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L222W of Hemagglutinin Affects the Receptor Binding Affinity of Avian Origin H3N2 Canine Influenza Virus

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Emergence of avian origin and equine origin canine influenza viruses (CIVs) in Asia and the United States brings important concerns. Humans are in closer and more frequent contact with dogs than other common hosts of influenza. Thus, CIV is a potential threat to human health. However, little is known about the determinants of CIV host tropism or the transmissibility of CIVs to humans. An amino acid change (W222L) was implicated in modifying hemagglutinin receptor binding by CIV. This was tested using reverse genetics, glycan microarray and virus histochemistry. Glycan microarray demonstrated that avian-origin CIV (H3N2-222W) bind predominantly to alpha-2, 3 linked glycans. Virus histochemistry indicated that rH3N2-222L had higher binding affinity with epithelial cilia of canine tracheal tissue and weaker binding with avian tracheal tissue. Ferret infection demonstrated that the avian-origin H3N2 CIV could cause infection and limited to rhinitis, suggesting that CIV could infect humans.

DEDICATION

I would like to dedicate this thesis to my father Xuecheng Yang, my mother-in-law Cuilan Liu, my wife Jingjun Lu, my son Junli and daughter Lucia.

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LIST OF SYMBOLS/TERMS

CIV: canine influenza A virus

SPF: specific pathogen free

LPAI: low pathogenic avian influenza

HPAI: highly pathogenic avian influenza

CTE: canine tracheal epithelial

HTEpC: human tracheal epithelial cell

NP: nucleoprotein

MAA: *Maackia amurensis*

SNA: *Sambucus nigra*

DAPI: 4',6-diamidino-2-phenylindole

FITC: Fluorescein isothiocyanate

MOI: multiplicity of infection

SD: standard deviation

HA: hemagglutinin

RFU: relative fluorescence unit

PBS: phosphate buffered saline

d.p.i.: day post inoculation

CHAPTER I

REVIEW OF RELEVANT LITERATURE

Influenza A virus

Influenza A virus, a member of *Orthomyxoviridae* family, is a negative sense, single stranded and segmented RNA virus. Influenza A viruses are differentiated from type B and type C influenza viruses, so called influenza B virus and influenza C virus, based on the major internal protein antigens: nucleoprotein (NP) and matrix (M1) proteins (Büchen-Osmond, 2006; Webster et al., 1992). The genome consists of eight segments with a genome size approximately 13.5 kilobases, and encodes 11 proteins: hemagglutinin (HA), neuraminidase (NA), RNA polymerases (PB2, PB1, PB1-F, and PA), nucleoprotein (NP), matrix proteins (M1 and M2), and non-structural proteins (NS1 and NS2, so called nuclear export protein (NEP)). Every protein plays special and specific role(s) for the infection and transmission of influenza A virus. HA binds to host cell receptors and fuses the viral envelope and host cell. NA cleaves terminal sialic acid from glycoproteins or glycolipids and functions to free virus particles from host cell receptors and usually is glycosylated. PB2 polymerase provides viral RNA-dependent RNA polymerase activity for initiation of viral mRNA transcription, whereas PB1 polymerase is responsible for elongation of the primed nascent viral mRNA. PA polymerase is required for replication and transcription of vRNA and endonuclease cleavage of the cap RNA primer and may also be involved in virus assembly. NP plays a

role in switching viral RNA polymerase activity from mRNA synthesis to cRNA and vRNA synthesis, and usually is phosphorylated. M1 and M2 proteins initiate progeny virus assembly and/or act as proton channels to control the pH of the Golgi apparatus. NS1 and NS2 are involved in virus replication (Webster et al., 1992).

Influenza A viruses are classified into different subtypes based on the serological cross reactions of their surface glycoproteins: HA and NA (Webster et al., 1992). The number of influenza A virus subtypes has recently expanded from H1 to H17 and N1 to N9, and the newly identified H17 was isolated from fruit bats in 2012 (Gewin, 2012; NHSChoices, 2012; Tong et al., 2012). The subtypes keep expanding which makes the ecology of influenza A virus more complicated.

Ecology of influenza A virus

The influenza viruses were first isolated and characterized in the last century. Influenza A viruses have been isolated from many species, such as humans, pigs, horses, dogs, cats, bats, sea mammals and domestic and wild birds (Tong et al., 2012; Webster et al., 1992). Influenza A viruses can cause acute or mild respiratory tract diseases in humans, horses, pigs, domestic poultry, and sea mammals (Geraci et al., 1982; Lipatov et al., 2004; Olsen et al., 2006; Webster et al., 1992) and may lead to sporadic, epidemic and pandemic diseases. However, in waterfowl, such as ducks, influenza viruses replicate preferentially in the cells lining the intestinal tract, cause no disease signs, and are excreted in high concentration in feces (Webster et al., 1992).

The avirulent nature of avian influenza infection in ducks may be the result of virus adaptation to this host over many centuries, establishing a reservoir which ensures the perpetuation of the virus (Peiris et al., 2007; Webster et al., 1978). However, the

emerging H5N1 highly pathogenic avian influenza viruses can lead to systematic infection and deaths in waterfowl (Wan et al., 2008).

Evidence suggests that the mammalian influenza viruses have derived evolutionarily from avian viruses (Webster et al., 1992). Host range restrictions limit avian to mammalian interspecies transmission. In particular, human influenza viruses do not replicate efficiently in birds and vice versa (Beare and Webster, 1991; Hinshaw et al., 1983; Webster et al., 1992). However, influenza A viruses can eventually have the ability for inter- and intraspecies transmission through evolution such as genomic reassortment, mutation, and or recombination (Fouchier et al., 2005; Hinshaw et al., 1980; Li et al., 2003; RÖHm et al., 1996; Webster et al., 1992; Webster et al., 1978).

Influenza A viruses cross species barriers or transmit to other hosts from avian reservoirs mainly through HA, which determines the binding specificity to host sialic acid receptors. For example, avian and equine influenza A viruses bind preferentially to those containing α2,3-N-acetylneuraminic acid-galactose linkages (α2,3NeuAcGal). Human influenza viruses bind preferentially sialyloligosaccharide receptors with α2,6-acetylneuraminic acid-galactose linkages (α2,6NeuAcGal) (Connor et al., 1994; Ito, 2000; Ito et al., 1997; Kumari et al., 2007; Rogers and D'Souza, 1989; Rogers and Paulson, 1983; Rogers et al., 1983; Stephenson et al., 2003; Vines et al., 1998; Viswanathan et al., 2010). Swine possess both α2,3 and α2,6 receptors and, therefore, are susceptible to infection with mammalian and avian viruses (Ito et al., 1998). As a result, swine can serve as intermediate hosts for adaptation of avian influenza viruses to replication in mammals and as "mixing vessels" to generate genetically novel viruses with pandemic potential (Bochner et al., 2005; Claas et al., 1998; Ma et al., 2009; Myers

et al., 2007; Scholtissek et al., 1985; Shinde et al., 2009; Smith et al., 2009; Webster et al., 1992).

However, since 1996, several reports indicate that avian influenza H7N7, H5N1 and H9N2 identified in humans were transmitted directly from birds (Banks et al., 1998; de Jong et al., 1997; Peiris et al., 2004; Peiris et al., 1999; Yuen et al., 1998). Although these viruses have failed to transmit within the human population, the concern is that avian influenza viruses still have the potential to cause pandemics (Fouchier et al., 2012; Gambotto et al., 2008).

Evolution of influenza A virus

Higher mutation rate is one of the major characteristics of RNA viruses, which facilitates the virus evolution. Influenza RNA polymerase lacks a proofreading function. This causes errors at the rate of 10^{-3}-10^{-4} base substitutions per position per virus generation, or about one base mutation in the HA gene per virus generation (Holland et al., 1982; Steinhauer and Holland, 1987). This mutation rate is very high compared to the much higher replication fidelity found among DNA polymerases (errors on the order of 1 in 10^9 bases per replication cycle). Some studies indicate that nucleotide mutations continuously occur in avian influenza genes; however, there is no strong evidence showing net accumulation of amino acid changes (Bean et al., 1992; Gorman et al., 1991; Gorman et al., 1990).

The evolution events of influenza A virus can be point mutation (amino acid substitution, insertion or deletion), gene reassortment, and occasional RNA recombination (Webster et al., 1992). Among these evolutionary events, reassortment is an important mechanism for generating diversity rapidly and effectively, facilitating

emergence of pandemic influenza viruses (Dugan et al., 2008; Obenauer et al., 2006). Reassortment can cause antigenic shift whereas mutations on HA can cause antigenic drift, which is driven by host immune pressure (Webster et al., 1992). In 1918, 1957 and 1968, influenza A viruses with a new HA gene (with or without an accompanying novel NA gene) spread globally to a population which lacked immunity to the novel HA antigen, causing pandemics with significant morbidity and mortality (Cox and Subbarao, 2000). At the beginning of this century, the 2009 H1N1 triple reassortant swine flu pandemic spread over more than 20 countries, causing approximately 18,000 deaths (Chen et al., 2010; Chowell et al., 2009; Louie et al., 2010).

Low pathogenic and highly pathogenic avian influenza

Based on the level of pathogenicity of influenza viruses established in chickens, avian influenza can be classified into low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) viruses. HPAI viruses cause severe systemic disease with high morbidity and mortality and are associated with some strains of H5 or H7 HA subtypes (Senne et al., 1996). Instead, LPAI viruses usually cause asymptomatic infection and/or mild disease; however, occasionally some LPAI strains can cause higher mortality accompanying secondary pathogens (Alexander, 2000). The switch of a LPAI virus into a HPAI virus occurs usually by the introduction of basic amino acid residues into HA0 cleavage site, which facilitates systemic replication (Olsen et al., 2006).

Public health threats caused by influenza A viruses

Influenza viruses bearing H1, H2 and H3 HA genes and N1 and N2 NA genes have circulated in the human population during the 20th century (Subbarao and Katz,

2000) and in the beginning of this century. H1N1 Spanish flu appeared in 1918 and circulated until 1957; H2N2 Asia flu emerged in 1957 and circulated until 1968; and H3N2 Hong Kong flu appeared in 1968 and continued to circulate until the beginning of the 21st century.

Emergence of novel influenza A viruses among the animal-human interface continues to pose a severe threat to public health. With the influenza A virus family continually expanding, the ecology of influenza viruses becomes more complicated. This is especially true in the case of the new viruses that have the potential to cause an epidemic or pandemic in a human population lacking protective immunity against the new hemagglutinin. Currently, the concern regarding the pandemic potential of H5N1 HPAI is increasing (Gambotto et al., 2008; Watanabe et al., 2011; WHO, 2012). Meanwhile, subtype H9N2 avian influenza has been reported to infect humans and, also, has been isolated from pigs (Butt et al., 2005; Choi et al., 2004; Peiris et al., 1999). Some studies have shown that recombination of H9N2 and H1N1 could pose a pandemic threat (Sun et al., 2011; Wan and Perez, 2007). The newly emerging H3N2 and H3N8 canine influenza viruses can be another potential threat (Crawford et al., 2005; Li et al., 2010; Song et al., 2008).

Key techniques for influenza research

Reverse genetics

Reverse genetics has been established and developed as one of the most powerful techniques for the study of influenza A viruses (Neumann and Kawaoka, 1999; Palese et al., 1996). In order to determine the function of each segment and each amino acid residue in the proteins or to select the desired virus, the very primary approach was co-

infection and followed with time-consuming selection of the desired reassortant virus and its gene constellation confirmation (Voeten et al., 1999). Although the reverse genetics approach in which cells are transfected with *in vitro* generated ribonucleoproteins, reduces the possible number of progeny viruses, an efficient selection method is required (Li et al., 1999). Plasmid-driven synthesis of viral RNA and proteins allows the generation of infectious influenza virus without the need for helper virus infection (Fodor et al., 1999; Hoffmann et al., 2000; Neumann and Kawaoka, 1999). An eight plasmid DNA transfection system was developed to allow the generation of 6+2 reassortant viruses (Hoffmann et al., 2002). The strategy is that a plasmid-based expression system is utilized and viral cDNA is inserted between the RNA polymerase I (pol I) promoter and terminator sequences in a vector, such as pHW2000, which provides a useful way to address the host specificity related to hemagglutinin or neuraminidase protein of influenza virus and the function of other segments of influenza viruses (Neumann and Kawaoka, 1999; Palese et al., 1996). Combining the technique of site-directed mutagenesis, theoretically, the function of any amino acid in hemagglutinin or other proteins can be determined through generating different reassortants and mutants.

Glycan microarray

Since the Consortium for Functional Glycomics (CFG) has developed two generations of high-throughput arrays to date, glycan microarray has been applied to determine the glycan binding profiles for H5N1 HPAI virus (Stevens et al., 2008; Stevens et al., 2006c), H2N2 and H3N2 pandemic influenza (Stevens et al., 2008), and H3N2 seasonal influenza viruses (Kumari et al., 2007) . Many other studies have demonstrated the advantages of solid-phase glycan arrays as a means of identifying glycan binding

specificity with high-throughput screening (Angeloni et al., 2005; Blixt et al., 2004; Bryan et al., 2004). As glycan libraries have been expanded, more specific binding information has been released (Blixt et al., 2004; Bochner et al., 2005; Guo et al., 2004; Schwartz-Albiez and Kniep, 2005; Stevens et al., 2006a).

Airway epithelial cell culture

In addition to animal models, airway epithelial cell culture is also used to investigate the physiology of airway epithelial and airway disease caused by influenza viruses and it can be used for in vitro/ex vivo studies on pharmacological and toxicological targets relevant to airway diseases. Tracheal/bronchial epithelium, mainly consisting of ciliated cells, goblet cells and basal cells, is the tissue initially attacked by the invading influenza viruses. For example, the infected chicken trachea shows necrosis and detachment of ciliated cells, suggesting that ciliated cells are targeted by AIV (Mo et al., 1997). To date, the *in vitro* models of airway epithelial cells have been established for various species, including human, monkey, chicken, mouse, hamster, and horse, and applied in the study of influenza virus (Gruenert et al., 1995; Lin et al., 2001; Niles et al., 1988; Quintana et al., 2011; Shen et al., 2010 ; Shen et al., 2011; Shibeshi et al., 2008; Sime et al., 1997; You et al., 2002; Zaffuto et al., 2008; Zhang et al., 2002). Both SAα2,3Gal and SAα2,6Gal molecules have been found on cells artificially differentiated from isolated human tracheal cells *in vitro* (Matrosovich et al., 2004). However, the anatomical study indicated that the tracheal epithelial cells mainly express SAα2,6Gal; SAα2,3Gal was only occasionally detected (Shinya et al., 2006). However, human airway epithelial (HAE) cell culture has been used to investigate avian influenza A virus H9N2 infection in humans. The study indicates that variation of site 226 in HA of H9N2

influenza viruses affects cell tropism and the replication of this virus in HAE cells (Wan and Perez, 2006).

Virus histochemistry attachment assay

Recently, a virus tissue attachment assay has been reported in the literature to address the pathogenicity and infectivity of influenza A viruses. For instance, a study showing HPAI H5N1 virus attached to the lower respiratory tract indicates that this technique is reliable (van Riel et al., 2006). Virus attachment assay suggests that the ability of wild birds to serve as hosts for influenza viruses shows big variations among species. This will provide valuable information to assess the interspecies transmission of influenza viruses in natural environments and better understand the ecology of influenza (Jourdain et al., 2011). Virus attachment studies showed that pdmH1N1 with D222G acquired dual receptor specificity for α2,6- and α2,3-linked sialic acids (Chutinimitkul et al., 2010).

The main objectives of this study

This study is to explore the molecular mechanisms of canine influenza infection, especially the factors within the aspects of both virus and host determining canine influenza host specificity, and to assess the risk of human infection of CIVs. The hypotheses of this study were that canines could have a unique set of glycan receptors determining canine influenza host tropisms and that some specific amino acid substitution in the receptor binding region of hemagglutinin protein of influenza A virus could be the cause that results in avian-origin influenza A viruses jumping to the canine. To test the hypothesis of this study, candidate residue in HA was identified using

computational biology, and reverse genetics approach was applied to generate recombinant viruses. Phenotypic analyses, glycan microarray and virus histochemistry assay were conducted to disclose the role of the specific residue of HA in host specificities. Ferret infection experiment was performed to assess the potentiality of human infection with CIV H3N2.

CHAPTER II

L222W OF HEMAGGLUTININ AFFECTS THE RECEPTOR BINDING AFFINITY OF AVIAN ORIGIN H3N2 CANINE INFLUENZA VIRUS

Abstract

Influenza A virus has been responsible for high economic losses and human suffering and remains a huge threat to public health. Recent emergence of an avian origin canine influenza virus (CIV) in Asia has extended the list of known strains and complicated influenza ecology. This study proposes to identify the molecular mechanisms determining host tropisms of canine influenza specifically, how avian influenza viruses adapted to the canine host. Through phylogenetic analysis, sequence alignment and computational modeling, the residue 222 in hemmaglutinin was predicted to play a critical role in host tropisms for H3N2 CIV. Recombinant viruses, rH3N2-222L, rH3N2-222W, were generated using reverse genetics. The infection of both rH3N2-222L and -222W viruses to primary canine tracheal epithelial cell culture indicated that rH3N2-222L had significantly higher infectivity than rH3N2-222W did. Virus histochemistry assay suggested that rH3N2-222L had higher binding affinity to epithelial cilia of canine tracheal tissue and weaker binding to avian tracheal tissue. However, rH3N2-222W virus bound stronger than 222L did on avian tracheal tissue. Glycan microarray demonstrated that avian-origin wild type virus and its recombinant strains had similar glycan binding patterns, and the majority of bindings were to alpha-2, 3 linked glycans which are

prevalent in the canine airway epithelial cells. Furthermore, the L222W substitution leads to decrease of binding affinity to a set of Neu5Aca2-3Galb1-4(Fuca-) or Neu5Aca2-3Galb1-3(Fuca-) type of glycans for avian-origin canine influenza viruses. This indicates that W222L in HA of H3N2 avian viruses could facilitate their host adaptation to the new canine host.

Introduction

Influenza A virus, a negative-strand segmented RNA virus, is a member of *Orthomyxoviridae* family. As one of the major causes for respiratory diseases in humans (Lipatov et al., 2004; Webster et al., 1992), influenza A virus can naturally infect birds, pigs, horses, sea mammals, and recently dogs (Crawford et al., 2005; Li et al., 2010; Song et al., 2008; Yoon et al., 2005) and bat (Tong et al., 2012). The serotype of influenza A virus is determined by its surface glycoprotein hemagglutinin (HA) and neuraminidase (NA). To date, 17 HA subtypes and 10 NA subtypes of influenza A virus have been identified (Tong et al., 2012). All known HA and NA subtypes have been detected in migratory waterfowl, which are the proposed natural reservoirs for influenza A viruses. Recently, two subtypes, H3N2 and H3N8, of influenza A viruses emerged in dogs in Asia and North America, respectively (Crawford et al., 2005; Li et al., 2010; Song et al., 2008; Yoon et al., 2005). The H3N2 canine influenza virus was genetically shown to be of avian origin whereas the H3N8 canine influenza virus of equine origin (Crawford et al., 2005; Li et al., 2010; Song et al., 2008; Yoon et al., 2005).

