Bat09-NA and the coding region from B/Russia/1969-NA. (**B**) mH1ss was constructed by introducing 64 of silent

substitutions into the coding region of mH1 to disrupt the remaining packaging signals in the PR8-HA coding region. mN1ss was constructed by introducing 90 of silent substitutions into the coding region of mN1 to disrupt the remaining packaging signals in the PR8-NA coding region. The mH1ss was referred as H17ps-H1ss and the mN1ss was referred as N10ps-N1ss in Table 5. The H18ps-H1ss and N11ps-N1ss have the HA and NA packaging regions from Bat10. (**C**) The wild type NS gene and the NS1 truncated NS gene from Bat09. NS1 truncated PR8-NS genes were constructed similarly. (**D**) Bat09 NP and NS coding regions flanked by putative cis-acting packaging regions from PR8 NP and NS. PR8 NP and NS coding regions flanked by putative cis-acting packaging regions from Bat NP and NS. (**E**) The pPol1-Bat-NS-Luc, pPol1-FluA-NS-Luc, and pPol1-FluB-NS-Luc reporter genes. doi:10.1371/journal.ppat.1004420.s002

Figure 19 Compatibility between RNPs and viral RNA promoters from different viruses

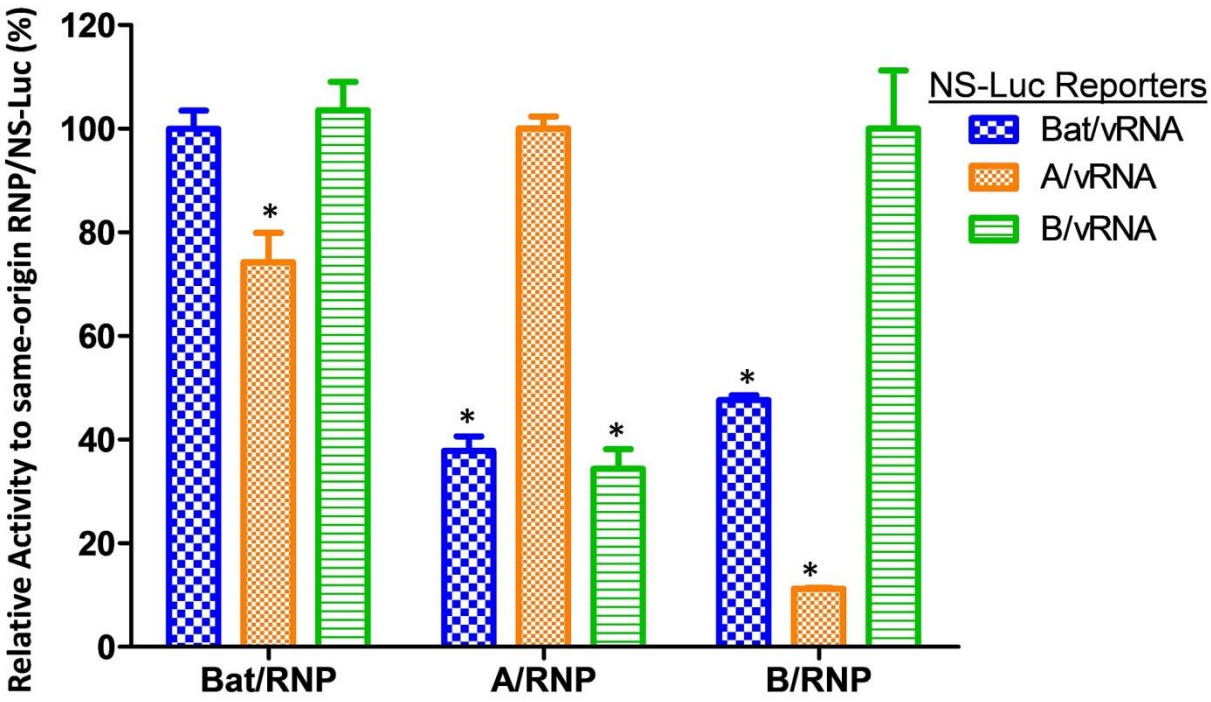

Figure 19Left, RNP from Bat09 and luciferase reporter flanked by NS non-coding regions from bat influenza virus, IAV, and IBV. Middle, RNP from influenza A and the three luciferase reporters. Right, RNP from IBV and the three luciferase reporters. Within each group of RNP, * indicates $P<0.05$, compared to the vRNA reporter from the same type of virus as the RNP. doi:10.1371/journal.ppat.1004420.s003