The H3N2 avian-origin CIV emerged in South Korea and China in 2006 (Li et al., 2010; Song et al., 2008). Under experimental conditions, this H3N2 virus can infect dogs and reproduce respiratory disease through nasal inoculation or close contacts (Kirkland et

al., 2010). Serological survey in 829 serum samples (361 farmed dogs and 468 pet dogs) collected between June and December 2007 across Korea showed that the canine populations investigated had a serum conversion rate of 19 to 100% with anti-H3N2 influenza viral antibody (Lee et al., 2009). In China, the seroconversion rate for H3N2 virus was about 6.7% (Li et al., 2010). These surveillance results suggested that H3N2 avian-origin CIV had become epidemic in canine populations in Korea and China.

Influenza A viruses bind and enter into host cells depending on the recognition of terminal sialic acid (SA) capped glycosylated molecules by HA (Horimoto and Kawaoka, 2005; Skehel and Wiley, 2008; Webster et al., 1992). Human influenza A virus preferentially binds to sialyloligosaccharides with N-acetyl sialic acid α 2,6 linked galactose (NeuAcα2,6Gal), whereas avian influenza virus (AIV) and equine influenza virus prefer NeuAcα2,3Gal (Daly et al., 2008; Viswanathan et al., 2010). The mutations in HA were frequently detected when AIV was transmitted and adapted from birds to mammals or even between mammalians. For example, G226L and G228S in the 220 loop of the HA receptor binding site can increase the capability for an AIV to infect humans (Bateman et al., 2008; Connor et al., 1994; Nobusawa and Nakajima, 1988; Vines et al., 1998; Wan and Perez, 2006; Xu et al., 2010). Compared to its avian precursor virus, the HA of H3N2 CIV has seven mutations, including T10A, D81N, L111T/N, A160T, D172N, W222L, and D489N (Li et al., 2010).

To date, little is known about the factors that facilitated the transmission of these virus strains from the avian to the canine host. This study aims to characterize the impact of W222L, a single amino acid substitution consistently detected in H3N2 canine influenza viruses, in the receptor binding region of canine influenza viruses. Our results

demonstrated this substitution could affect the receptor binding affinity of avian origin H3N2 canine influenza virus.

Materials and Methods

Hemagglutinin protein sequence data

The predicted HA protein sequences, five from H3N2 CIVs isolated between 2006 and 2010, fifteen from H3N2 and H3N8 avian viruses isolated between 1992 and 2009, eight from H3N8 CIVs isolated between 2004 and2008 and six from equine H3N8 isolated between 1986 and 2010 were obtained from NCBI. These sequences were used to characterize the amino acid mutations in HA.

Cell lines, influenza A viruses, and virus propagation

Both Madin-Darby Canine Kidney (MDCK) and human embryonic kidney 293T cells were purchased from American Type Culture Collection (Manassas, VA). Both cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO/BRL, Grand Island, NY), supplemented with 5-10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), penicillin–streptomycin, and amphotericin B (GIBCO/BRL, Grand Island, NY), at 37 °C with 100% humidity and 5% CO_2 atmosphere. Tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (TPCK) (1 μg/ml) (Worthington Biochemical Corporation, Lakewood, New Jersey) was used during influenza infection, and 0.25% Trypsin-Ethylenediamine Tetraacetic Acid (EDTA) (GIBCO/BRL, Grand Island, New York) was used to suspend the cells.

An avian-origin canine influenza A virus, A/canine/Guangdong/1/2006(H3N2) was used in this study. Virus was propagated in MDCK cells as described above for

about 72 h. The harvested cultures were clarified by low speed centrifugation (1,800×g) for 10 min, and the supernatants were aliquoted and stored at -80°C.

Molecular cloning and mutagenesis

Viral RNA was isolated from supernatant from above using RNeasy Mini kit (Qiagen, Valencia, CA). The full-length HA and NA genes were amplified using SuperScript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen, Grand Island, NY) and then cloned into pHW2000 vector (Hoffmann et al., 2000). Mutagenesis of L222W on HA genes was performed using QuikChange® II XL site-directed mutagenesis kit (Agilent, La Jolla, CA) according to the instruction of manufacturer. The primers used for cloning and mutagenesis are listed in Table 1. The cloned HA genes and mutated genes were confirmed by Sanger sequencing at the Cornell University Life Sciences Core Laboratories Center.

Generation of recombinant viruses using reverse genetics

The two plasmids containing HA and NA of H3N2 canine influenza virus and six plasmids containing internal genes of A/Puerto Rico/8/34 were co-transfected into 293T cells. After culture at 37°C for 8 h, the DNA-transfection mixture was replaced by 2 ml of Opti-MEM with penicillin and streptomycin antibiotics. After 48 hours of transfection, the suspension medium was used to inoculate 9-day-old SPF embryonated eggs that were maintained at 37 °C for 48 h. The rescued viruses were harvested for serological assays after confirmation of expected sequences. Two recombinant viruses were produced, A/canine/Guangdong/1/2006 (H3N2) HA/NA×PR8 (designated as rH3N2 222L) and A/canine/Guangdong/1/2006 (H3N2) HA-L222W/NA×PR8 (rH3N2-222W) (Table 2).

Hemagglutination (HA) assay

The HA assay was carried out as described elsewhere (Masurel et al., 1981). Briefly, 50 µl volumes of the culture supernatants were serially diluted 2-fold in phosphate-buffered saline (PBS) in round bottom plates. Subsequently, 50 µl of a 0.5% suspension of chicken erythrocytes were added to each well. The plates were incubated at 37°C for 30 min before recording the HA titers.

Virus growth curve

Confluent MDCK cells were infected with H3N2 WT, rH3N2-222L and rH3N2-222W at an MOI of 0.01 PFU/cell and incubated for different time periods in the presence of TPCK-trypsin (1 µg/ml). Virus in the supernatant was titrated (log_{10}PFU/ml) at the specific times post-infection.

Canine tracheal epithelial (CTE) cell line preparation

CTE cell cultures were prepared based on the reported procedures for other animal tracheal epithelial cell culture (Busch et al., 2008; Gray et al., 1996; Quintana et al., 2011; Schroth et al., 1999; Shen et al., 2011; Shibeshi et al., 2008; Sime et al., 1997; Wu et al., 1985). Briefly, the tracheal tissues from a healthy beagle dog were excised within 30 min after euthanasia. The trachea was sliced longitudinally to expose the mucosa, which was washed thoroughly with PBS containing penicillin and streptomycin. Mucosal membrane, a source of primary cells, was peeled off, cut into small pieces, and washed with cold PBS containing antibiotics in 50 ml conical tube. After washing, tracheal membrane pieces were digested by 0.25% trypsin-EDTA for 2 h at 37°C or were digested in DMEM with 1.4 mg/ml pronase and 0.1mg/ml deoxyribonuclease I (Roche

Applied Science, Indianapolis, IN) in 50ml tubes for 20 h at 4°C. Inactivated FBS was added to a final concentration of 10% to inactivate the enzyme activity. The solution was sieved by 40μm cell strainer (BD Biosciences, San Jose, CA) and centrifuged at 220 × g for 10 min, and the supernatant was discarded. The cell pellets were suspended in DMEM with 5% FBS, and then incubated at the atmosphere with 100% humidity and 5% CO_2 for 3 h to permit the attachment of contaminating fibroblasts to the dish or flask surface. After incubation, supernatant was transferred into a 50 ml tube after passing through a 40 μm cell strainer, centrifuged at 220×g for 10 min at 4°C, and re-suspended into T75 flask and/or 8-well slide (EMD Millipore, Billerica, MA) with BEGM bronchial epithelial cell growth medium (Lonza Walkersville Inc., Walkersville, MD). BEGM medium was changed every other day. To make the stock of CTE cells, 70%-80% confluent cells were trypsinized with subculture reagents according to the instructions of the manufacturer (Lonza Walkersville Inc., Walkersville, MD), frozen, and stored at -153°C.

The growth dynamics of CTE cells were measured by cell counting manually. The cultures were harvested by trypsin digestion suspended in trypan blue solution and counted on a hemacytometer.

Immunocytostaining and confocal imaging to characterize canine influenza infection on CTE cells

The confluent CTE cells were washed with BEBM medium, and inoculated with reassortant wild type or mutant virus (Table 2) at a multiplicity of infection (MOI) of 2, 1 and 0.1, at 37°C in cell culture chamber. After 1 h incubation, the inoculums were pipetted off, and the cells were incubated with BEBM media after three times of washing. After 7 h incubation, infected cells were fixed in 4% cold paraformaldehyde, then

permeabilized using 0.3% Triton X-100 and blocked using BSA blocking solution. Immunocytochemistry was performed using anti-influenza NP (Millipore, Temecula, CA) as primary antibody and goat anti-mouse IgG (H+L) FITC conjugated (Millipore, Temecula, California) as secondary antibody. Texas Red Conjugated Maackia amurensis Lectin was used to determine cell type according to the instruction of manufacturer (EY Laboratorie, Inc. San Mateo, CA). Blue 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-aldrich, Saint Louis, MO) was applied for nuclear staining. The number of immunocytochemistry staining positive cells was manually counted from at least eight individual fields. Images were taken using confocal microscope (LSM510 Meta, Zeiss) with objective lens (40×).

Virus purification and concentration for glycan microarray

The glycan binding profiles of H3N2 canine influenza wild type strains and two recombinants were analyzed. The influenza viruses were propagated in MDCK cells using T175 flasks. Cell debris was removed after centrifugation with 5,000 × g for 10 min at 4°C. To purify the viruses from the supernatants, 25% sucrose cushioned NTE buffer was used to concentrate and purify viruses through a centrifugation at 80,000×g at 4°C for 2~3 h using Sorvall Ultra Pro 80 centrifuge (DuPont, New York, NY) (Bradley et al., 2011). Pellets were dissolved in PBS with 0.05 mM EDTA. The titrations of six viruses were determined using HA assay prior to labeling and after labeling.

Virus labeling, glycan microarray hybridization and data analysis

For every 150 µl (>1.0 × 10^5 HAU/ml) influenza virus, 15 µl 1.0 M $NaHCO_3$ (pH 9.0) and 25 µg of dessicated Alexa Fluor-488 succinimidyl ester (Invitrogen, Grand

Island, NY) resuspended in 25 µl of H_2O were added, in a final ratio of about 0.005 µg Alexa per HAU. A 200 µl of labeled virus was relocated into mini-dialysis units and dialyzed against 500 ml PBS with 1 mM EDTA for 1 h with a stir bar in 4˚C cold room. The dialysis buffer was changed every four hours for at least four times. After dialysis, the samples were transferred to clean tubes and stored at 4˚C until glycan microarray hybridization (Bradley et al., 2011; Kumari et al., 2007; Stevens et al., 2010).

The version 5.0 glycan slides from the Consortium of Functional Glycomics used in this study have 611 glycans, including more than100 α2, 3 and α2, 6 linked sialic acids. A volume of 25 µl dialyzed virus was diluted 4 fold with PBS, and 70 µl diluted viruses were loaded to the printed surface of a rehydrated glycan slide and incubated in a high humidity chamber at 4°C for 1 h. The slide was washed and then drained under slide spinner. The binding image was read in a PerkinElmer ProScanArray scanner and analyzed using Imagene version 6.0 image analysis software (BioDiscovery, Hawthorne, CA).

Relative fluorescence unit (RFU) data was normalized by adjusting the total RFU to the same level across experiments. A threshold 2,000 RFU was used to floor the samples, and only the glycans with at least 2,000 RFU were statistically analyzed. The Wilcoxon signed rank test was used to compare the glycan binding patterns among canine H3N2 wild type, rH3N2 222L, and rH3N2-222W viruses.

Tissue preparation, virus inactivation, labeling and attachment

The canine, avian, and equine tracheal tissues were collected from healthy animals without histological lesions or evidence of respiratory tract infection. The tissues were fixed in 10% phosphate buffered formalin for 24 h at 4ºC with gentle shaking (van

Riel et al., 2006; Xu et al., 2010). Paraffin-embedded tissue sections were prepared by Diagnostic Laboratory Services, College of Veterinary Medicine, Mississippi State University. The tissue sections were deparaffinized by incubation at 75ºC for 15 min, immersed in xylene for 15 min, then rehydrated by using graded ethanol prior to using in the binding assay.

Viruses were inactivated by incubating with 10% formalin (volume/volume = 1) at room temperature for 1 h, and then dialyzed in PBS buffer at 4 ºC overnight. After dialysis, viruses were mixed with equal volume of 0.1 mg/ml fluorescein isothiocyanate (FITC) (Sigma, Saint Louis, MO) in 0.5 M bicarbonate buffer (pH 9.5), incubated for 1 h at room temperature, then dialyzed against PBS with stirring overnight at 4ºC to remove unbound FITC. FITC-labeled viruses were diluted with PBS to 50-100 HAU per 50 µl. The viruses were incubated with tissue sections overnight at 4ºC after 3% H_2O_2 quenching the endogenous peroxidase and blocking reagent applied to block the tissue. PBS or Tris-HCl sodium buffer (TN) with Tween-20 was used to wash slides three times to remove unbound viruses, followed by incubation with polyclonal rabbit anti-FITC/HRP (DakoCytomation, Carpinteria, CA) diluted 1:50 in PBS at room temperature for 1 h. The signal was amplified with a tyramide signal amplification system according to the instructions of the manufacturer (Perkin Elmer, Boston, MA). Then tissue sections were developed with Aminoethylcarbazole (AEC) (ENZO Life Sciences, Inc., Farmingdale, NY) for influenza antigen (red) and with hematoxylin (Surgipath® SelectTech™, Leica) for nuclear counterstaining (blue-violet) (van Riel et al., 2006; Xu et al., 2010). Negative controls were processed the same except the slides were not

incubated with FITC-labeled virus. Binding of labeled viruses to MDCK cells were used to evaluate differences of binding affinity and evaluate labeling efficiency.

Tubulin staining of tracheal epithelial cilia

Deparaffinized tissue sections were treated with 3% H_2O_2 to quench endogenous peroxidase, followed by incubation with blocking reagent for 30 min at room temperature. After three washes, the sections were incubated with anti-β-Tubulin (Sigma) 1:500 dilution in PBS solution at room temperature for 1 h, and washed three times using PBS buffer. Then the slides were incubated with 1:100 diluted goat anti-mouse IgG (Fab Specific)-Peroxidase (Sigma) was incubated with slides for 30 min. The sections were developed with VECTOR SG substrate (Vector Laboratories, Inc., Burlingame, CA) and tissues were counterstained with hematoxylin.

Lectin staining of tissue section

The Dig Glycan Differentiation Kit was used to label lectin in tissue sections according to manufacturer's manual (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, IN). Briefly, after deparaffinization, the tissue sections were incubated in the blocking solution at 4ºC for 30 min, and then washed twice with 50 ml TBS for 5 min and once in buffer 1, for 10 min. The tissue sections were incubated in lectin solution and then washed three times in 50 ml TBS, 10 min each time. Then the sections were immersed into the staining solution. After development of the gray color within a few minutes, the sections were rinsed at least three times with double distilled water. Hematoxylin was applied for tissue nuclear counterstaining. The slide was then mounted and photographed or imaged.

Image analysis

Representative images of virus attachment were taken using an Olympus BX51 microscope with a 40× objective lens. Virus-tissue binding signal strength was evaluated based on the whole tissue section at three replicates. The strongest binding signal was designated as "+++++", moderate binding was designated as "+++", weak binding was labeled as "+", "-" indicated no detectable virus binding signal.

Results

W222L mutations are conserved in HA of H3N2 canine influenza viruses

Leucine (L) is the consistent amino acid residue at site 222 of HA in both H3N2 and H3N8 CIVs compared to tryptophan (W) in their corresponding precursor HAs in avian influenza viruses and equine influenza viruses, respectively (Figure 1). There are no other consistent amino-acid substitutions around the receptor binding regions (Appendix A), such as 130 loop, 190 loop, and 220 loop, between avian or equine and canine. Based on this observation, we hypothesize that W222L in HA could facilitate host adaptation of H3 influenza A viruses from avian to canine and from equine to canine. To determine the role of site 222 on receptor binding affinity, we performed a retrospective study and generated canine influenza viral mutants carrying the amino acid feature (W) at residue 222 in their precursor HA genes in avian. Two recombinant viruses were generated, rH3N2-222L (canine-like) and rH3N2-222W (avian-like).

Growth curve of canine influenza virus and HA-L222W recombinants.

Growth curve was generated to compare the growth characteristics of H3N2 CIV seed virus and recombinant viruses, rH3N2-222L and rH3N2-222W (Figure 2). The

recombinant viruses, with PR8 backbones, grew well compared to their HA and NA precursor virus, rH3N2-222L yielded ~0.7 log10 more viruses than rH3N2-222W at the first replication cycle and H3N2 CIV was unable to grow to high titer in MDCK cells (Figure 2).

L222W substitution affects the receptor binding affinities to red blood cells

HA assays were performed to compare the binding affinities to erythrocytes between the purified recombinants and corresponding parent wild type strain. The result showed that rH3N2-222W had reduced binding affinity with erythrocytes of horse, guinea pig and beagle, respectively (Table 3).

L222W affects the infectivity of H3N2 but not H3N8 in canine tracheal epithelial (CTE) cells

Primary CTE cells were prepared from fresh canine tracheal tissue. The growth curve determined using daily counts demonstrated that CTE cells began exponential growth after 3 days post seeding (Figure 3). The cells were approximately 90% confluent at day 7 post cell seeding (Figure 3A), and these cells were used to study the infectivity of both rH3N2-222L and rH3N2-222W.

The CTE cells were inoculated with rH3N2-222L and rH3N2-222W at a multiplicity of inflection (MOI) of 0.1 or 1.0 and at 7 h post-infection (h.p.i.), which approximates one replication cycle, the infected cells were fixed and immunocytostained. The average number of infected cells from the experiments with 0.1 MOI CIV were significantly more in cells infected with rH3N2-222L than cultures infect with rH3N2-222W ($p<0.001$) (Figure 4; 5). Similar results were observed when MOI was increased from 0.1 to 1.0 (Figure 6).

To reveal the cell types that the CIV infected, Texas red-MAA was used to stain ciliated cells red. Our results showed that the majority of the infected cells were ciliated cells (0.88±0.089, n=3,024), indicating that ciliated cell was the primary target cell for canine influenza infection.

L222W affects the binding affinities of CIVs in avian, canine but not equine tracheal epithelial cells

Before comparing the binding affinities of CIV to various tracheal epithelial cells, anti-β-tubulin was used to confirm the presence of ciliated cells in the experimental tissues. Ciliated cells of all the three different tracheal tissue sections were consistently labeled blue (or dark color) by anti-β-tubulin, indicating the presence of ciliated cells (Figure 7). The types of receptors on avian, canine, and equine tracheal epithelial cells were further characterized using two types of immunochemical labeling: Dig-MAA specific for sialic acid α2,3 galactose and Dig-SNA specific for Neu5Ac α2,6 Gal/GalNAc. Our results showed that ciliated cells of avian, canine and equine tracheal sections were bound strongly by Dig-MAA lectin but barely bound by Dig-SNA (Figure 7). This suggested that α-2, 3 linked glycoproteins are predominant on avian, canine, equine epithelial cells of tracheal tissues.

To compare the CIV binding affinities to tracheal tissues, 50-100 HAU FITC-labeled CIVs were incubated with deparaffinized slides. rH3N2-222L bound much stronger to canine tracheal epithelial cilia than to avian tracheal epithelial cilia, whereas rH3N2-222W had a stronger binding avidity to avian tracheal epithelial cilia than canine tracheal epithelial cilia (Figure 8; Table 4). This indicates that L222W substitution changes the receptor binding affinity of avian origin canine influenza viruses. Both

recombinant viruses demonstrated no detectable attachment to equine tracheal epithelial cells, suggesting that H3N2 canine influenza virus might not infect horse through the trachea (Figure 8). This finding should be confirmed by evaluating the attachment of the original seed virus.

L222W affecting a small set of glycan binding for H3N2 and H3N8 CIVs

Glycan microarray was used to identify the impacts of L222W on the glycan binding specificity. The glycan binding profile demonstrated that H3N2 CIV, rH3N2 -222L and rH3N2-222W had the highest affinity to α-2,3 linked glycans (Figure 9; Appendix B; Appendix C), which confirmed the tissue binding experiments conducted above (Figure 7; 8).

Further analyses showed that rH3N2-222L had reduced affinity for 10 Neu5Aca2-3GalNAcb1-4GlcNAcb- and Neu5Gca2-3Galb1-4(Fuca1-3) GlcNAcb-type glycans compared to H3N2 and the corresponding mutant rH3N2-222W (Figure 10; Table 5).

Discussion

The molecular mechanism of how avian-origin canine virus H3N2 transmitted and adapted to the canine host needs to be understood. Both canine H3N2 and H3N8 had one common substitution on HA receptor binding region (Crawford et al., 2005; Li et al., 2010; von Grotthuss and Rychlewski, 2006). A 3D model shows that site 222 of HA is exposed to the serum and probably participates in host recognition (von Grotthuss and Rychlewski, 2006). The substitution of tryptophan with leucine at site 222 in HA of both H3N2 avian and H3N8 equine represents a non-conservative change close to the sialic acid-binding pocket, which suggests a potential modulating function in adaptation

of equine influenza virus to canine receptor(Payungporn et al., 2008). Although the host specificity of influenza virus is known to depend mainly on the surface protein, especially the viral hemagglutinin receptor binding region (Crawford et al., 2005; von Grotthuss and Rychlewski, 2006), the contribution of some specific amino acid residues, such as 226 and 228, remains to be determined. In this study, we aligned the predicted protein sequences of HA of avian H3N2, H3N8 and equine H3N8 and both H3N2 and H3N8 canine viruses from NCBI and found that amino acid residue 222 in the receptor binding region in HA had, most consistently, substitution from 222W in avian and equine to 222L in canine, which is consistent with the publications (Crawford et al., 2005; Li et al., 2010). Both residue 226 and 228 in HA, thought to be important for virus host specificity in the receptor region, did not show amino acid changes among avian, equine or canine viruses (Figure 1). This could indicate that site 222 of HA could play an important role in the CIV host adaptation.

Phenotypic determination of viral binding affinity was performed using different red blood cell lines. Reassortant rH3N2-222L and mutant rH3N2-222W viruses were subjected to the receptor binding affinity comparison. Five different animal red blood cells were applied (Table 2). The HAU data indicated that rH3N2-222L and its HA mutant rH3N2-222W had obvious binding specificity differences on different red blood cells (Table 3). The data of hemagglutination assay gave us an indication that L222W substitution changed the viral receptor binding affinity.

However, establishment of primary CTE cells for influenza virus infection is not trivial. Although human and some other animal airway epithelial cell cultures were established (Gruenert et al., 1995; Lin et al., 2001; Niles et al., 1988; Shen et al., 2010 ;

Shen et al., 2011; Shibeshi et al., 2008; Sime et al., 1997; You et al., 2002; Zaffuto et al., 2008; Zhang et al., 2002), for canine airway epithelial cells, different methods were tried from tissue dissociation, growth medium components to cell subpassage. The challenge is that CTE P0 cells grew well on tissue culture flask surface, but most cells did not attach to the tissue culture flask surface any more once they were suspended by dissociation medium. Even with changes of tissue dissociation enzyme and medium component, it was still difficult to get the passage 1 (P1) cell and set up the air-liquid interface culture, which limited the experiment. Therefore, in this study, the P0 generation of the primary canine tracheal epithelial cells was used to perform the virus infection. Infection experiments performed using P0 epithelial cell culture were reported somewhere (Busch et al., 2008; Shen et al., 2010). The method of generating primary canine tracheal epithelial cells (Quintana et al., 2011) was tried, but the cells did not grow well in this experiment.

In this study, the primary CTE cell culture was applied to conduct the infectivity experiment. Although in vivo system is the best for studying infectivity and pathogenicity of influenza viruses, the application of in vitro cell cultures offers the opportunity to study the mechanisms of infection and host responses under controlled conditions. P0 CTE cell culture was infected by recombinant viruses, rH3N2-222L and rH3N2-222W, separately. The immunocytochemistry staining suggested that CTE cells could be infected and most of the infected cells were α2,3-linked ciliated cells (Figure 4; 6) stained by MAA (Wang and Cummings, 1988), and there was a significant difference of infectivity between rH3N2-222L and rH3N2-222W in CTE cell culture. Quantified data indicated that most of the infected cells were MAA stained and when viral load

increased, non-ciliated cells were infected (Figure 6). Therefore, the canine influenza viruses have the potentiality to infect other cell lines in the tracheal epithelium according to the H3N2 infection data.

To further understand the molecules which may be involved in the virus host receptor recognition and receptor binding in vivo, glycan microarray was performed by using the new version of glycan slide which includes 611 active printed glycans. Virus glycan binding data suggested that HA of H3N2 canine influenza viruses preferentially bound with sialic acid α2,3-linked glycan moieties, especially on Neu5Aca2-3GalNAcb1-4GlcNAcb- and Neu5Gca2-3Galb1-4(Fuca1-3)GlcNAcb- structures, and these molecules could be the major glycoproteins in canine tracheal epithelial ciliated cells (Oshansky et al., 2011). These data reflect the canine influenza virus binding profile, although the printed glycans on the slides could not represent all glycans which canine respiratory tract tissue may have. Glycan arrays have been applied to determine the virus glycan binding affinity and specificity which could provide good reference for real situation (Bradley et al., 2011; Stevens et al., 2006b).

Similar to host infection by influenza virions, influenza viruses attach to host cells through binding of the surface proteins, mainly hemagglutinin, to receptors which are sialosaccharides on the host cells. The virus attachment pattern could help to understand the pathogenesis and infectivity of viruses (van Riel et al., 2006). Virus attachment assays have been accepted to investigate the infectivity and pathogenicity of some newly isolated or mutant viruses (Chutinimitkul et al., 2010; van Riel et al., 2006; Xu et al., 2010). It is more economical and efficient to use this technique to characterize the unknown viruses or mutants before doing animal experiments. In this study, virus-

tracheal tissue attachment assay disclosed some important information and confirmed the data obtained from both CTE cell culture infection and glycan microarray. H3N2 CIV recombinant viruses bind avian and canine tracheal epithelial ciliated cells which predominantly express α2,3-sialic acid linked glycans. L222W in HA of H3N2 showed different binding affinity on specific tracheal tissues. Both rH3N2-222L and rH3N8-222W barely bound with equine tracheal tissue, which indicate that H3N2 could not transmit to equine or could transmit to equine through other tissues, such as turbinate and larynx. Further virus binding experiments could be conducted to confirm the binding pattern of canine H3N2 seed viruses with these tracheal tissues and comparison with recombinant viruses.

Based on our data, more tissues from avian, canine and equine origin, such as turbinate, larynx, lung, liver, could be included to confirm the real scenario of infectivity and pathogenicity of H3N2 canine virus. In order to understand whether the other mutation sites of HA of canine H3N2 could affect virus attachment pattern, more recombinant viruses will be generated to conduct attachment assay, which may give a clearer picture about the role of each mutation in HA of H3N2 canine viruses. Moreover, the mutations in NA and internal genes were considered to affect influenza host tropisms, as well (Li et al., 2010; Rivailler et al., 2010; Subbarao et al., 1993). Future studies need to cover the function of internal genes on host specificities.

Table 1 Primers used in cloning HA and NA full length genes and constructing mutants.

Virus	Genes	Primer name	Sequence*
A/canine/Guangdong/1/2006(H3N2)			
	HA	Forward, YW10C	5'-TATTGGTCTCAGGGAGCAAAAGCAGGGG-3'
	HA	Reverse, YW11C	5'-ATGGTCTCCTATTAGTAGAAACAAGGGTGTTT-3'
	HA-L222W	Forward, YW42F	5'-CATTGGATCTAGACCCTGGGTAAGGGGCCAATCTG-3'
	HA-L222W	Reverse, YW43R	5'-CAGATTGGCCCCTTACCCAGGGTCTAGATCCAATG-3'
	NA	Forward, YW6C	5'-TATTCGTCTCAGGGAGCAAAAGCAGGAGT-3'
	NA	Reverse, YW7	5'- ATCGTCTCCTATTAGTAGAAACAAGGAGTTTTTTTGAAC-3'

*: underlined part was BsmBI restriction site.

Table 2 Recombinant viruses generated in this study

Strain name	HA and NA	Internal genes	222*
rH3N2-222L	A/canine/Guangdong/1/2006(H3N2)	A/PR/8/1934(H1N1)	L (canine)
rH3N2-222W	A/canine/Guangdong/1/2006(H3N2)	A/PR/8/1934(H1N1)	W (avian)

*: The predominant amino acid residue in the H3 proteins of influenza A viruses in the host species listed in the parenthesis.

Table 3 L222W of HA changed the receptor binding affinity of canine influenza virus H3N2

Virus stain	HA titer				
	Turkey	Chicken	Guinea pig	Beagle	Horse
H3N2 WT	128	64	128	32	53.3±18.5
rH3N2-222L	128	64	128	32	64
rH3N2-222W	128	85.3±37*	64	16	12±4.4

*, standard deviation (SD)

Hemagglutination assay was conducted on purified viruses according to the method described in Materials and Methods. All of the virus titers were adjusted to the same HA value based on the agglutination in turkey red blood cells. Each assay was conducted at three biological replicates.

Table 4 Attachment of recombinant virus to tracheal tissues.

Tissue	Recombinant viruses	
	rH3N2-222L	rH3N2-222W
Avian	+++	++++
Canine	+++++	++++
Equine	-	-

The mean abundance of cells and strength of signals to which virus attached were scored as follows: -, no attachment detected; +, attachment to rare or few cells; +++, attachment to a moderate number of cells; +++++, attachment to many cells with strong signals. (The scores are median scores of three individual repeats).

Table 5 Glycans on the microarray showed obvious changes in the HA-222L wild type strain over 222W mutant

Glycan no.	Sugar name	Normalized signal (RFU)		222L/222W
		rH3N2-222L	rH3N2-222W	
283	Neu5Gca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	6563	103	63.89
377	Neu5Aca2-3Galb1-4GlcNAcb1-3GalNAc-Sp14	2142	764	2.81
256	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp8	6566	617	10.64
255	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	3744	1131	3.31
257	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb-Sp8	6235	2514	2.48
258	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4GlcNAcb-Sp8	10128	1916	5.28
254	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	7615	2576	2.96
535	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-2Mana-Sp0	8680	2642	3.28
253	Neu5Aca2-3Galb1-4(Fuca1-3)(6S)GlcNAcb-Sp8	9783	3495	2.80
240	Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb-Sp8	6808	1338	5.09

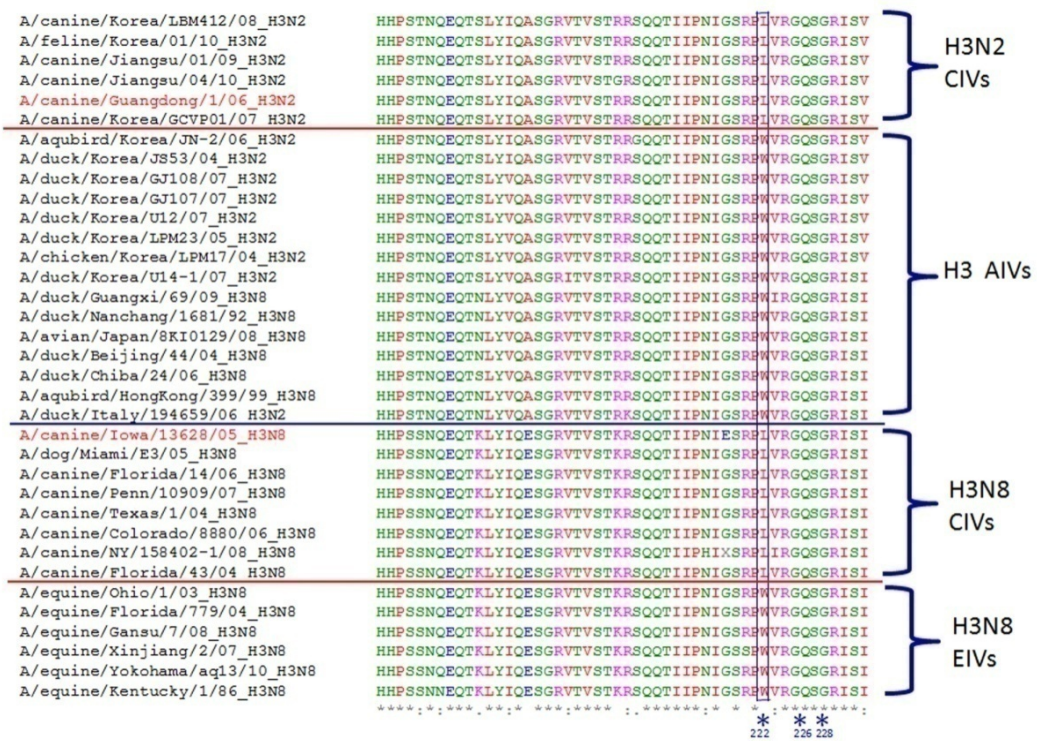

Figure 1 Comparison of receptor binding region of hemagglutinin sequences.

Predicted protein sequences of hemagglutinin of some representative avian influenza viruses H3N2 and H3N8, equine H3N8, canine influenza viruses H3N2 and H3N8 from 1986 to2010, were downloaded from NCBI and aligned using ClustalW2. Residue 222 was highlighted in frame and residues 226 and 228 were highlighted with *. A/canine/Guangdong/1/2006 (H3N2) was applied in this study.

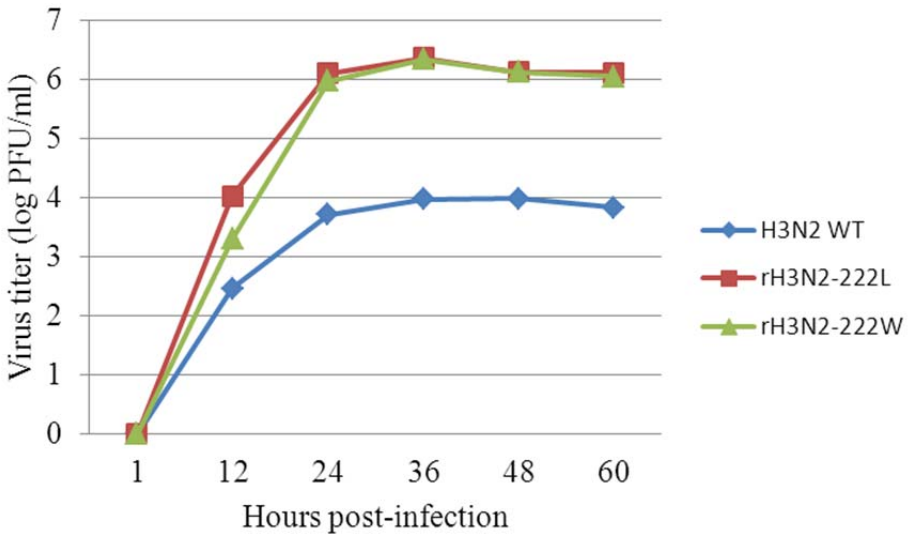

Figure 2 Growth curve of H3N2 CIV and recombinant viruses.

MDCK cells were infected with the recombinant viruses at an MOI of 0.01 PFU/cell. Virus in the supernatant was titrated (log PFU/ml) at the indicated times post-infection.

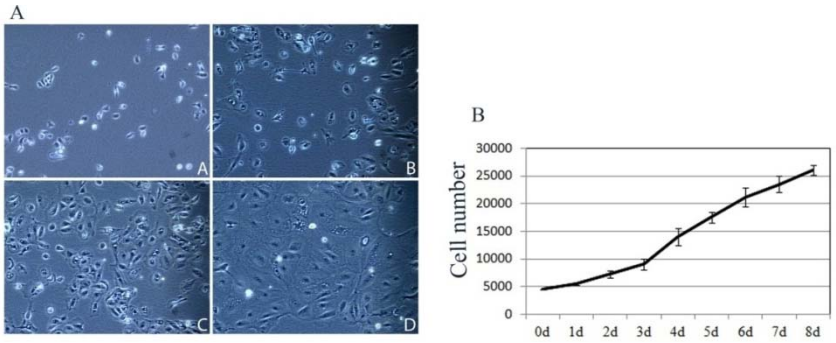

Figure 3 The morphology of primary canine tracheal epithelial cells.

A, CTE cells growing in BEGM medium on surface of tissue culture flask at different growing stages were shown in panels, (A) 1d, (B) 3d, (C) 4d, (D), 7d. B, Growth dynamic curve of canine tracheal epithelial cell. (magnification, 100×).

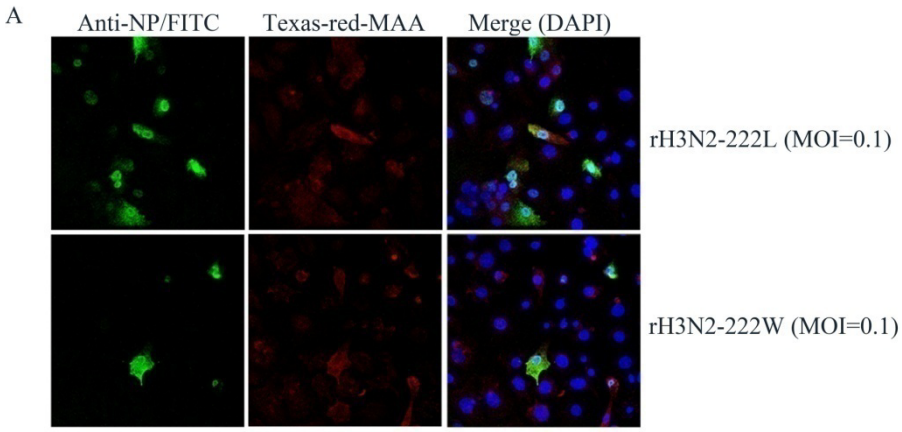

Figure 4 Infection of CTE cells with generated recombinant viruses at an MOI=0.1.

Primary canine tracheal epithelial cells were inoculated with recombinant viruses, rH3N2-222L and rH3N2-222W, respectively, at MOI=0.1, for 1 h adsorption at 37°C. After 7 h.p.i., the expression of viral proteins in CTE cells was detected by anti-influenza NP and goat anti-mouse IgG (H+L) FITC conjugated secondary antibodies. The infected cells were further characterized by staining for cells expressing α2,3 linked glycans with marker Texas red MAA (red), and nuclei of cells were counterstained by DAPI (blue).