Figure 20 Sequence alignment of PR8-HA, mH1, mH1ss and PR8-NA, mN1, mN1ss

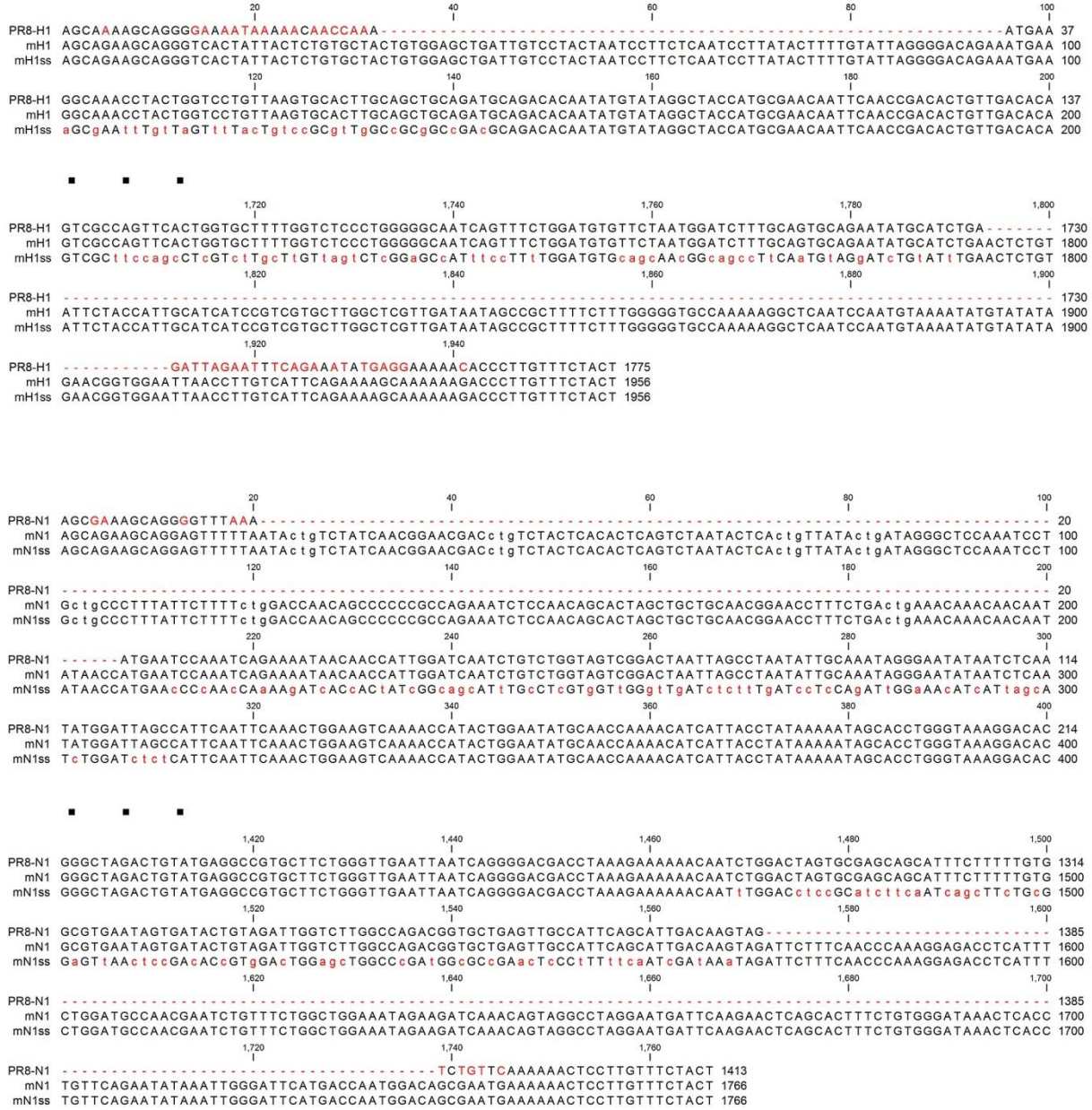

Table 7 Rescue result of reassortment between Bat09 and PR8

	PB2	PB1	PA	HA	NP	NA	M	NS	Rescue*
1	Bat	Bat	Bat	Bat	Bat	Bat	Bat	Bat	Neg
2	PR8	Bat	Bat	Bat	Bat	Bat	Bat	Bat	Neg
3	Bat	PR8	Bat	Bat	Bat	Bat	Bat	Bat	Neg
4	Bat	Bat	PR8	Bat	Bat	Bat	Bat	Bat	Neg
5	Bat	Bat	Bat	PR8	Bat	Bat	Bat	Bat	Neg
6	Bat	Bat	Bat	Bat	PR8	Bat	Bat	Bat	Neg
7	Bat	Bat	Bat	Bat	Bat	PR8	Bat	Bat	Neg
8	Bat	Bat	Bat	Bat	Bat	Bat	PR8	Bat	Neg
9	Bat	Bat	Bat	Bat	Bat	Bat	Bat	PR8	Neg
10	Bat	Bat	Bat	PR8	Bat	PR8	Bat	Bat	Neg
11	Bat	Bat	Bat	Bat	Bat	Bat	PR8	PR8	Neg
12	PR8	PR8	PR8	Bat	Bat	Bat	Bat	Bat	Neg
13	Bat	Bat	Bat	Bat	PR8	Bat	PR8	PR8	Neg
14	PR8	PR8	PR8	Bat	PR8	Bat	Bat	Bat	Neg
15	PR8	PR8	PR8	Bat	PR8	Bat	PR8	PR8	Neg
16	PR8	PR8	PR8	PR8	PR8	PR8	PR8	PR8	++++
17	Bat	PR8	PR8	PR8	PR8	PR8	PR8	PR8	Neg
18	PR8	Bat	PR8	PR8	PR8	PR8	PR8	PR8	Neg
19	PR8	PR8	Bat	PR8	PR8	PR8	PR8	PR8	Neg
20	PR8	PR8	PR8	Bat	PR8	PR8	PR8	PR8	Neg
21	PR8	PR8	PR8	PR8	Bat	PR8	PR8	PR8	Neg
22	PR8	PR8	PR8	PR8	PR8	Bat	PR8	PR8	Neg**
23	PR8	PR8	PR8	PR8	PR8	PR8	Bat	PR8	Neg
24	PR8	PR8	PR8	PR8	PR8	PR8	PR8	Bat	Neg
25	PR8	PR8	PR8	Bat	PR8	Bat	PR8	PR8	Neg
26	PR8	PR8	PR8	PR8	PR8	PR8	Bat	Bat	Neg
27	Bat	Bat	Bat	PR8	PR8	PR8	PR8	PR8	Neg
28	PR8	PR8	PR8	PR8	Bat	PR8	Bat	Bat	Neg
29	Bat	Bat	Bat	PR8	Bat	PR8	PR8	PR8	Neg

* Rescue efficiency definition described in Table 1.

** This virus can occasionally be rescued, and mutations were found in PR8-HA, which compensates for the lack of neuraminidase activity from the Bat09-NA. The virus lost the Bat09-NA in a few passages.

Table 8 Co-infection results for reassortment between Bat09:mH1mN1 and Bat10:mH1mN1

	PB2	PB1	PA	NP	M	NS	HA	NA	No. of Plaques*
1	Bat09	Bat09	Bat09	Bat09	Bat09	Bat09	H17ps-H1	N10ps-N1	39
2	Bat10	Bat09	Bat10	Bat09	Bat10	Bat09	H17ps-H1	N10ps-N1	1
3	Bat10	Bat09	Bat10	Bat09	Bat10	Bat10	H18ps-H1	N10ps-N1	1
4	Bat10	Bat10	Bat10	Bat09	Bat10	Bat10	H17ps-H1	N10ps-N1	2
5	Bat10	Bat10	Bat10	Bat10	Bat09	Bat09	H18ps-H1	N10ps-N1	1
6	Bat09	Bat10	Bat10	Bat10	Bat10	Bat09	H17ps-H1	N11ps-N1	1
7	Bat10	Bat10	Bat10	Bat10	Bat10	Bat10	H17ps-H1	N10ps-N1	1
8	Bat10	Bat10	Bat10	Bat10	Bat10	Bat10	H18ps-H1	N10ps-N1	1
9	Bat10	Bat10	Bat10	Bat10	Bat10	Bat10	H17ps-H1	N11ps-N1	1
10	Bat10	Bat10	Bat10	Bat09	Bat10	Bat10	H18ps-H1	N11ps-N1	2
11	Bat10	Bat10	Bat10	Bat10	Bat10	Bat09	H18ps-H1	N11ps-N1	13
12	Bat10	Bat10	Bat10	Bat10	Bat10	Bat10	H18ps-H1	N11ps-N1	45

* Totally 108 plaques were purifies for genotyping.