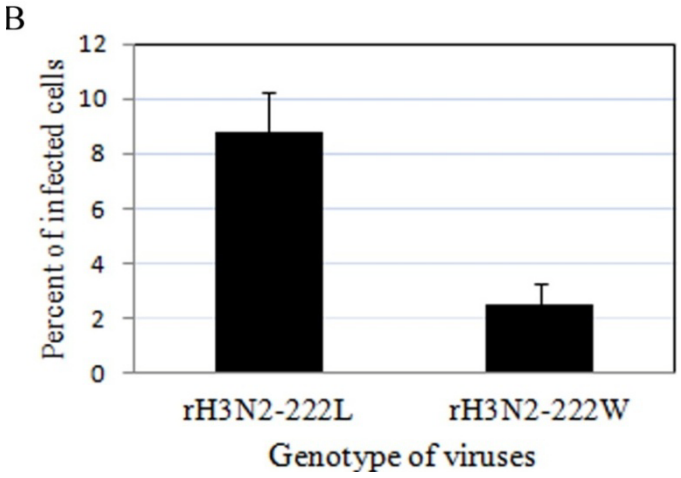

Figure 5 Infection of CTE cells with generated recombinant viruses.

The infected cells were counted in at least eight individual fields from each infection. The ratio of infected cells was calculated.

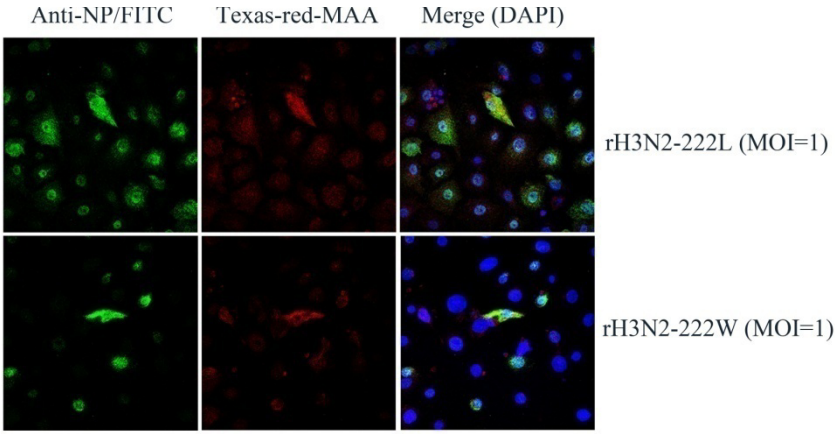

Figure 6 Infection of CTE cells with generated recombinant viruses at an MOI=1.

Primary canine tracheal epithelial cells were inoculated with recombinant viruses, rH3N2-222L and rH3N2-222W, respectively, at MOI=1, for 1 h incubation at 37°C. After 7 h.p.i., the expression of viral proteins was determined by anti-influenza NP primary antibody and goat anti-mouse IgG (H+L) FITC conjugated secondary antibody. The infected cells were further characterized by staining cells expressing α2,3 linked glycans with marker Texas red MAA (red) and nuclei of cells were counterstained by DAPI (blue).

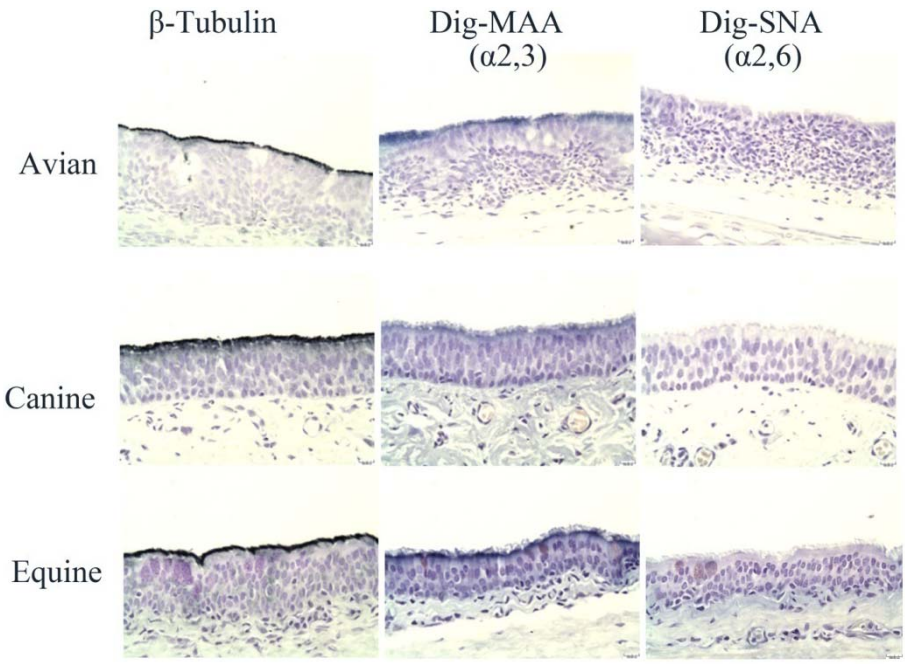

Figure 7 Determination of epithelial cell types of tracheal tissues from avian (chicken), canine (dog) and equine (horse).

β-tubulin was applied to identify the cilia, which were stained gray to black color with the development of VECTOR SG substrate; Dig-MAA, designated as marker of α2,3-SA linked glycoproteins; Dig-SNA, designated marker of α2,6-SA linked glycoproteins, both were developed with NBT or BCIP solution to produce gray color. Cell nuclei were counterstained to blue color by hematoxylin.

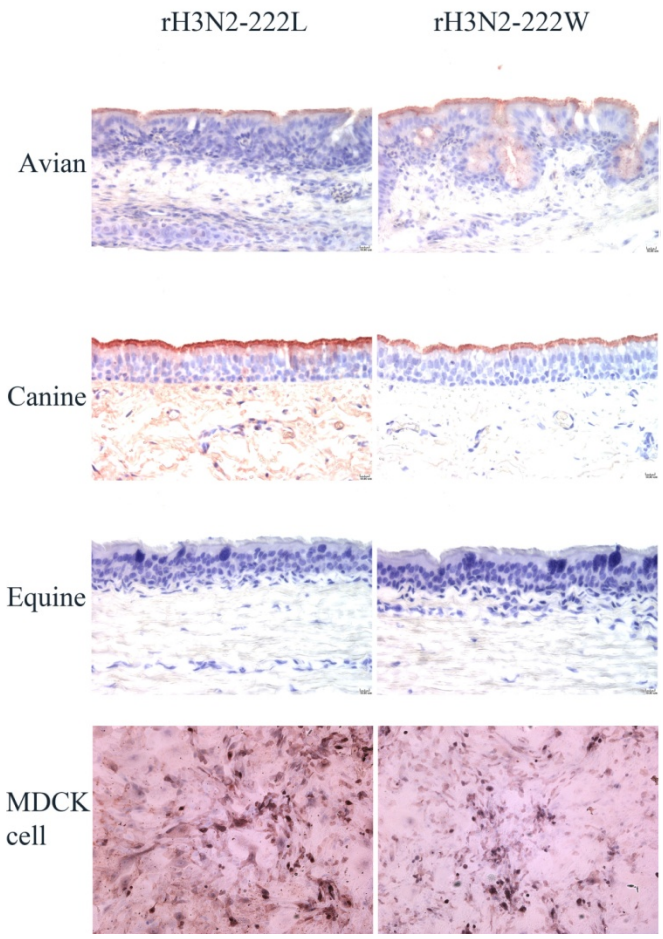

Figure 8 Attachment of two recombinant viruses to tracheal tissues.

Formalin fixed, paraffin embedded histological sections of tracheal tissues from pathogen free avian (chicken), canine (dog) and equine (horse) were deparaffinized before the binding assay. In the tracheal section, viruses-visible as different level of red-brown staining were attached only to epithelial cilia of avian, canine and equine. The panels were chosen to reflect binding pattern in the tissue sections from three biological repeats, but small differences between the single panels and overall view may exist.

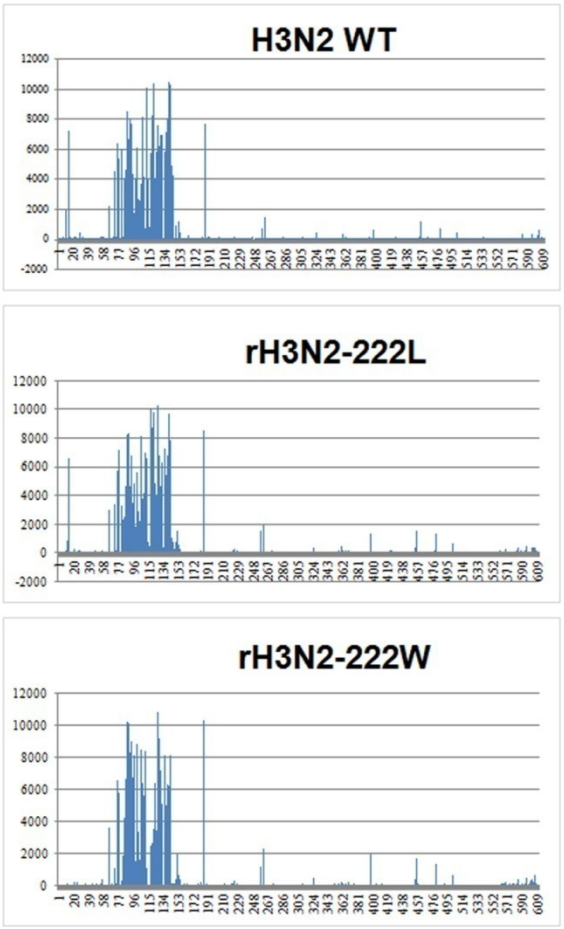

Figure 9 Glycan receptor binding profiles of canine influenza virus and its HA reassortants.

Purified and concentrated viruses were subjected to labeling, dialysis and glycan binding (version 5.0). Similar glycan binding patterns are shown between seed canine H3N2, rH3N2-222L and rH3N2-222W viruses. Glycans shown in the graph were re-ordered according to the glycan name from Z to A

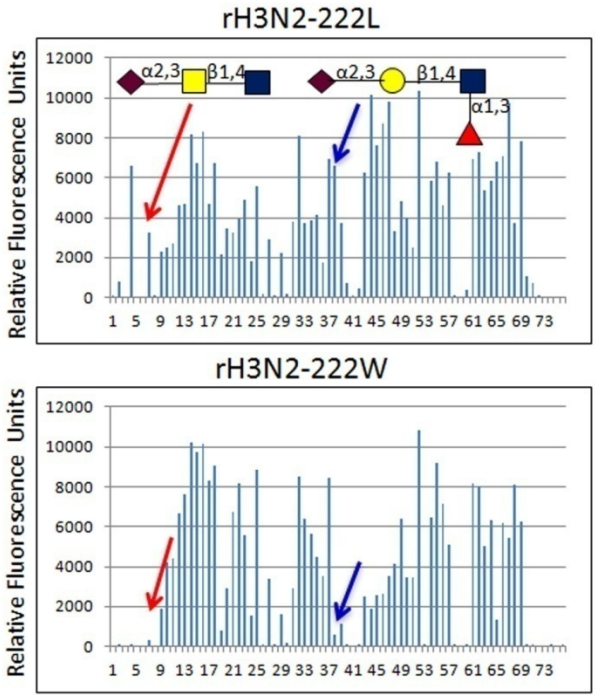

Figure 10 The impact of L222W on the glycan binding affinity of canine influenza viruses.

Comparison of the binding affinity of canine influenza viruses between 222L and 222W to all alpha 2,3 glycans on the microarray.

CHAPTER III

RISK ASSESSMENT OF CANINE INFLUENZA VIRUS H3N2 INFECTION IN HUMANS

Abstract

Primary human tracheal epithelial cells (HTEpCs) were infected with canine virus, A/canine/Guangdong/1/2006 (H3N2). Preliminary immunocytochemistry data showed that this canine virus could infect HTEpCs with α2,6-sialic acid linked glycans with higher multiplicity of infection. The pilot ferret infection experiment performed through nasal inoculation with canine influenza virus A/canine/Guangdong/1/ 2006(H3N2) demonstrated mild symptoms including sneezing, but no obvious body temperature elevation was detected after virus inoculation. Virus shedding was detected in the nasal washings for 6 days post inoculation (d.p.i.) with the titers peaking on 3 to 4 d.p.i.. High virus titers were detected in homogenized turbinates of infected ferrets and no virus shedding were detected in mock infected ferrets at 5 d.p.i.. Hemagglutination inhibition (HI) assays indicated that seroconversion of the infected ferrets during the convalescent stage on 14 d.p.i. with high HI (HI=640-1280) values. Histopathological examination of tissues at 5 d.p.i. indicated that H3N2 CIV inoculated ferrets developed rhinitis and bronchiolitis. No viral shedding, pathological lesions or seroconversion was detected in the mock infected group. The pilot experimental result demonstrated that the avian-origin H3N2 canine influenza virus could cause infection and mild pathogenicity in

ferrets and this virus may potentially have the ability to infect human beings through mutation and adaptation. Virus tissue attachment also indicated that CIV H3N2 might have the potential to infect humans. Cross reactions were detected between H3 canine viruses and human H3N2 antisera by HI assay, which could complicate the surveillance of canine and human influenza viruses. Taken together, canine H3N2 is low pathogenic to canine but presents a potential threat to public health, and it is possible that this virus will mutate and/or reassert with other influenza A viruses among human-canine interface, and generate a pandemic or epidemic influenza strain.

Introduction

Influenza A viruses have caused loss of human lives and large economic burdens around the world and continue to present a huge challenge to both animal and human health. As a zoonotic pathogen, influenza A viruses have a wide range of natural host reservoirs, such as avian, swine, equine, and sea mammals. Most of the influenza viruses infect humans through genomic reassortment, mutation, adaptation and evolution of influenza viruses from birds. The toll of the past four recorded pandemic influenza viruses are as follows: more than 40 million people were killed globally in the 1918 catastrophic avian origin H1N1 Spanish influenza (Patterson and Pyle, 1991); H2N2 avian-origin Asian flu caused more than 1 million deaths globally from 1957 to 1968 (Kilbourne, 2006); H3N2 avian-origin Hong Kong flu of 1968 killed about a half million people globally (Guan et al., 2010; Kilbourne, 2006); the most recent swine-origin 2009 H1N1 pandemic caused about 55 million infections and more than 11,000 deaths in the United States, mostly in children and young adults (Reed et al., 2009).

One of the major challenges of influenza research is to choose a suitable animal model that accurately reflects the disease and the protective immune response to influenza infection in humans. Ferrets are susceptible to infection with human influenza viruses and an important model to study human influenza virus pathogenicity, transmissibility, attenuation, immunogenicity, and protective efficacy of the vaccine strains and for evaluation of antiviral drugs. Ferrets also have similarities to humans in lung physiology, airway morphology, and cell types present in the respiratory tract, including the distribution of the α2,6 sialic acid lineage receptor for human influenza viruses (Plopper et al., 1980; van Riel et al., 2007). Also ferrets develop some of the symptoms of influenza that are observed in humans (Matsuoka et al., 2009).

Recently, H3N8 equine-origin CIV has been identified in canine populations in North America and Europe (Crawford et al., 2005), and an H3N2 avian-origin influenza virus has caused epidemics in dog populations in Asia (Li et al., 2010; Song et al., 2008). The emergence and spread of influenza viruses in dogs have broadened the host range of influenza A viruses and complicated influenza ecology. With the pet dog as its natural host, CIV shortens the distance between animals and humans more than ever before at the animal-human interface. Thus, CIVs are presenting potentially emerging threats to public health, and the risk of these CIVs to human health has not been evaluated. This study investigated the potential of CIV to infect humans by evaluating of the susceptibility of cultured human airway epithelial cells and the susceptibility of ferrets to CIV.

Materials and Methods

HTEpC culture

Primary human tracheal epithelial cell line (HTEpC) was purchased from Cell Applications, Inc. (San Diego, California). Cell maintenance and propagation was conducted based on the instruction of the manufacturer. The cells were proliferated in T-75 flasks with growth medium from Cell Applications, Inc., and stored in -150°C freezer or liquid nitrogen. Passage 2 cells were expanded similarly. Upon reaching 70% to 80% confluence, the cells were trypsinized and subpassaged; P5 cells were applied to the infection experiment.

HTEpC infection and double staining

HTEpC cells were grown in 8-well slides and upon confluence were washed with growth medium and inoculated with A/canine/Guangdong/1/2006 (H3N2) seed virus at a multiplicity of infection (MOI) of 1.0 and 10. After 1 h of incubation at 37°C, the inoculum was removed, and the cells were washed with growth medium and incubated at 37°C in an atmosphere of 5% CO_2. At 6-8 h.p.i, the cells were fixed with 4% paraformaldehyde and processed for immunocytostaining. After being washed with PBS and blocked with 1% BSA in PBST, the virus-infected cells were incubated with monoclonal antibody anti-influenza NP, and secondary antibody, FITC-conjugated goat anti-mouse IgG (H+L) (Millipore, Temecula, California). Fluorescent labeled lectins, Texas red-MAA and Texas red-SNA, were applied to stain the cells according to the instruction. DAPI (Sigma-Aldrich, Saint Louis, Missouri) was applied for nuclear staining. The cultures were mounted and photographed for representative images. To determine the number of infected cells and the tropisms of the viruses, the stained

cultures were observed at 400× magnification under confocal or at 100× with Olympus BX-51microscope. Each inoculation and staining was repeated at least two times with different lots of HTEpC cultures.

Ferret infection experiment

The ferret infection experiment was approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC). Four specific-pathogen-free ferrets (4 month old, females) and that were confirmed to be serologically negative for influenza virus were purchased (Triple F Farms, Inc., Sayre, Pennsylvania). The ferrets were acclimatized for one week. Then two ferrets were intranasally inoculated with 10^4 PFU of A/canine/Guangdong/1/2006 (H3N2) seed virus (in volume of 1 ml) according to the method (Kalthoff et al., 2008). Two ferrets were used as controls by inoculation with I ml PBS buffer. The experimental design is illustrated in the flowchart (Figure 11). After virus inoculation, body temperature, nasal washes and fecal swabs were taken every day (until 14 d.p.i.). Fecal swabs were collected from the ferrets using absorbent cotton swabs (Fisher Scientific, Pittsburgh, PA). One virus exposed ferret and one control ferret were euthanatized on 5 d.p.i., then the remaining ferrets were euthanatized on 14 d.p.i. Ten tissues, including trachea, nasal turbinate, lung, liver, spleen, colon, small intestine, kidney, brain, and heart, were collected from euthanatized ferrets. Samples for virology were stored at -80°C. Virus shedding after inoculation collected from nose was determined using plaque assay. The sera were collected and hemagglutination inhibition assay was conducted to determine the antibody generation.

Ferret anesthesia and sample collection

Ketamine and xylazine were used to anesthetize ferrets, followed with nasal wash and fecal swab every day until 14 d.p.i. Briefly, 1ml PBS with antibiotics in plastic feeding tube (Instech Laboratories, Inc. Plymouth Meeting, Pennsylvania) was used to tease the nose of the ferret, petri-dish or weighing boat was used to collect the nasal discharge, and recover the washings. These were transferred to centrifuge tubes which were stored in -80˚C. Oxygen and isoflurane were applied while bleeding the ferret, 1ml blood was collected from each ferret every other day post virus inoculation.

Specimen collection for histology

Tissues for histology were fixed in 10% neutral buffered formalin and embedded in paraffin; 5-µm sections were stained with hematoxylin and eosin for histopathologic examination (conducted by Diagnosis Laboratory Services, College of Veterinary Medicine, Mississippi State University). The infection of CIVs was evaluated using immunohistochemistry staining.

Tissue homogenization

Frozen tissues from each of the 4 ferrets were thawed and homogenized in 10 volumes of PBS (pH7.4) with 100 U of penicillin and 100 µg of streptomycin per 100 ml. Briefly, 1ml PBS was added to 100 mg tissue, and ground thoroughly, followed by collecting sample to centrifuge tube, separately. Solid debris was removed by centrifugation at 2000×g for 15 min, and supernatants were used to conduct plaque assays to determine the viral titer of tissues infected by H3N2 CIV.

Plaque assay

Suspended MDCK cells were sub-passaged to 6-well or 12-well tissue culture plate. Cells were grown at 37ºC in a humidified atmosphere containing 5% CO_2. Duplicate monolayers of MDCK cells were washed extensively with PBS before and after adsorption of virus as described (Gaush and Smith, 1968) with minor modifications. Serial 10-fold dilutions of virus stocks were prepared. Then the plates were incubated at 37ºC for 1 h. During this period, the 1.2% agarose gel (autoclaved) was melted and mixed with 42°C pre-warmed 2x serum free DMEM medium containing 100 U of penicillin and 100 µg of streptomycin per 100 ml. After the virus absorbance, the inoculums were aspirated and wells were washed twice with PBS, and 3 ml agarose medium was added to each well (6-well plate). Then the plate was covered and allowed to stand in the hood undisturbed for 15 min. Plates were incubated in the CO_2 incubator at 37°C for 72 h, then fixed in 10% formalin for 30 min at room temperature. Agarose gel was removed from the plate using a spatula and followed with PBS washing for at least two times. Crystal violet was applied to stain the wells at room temperature for 30 min. Staining solution was removed and plates were washed three times. Plates dried in the hood and plaques were counted and the viral titers were calculated as numbers of PFU/ml or PFU/g tissue.

HI assay

HI test was performed in 96-dome well microtiter plates following the method described elsewhere (WHO, 1982). Briefly, the serum samples were incubated with receptor-destroying enzyme (DENKA SEIKEN Co. LTD, Accurate Chemical & Scientific Corp., Westbury, New York) to remove nonspecific inhibitors of

hemagglutination. Antiserum (1 part) and receptor-destroying enzyme (3 parts) were mixed together for 16 h at 37ºC incubation before heat inactivation for 30 min at 56ºC. Each testing antiserum was diluted to 10 fold with saline buffer before use, and a two-fold serial antiserum dilution was performed in PBS. Then 25µl virus with HAU=8 (50 µl) was added to each corresponding well, gently shaking the plate, the plate was placed in a 37 ºC incubator for 30 min. This was followed by adding 50 µl of 0.5% turkey red blood cells to each well including control well. The plate was held at room temperature for 40 min or 37ºC for 30 min and agglutination endpoints were determined and used to calculate the HI titer. On the basis of assay results from serum of uninfected specific-pathogen-free ferrets with HI titers < 20, HI titers ≥ 20 were considered as evidence of seroconversion. All HI assays were performed in three replicates.

Preparation of turkey red blood cells

Fresh turkey blood was obtained from healthy turkey in the Department of Poultry Sciences, MSU. The collected blood was mixed with Alsever's solution (1:1 volume ratio) in 50 ml polypropene centrifuge tube (Thomas Scientific, Swedesboro, NJ) by gently inverting tube to prevent blood from agglutinating. Sterilized PBS pH 7.4 was applied to wash the blood with gentle rocking, followed by centrifugation at 4°C for 10 min at 3000 rpm (Eppendorf, Hauppauge, NY). The supernatant and the white blood cell layer were removed, PBS was added to wash the cells again, and the wash was repeated three times. To make 0.5% tRBC, 250 µl pure tRBC was added to 50 ml PBS, and mixed gently, at which point the cells were ready for use.

Tissue preparation for virus histochemistry assay

Ferret tissues, trachea, larynx were fixed and processed according to the method mentioned in Materials and Methods of Chapter II.

Results

HTEpC infected by canine influenza viruses

Immunocytochemistry stain of HTEpC inoculated with H3N2 CIV showed that HTEpC culture (P5) was infected by A/canine/Guangdong/1/2006 (H3N2) at MOI of 1 (Figure 12) and 10 (Figure 13). While at MOI=1, most of the infected cells were MAA labeled. This indicated that canine viruses mainly infected HTEpC cells with α2,3 SA linked glycoprotein receptors. When MOI increased to 10, many cells were infected with virus and most of the infected cells were labeled by MAA. However, some infected cells stained by Texas-red-SNA (Figure 13), indicating canine influenza viruses had the ability to bind to both α2,3 linked SA and α2,6 linked SA receptors of human tracheal epithelial cells.

Pathogenesis of H3N2 canine influenza viruses in ferrets

Two infected ferrets demonstrated mild symptoms including sneezing but no body temperature elevation was detected. In comparison no changes were seen in two ferrets that were mock infected. Viruses were detected in the nasal washings until 6 d.p.i. with the viral titers peaked on 3 to 5 d.p.i. (Figure 14 A). No virus was detected in any fecal swab samples. High viral titer was detected in homogenized turbinate sample collected on 5 d.p.i., but not those on 14 d.p.i. No virus was detected in the other nine tissues collected at both 5 d.p.i. and 14 d.p.i. (Figure 14 B).

HI assay with the sera collected on14 d.p.i. indicated the seroconversion of the infected ferrets (Figure 15). Histopathological examination on 5 d.p.i. demonstrated that the infected ferrets developed rhinitis and bronchiolitis (Figure 16), this result was consistent with the virus loads found in the tissues. Immunostaining of other tissues from infected ferret, lung, liver, spleen, intestine, kidney, colon, brain, heart was negative. No viral shedding, pathological lesions or seroconversion was detected in the mock inoculated ferrets. In summary, our results demonstrated that the avian-origin H3N2 canine influenza virus could cause infection and mild pathogenicity in ferrets.

Canine H3 cross reacts with historical human H3N2 seasonal influenza viruses in the middle of 1990s.

HI results showed there was a weak cross reaction between H3N8 and H3N2 canine influenza viruses and their corresponding ferret antisera. With twelve antisera against human H3N2 seasonal influenza viruses, a weak cross reaction was detected between H3N2 canine influenza virus with both A/Nanchang/933/1995(H3N2) and A/Sydney/5/1997(H3N2) ferret antisera (Table 6).

H3N2 canine influenza virus binds to ferret tracheal epithelial ciliated cells

Virus attachment assay indicated that H3N2 virus moderately bound ferret tracheal epithelial ciliated cells (Figure 17), confirming that H3N2 virus has the potential to infect ferrets, as shown above.

Discussion

In human airway epithelial cells, both ciliated cells and goblet cells expressed α2,6 linked SA, indicating that differentiated human airway epithelial cell cultures are

suitable for the evaluation of infectivity and/or pathogenicity of some human strains (Ibricevic et al., 2006). In this study, human tracheal epithelial cell culture could be infected by H3N2 canine influenza A virus, and the majority of the infected cells had surface receptor bearing SAα2,3Gal (MAA stained) in assays with low virus inoculation doses. Previous studies indicate that SAα2,3GAL receptors are found in the human airway or airway cell cultures (Shinya et al., 2006; Wan and Perez, 2007). Glycan microarray data suggest that H3N2 CIV, like avian viruses, preferentially bind to SAα2,3Gal receptors. We also found that at high virus loads, some cells bearing SAα2,6Gal (SNA stained) linked receptors could be infected, indicating binding on the normal receptor for human influenza viruses (Matrosovich et al., 2004).

In our animal infection experiment, the first generation of proliferated seed virus was used to inoculate ferrets. After inoculation, the ferrets did not show obvious body temperature elevation and clinical signs in the infection group were limited to mild sneezing. Virus shedding from nasal washings was detected from 1 d.p.i. to 6 d.p.i., and nasal turbinate was determined to have high virus propagation at 5 d.p.i. Combining the infectivity data with seroconversion and pathologic findings in the nasal cavity, CIV H3N2 can infect ferrets and cause mild disease. The reason for the mild disease could be the low dose of virus. Other studies have used higher doses in ferrets ($\geq 10^6$ $TCID_{50}$ or EID_{50} or PFU) (WHO, 2009) and in mice ($\geq 10^5$ $TCID_{50}$ or EID_{50}) (Kim et al., 2012). To understand the transmissibility and pathogenicity of CIV H3N2, and the related HA mutants, respectively, transmission experiments could be performed in dogs and/or ferrets.

Virus attachment assays provided a useful tool to evaluate the possibilities of infectivity or pathogenicity of influenza A viruses (Jourdain et al., 2011; Kimble et al., 2010; van Riel et al., 2007; Xu et al., 2010). In this study, rH3N2-222L (canine-like) showed moderate binding to ferret tracheal epithelial cilia (Figure 17). This suggests that H3N2 CIV can infect the ferret's trachea. However, homogenized tracheal tissue from a CIV H3N2-inoculated ferret did not show detectable virus at 5 d.p.i.. The reasons may be the low amount of virus in the infected ferret, inability of the plaque assay to detect virus, or the low amount of inoculated virus, or the late date of euthanasia (5 d.p.i.) which exceeded the time for virus detection.

Hemagglutination inhibition assays indicate there was not any or just weak cross reactivity between H3N2 and H3N8 canine influenza viruses with ferret sera against contemporary seasonal influenza viruses. This result showed that canine influenza viruses could have the potential to infect humans and cause disease concern. Because of the cross reactivity, the serological surveillance of CIVs and human H3N2 viruses will become more complicated.

Table 6 Cross reactivity of ferret antibodies to canine H3 viruses and human historical H3 viruses using HI assay.

Virus strain	Sera generated from ferret						
	CIV H3N2 2006	CIV H3N8 2005	Bangkok 79	Philipine 82	Cane 84	Mississippi 85	Leningard 86
CIV H3N2 2006	1280	40	<20	<20	<20	<20	<20
CIV H3N8 2005	40	1280	<20	<20	<20	<20	<20
	Sichuan 87	Sichuan 89	Ann arbor 93	Johannesburg 94	Nanchang 95	Sydney 97	Wisconsin 05
CIV H3N2 2006	27	<20	<20	<20	20	40	<20
CIV H3N8 2005	<20	<20	<20	<20	<20	40	<20

Canine influenza viruses H3N2 and H3N8 were applied to react with antiserum generated from ferret against human H3N2 influenza viruses, respectively. HI < 20 was designated as negative and HI ≥ 20 was designated as positive.

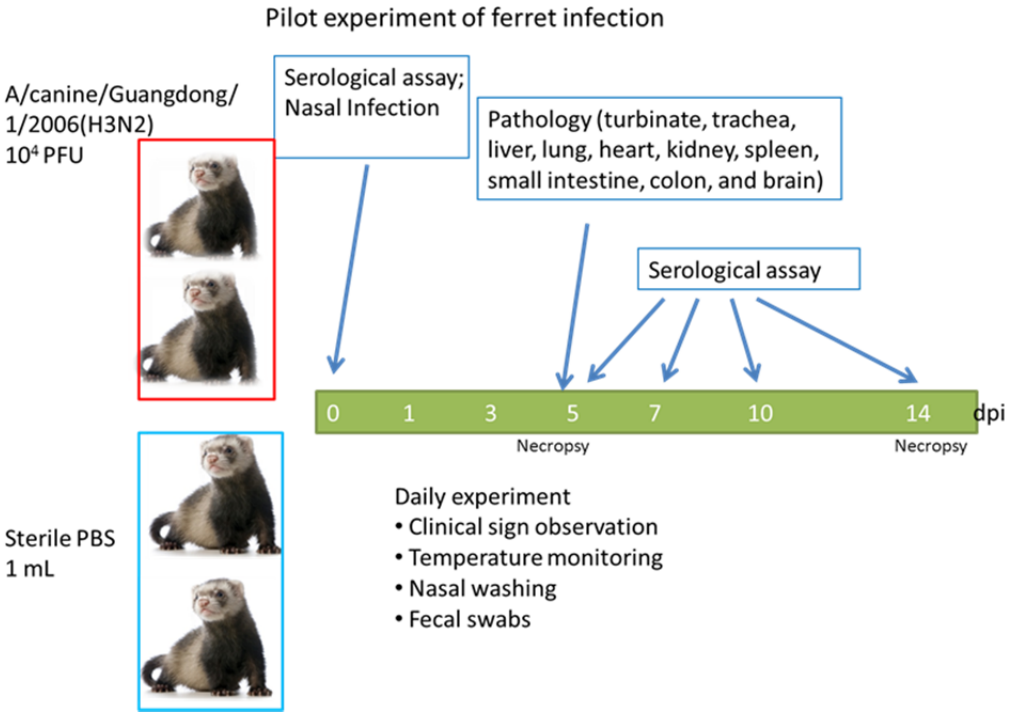

Figure 11 Flowchart of the experimental design for the ferret infection experiment.

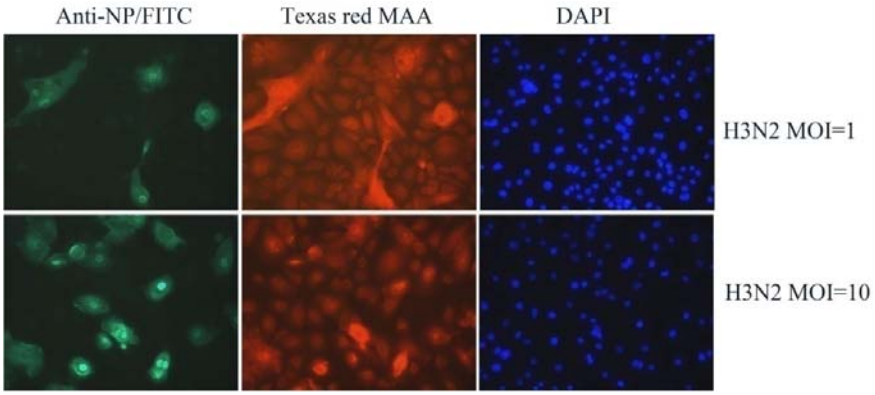

Figure 12 HTEpC infected by CIV H3N2 at MOI=1 and 10 with Texas-red-MAA lectin stain.

Primary human tracheal epithelial cells were inoculated with CIV virus H3N2 at MOI=1 and 10, respectively, for 1 h incubation at 37°C. After 7 h.p.i., the expression of viral proteins was determined by anti-influenza NP primary antibody and goat anti-mouse IgG (H+L) FITC conjugated secondary antibody. The infected cells were characterized by staining with lectin marker, Texas red MAA (red) and cell nuclei were counterstained by DAPI (blue). Images were captured with microscope, Olympus BX-51, 200×.

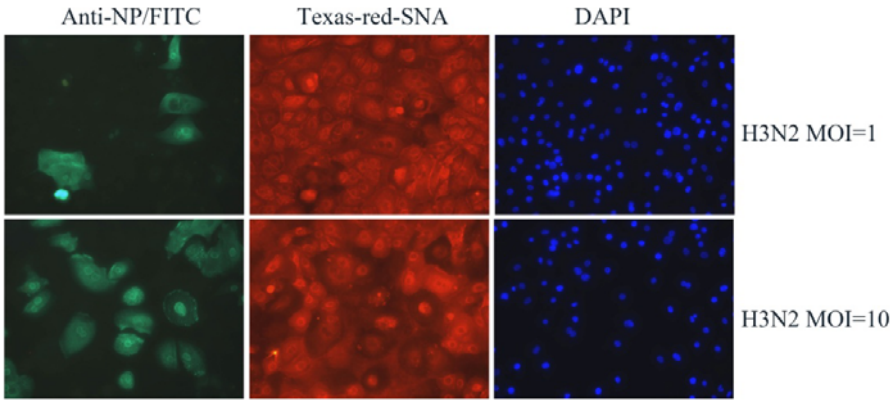

Figure 13 HTEpC infected by CIV H3N2 at MOI=1 and 10 with Texas-red-SNA lectin stain.

Primary human tracheal epithelial cells were inoculated with CIV virus H3N2 at MOI=1 and 10, respectively, for 1 h incubation at 37°C. After 7 h.p.i., the expression of viral proteins was determined by anti-influenza NP primary antibody and goat anti-mouse IgG (H+L) FITC conjugated secondary antibody. The infected cells were characterized by staining with lectin, marker Texas red SNA (red), and nuclei were counterstained by DAPI (blue). Images were captured with microscope, Olympus BX-51, 200×.

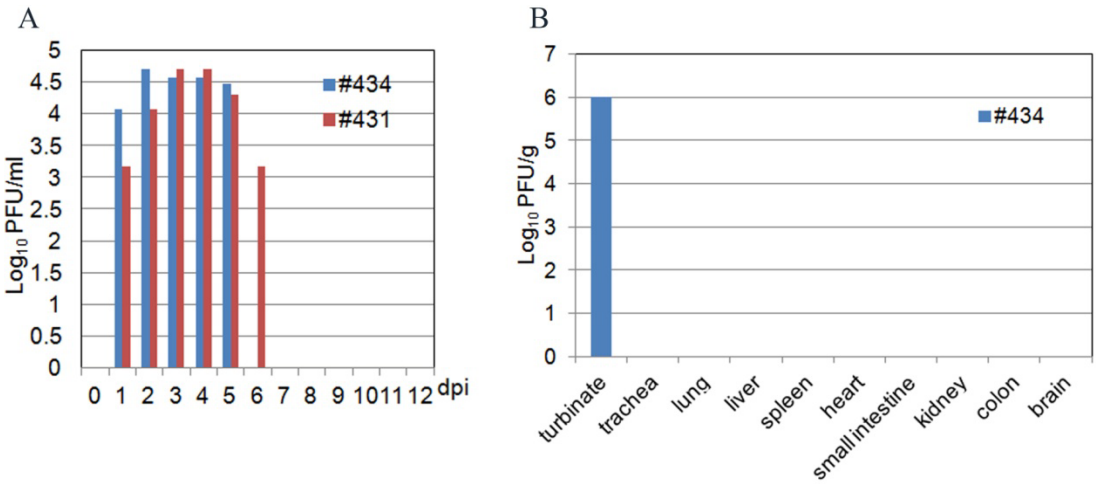

Figure 14 Infectivity of canine influenza virus H3N2 inoculated to ferret.

A, Virus titers were determined by plaque assay from nasal washings after CIV H3N2 inoculation. B, Virus titration of homogenized ferret tissues at 5 d.p.i.