Figure 21 Generation of viruses relevant to Bat09

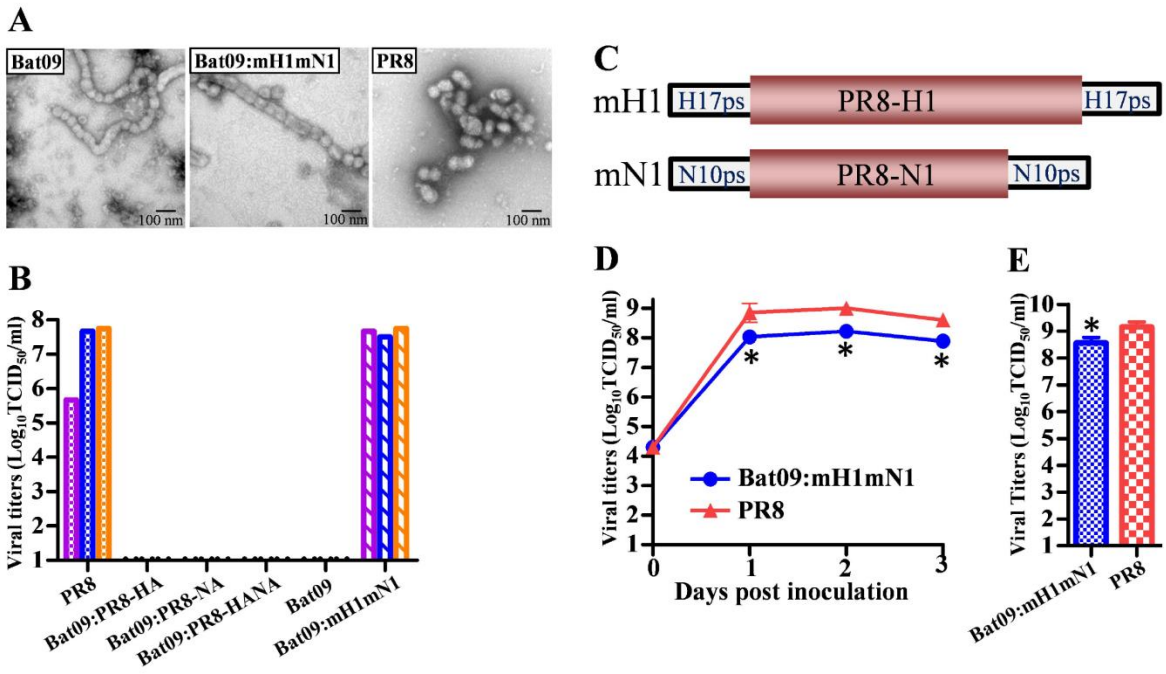

Figure 21 (A) EM picture of Bat09 (left), Bat09:mH1mN1 (middle), and PR8 (Benfield et al.). (B) Viral titers in transfection supernatants of Bat09 and PR8-HA/NA reassortants, and Bat09:mH1mN1. Each bar represents an independent rescue experiment. (C) The mH1 contains PR8-HA coding region and Bat09-HA packaging region (start codon removed). The mN1 was constructed using the same strategy. (See Fig. S2A for details.) (D) Bat09:mH1mN1 and PR8 replication kinetics in MDCK cells. (E) Peak titers of the viruses in embryonated chicken eggs. *, $P < 0.05$, compared to PR8. doi:10.1371/journal.ppat.1004420.g001

Figure 22 Pathogenicity of Bat09:mH1mN1 and PR8 viruses in mice

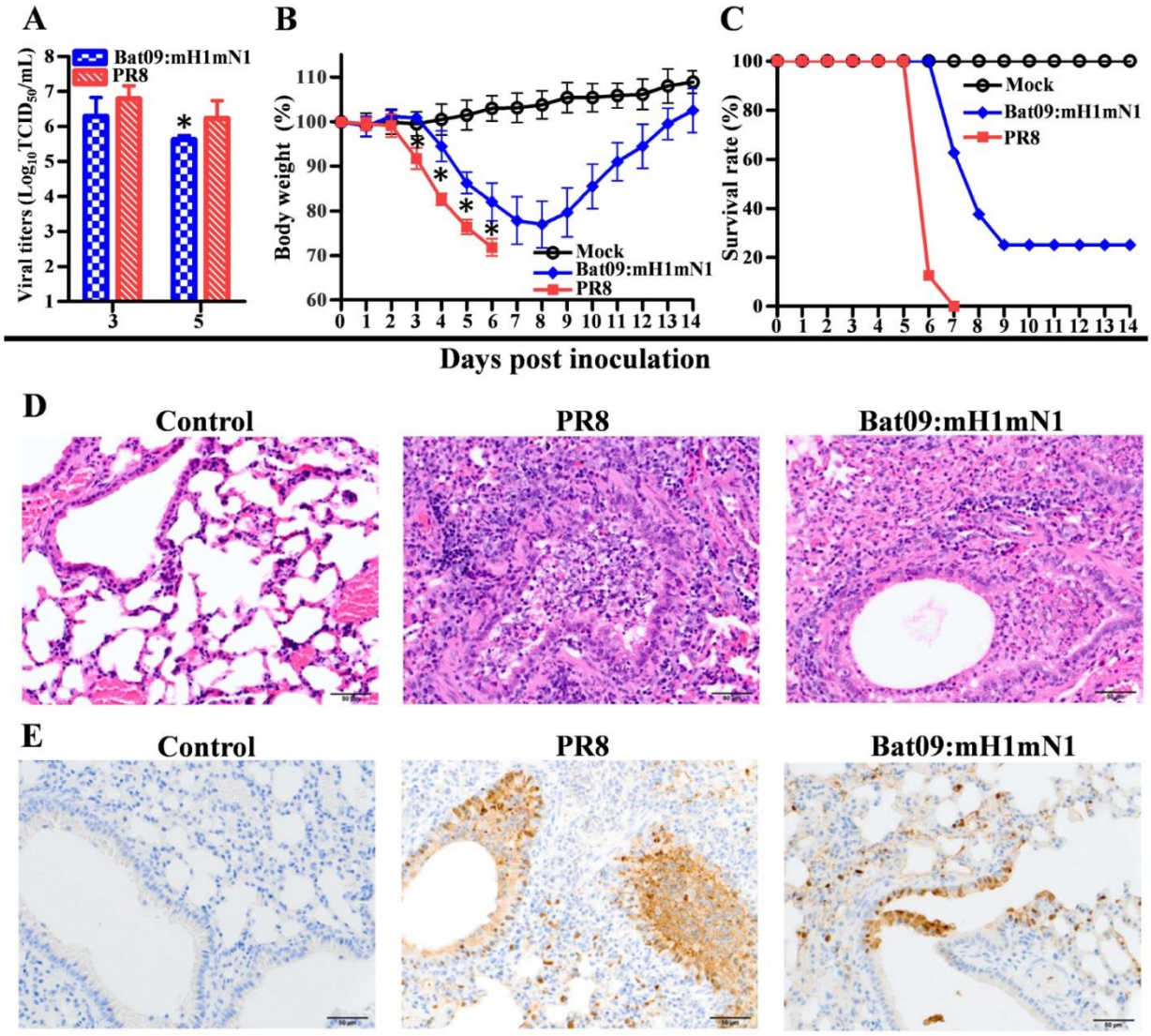

Figure 22 (A) Virus titers of Bat09:mH1mN1 and PR8 in mouse lungs at 3 and 5 dpi. Each mouse was intranasally inoculated with 10^3 $TCID_{50}$ of each virus. (B) Mouse weight on each day post inoculation was represented as a percentage of the weight on day 0 (100%). Each mouse was intranasally inoculated with 10^4 $TCID_{50}$ of Bat09:mH1mN1 or PR8. (C) Survival rate of the mice inoculated with 10^4 $TCID_{50}$ of virus. (D) H&E staining of microscopic lung sections from mice inoculated with 10^3 $TCID_{50}$ of virus at 5 dpi. (E) IHC staining of lung sections at 5 dpi. *, $P < 0.05$, Bat09:mH1mN1 compared to PR8. doi:10.1371/journal.ppat.1004420.g002

Figure 23 Diagram of the Bat09:mH3mN2 construct, and pathogenicity of Bat09:mH3mN2 and TX98 viruses in mice