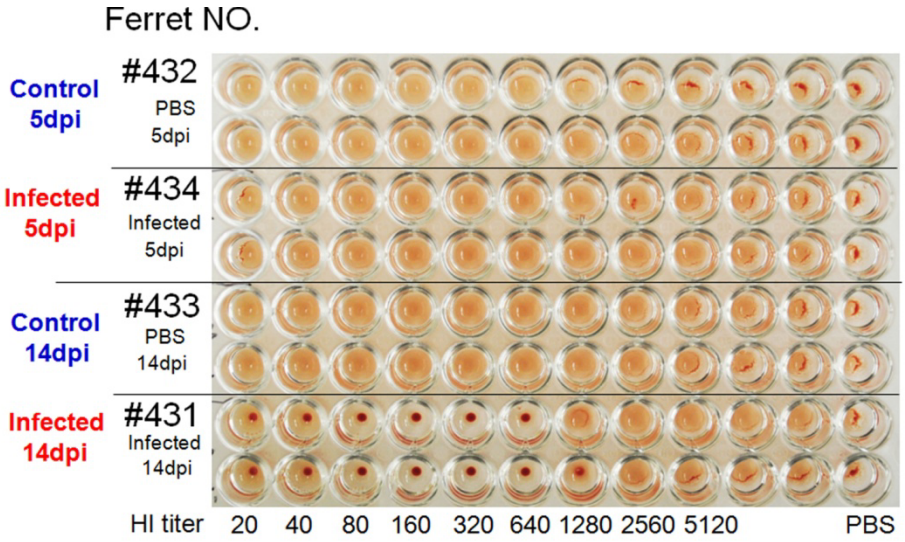

Figure 15 Seroconversion detected in ferret after CIV H3N2 infection.

Sera were collected from ferrets euthanized at both 5 d.p.i. and 14 d.p.i., and standard HI assay was applied to determine the antibody generation after canine H3N2 infection. At 5 d.p.i. antibody could not be detectable based on this experiment, however, the generated antibody can be detected at the convalescent stage (14 d.p.i.).

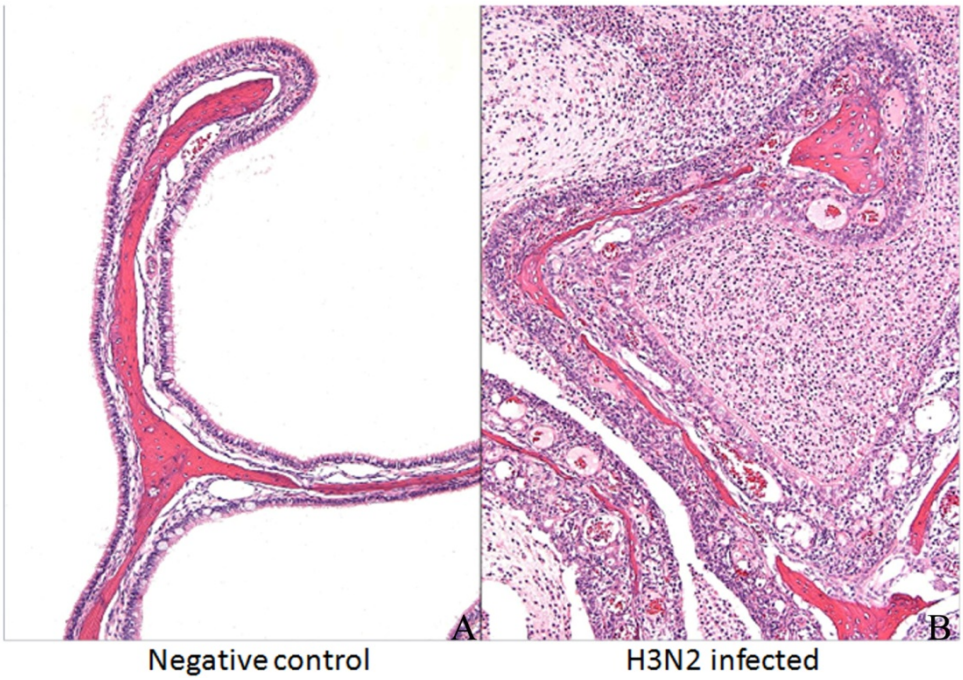

Figure 16 Histological sections demonstrating the rhinitis that developed after CIV H3N2 infection.

The ferrets were inoculated with 10^4 PFU H3N2 avian origin canine influenza virus and PBS as mock infected control. One set of ferrets was euthanized at 5 d.p.i. Multiple sections of nasal cavity were examined at different levels. The negative control was normal (A). The infected ferret was shown to have rhinitis with necrosis and atrophy, and severe mucopurulent discharge (B). The nasal cavity in the infected ferret is filled with neutrophils admixed with proteinaceous fluid, necrotic cellular debris and mucus. This exudate fills the interstices between nasal turbinates. Sections were from formalin fixed paraffin embedded tissues and stained with hematoxylin and eosin.

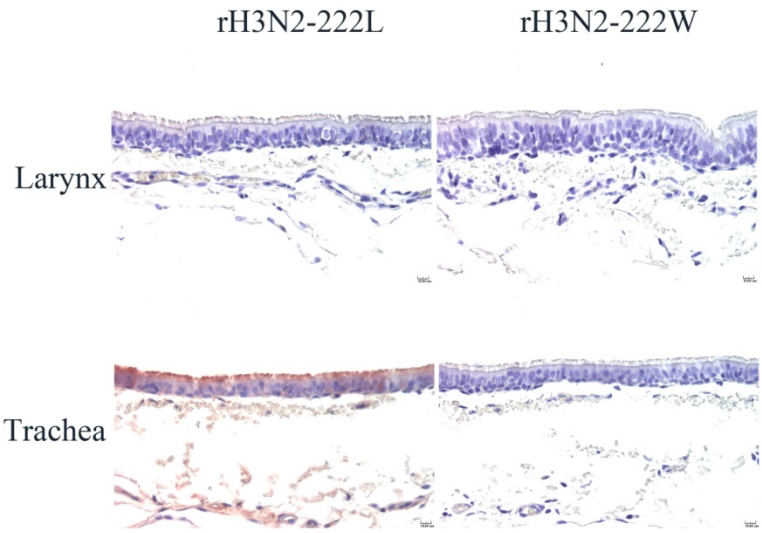

Figure 17 Attachment of recombinant viruses to ferret tissues.

Trachea and larynx were obtained from pathogen free ferret. Tissues were formalin-fixed and paraffin-embedded before virus histochemistry assay.

CHAPTER IV

CONCLUSIONS

Computational analysis and sequence alignment indicate that receptor binding site 222 in HA of H3N2 CIV could play an important role in host specificity. Reverse genetics and site-directed mutagenesis approaches were applied to generate H3N2 reassortant and mutant viruses in this study.

Different binding affinities were shown between reassortant and mutant canine H3N2 viruses using hemagglutination assay with different erythrocytes. Infectivity of rH3N2-222L reassortant and rH3N2-222W mutant indicated that virus containing 222L in HA has significantly higher infectivity to primary canine tracheal epithelial cell culture. Profiles of different genotypes of recombinant viruses showed similar glycan binding patterns and all of them preferentially bound with α2,3-linked glycans through glycan microarray. The same set of specific glycans, especially Neu5Aca2-3Galb1-4(Fuca-), Neu5Aca2-3Galb1-3(Fuca-) and Neu5Gca2-3Galb1-4(Fuca-) were shown to have different binding affinity between 222L and 222W of HA. This could be the main mechanism for avian viruses adapting to the canine which required some specific glycans with increased binding affinity.

Virus attachment assay suggests that L222W substitution in HA of CIV H3N2 virus has different binding affinity pattern on tracheal tissue from different species, including avian, canine and equine.

Human tracheal epithelial cell cultures were infected by CIVs H3N2. Pilot ferret infection experiment indicated that CIV H3N2 could infect the ferret and cause pathologic changes, and immune response was induced upon virus infection.

Canine H3 viruses share similar epitopes with human historical H3 viruses which could interfere with the serologic surveillance of CIV H3N2 and H3N8 and human H3N2 viruses.

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APPENDIX A

ALIGNMENT OF PREDICTED PROTEIN SEQUENCES OF HA OF AVIAN AND CANINE H3N2 AND EQUINE AND CANINE H3N8 DOWNLOADED FROM NCBI

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A/canine/Korea/LBM412/08_H3N2       ------------------QNLPGNENNAATLCLGHHAVPNGTIVKTITDD 32
A/feline/Korea/01/10_H3N2           MKTVIALS--CIFCLAFGQNLPGNENNAATLCLGHHAVPNGTIVKTITDD 48
A/canine/Jiangsu/01/09_H3N2         MKTVIALS--YIFCLAFGQNLPGNENNAATLCLGHHAVPNGTIVKTITDD 48
A/canine/Jiangsu/04/10_H3N2         MKTVIALS--YIFCLAFGQNLPGNENNAATLCLGHHAVPNGTIVKTITDD 48
A/canine/Guangdong/1/06_H3N2        MKTVIALS--YIFCLAFGQNLPGNENNAATLCLGHHAVPNGTIVKTITDD 48
A/canine/Korea/GCVP01/07_H3N2       MKTVIALS--YIFCLAFGQNLPGNENNAATLCLGHHAVPNGTIVKTITDD 48
A/aqubird/Korea/JN-2/06_H3N2        MKTVIALS--YIFCLAFGQNLPGNENNTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Korea/JS53/04_H3N2           MKTVIALS--YIFCLAFGQNLSGNENNTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Korea/GJ108/07_H3N2          MKTVIALG--YIFCLAFGQNLPESENNTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Korea/GJ107/07_H3N2          ----IALS--YIFCLAFGQNLPESENNTATLCLGHHAVPNGTIVKTITDD 44
A/duck/Korea/U12/07_H3N2            MKTAIALS--YILCLASGQNLPGSENNTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Korea/LPM23/05_H3N2          MKTVIALS--CIFCLAFGQNLPGNENNTATLCLGHHAVPNGTIVKTITDD 48
A/chicken/Korea/LPM17/04_H3N2       MKTVIAFS--YIFCLAFGQNLPGNENNTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Korea/U14-1/07_H3N2          MKTVIALS--YIFCLAFGQNLPGNENNTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Guangxi/69/09_H3N8           MKTVIALS--YIFCLAFGQNLPENDNSTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Nanchang/1681/92_H3N8        MKTVIALS--YIFCLAFGQDLSGNDNSTATLCLGHHAVPNGTIVKTITDD 48
A/avian/Japan/8KI0129/08_H3N8       MKTVIALS--YIFCLAFGQDLPGNDNSTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Beijing/44/04_H3N8           MKTVIALS--YIFCLAFGQDLPGNDNSTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Chiba/24/06_H3N8             MKTVIALS--YIFCLTFGQDLPGNDNSTATLCLGHHAVPNGTIVKTITDD 48
A/aqubird/HongKong/399/99_H3N8      MKTVIALS--YILCLAFGQDLPGIDNSTATLCLGHHAVPNGTIVKTITDD 48
A/duck/Italy/194659/06_H3N2         MKTIIALS--YIFCLAFGQDLPGSDNSTATLCLGHHAVPNGTIVKTITDD 48
A/canine/Iowa/13628/05_H3N8         MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTMSDD 47
A/dog/Miami/E3/05_H3N8              MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTMSDD 47
A/canine/Florida/14/06_H3N8         MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKIMSDD 47
A/canine/Penn/10909/07_H3N8         MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTMSDD 47
A/canine/Texas/1/04_H3N8            MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTMSDD 47
A/canine/Colorado/8880/06_H3N8      MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTMSDD 47
A/canine/NY/158402-1/08_H3N8        MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTMSDD 47
A/canine/Florida/43/04_H3N8         ----------LLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTMSDD 39
A/equine/Ohio/1/03_H3N8             MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTISDD 47
A/equine/Florida/779/04_H3N8        MKTTIIL--ILLTHWAYSQN-PISGNNTATLCLGHHAVANGTLVKTISDD 47
A/equine/Gansu/7/08_H3N8            MKTTIIL--ILLTHWAYSQN-PISNNNTATLCLGHHAVANGTLVKTISDD 47
A/equine/Xinjiang/2/07_H3N8         MKTTIIL--ILLTHWAYSQN-PISNNNTATLCLGHHAVANGTLVKTISDD 47
A/equine/Yokohama/aq13/10_H3N8      MKTTIIFNFILLTHWAYSQN-PISNNNTATLCLGHHAVANGTLVKTISDD 49
A/equine/Kentucky/1/86_H3N8         MKTTIIL--ILLTHWVYSQN-PTSGNNTATLCLGHHAVANGTLVKTITDD 47
                                                    *: .   *.:**********.***:** ::**

A/canine/Korea/LBM412/08_H3N2       QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQNE 82
A/feline/Korea/01/10_H3N2           QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQNE 98
A/canine/Jiangsu/01/09_H3N2         QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQNE 98
A/canine/Jiangsu/04/10_H3N2         QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQNE 98
A/canine/Guangdong/1/06_H3N2        QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQNE 98
A/canine/Korea/GCVP01/07_H3N2       QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQNE 98
A/aqubird/Korea/JN-2/06_H3N2        QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVLQDE 98
A/duck/Korea/JS53/04_H3N2           QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 98
A/duck/Korea/GJ108/07_H3N2          QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 98
A/duck/Korea/GJ107/07_H3N2          QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 94
A/duck/Korea/U12/07_H3N2            QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 98
A/duck/Korea/LPM23/05_H3N2          QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 98
A/chicken/Korea/LPM17/04_H3N2       QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 98
A/duck/Korea/U14-1/07_H3N2          QIEVTNATELVQNSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 98
A/duck/Guangxi/69/09_H3N8           QIEVTNATELVQSSSTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 98
A/duck/Nanchang/1681/92_H3N8        QIEVTNATELVQSSSKGKICNNPHRILDGRDCTLVDALLGDPHCDVFQDE 98
A/avian/Japan/8KI0129/08_H3N8       QIEVTNATELVQSSSTGKICNNPHRILDGRDCTLMDALLGDPHCDVFQDE 98
A/duck/Beijing/44/04_H3N8           QIEVTNATELVQSSSTGKICNNPHRILDGRGCTLIDALLGDPHCDVFQDE 98
A/duck/Chiba/24/06_H3N8             QIEVTNATELVQSSSTGKICNNPHRILDGRDCTLIDALLGDPHCDVFQDE 98
A/aqubird/HongKong/399/99_H3N8      QIEVTNATELVQSSSTGKICNNPHRILDGRDCTLIDALLGDPHCDVFQDE 98
A/duck/Italy/194659/06_H3N2         QIEVTNATELVQSSLTGKICNNPHKILDGRDCTLIDALLGDPHCDVFQDE 98
A/canine/Iowa/13628/05_H3N8         QIEVTNATELVQSISMGKICNKSYRILDGRNCTLIDAMLGDPHCDALQYE 97
A/dog/Miami/E3/05_H3N8              QIEVTNATELVQSISMGKICNKSYRILDGRNCTLIDAMLGDPHCDAFQYE 97
A/canine/Florida/14/06_H3N8         QIEVTNATELVQSISMGKICNKSYRILDGRNCTLIDAMLGDPHCDAFQHE 97
A/canine/Penn/10909/07_H3N8         QIEVTNATELVQSISMGKICNKSYRILDGRNCTLIDAMLGDPHCDAFQYE 97
A/canine/Texas/1/04_H3N8            QIEVTNATELVQSISMGKICNKSYRILDGRNCTLIDAMLGDPHCDAFQYE 97
A/canine/Colorado/8880/06_H3N8      QIEVTNATELVQSISMGKICNKSYRILDGRNCTLIDAMLGDPHCDAFQYE 97
A/canine/NY/158402-1/08_H3N8        QIEVTNATELVQSISMGKICNKSYRVLDGRNCTLIDAMLGDPQCDAFQYE 97
A/canine/Florida/43/04_H3N8         QIEVTNATELVQSISMGKICNKSYRILDGRNCTLIDAMLGDPHCDAFQYE 89
A/equine/Ohio/1/03_H3N8             QIEVTNATELVQSISMGKICNNSYRILDGRNCTLIDAMLGDPHCDAFQYE 97
A/equine/Florida/779/04_H3N8        QIEVTNATELVQSISMGKICNNSYRILDGRNCTLIDAMLGDPHCDAFQYE 97
A/equine/Gansu/7/08_H3N8            QIEVTNATELVQSISMGKICNNSYRILDGRNCTLIDAMLGDPHCDVFQYE 97
A/equine/Xinjiang/2/07_H3N8         QIEVTNATELVQSITMGKICNNSYRILDGRNCTLIDAMLGDPHCDVFQYE 97
A/equine/Yokohama/aq13/10_H3N8      QIEVTNATELVQSISMGKICNNSYRILDGRNCTLIDAMLGDPHCDVFQYE 99
A/equine/Kentucky/1/86_H3N8         QIEVTNATELVQSISIGKICNNSYRVLDGRNCTLIDAMLGDPHCDVFQYE 97
                                    ************.   *****:.::*****.***:**:****:**.:* *
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A/canine/Korea/LBM412/08_H3N2       TWDLFVERSNAFSNCYPYDVPDYASLRSIVASSGTLEFITEGFTWAGVTQ 132
A/feline/Korea/01/10_H3N2           TWDLFVERSNAFSNCYPYDVPDYASLRSIVASSGTLEFITEGFTWAGVTQ 148
A/canine/Jiangsu/01/09_H3N2         TWDLFVERSNAFSNCYPYDVPDYASLRSIVASSGTLEFITEGFTWAGVTQ 148
A/canine/Jiangsu/04/10_H3N2         TWDLFVERSNAFSNCYPYDVPDYASLRSIVASSGTLEFITEGFTWAGVTQ 148
A/canine/Guangdong/1/06_H3N2        TWDLFVERSNAFSNCYPYDVPDYASLRSIVASSGTLEFITEGFTWAGVTQ 148
A/canine/Korea/GCVP01/07_H3N2       TWDLFVERSNAFSNCYPYDVPDYASLRSIVASSGTLEFITEGFTWTGVTQ 148
A/aqubird/Korea/JN-2/06_H3N2        TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWAGVTQ 148
A/duck/Korea/JS53/04_H3N2           TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWAGVTQ 148
A/duck/Korea/GJ108/07_H3N2          TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTGVTQ 148
A/duck/Korea/GJ107/07_H3N2          TWDLFVERSNAFSNCYPYDVPNYASLRSLVASSGTLEFITEGFTWTGVTQ 144
A/duck/Korea/U12/07_H3N2            TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTGVTQ 148
A/duck/Korea/LPM23/05_H3N2          TWDLFVERSNAFSSCYPYDVPDYASLRSLVASSGTLEFITEGFTWAGVTQ 148
A/chicken/Korea/LPM17/04_H3N2       TWDLFVERSNAFSSCYPYDVPDYASLRSLVASSGTLEFITEGFTWAGVTQ 148
A/duck/Korea/U14-1/07_H3N2          TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFNWAGVTQ 148
A/duck/Guangxi/69/09_H3N8           TWDLFVERSSAFSNCYPYDVPDYASLRSLIASSGTLEFITEGFTWAGVTQ 148
A/duck/Nanchang/1681/92_H3N8        TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTGVTQ 148
A/avian/Japan/8KI0129/08_H3N8       TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTGVTQ 148
A/duck/Beijing/44/04_H3N8           TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTGVTQ 148
A/duck/Chiba/24/06_H3N8             TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTGVTQ 148
A/aqubird/HongKong/399/99_H3N8      TWDLFVERSNAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTGVTQ 148
A/duck/Italy/194659/06_H3N2         TWDLFVERSNAFSNCYPYDVPDYASLRALVATSGTLEFITEGFTWNGVTQ 148
A/canine/Iowa/13628/05_H3N8         SWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTVEFTAEGFTWTGVTQ 147
A/dog/Miami/E3/05_H3N8              SWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTVEFTAEGFTWTGVTQ 147
A/canine/Florida/14/06_H3N8         SWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTVEFTAEGFTWTGVTQ 147
A/canine/Penn/10909/07_H3N8         SWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTVEFTAEGFTWTGVTQ 147
A/canine/Texas/1/04_H3N8            SWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTVEFTAEGFTWTGVTQ 147
A/canine/Colorado/8880/06_H3N8      SWDLFIERSNAFSNCYPYDIPDYASLRSIVASSGTVEFTAEGFTWTGVTQ 147
A/canine/NY/158402-1/08_H3N8        SWDLFIERSNAFSNCYPYDIPDYASLRSIVASSGTVEFTAEGFTWTGVTQ 147
A/canine/Florida/43/04_H3N8         SWDLFIERSNAFSNCYPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQ 139
A/equine/Ohio/1/03_H3N8             NWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQ 147
A/equine/Florida/779/04_H3N8        NWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQ 147
A/equine/Gansu/7/08_H3N8            NWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQ 147
A/equine/Xinjiang/2/07_H3N8         NWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQ 147
A/equine/Yokohama/aq13/10_H3N8      NWDLFIERSSAFSNCYPYDILDYASLRSIIASSGTLEFTAEGFTWTGVTQ 149
A/equine/Kentucky/1/86_H3N8         NWDLFIERSSAFSNCYPYDIPDYASLRSIVASSGTLEFTAEGFTWTGVTQ 147
                                    .****:***.***.*****: :*****:::*:***:** :***.* ****

A/canine/Korea/LBM412/08_H3N2       NGGSGACKKGPANGFFSRLNWLTKSGNTYPVLNVTMPNNNNFDKLYIWGV 182
A/feline/Korea/01/10_H3N2           NGGSGACKRGPANGFFSRLNWLTKSGNTYPVLNVTMPNNNNFDKLYIWGV 198
A/canine/Jiangsu/01/09_H3N2         NGGSGACKRGPANGFFSRLNWLTKSGNTYPVLNVTMPNNNNFDKLYIWGV 198
A/canine/Jiangsu/04/10_H3N2         NGGSGACKRGPANGFFSRLNWLTKSGNTYPVLNVTMPNNNNFDKLYIWGV 198
A/canine/Guangdong/1/06_H3N2        NGGSGACKRGPANGFFSRLNWLTKSGNTYPVLNVTMPNNNNFDKLYIWGV 198
A/canine/Korea/GCVP01/07_H3N2       NGGSGACKRGPANGFFSRLNWLTKSGNTYPVLNVTMPNNNNFDKLYIWGV 198
A/aqubird/Korea/JN-2/06_H3N2        NGGSGACKRGPANGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Korea/JS53/04_H3N2           NGGSGACKRGPANGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Korea/GJ108/07_H3N2          NGGSGACKRGPANGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Korea/GJ107/07_H3N2          NGGSGACKRGPANGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 194
A/duck/Korea/U12/07_H3N2            NGGSGACKRGPANGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Korea/LPM23/05_H3N2          NGGSGACKRGPANGFFSRLNWLTKSGNAYPLLNVTMPNNDNFDKLYIWGV 198
A/chicken/Korea/LPM17/04_H3N2       NGGSGACKRGPANGFFSRLNWLTKSGNAYPLLNVTMPNNDNFDKLYIWGV 198
A/duck/Korea/U14-1/07_H3N2          NGGSGACKRGPANGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Guangxi/69/09_H3N8           NGGSGACKRGPANGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Nanchang/1681/92_H3N8        NGGSNACKRGPANGFFSRLNWLTKSGNAYPMLNVTMPNNDNFDKLYIWGV 198
A/avian/Japan/8KI0129/08_H3N8       NGGSNACKRGPASGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Beijing/44/04_H3N8           NGGSNACKRGPDRGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Chiba/24/06_H3N8             NGGSNACKRGPASGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/aqubird/HongKong/399/99_H3N8      NGGSSACKRGPASGFFSRLNWLTKSGNAYPVLNVTMPNNDNFDKLYIWGV 198
A/duck/Italy/194659/06_H3N2         NGGSGACKRGPANGFFSRLNWLTKSGNAYPVLNVTMPNSDNFDKLYIWGV 198
A/canine/Iowa/13628/05_H3N8         NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/dog/Miami/E3/05_H3N8              NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/canine/Florida/14/06_H3N8         NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/canine/Penn/10909/07_H3N8         NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/canine/Texas/1/04_H3N8            NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/canine/Colorado/8880/06_H3N8      NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/canine/NY/158402-1/08_H3N8        NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/canine/Florida/43/04_H3N8         NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 189
A/equine/Ohio/1/03_H3N8             NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/equine/Florida/779/04_H3N8        NGRSGACKRGSADSFFSRLNWLTKSGSSYPTLNVTMPNNKNFDKLYIWGI 197
A/equine/Gansu/7/08_H3N8            NGRSGACKRGSADSFFSRLNWLTKSGNSYPTLNVTMPNNKNFDKLYIWGI 197
A/equine/Xinjiang/2/07_H3N8         NGRSGACKRGSADSFFSRLNWLTKSGNSYPTLNVTMPNNKNFDKLYIWGI 197
A/equine/Yokohama/aq13/10_H3N8      NGRSGACKRGSADSFFSRLNWLTKSGNSYPTLNVTMPNNKNFDKLYIWGI 199
A/equine/Kentucky/1/86_H3N8         NGRSGACKRGSADSFFSRLNWLTKSGNSYPTLNVTMPNNNNFDKLYIWGI 197
                                    ** *.***:*.  .************.:** *******..*********:
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A/canine/Korea/LBM412/08_H3N2       HHPSTNQEQTSLYIQASGRVTVSTRRSQQTIIPNIGSRPLVRGQSGRISV 232
A/feline/Korea/01/10_H3N2           HHPSTNQEQTSLYIQASGRVTVSTRRSQQTIIPNIGSRPLVRGQSGRISV 248
A/canine/Jiangsu/01/09_H3N2         HHPSTNQEQTSLYIQASGRVTVSTRRSQQTIIPNIGSRPLVRGQSGRISV 248
A/canine/Jiangsu/04/10_H3N2         HHPSTNQEQTSLYIQASGRVTVSTGRSQQTIIPNIGSRPLVRGQSGRISV 248
A/canine/Guangdong/1/06_H3N2        HHPSTNQEQTSLYIQASGRVTVSTRRSQQTIIPNIGSRPLVRGQSGRISV 248
A/canine/Korea/GCVP01/07_H3N2       HHPSTNQEQTSLYIQASGRVTVSTRRSQQTIIPNIGSRPLVRGQSGRISV 248
A/aqubird/Korea/JN-2/06_H3N2        HHPSTNQEQTSLYIQASGRVTVSTRRGQQTIIPNIGSRPWVRGQSGRISV 248
A/duck/Korea/JS53/04_H3N2           HHPSTNQEQTSLYIQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISV 248
A/duck/Korea/GJ108/07_H3N2          HHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISV 248
A/duck/Korea/GJ107/07_H3N2          HHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISV 244
A/duck/Korea/U12/07_H3N2            HHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISV 248
A/duck/Korea/LPM23/05_H3N2          HHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISV 248
A/chicken/Korea/LPM17/04_H3N2       HHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISV 248
A/duck/Korea/U14-1/07_H3N2          HHPSTNQEQTSLYVQASGRITVSTRRSQQTIIPNIGSRPWVRGQSGRISI 248
A/duck/Guangxi/69/09_H3N8           HHPSTNQEQTNLYVQASGRVTVSTRRSQQTIIPNIGSRPWIRGQSGRISI 248
A/duck/Nanchang/1681/92_H3N8        HHPSTNQEQTNLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISI 248
A/avian/Japan/8KI0129/08_H3N8       HHPSTNQEQTNLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISI 248
A/duck/Beijing/44/04_H3N8           HHPSTNQEQTNLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISI 248
A/duck/Chiba/24/06_H3N8             HHPSTNQEQTSLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISI 248
A/aqubird/HongKong/399/99_H3N8      HHPSTNQEQTNLYVQASGRVTVSTRRSQQTIIPNIGSRPWVRGQSGRISI 248
A/duck/Italy/194659/06_H3N2         HHPSTNQEQTNLYVQASGRVTVSTRKSQQTIIPNIGSRPWVRGQSGRISI 248
A/canine/Iowa/13628/05_H3N8         HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIESRPLVRGQSGRISI 247
A/dog/Miami/E3/05_H3N8              HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPLVRGQSGRISI 247
A/canine/Florida/14/06_H3N8         HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPLVRGQSGRISI 247
A/canine/Penn/10909/07_H3N8         HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPLVRGQSGRISI 247
A/canine/Texas/1/04_H3N8            HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPLVRGQSGRISI 247
A/canine/Colorado/8880/06_H3N8      HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPLVRGQSGRISI 247
A/canine/NY/158402-1/08_H3N8        HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPHIXSRPLIRGQSGRISI 247
A/canine/Florida/43/04_H3N8         HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPLVRGQSGRISI 239
A/equine/Ohio/1/03_H3N8             HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPWVRGQSGRISI 247
A/equine/Florida/779/04_H3N8        HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPWVRGQSGRISI 247
A/equine/Gansu/7/08_H3N8            HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPWVRGQSGRISI 247
A/equine/Xinjiang/2/07_H3N8         HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSSPWVRGQSGRISI 247
A/equine/Yokohama/aq13/10_H3N8      HHPSSNQEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPWVRGQSGRISI 249
A/equine/Kentucky/1/86_H3N8         HHPSSNNEQTKLYIQESGRVTVSTKRSQQTIIPNIGSRPWVRGQSGRISI 247
                                    ****:*:***.**:* ***:**** :.******:* * * :*********:

A/canine/Korea/LBM412/08_H3N2       YWTIVKPGDVLVINSNGNLIAPRGYFKMRIGKSSIMRSDAPIDTCISECI 282
A/feline/Korea/01/10_H3N2           YWTIVKPGDVLVINSNGNLIAPRGYFKMHIGKSSIMRSDAPIGTCISECI 298
A/canine/Jiangsu/01/09_H3N2         YWTTVKPGDVLVINSNGNLIAPRGYFKMHIGKSSIMRSDAPIDTCISECI 298
A/canine/Jiangsu/04/10_H3N2         YWTTVKPGDVLVINSNGNLIAPRGYFKMHIGKSSIMRSDAPIDTCISECI 298
A/canine/Guangdong/1/06_H3N2        YWTIVKPGDVLVINSNGNLIAPRGYFKMRIGKSSIMRSDAPIDTCISECI 298
A/canine/Korea/GCVP01/07_H3N2       YWTIVKPGDVLVINSNGNLIAPRGYFKMRIGKSSIMRSDAPIDTCISECI 298
A/aqubird/Korea/JN-2/06_H3N2        YWTIVKPGDVLVINSNGNLIAPRGYFKMRIGKSSIMRSDAPIDTCISECI 298
A/duck/Korea/JS53/04_H3N2           YWTIVKPGDVLVINSNGNLIAPRGYFKMRIGKSSIMRSDAPIDTCISECI 298
A/duck/Korea/GJ108/07_H3N2          YWTVVKPGDVLVINSNGNLIAPRGYFKMRVGKSSIMRSDAPIDTCISECI 298
A/duck/Korea/GJ107/07_H3N2          YWTVVKPGDVLVINSNGNLIAPRGYFKMRVGKSSIMRSDAPIDTCISECI 294
A/duck/Korea/U12/07_H3N2            YWTVVKPGDVLVINSNGNLIAPRGYFKMRVGKSSIMRSDAPIDTCISECI 298
A/duck/Korea/LPM23/05_H3N2          YWTVVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMKSDAPIDTCISECI 298
A/chicken/Korea/LPM17/04_H3N2       YWTVVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMKSDAPIDTCISECI 298
A/duck/Korea/U14-1/07_H3N2          YWTVVKPGDILVINSNGNLIAPRGYFKMRIGKSSIMRSDAPIDTCISECI 298
A/duck/Guangxi/69/09_H3N8           YWTVVKPGDVLVINSNGNLIAPRGYFKIRIGKSSIMRSDAPIDTCISECI 298
A/duck/Nanchang/1681/92_H3N8        YWTIVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECI 298
A/avian/Japan/8KI0129/08_H3N8       YWTIVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECI 298
A/duck/Beijing/44/04_H3N8           YWTVVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECI 298
A/duck/Chiba/24/06_H3N8             YWTIVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECI 298
A/aqubird/HongKong/399/99_H3N8      YWTIVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECI 298
A/duck/Italy/194659/06_H3N2         YWTIVKPGDVLVINSNGNLIAPRGYFKMRTGKSSIMRSDAPIDTCISECI 298
A/canine/Iowa/13628/05_H3N8         YWTIVKPGDILMINSNGNLVAPRGYFKLNTGKSSVMRSDVPIDICVSECI 297
A/dog/Miami/E3/05_H3N8              YWTIVKPGDILMINSNGNLVAPRGYFKLNTGKSSVMRSDVPIDICVSECI 297
A/canine/Florida/14/06_H3N8         YWTIVKPGDILMINSNGNLVAPRGYFKLNTGKSSVMRSDVPIDICVSECI 297
A/canine/Penn/10909/07_H3N8         YWTIVKPGDILMINSNGNLVAPRGYFKLNTGKSSVMRSDVPIDICVSECI 297
A/canine/Texas/1/04_H3N8            YWTIVKPGDILMINSNGNLVAPRGYFKLNTGKSSVMRSDVPIDICVSECI 297
A/canine/Colorado/8880/06_H3N8      YWTIVKPGDILMINSNGNLVAPRGYFKLNTGKSSVMRSDVPIDICVSECI 297
A/canine/NY/158402-1/08_H3N8        YWTIVKPGDILMINSNGNLVAPRGYFKLNPGKSSVMRSDVPIDICVSECI 297
A/canine/Florida/43/04_H3N8         YWTIVKPGDILMINSNGNLVAPRGYFKLKTGKSSVMRSDAPIDICVSECI 289
A/equine/Ohio/1/03_H3N8             YWTIVKPGDILMINSNGNLVAPRGYFKLKTGKSSVMRSDVPIDICVSECI 297
A/equine/Florida/779/04_H3N8        YWTIVKPGDILMINSNGNLVAPRGYFKLKTGKSSVMRSDVPIDICVSECI 297
A/equine/Gansu/7/08_H3N8            YWTIVKPGDILMINSNGNLVAPRGYFKLKTGKSSVMRSDVPIDICVSECI 297
A/equine/Xinjiang/2/07_H3N8         YWTIVKPGDILMINSNGNLVAPRGYFKLKTGKSSVMRSDVPIDICVSECI 297
A/equine/Yokohama/aq13/10_H3N8      YWTIVKPGDILMINSNGNLVAPRGYFKLKTGKSSVMRSDVPIDICVSECI 299
A/equine/Kentucky/1/86_H3N8         YWTIVKPGDTLMINSNGNLVAPRGYFKLRTGKSSVMRSDAPIDTCVSECI 297
                                    *** ***** *:*******:*******:. ****:*:**.**. *:****
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A/canine/Korea/LBM412/08_H3N2       TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPERQTRGLF 332
A/feline/Korea/01/10_H3N2           TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPERQTRGLF 348
A/canine/Jiangsu/01/09_H3N2         TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPERQTRGLF 348
A/canine/Jiangsu/04/10_H3N2         TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPERQTRGLF 348
A/canine/Guangdong/1/06_H3N2        TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/canine/Korea/GCVP01/07_H3N2       TPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPERQTRGLF 348
A/aqubird/Korea/JN-2/06_H3N2        TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Korea/JS53/04_H3N2           TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNIPEKQTRGLF 348
A/duck/Korea/GJ108/07_H3N2          TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Korea/GJ107/07_H3N2          TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 344
A/duck/Korea/U12/07_H3N2            TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Korea/LPM23/05_H3N2          TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/chicken/Korea/LPM17/04_H3N2       TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Korea/U14-1/07_H3N2          TPNGSIPNEKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Guangxi/69/09_H3N8           TPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Nanchang/1681/92_H3N8        TPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/avian/Japan/8KI0129/08_H3N8       TPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Beijing/44/04_H3N8           TPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Chiba/24/06_H3N8             TPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/aqubird/HongKong/399/99_H3N8      TPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/duck/Italy/194659/06_H3N2         TPNGSIPNDKPFQNVNKITYGACPKYVKQNTLKLATGMRNVPEKQTRGLF 348
A/canine/Iowa/13628/05_H3N8         TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQTRGIF 347
A/dog/Miami/E3/05_H3N8              TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQTRGIF 347
A/canine/Florida/14/06_H3N8         TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQTRGIF 347
A/canine/Penn/10909/07_H3N8         TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQTRGIF 347
A/canine/Texas/1/04_H3N8            TPNGSIPNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQTRGIF 347
A/canine/Colorado/8880/06_H3N8      TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQTRGIF 347
A/canine/NY/158402-1/08_H3N8        TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQTRGIF 347
A/canine/Florida/43/04_H3N8         TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQTRGIF 339
A/equine/Ohio/1/03_H3N8             TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQIRGIF 347
A/equine/Florida/779/04_H3N8        TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQIRGIF 347
A/equine/Gansu/7/08_H3N8            TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQIRGIF 347
A/equine/Xinjiang/2/07_H3N8         TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQIRGIF 347
A/equine/Yokohama/aq13/10_H3N8      TPNGSISNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQIRGIF 349
A/equine/Kentucky/1/86_H3N8         TPNGSIPNDKPFQNVNKVTYGKCPKYIRQNTLKLATGMRNVPEKQIRGIF 347
                                    ******.*:********:*** ****::*************:**:* **:*