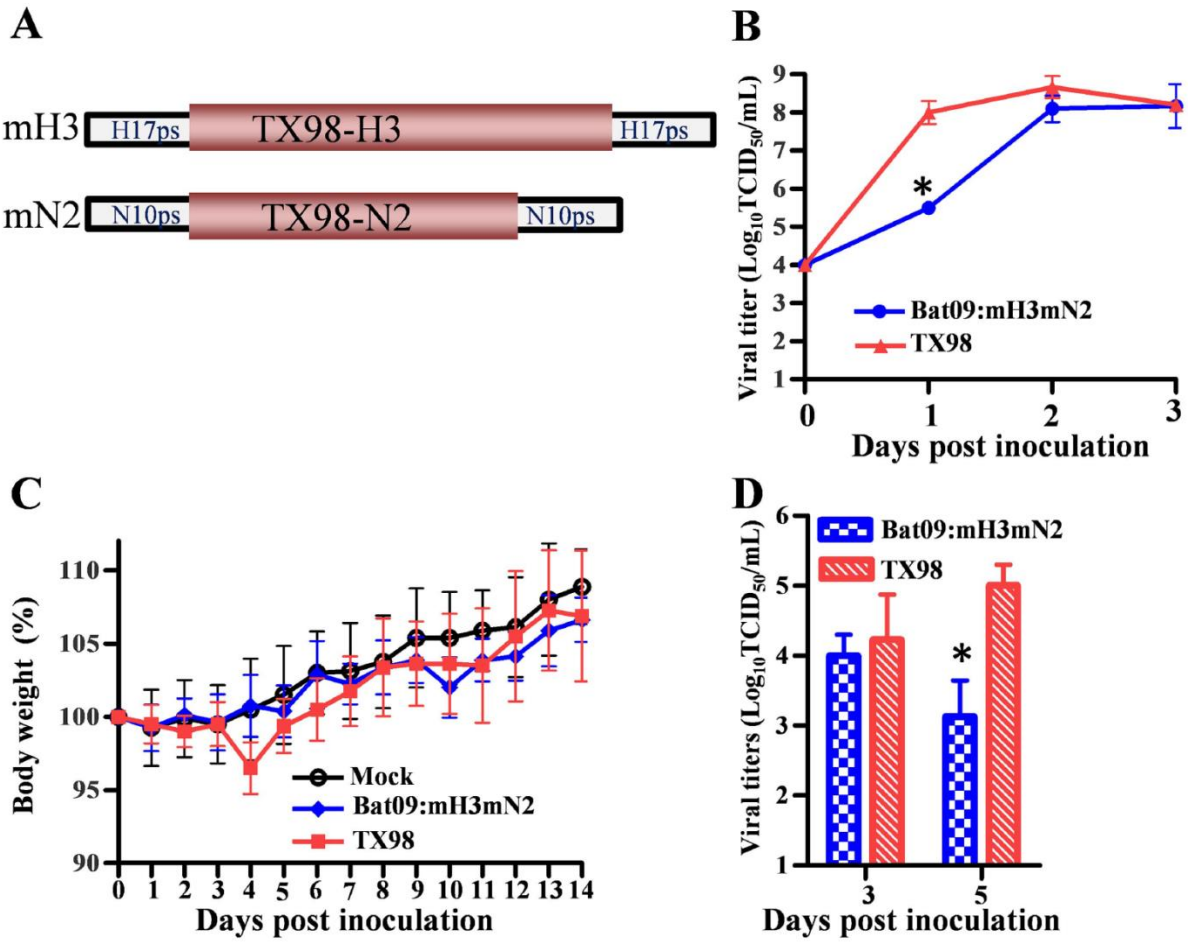

Figure 23 (A) Modified HA (mH3) and modified NA (mN2). To construct the mH3, TX98-HA coding region was flanked by the putative packaging regions from Bat09-HA and all potential start codons in the Bat09-HA 5' packaging region were mutated. To construct the mN1, PR8-NA coding region was flanked by the putative packaging regions from Bat09-NA and all start codons in the Bat09-NA 5' packaging region were mutated. (B) Bat09:mH3mN2 and TX98 replication kinetics in MDCK cells. MDCK cells were inoculated at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell with the Bat09:mH3mN2 or TX98 viruses. (C) Weight loss of mice mock-infected or infected with Bat09:mH3mN2 or TX98 viruses. Each mouse was intranasally inoculated with 3×10^5 $TCID_{50}$ of each virus. (D) Virus titers of Bat09:mH3mN2 and TX98 viruses in mouse lungs at 3 and 5 dpi. Each mouse was intranasally inoculated with 3×10^4 $TCID_{50}$ of each virus. *, $P < 0.05$, Bat09:mH3mN2 compared to TX98. doi:10.1371/journal.ppat.1004420.g003

Figure 24 *In vitro* property of Bat-NS1 mutants

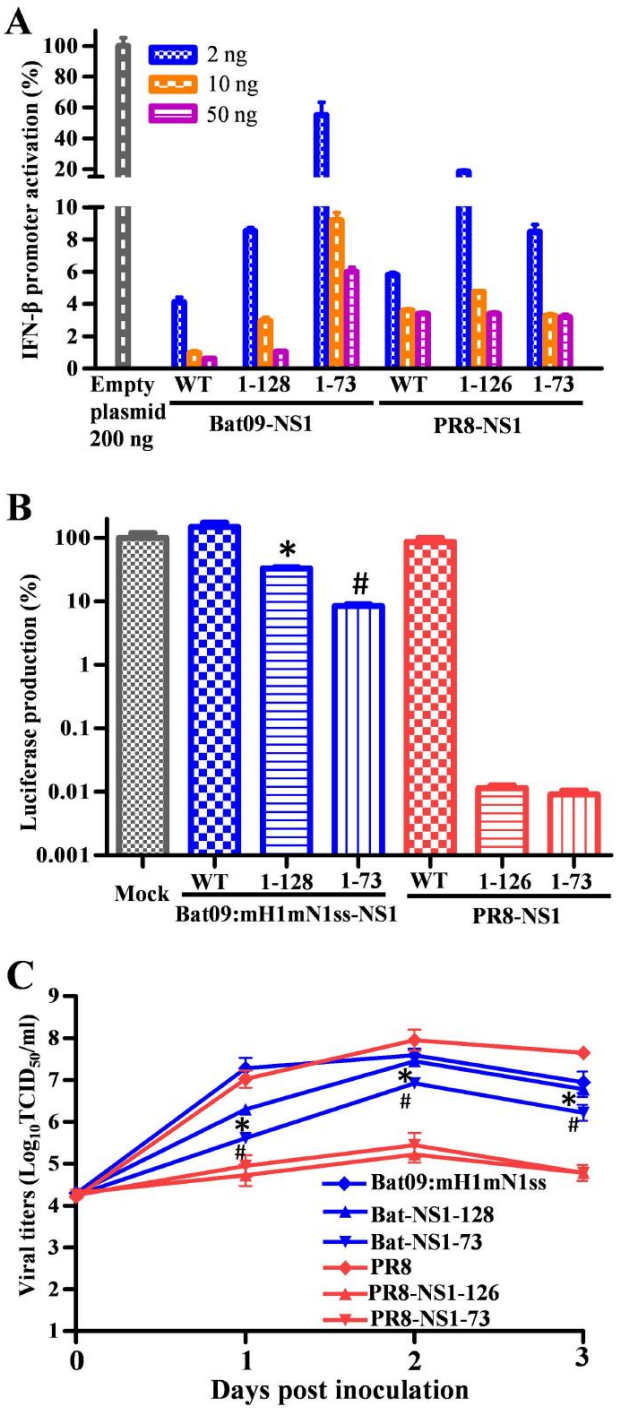

Figure 24 (A) Luciferase reporter mediated assay to quantitate the NS1 protein inhibition effects on interferon-b promoter activation. (B) VSV-luciferase mediated bioassay to quantitate the inhibitory effects on VSV virus infection resulted from the immune response induced by the different NS1 WT or truncated viruses. (C) Virus replication kinetics in human lung epithelial Calu-3 cells. * or #, P,0.05, Bat-NS1-128 compared to PR8-NS1-126 (*) and Bat-NS1-73 compared to PR8-NS1-73 (#) are shown in (B) and (C). doi:10.1371/journal.ppat.1004420.g004

Figure 25 Pathogenicity of Bat-NS1 mutants in mice

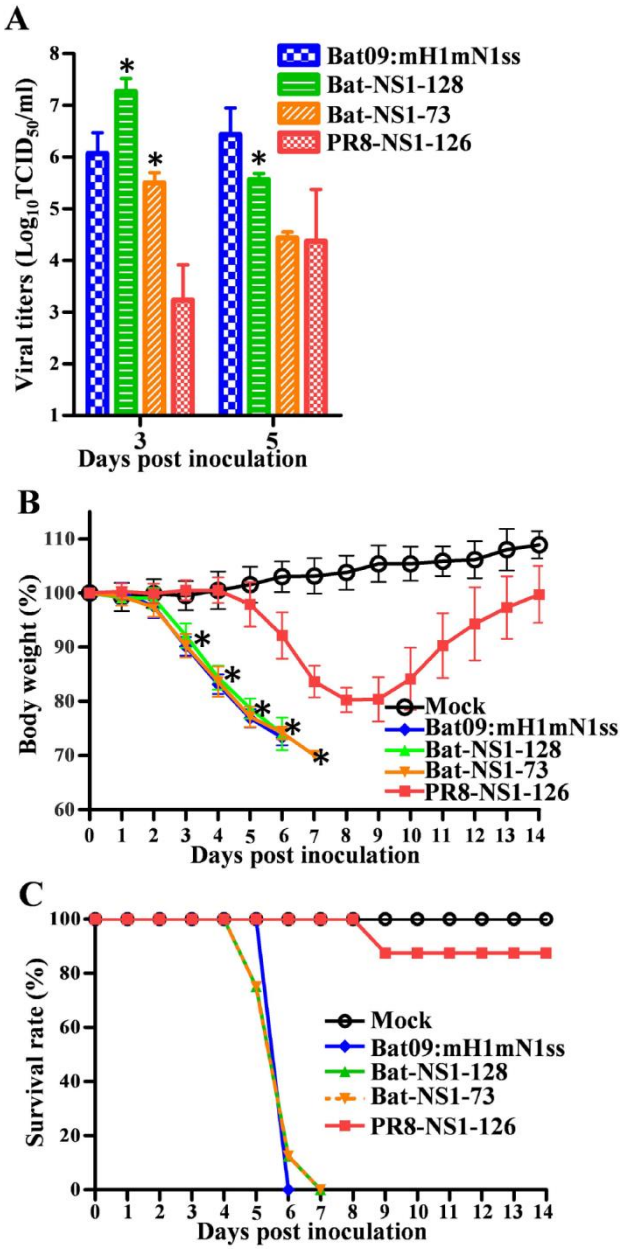

Figure 25 (A) Virus titers of Bat09:mH1mN1ss and NS1 mutants in mouse lungs at 3 and 5 dpi. Each mouse was intranasally inoculated with 104 TCID50 of each virus. (B) Mouse weight on each day post inoculation was represented as a percentage of the weight on day 0 (100%). Each mouse was intranasally inoculated with 105 TCID50 of the indicated viruses. (C) Survival rate of the mice inoculated with 105 TCID50 of the viruses. Higher virus doses were used in this experiment based on the PR8-NS1-126 control virus, which caused significant weight loss but had low mortality at 105 TCID50 so the attenuation of Bat-NS1 truncated viruses can be appropriately compared to it. *, P,0.05, truncated Bat09:mH1mN1ss-128 and Bat09:mH1mN1ss-73 compared to PR8-NS1-126. doi:10.1371/journal.ppat.1004420.g005

Figure 26 Pathogenicity of Bat-PB2 mutants in mice

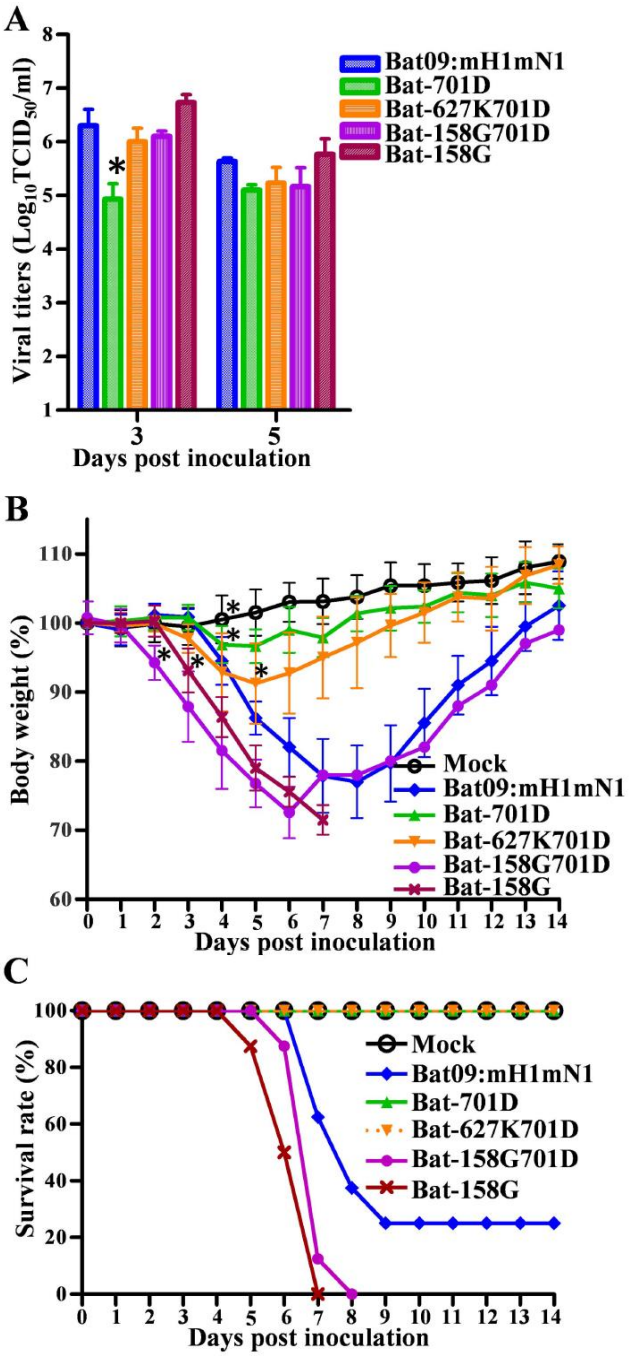

Figure 26 (A) Virus titers of Bat09:mH1mN1 and PB2 mutants in mouse lungs at 3 and 5 dpi. Each mouse was intranasally inoculated with 10^3 $TCID_{50}$ of each virus. (B) Mouse weight on each day post inoculation was represented as a percentage of the weight on day 0 (100%). Each mouse was intranasally inoculated with 10^4 $TCID_{50}$ of the indicated viruses. (C) Survival rate of the mice inoculated with 10^4 $TCID_{50}$ of the viruses. *, $P < 0.05$, PB2 mutants compared to Bat09:mH1mN1. For mouse body weight, * is only marked on the first day of each group that is significantly different from Bat09:mH1mN1. doi:10.1371/journal.ppat.1004420.g006

Figure 27 Polymerase activity of Bat09 with wild type and mutant PB2

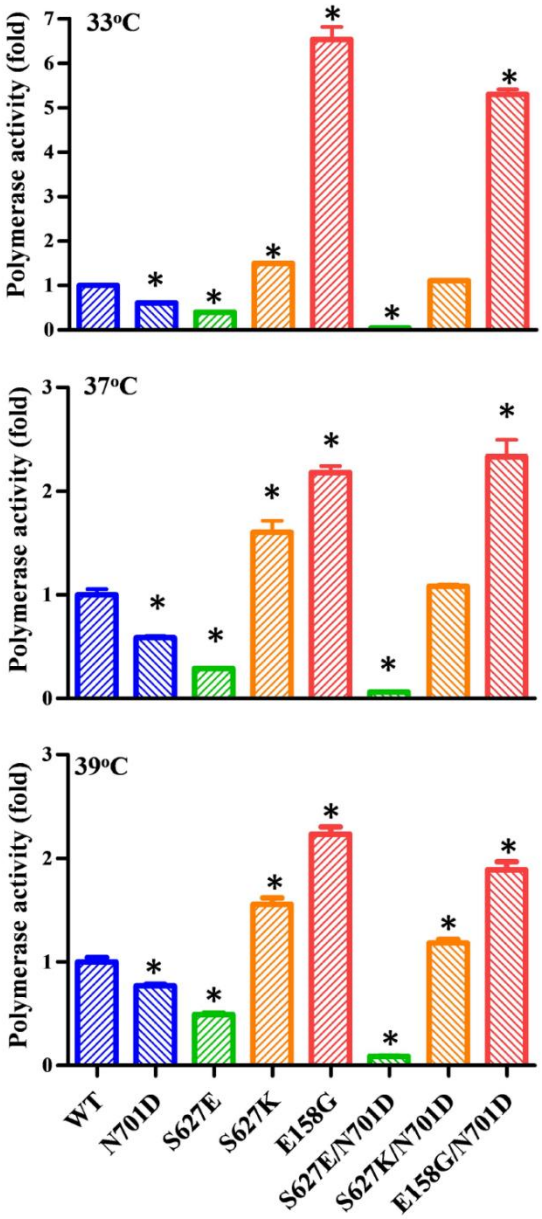

Figure 27 A luciferase mediated mini-genome replication assay was performed at 33uC, 37uC, and 39uC by co-transfecting 293T cells with Bat09 PB2 (WT or mutant), PB1, PA, NP, and a vRNA-like luciferase reporter. Relative luciferase activity were determined to represent the viral polymerase activity. *, $P < 0.05$, compared to WT. doi:10.1371/journal.ppat.1004420.g007

Figure 28 Compatibility of the PB2, PB1, PA and NP originated from Bat09 and other influenza viruses.

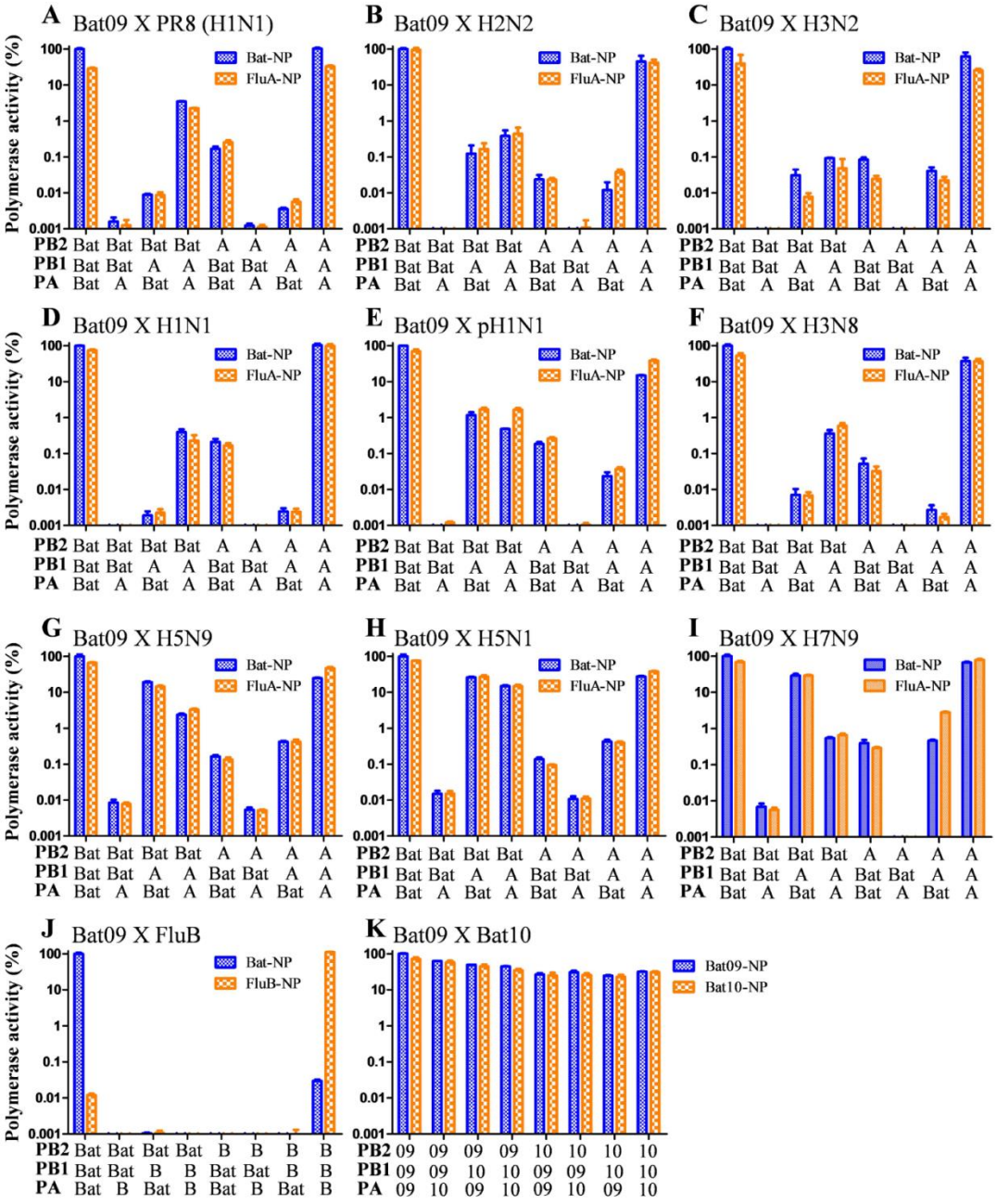

Figure 28 A mini-genome replication assay was used to determine the compatibility of the different RdRp components. (A–I) PB2, PB1, PA, and NP from Bat09 and various influenza A viruses as indicated. (J) PB2, PB1, PA and NP from Bat09 and B/Russia/69. (K) PB2, PB1, PA and NP from Bat09 and Bat10. The vRNA reporters used for the compatibility test between Bat09 and IAVs (Fig. 8A–I) were an equal ratio of pPolI-Bat-NS-Luc and pPolI-FluA-NS-Luc. For compatibility test between Bat09 and IBV (Fig. 8J) the vRNA reporters used were pPolI-Bat-NS-Luc and pPolI-FluB-NS-Luc. For compatibility test between Bat09 and Bat10 (Fig. 8K) only the pPolI-Bat-NS-Luc plasmid was used. doi:10.1371/journal.ppat.1004420.g008

Table 9 Rescue efficiency of PB2, PB1, PA reassortants between Bat09:mH1mN1 and PR8

	PB2	PB1	PA	NP,M,NS	HA,NA	Rescue*
1	Bat	Bat	Bat	Bat	mH1,mN1	++++
2	PR8	Bat	Bat	Bat	mH1,mN1	Neg
3	Bat	PR8	Bat	Bat	mH1,mN1	Neg
4	Bat	Bat	PR8	Bat	mH1,mN1	Neg
5	PR8	PR8	Bat	Bat	mH1,mN1	Neg
6	PR8	Bat	PR8	Bat	mH1,mN1	Neg
7	Bat	PR8	PR8	Bat	mH1,mN1	Neg
8	PR8	PR8	PR8	Bat	mH1,mN1	Neg
9	Bat	Bat	Bat	PR8	PR8	Neg
10	PR8	Bat	Bat	PR8	PR8	Neg
11	Bat	PR8	Bat	PR8	PR8	Neg
12	Bat	Bat	PR8	PR8	PR8	Neg
13	PR8	PR8	Bat	PR8	PR8	Neg
14	PR8	Bat	PR8	PR8	PR8	Neg
15	Bat	PR8	PR8	PR8	PR8	Neg
16	PR8	PR8	PR8	PR8	PR8	++++

* Rescue efficiency definition.
Very easy (++++): P0 viral titer 10^6-10^8 $TCID_{50}$/ml, or severe CPE observed in P1 within 1 dpi;
Moderate (+++):P0 titer 10^4-10^6 $TCID_{50}$/ml, or obvious CPE observed in P1 within 2 dpi;
Difficult (++):P0 titer 10^2-10^4 $TCID_{50}$/ml, or weak CPE observed in P1 within 4 dpi;
Very difficult (+): P0 titer lower than 10^2 $TCID_{50}$/ml, or CPE not observed until P2/P3;
Negative (Neg): rescue failed, no CPE observed through passage 3.
For each combination, the rescue was repeated at least 3 times.

Table 10 Rescue efficiency of internal gene reassortants between Bat09:mH1mN1 and PR8

	Pols*	NP	M	NS	HA,NA	Rescue**
1	Bat	Bat	Bat	Bat	mH1,mN1	++++
2	Bat	Bat	Bat	PR8	mH1,mN1	Neg
3	Bat	Bat	PR8	Bat	mH1,mN1	+++
4	Bat	Bat	PR8	PR8	mH1,mN1	Neg
5	Bat	PR8	Bat	Bat	mH1,mN1	Neg
6	Bat	PR8	Bat	PR8	mH1,mN1	Neg
7	Bat	PR8	PR8	Bat	mH1,mN1	Neg
8	Bat	PR8	PR8	PR8	mH1,mN1	Neg
9	PR8	Bat	Bat	Bat	mH1,mN1	Neg
10	PR8	Bat	Bat	PR8	mH1,mN1	Neg
11	PR8	Bat	PR8	Bat	mH1,mN1	Neg
12	PR8	Bat	PR8	PR8	mH1,mN1	Neg
13	PR8	PR8	Bat	Bat	mH1,mN1	Neg
14	PR8	PR8	Bat	PR8	mH1,mN1	Neg
15	PR8	PR8	PR8	Bat	mH1,mN1	Neg
16	PR8	PR8	PR8	PR8	mH1,mN1	Neg
17	Bat	Bat	Bat	Bat	PR8	Neg
18	Bat	Bat	Bat	PR8	PR8	Neg
19	Bat	Bat	PR8	Bat	PR8	Neg
20	Bat	Bat	PR8	PR8	PR8	Neg
21	Bat	PR8	Bat	Bat	PR8	Neg
22	Bat	PR8	Bat	PR8	PR8	Neg
23	Bat	PR8	PR8	Bat	PR8	Neg
24	Bat	PR8	PR8	PR8	PR8	Neg
25	PR8	Bat	Bat	Bat	PR8	Neg
26	PR8	Bat	Bat	PR8	PR8	Neg
27	PR8	Bat	PR8	Bat	PR8	Neg
28	PR8	Bat	PR8	PR8	PR8	Neg
29	PR8	PR8	Bat	Bat	PR8	Neg
30	PR8	PR8	Bat	PR8	PR8	Neg
31	PR8	PR8	PR8	Bat	PR8	Neg
32	PR8	PR8	PR8	PR8	PR8	++++

* Pols = Co-transfection of PB1, PB2, and PA reverse genetics plasmids.

** Rescue efficiency defined in Table 1. For each combination, the rescue was repeated at least 3 times.

Table 11 Rescue efficiency of reassortants with NP and NS containing modified packaging signals

	PB2	PB1	PA	NP	M	NS	HA,NA	Rescue*
1	Bat	Bat	Bat	Bat	Bat	Bat	mH1,mN1	++++
2	Bat	Bat	Bat	Batps-PR8-NP	Bat	Bat	mH1,mN1	Neg
3	Bat	Bat	Bat	Bat	Bat	Batps-PR8-NS	mH1,mN1	Neg
4	Bat	Bat	Bat	Batps-PR8-NP	Bat	Batps-PR8-NS	mH1,mN1	Neg
5	Bat	Bat	Bat	PR8ps-Bat-NP	Bat	Bat	mH1,mN1	Neg
6	Bat	Bat	Bat	Bat	Bat	PR8ps-Bat-NS	mH1,mN1	+
7	PR8	PR8	PR8	PR8ps-Bat-NP	PR8	PR8	PR8	Neg
8	PR8	PR8	PR8	PR8	PR8	PR8ps-Bat-NS	PR8	Neg
9	PR8	PR8	PR8	PR8ps-Bat-NP	PR8	PR8ps-Bat-NS	PR8	Neg
10	PR8	PR8	PR8	Batps-PR8-NP	PR8	PR8	PR8	Neg
11	PR8	PR8	PR8	PR8	PR8	Batps-PR8-NS	PR8	+++
12	PR8	PR8	PR8	PR8	PR8	PR8	PR8	++++

* Rescue efficiency defined in Table 1.

For each combination, the rescue was repeated at least 3 times.

Table 12 Rescue efficiency of reassortants containing HA and NA packaging single from other viruses

	Internal	HA	NA	Rescue*
1	Bat	mH1	mN1	++++
2	Bat	PR8	mN1	++
3	Bat	mH1	PR8	+
4	Bat	PR8	PR8	Neg
5	PR8	PR8	PR8	++++
6	PR8	mH1	PR8	Neg
7	PR8	PR8	mN1	+++
8	PR8	mH1	mN1	Neg
9	PR8	mH1ss	PR8	Neg
10	PR8	PR8	mN1ss	+++
11	PR8	mH1ss	mN1ss	Neg
12	PR8	PR8	Batps/B-NA	+++

*Rescue efficiency definition described in Table 1.
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* Rescue efficiency defined in Table 1.

Table 13 Rescue efficiency of reassortants between Bat09:mH1mN1ss and Bat10:mH1mH1ss

Table 5. Rescue efficiency of reassortants between Bat09:mH1mN1ss and Bat10:mH1mH1ss.

	PB2	PB1	PA	NP	M	NS	HA	NA	Rescue*
1	Bat09	Bat09	Bat09	Bat09	Bat09	Bat09	H17ps-H1ss	N10ps-N1ss	++++
2	Bat10	Bat09	Bat09	Bat09	Bat09	Bat09	H17ps-H1ss	N10ps-N1ss	+++
3	Bat09	Bat10	Bat09	Bat09	Bat09	Bat09	H17ps-H1ss	N10ps-N1ss	++
4	Bat09	Bat09	Bat10	Bat09	Bat09	Bat09	H17ps-H1ss	N10ps-N1ss	+
5	Bat09	Bat09	Bat09	Bat10	Bat09	Bat09	H17ps-H1ss	N10ps-N1ss	+++
6	Bat09	Bat09	Bat09	Bat09	Bat10	Bat09	H17ps-H1ss	N10ps-N1ss	++++
7	Bat09	Bat09	Bat09	Bat09	Bat09	Bat10	H17ps-H1ss	N10ps-N1ss	++++
8	Bat09	Bat09	Bat09	Bat09	Bat09	Bat09	H18ps-H1ss	N10ps-N1ss	++++
9	Bat09	Bat09	Bat09	Bat09	Bat09	Bat09	H17ps-H1ss	N11ps-N1ss	++++
10	Bat09	Bat09	Bat09	Bat09	Bat09	Bat09	H18ps-H1ss	N11ps-N1ss	+++
11	Bat10	Bat10	Bat10	Bat10	Bat10	Bat10	H18ps-H1ss	N11ps-N1ss	++++
12	Bat09	Bat10	Bat10	Bat10	Bat10	Bat10	H18ps-H1ss	N11ps-N1ss	+
13	Bat10	Bat09	Bat10	Bat10	Bat10	Bat10	H18ps-H1ss	N11ps-N1ss	+++
14	Bat10	Bat10	Bat09	Bat10	Bat10	Bat10	H18ps-H1ss	N11ps-N1ss	++
15	Bat10	Bat10	Bat10	Bat09	Bat10	Bat10	H18ps-H1ss	N11ps-N1ss	+++
16	Bat10	Bat10	Bat10	Bat10	Bat09	Bat10	H18ps-H1ss	N11ps-N1ss	+++
17	Bat10	Bat10	Bat10	Bat10	Bat10	Bat09	H18ps-H1ss	N11ps-N1ss	++++
18	Bat10	Bat10	Bat10	Bat10	Bat10	Bat10	H17ps-H1ss	N11ps-N1ss	+++
19	Bat10	Bat10	Bat10	Bat10	Bat10	Bat10	H18ps-H1ss	N10ps-N1ss	+++
20	Bat10	Bat10	Bat10	Bat10	Bat10	Bat10	H17ps-H1ss	N10ps-N1ss	+++

* Rescue efficiency defined in Table 1.

Chapter 5 General discussion and conclusion

IAVs continue to pose a threat to animal and public health after being identified nearly a century ago. Over the past century, the virus has continually been evolving to adapt and maintain itself in various animal species. Intensive influenza vaccines have been used in humans; however, novel influenza viruses continue to emerge and cause epidemics and pandemics. One such example is A(H1N1)pdm09 which emerged as the first pandemic in this century and killed about 18,000 people due to insufficient immunity against the virus (WHO, 2010).

Usually, IAVs show a restricted host range. Cross-species transmission may occur if mutation occurs in the host range determining genes of the virus, or after reassortment with a different virus. A pandemic may occur when IAVs obtain human-to-human transmissibility and specific immunity is lacking in the human population. Emergence of novel IAVs in animal hosts poses a significant threat to public health and calls for more attention. Therefore, the objectives of this research project are to assess pathogenicity and transmissibility of novel influenza viruses that have emerged in swine and bats.

Soon after the emergence of A(H1N1)pdm09 virus in humans, this virus transmitted to swine and reassorted with endemic swine IAVs worldwide. Ten different genotypes of H3N2 subtype swine IAVs containing at least one gene from A(H1N1)pdm09 have been reported in US swine herds (Kitikoon et al., 2013). In chapter two, we identified seven novel reassortant swine H3N2 IAVs containing 3 to 5 genes from A(H1N1)pdm09 in Kansas, US. The reassortant viruses with different genome patterns have also been reported in other areas of the US (Cox et al., 2011; Ducatez et al., 2011). Additionally an H3N2 variant with M gene from A(H1N1)pdm09 has caused more than 300 human infections in the US, suggesting that the ressortant viruses between A(H1N1)pdm09 and endemic swine IAVs pose a great threat to

public health. Recently, studies showed that the internal genes of A(H1N1)pdm09 have persisted in pig herds in Southern China and continually reassort with swine IAVs (Liang et al., 2014). These results warrant intensive surveillance of swine IAVs in pigs and systemic analysis of swine influenza isolates, especially novel reassortant viruses.

Novel reassortant H3N2 swine viruses with genes from 2009 pandemic virus have been isolated and characterized. One study showed that neither swine H3N2 variant or reassortant swine H3N2 virus with genes from A(H1N1)pdm09 showed increased virulence compared with endemic swine H3N2 virus (Kitikoon et al., 2012). Another study identified nine reassortant viruses representing seven reassortant patterns. The pathogenicity of a reassortant virus with PA, NP, and M gene from pandemic were evaluated, compared with the A(H1N1)pdm09 strain and an endemic swine H3N2 strain in ferrets. The result suggested that A(H1N1)pdm09 genes offered no enhancement to viral virulence (Ducatez et al., 2011).

In our study, the pathogenicity study indicated that the reassortant H3N2 viruses with genes from A(H1N1)pdm09 and NA gene from recent human cluster showed increased virulence and transmission. This result is consistent with surveillance data in the field. The swine variant H3N2 virus has caused around 400 human infections, 18 hospitalization and one human death (CDC, 2014). Moreover, the virus that caused human infections also contained the NA gene that belongs to the recent human virus. Our study also found that the reassortant viruses bearing an avian-like HA receptor preference (HA 228G) showed lower transmission compared with other viruses with human-like HA receptor preference HA. It is known that the HA receptor preference affects viral transmission and the effect of HA receptor binding preference to viral transmission has also been found in studies with H1N1 and H7N9 viruses in different animal models (Liu et al., 2012b; Lorusso et al., 2014; Watanabe et al., 2011).

The genome sequences of two influenza A-like viruses have been identified in bat specimens in South America. However, until now no live virus has been isolated or cultured. Discovery of these influenza A-like bat virus sequences has raised public health concerns. However, the characteristics of the bat influenza virus remain unknown due to no available cultivable virus . In chapter four, we observed the bat influenza virus particles generated by reverse genetics. The virus showed similar morphology as classical IAVs; however, the rescued bat influenza virus cannot grow in embryonated chicken eggs and different cell lines derived from many species including canine (MDCK), mink (Mv1-Lu), swine (ST), African green monkey (Vero), human (A549, Calu-3), and free-tailed bat (Tadarida brasiliensis, Tb1Lu). Based on their crystal structures, both H17 and H18 subtype HAs cannot bind canonical sialic acid receptors (Sun et al., 2013; Tong et al., 2013; Wu et al., 2014), which may be the reason why these viruses are unable to grow on chicken embryos and substrates used for canonical influenza A viruses (Zhou et al., 2014b). The results suggest that the bat influenza viruses should be viable viruses, but identification of the host receptors and a special cell line is necessary to support the virus replication.

Although we were unable to culture the wild-type bat virus, we could rescue a modified bat-influenza H17N10 virus that had the HA and NA coding regions replaced with those of A/PR/8/1934 (H1N1). This modified bat-influenza virus replicated efficiently *in vitro* and in mice, resulting in severe disease and this result has been confirmed by the other groups (Juozapaitis et al., 2014). These data indicate that the internal genes of bat influenza virus are functional and compatible with the HA and NA genes of classical IAVs. The crystal structure of RNP of bat influenza virus has been solved, providing more evidence of viability of bat influenza viruses (Pflug et al., 2014). Although the chimeric bat influenza virus is virulent to mice,

additional studies using a chimeric bat-influenza virus that had the HA and NA of A/swine/Texas/4199-2/1998 (H3N2) showed that the PR8 HA and NA contributed to the pathogenicity in mice.

The NS1 protein of IAVs has the ability to inhibit the host IFN response, and truncation of the NS1 C terminal attenuates the pathogenicity of virus. However, mice experiments revealed that the truncation of Bat09 NS1 had minimal effects on the viral pathogenesis compared to the truncation of PR8 NS1. The PB2 of IAV plays an important role in replication and virulence. Our results showed that bat-influenza PB2 is also a virulence determinant. Substitution of N701D attenuated the virus, and the E158G substitution enhanced virulence in the mouse model, which is similar with classical IAVs. This data suggest that the pathogenicity of bat influenza virus is a polygenic trait.

Reassortment will occur when different IAVs coinfect the same cell, which is important in the evolution of IAVs and generation of panzootic and pandemic strains. Our coinfection studies demonstrated that no reassortment occurred between chimeric bat influenza viruses and classical influenza A viruses, which has also been confirmed by other groups (Juozapaitis et al., 2014). The low homology of sequences between bat influenza viruses and canonical IAVs may be a reason for such incompatibility. Moreover, the PB1 and PA subunits of the polymerase of bat influenza viruses do not support the polymerase function of canonical IAVs (Juozapaitis et al., 2014; Zhou et al., 2014b). The limited ability of bat influenza viruses to reassort with influenza A and B viruses indicates the low zoonotic risk of bat influenza viruses to public health.

Taken together, our studies indicate that the novel reassortant swine IAVs with increased virulence and transmission will pose a severe challenge to the swine industry in controlling the disease. The chimeric bat influenza virus can be rescued and propagated in culture substrates and

used as a tool to study novel bat influenza virus. In addition, bat influenza viruses have limited reassortant compatibility with the influenza A and B viruses, suggesting the bat influenza viruses pose little pandemic threat.

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