A/canine/Korea/LBM412/08_H3N2       GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 382
A/feline/Korea/01/10_H3N2           GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/canine/Jiangsu/01/09_H3N2         GAIAGSIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/canine/Jiangsu/04/10_H3N2         GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/canine/Guangdong/1/06_H3N2        GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/canine/Korea/GCVP01/07_H3N2       GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/aqubird/Korea/JN-2/06_H3N2        GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Korea/JS53/04_H3N2           GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Korea/GJ108/07_H3N2          GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Korea/GJ107/07_H3N2          GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 394
A/duck/Korea/U12/07_H3N2            GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Korea/LPM23/05_H3N2          GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/chicken/Korea/LPM17/04_H3N2       GAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Korea/U14-1/07_H3N2          GAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Guangxi/69/09_H3N8           GAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Nanchang/1681/92_H3N8        GAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/avian/Japan/8KI0129/08_H3N8       GAKAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Beijing/44/04_H3N8           GAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Chiba/24/06_H3N8             GAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/aqubird/HongKong/399/99_H3N8      GAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/duck/Italy/194659/06_H3N2         GAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN 398
A/canine/Iowa/13628/05_H3N8         GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/dog/Miami/E3/05_H3N8              GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/canine/Florida/14/06_H3N8         GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/canine/Penn/10909/07_H3N8         GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/canine/Texas/1/04_H3N8            GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/canine/Colorado/8880/06_H3N8      GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/canine/NY/158402-1/08_H3N8        GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/canine/Florida/43/04_H3N8         GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 389
A/equine/Ohio/1/03_H3N8             GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/equine/Florida/779/04_H3N8        GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
A/equine/Gansu/7/08_H3N8            GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQTAIDQINEKLN 397
A/equine/Xinjiang/2/07_H3N8         GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQTAIDQINEKLN 397
A/equine/Yokohama/aq13/10_H3N8      GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQTAIDQINEKLN 399
A/equine/Kentucky/1/86_H3N8         GAIAGFIENGWEGMVDGWYGFRYQNSEGTGQAADLKSTQAAIDQINGKLN 397
                                    ** ** ********:*******:****************:****** ***
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A/canine/Korea/LBM412/08_H3N2        RVIEKTNEKFHQIEKEFSEVEGRIQDLERYVEDTKVDLWSYNAELLVALE 432
A/feline/Korea/01/10_H3N2            RVIEKTNEKFHQIEKEFSEVEGRIQGLERYVEDTKVDLWSYNAELLVALE 448
A/canine/Jiangsu/01/09_H3N2          RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALE 448
A/canine/Jiangsu/04/10_H3N2          RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALE 448
A/canine/Guangdong/1/06_H3N2         KVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALE 448
A/canine/Korea/GCVP01/07_H3N2        RVIEKTNEKFHQIEKEFSEVEGRIQDLERYVEDTKVDLWSYNAELLVALE 448
A/aqubird/Korea/JN-2/06_H3N2         RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALE 448
A/duck/Korea/JS53/04_H3N2            RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALE 448
A/duck/Korea/GJ108/07_H3N2           RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALE 448
A/duck/Korea/GJ107/07_H3N2           RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALE 444
A/duck/Korea/U12/07_H3N2             RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALE 448
A/duck/Korea/LPM23/05_H3N2           RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/chicken/Korea/LPM17/04_H3N2        RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/duck/Korea/U14-1/07_H3N2           RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/duck/Guangxi/69/09_H3N8            RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/duck/Nanchang/1681/92_H3N8         RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/avian/Japan/8KI0129/08_H3N8        RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/duck/Beijing/44/04_H3N8            RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/duck/Chiba/24/06_H3N8              RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/aqubird/HongKong/399/99_H3N8       RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/duck/Italy/194659/06_H3N2          RVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 448
A/canine/Iowa/13628/05_H3N8          RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/dog/Miami/E3/05_H3N8               RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/canine/Florida/14/06_H3N8          RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/canine/Penn/10909/07_H3N8          RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/canine/Texas/1/04_H3N8             RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/canine/Colorado/8880/06_H3N8       RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/canine/NY/158402-1/08_H3N8         RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/canine/Florida/43/04_H3N8          RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 439
A/equine/Ohio/1/03_H3N8              RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/equine/Florida/779/04_H3N8         RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/equine/Gansu/7/08_H3N8             RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/equine/Xinjiang/2/07_H3N8          RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
A/equine/Yokohama/aq13/10_H3N8       RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 449
A/equine/Kentucky/1/86_H3N8          RVIERTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE 447
                                     :***:********************.**:******:**************

A/canine/Korea/LBM412/08_H3N2        NQNTIDLTDSEMNKLFEKTRRQ---------------------------- 454
A/feline/Korea/01/10_H3N2            NQNTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/canine/Jiangsu/01/09_H3N2          NQNIIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/canine/Jiangsu/04/10_H3N2          NQNTIDLTDSEMNKLFEKTRGQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/canine/Guangdong/1/06_H3N2         NQNTIDLTDSEMNKLFEKTRKQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/canine/Korea/GCVP01/07_H3N2        NQKTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/aqubird/Korea/JN-2/06_H3N2         NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/duck/Korea/JS53/04_H3N2            NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/duck/Korea/GJ108/07_H3N2           NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/duck/Korea/GJ107/07_H3N2           NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 494
A/duck/Korea/U12/07_H3N2             NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/duck/Korea/LPM23/05_H3N2           NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACMESIR 498
A/chicken/Korea/LPM17/04_H3N2        NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACMESIR 498
A/duck/Korea/U14-1/07_H3N2           NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/duck/Guangxi/69/09_H3N8            NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNICIESIR 498
A/duck/Nanchang/1681/92_H3N8         NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/avian/Japan/8KI0129/08_H3N8        NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/duck/Beijing/44/04_H3N8            NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/duck/Chiba/24/06_H3N8              NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/aqubird/HongKong/399/99_H3N8       NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/duck/Italy/194659/06_H3N2          NQHTIDLTDSEMNKLFEKTRRQLRENAEDMGNGCFKIYHKCDNACIESIR 498
A/canine/Iowa/13628/05_H3N8          NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIESIR 497
A/dog/Miami/E3/05_H3N8               NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIESIR 497
A/canine/Florida/14/06_H3N8          NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIESIR 497
A/canine/Penn/10909/07_H3N8          NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGDGCFKIYHKCDNACIESIR 497
A/canine/Texas/1/04_H3N8             NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIESIR 497
A/canine/Colorado/8880/06_H3N8       NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIESIR 497
A/canine/NY/158402-1/08_H3N8         NQHTIDLTDAGMNKLFEKTRRQLRENAEDMGDGCFKIYHKCDNACIESIR 497
A/canine/Florida/43/04_H3N8          NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIR 489
A/equine/Ohio/1/03_H3N8              NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIR 497
A/equine/Florida/779/04_H3N8         NQHTIDLTDAEKNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIR 497
A/equine/Gansu/7/08_H3N8             NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIR 497
A/equine/Xinjiang/2/07_H3N8          NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIR 497
A/equine/Yokohama/aq13/10_H3N8       NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIR 499
A/equine/Kentucky/1/86_H3N8          NQHTIDLTDAEMNKLFEKTRRQLRENAEDMGGGCFKIYHKCDNACIGSIR 497
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A/canine/Korea/LBM412/08_H3N2       --------------------------------------------------
A/feline/Korea/01/10_H3N2           NGTYDHNIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/canine/Jiangsu/01/09_H3N2         NGTYDHNIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/canine/Jiangsu/04/10_H3N2         NGTYDHNIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/canine/Guangdong/1/06_H3N2        NGTYDHNIYRNEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/canine/Korea/GCVP01/07_H3N2       NGTYDHNIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/aqubird/Korea/JN-2/06_H3N2        NGTYDHDIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Korea/JS53/04_H3N2           NGTYDHDIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Korea/GJ108/07_H3N2          NGTYDHDIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Korea/GJ107/07_H3N2          NGTYDHDIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCIVLL 544
A/duck/Korea/U12/07_H3N2            NGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Korea/LPM23/05_H3N2          NGTYDHDIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/chicken/Korea/LPM17/04_H3N2       NGTYDHDIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Korea/U14-1/07_H3N2          NGTYDHDIYRDEAVNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Guangxi/69/09_H3N8           NGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCAVLL 548
A/duck/Nanchang/1681/92_H3N8        NGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/avian/Japan/8KI0129/08_H3N8       NGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Beijing/44/04_H3N8           NGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Chiba/24/06_H3N8             NGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/aqubird/HongKong/399/99_H3N8      NGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/duck/Italy/194659/06_H3N2         NGTYDHDIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLLCVVLL 548
A/canine/Iowa/13628/05_H3N8         TGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/dog/Miami/E3/05_H3N8              TGTYDHYIYKDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/canine/Florida/14/06_H3N8         TGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/canine/Penn/10909/07_H3N8         TGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/canine/Texas/1/04_H3N8            TGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/canine/Colorado/8880/06_H3N8      TGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/canine/NY/158402-1/08_H3N8        TGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/canine/Florida/43/04_H3N8         TGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 539
A/equine/Ohio/1/03_H3N8             NGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/equine/Florida/779/04_H3N8        NGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/equine/Gansu/7/08_H3N8            NGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/equine/Xinjiang/2/07_H3N8         NGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547
A/equine/Yokohama/aq13/10_H3N8      NGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 549
A/equine/Kentucky/1/86_H3N8         NGTYDHYIYRDEALNNRFQIKGVELKSGYKDWILWISFAISCFLICVVLL 547

A/canine/Korea/LBM412/08_H3N2       ------------------
A/feline/Korea/01/10_H3N2           GFIMWACQRGNIRCNICI 566
A/canine/Jiangsu/01/09_H3N2         GFIMWACQRGNIRCNICI 566
A/canine/Jiangsu/04/10_H3N2         GFIMWACQRGNIRCNICI 566
A/canine/Guangdong/1/06_H3N2        GFIMWACQRGNIRCNICI 566
A/canine/Korea/GCVP01/07_H3N2       GFIMWACQRGNIRCNICI 566
A/aqubird/Korea/JN-2/06_H3N2        GFIMWACQRGNIRCNICI 566
A/duck/Korea/JS53/04_H3N2           GFIMWACQRGNIRCNICI 566
A/duck/Korea/GJ108/07_H3N2          GFIMWACQRGNIRCNI-- 564
A/duck/Korea/GJ107/07_H3N2          GFIMWACQRGNIRCNICI 562
A/duck/Korea/U12/07_H3N2            GFIMWACQRGNIRCNICI 566
A/duck/Korea/LPM23/05_H3N2          GFIMWACQRGNIRCNICI 566
A/chicken/Korea/LPM17/04_H3N2       GFIMWACQRGNIRCNICI 566
A/duck/Korea/U14-1/07_H3N2          GFIMWACQRGNIRCNICI 566
A/duck/Guangxi/69/09_H3N8           GFIMWSCQRGNIRCNICI 566
A/duck/Nanchang/1681/92_H3N8        GFIMWACQRGNIRCNICI 566
A/avian/Japan/8KI0129/08_H3N8       GFIMWACQRGNIRCNICI 566
A/duck/Beijing/44/04_H3N8           GFIVWACQRGNIRCNICI 566
A/duck/Chiba/24/06_H3N8             GFIMWACQRGNIRCNICI 566
A/aqubird/HongKong/399/99_H3N8      GFIMWACQRGNIRCNICI 566
A/duck/Italy/194659/06_H3N2         GFIMWACQRGNIRCNICI 566
A/canine/Iowa/13628/05_H3N8         GFIMWACQKGNIRCNICI 565
A/dog/Miami/E3/05_H3N8              GFIMWACQKGNIRCNICI 565
A/canine/Florida/14/06_H3N8         GFIMWACQKGNIRCNICI 565
A/canine/Penn/10909/07_H3N8         GFIMWACQKGNIRCNICI 565
A/canine/Texas/1/04_H3N8            GFIMWACQKGNIRCNICI 565
A/canine/Colorado/8880/06_H3N8      GFIMWACQKGNIRCNICI 565
A/canine/NY/158402-1/08_H3N8        GFIMWACQKGNIRCNICI 565
A/canine/Florida/43/04_H3N8         GFIMWACQRGNIRCNICI 557
A/equine/Ohio/1/03_H3N8             GFIMWACQKGNIRCNICI 565
A/equine/Florida/779/04_H3N8        GFIMWACQKGNIRCSICI 565
A/equine/Gansu/7/08_H3N8            GFIMWACQKGNIRCNICI 565
A/equine/Xinjiang/2/07_H3N8         GFIMWACQKGNIRCNICI 565
A/equine/Yokohama/aq13/10_H3N8      GFIMWACQKGNIRCNICI 567
A/equine/Kentucky/1/86_H3N8         GFIMWACQKGNIRCNICI 565
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APPENDIX B

BINDING AFFINITY OF H3N2 CIV AND RECOMBINANT VIRUSES TO ALPHA 2,3 LINKED GLYCANS IN GLYCAN MICROARRAY

No	Glycan no	Sugar name	Normalized signal (RFU)			222L/222W
			H3N2 WT	rH3N2-222L	rH3N2-222W	
1	284	Neu5Gca2-3Galb1-4GlcNAcb-Sp0	69	126	58	2.16
2	544	Neu5Gca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Gca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp24	1908	811	117	6.96
3	285	Neu5Gca2-3Galb1-4Glcb-Sp0	54	55	71	0.78
4	283	Neu5Gca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	7263	6563	103	63.89
5	282	Neu5Gca2-3Galb1-3GlcNAcb-Sp0	65	64	64	1.01
6	281	Neu5Gca2-3Galb1-3(Fuca1-4)GlcNAcb-Sp0	144	54	63	0.86
7	238	Neu5Aca2-3GalNAcb1-4GlcNAcb-Sp0	6084	3243	300	10.82
8	237	Neu5Aca2-3GalNAca-Sp8	229	87	68	1.28
9	246	Neu5Aca2-3Galb-Sp8	3403	2270	1917	1.18
10	261	Neu5Aca2-3Galb1-4GlcNAcb-Sp8	4079	2513	4225	0.59
11	260	Neu5Aca2-3Galb1-4GlcNAcb-Sp0	2527	2713	4428	0.61
12	480	Neu5Aca2-3Galb1-4GlcNAcb1-6GalNAca-Sp14	4619	4604	6681	0.69
13	472	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	1763	4697	7628	0.62
14	463	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	8569	8197	10239	0.80
15	462	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	6672	6715	9736	0.69
16	318	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	8057	8292	10132	0.82
17	289	Neu5Aca2-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	2064	4650	8330	0.56
18	461	Neu5Aca2-3Galb1-4GlcNAcb1-4Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	7759	6735	9029	0.75
19	377	Neu5Aca2-3Galb1-4GlcNAcb1-3GalNAc-Sp14	3185	2142	764	2.81
20	442	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb-Sp8	4372	3431	2935	1.17
21	262	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	1581	3250	6737	0.48
22	597	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	1749	3905	8143	0.48
23	601	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	4132	4877	5538	0.88
24	593	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3GalNAca-Sp14	3230	1822	1515	1.20
25	259	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	6145	5570	8851	0.63

26	608	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	87	144	93	1.55
27	604	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	2674	2879	3376	0.85
28	219	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	31	119	97	1.22
29	296	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-3GlcNAcb-Sp0	2618	2237	1618	1.38
30	479	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana-Sp0	47	206	148	1.38
31	327	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	3710	3822	2923	1.31
32	326	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	8171	8103	8491	0.95
33	484	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24	5063	3758	6379	0.59
34	460	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	4148	3836	5633	0.68
35	265	Neu5Aca2-3Galb1-4Glcb-Sp8	2122	4113	4484	0.92
36	264	Neu5Aca2-3Galb1-4Glcb-Sp0	697	1733	3552	0.49
37	242	Neu5Aca2-3Galb1-4(Neu5Aca2-3Galb1-3)GlcNAcb-Sp8	5371	6932	8411	0.82
38	256	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp8	10128	6566	617	10.64
39	255	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	3981	3744	1131	3.31
40	338	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAc-Sp14	982	754	79	9.49
41	300	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-6(Galb1-3)GalNAca-Sp14	381	85	44	1.95
42	379	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3GalNAca-Sp14	852	417	75	5.54
43	257	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb-Sp8	5737	6235	2514	2.48
44	258	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4GlcNAcb-Sp8	8271	10128	1916	5.28
45	254	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	7059	7615	2576	2.96
46	535	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-2Mana-Sp0	10377	8680	2642	3.28
47	253	Neu5Aca2-3Galb1-4(Fuca1-3)(6S)GlcNAcb-Sp8	8764	9783	3495	2.80
48	252	Neu5Aca2-3Galb1-4(6S)GlcNAcb-Sp8	4063	3318	4147	0.80
49	251	Neu5Aca2-3Galb1-3GlcNAcb-Sp8	5659	4849	6400	0.76
50	250	Neu5Aca2-3Galb1-3GlcNAcb-Sp0	5870	3982	3456	1.15
51	492	Neu5Aca2-3Galb1-3GlcNAcb1-6GalNAca-Sp14	721	2500	3476	0.72

52	475	Neu5Aca2-3Galb1-3GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp19	7631	10317	10802	0.96
53	530	Neu5Aca2-3Galb1-3GlcNAcb1-4Galb1-4Glcb-Sp0	19	59	134	0.44
54	400	Neu5Aca2-3Galb1-3GlcNAcb1-3GalNAca-Sp14	6264	5845	6450	0.91
55	248	Neu5Aca2-3Galb1-3GlcNAcb1-3Galb1-4GlcNAcb-Sp0	6943	6800	9189	0.74
56	295	Neu5Aca2-3Galb1-3GlcNAcb1-3Galb1-3GlcNAcb-Sp0	6422	4649	7167	0.65
57	527	Neu5Aca2-3Galb1-3GlcNAcb1-2Mana-Sp0	7006	6269	5060	1.24
58	397	Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAc-Sp19	37	82	118	0.70
59	487	Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAc-Sp21	12	21	51	0.42
60	413	Neu5Aca2-3Galb1-3GalNAcb1-4(Neu5Aca2-8Neu5Aca2-3)Galb1-4Glcb-Sp0	392	362	104	3.48
61	235	Neu5Aca2-3Galb1-3GalNAcb1-4(Neu5Aca2-3)Galb1-4Glcb-Sp0	5858	6952	8144	0.85
62	247	Neu5Aca2-3Galb1-3GalNAcb1-3Gala1-4Galb1-4Glcb-Sp0	7200	7245	8047	0.90
63	224	Neu5Aca2-3Galb1-3GalNAca-Sp8	8059	5380	5011	1.07
64	225	Neu5Aca2-3Galb1-3GalNAca-Sp14	5914	5829	6330	0.92
65	240	Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb-Sp8	10500	6808	1338	5.09
66	241	Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	4929	7092	6188	1.15
67	332	Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-3(Fuca1-4)GlcNAcb-Sp0	10291	9714	5430	1.79
68	239	Neu5Aca2-3Galb1-3(6S)GlcNAc-Sp8	1952	3755	8089	0.46
69	243	Neu5Aca2-3Galb1-3(6S)GalNAca-Sp8	4887	7815	6222	1.26
70	46	Neu5Aca2-3(6S)Galb1-4GlcNAcb-Sp8	4309	1047	133	7.89
71	231	Neu5Aca2-3(6S)Galb1-4(Fuca1-3)GlcNAcb-Sp8	2180	745	131	5.70
72	359	KDNa2-3Galb1-4Glc-Sp0	19	86	59	1.47
73	207	KDNa2-3Galb1-4GlcNAcb-Sp0	45	48	31	1.55
74	357	KDNa2-3Galb1-4(Fuca1-3)GlcNAc-Sp0	14	42	97	0.43
75	206	KDNa2-3Galb1-3GlcNAcb-Sp0	18	43	43	1.00
76	360	KDNa2-3Galb1-3GalNAca-Sp14	6	55	143	0.38

APPENDIX C

BINDING AFFINITY OF H3N2 CIV AND RECOMBINANT VIRUSES TO GLYCANS IN GLYCAN MICROARRAY (RFU value ≥ cutoff)

Glycan No	Sugar Name	Normalized Signal (RFU)			Ratio
		H3N2 WT	222L	222W	
283	Neu5Gca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	7263	6563	103	63.89
319	Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	2224	2948	3654	0.81
273	Neu5Aca2-6Galb1-4Glcb-Sp0	4522	3342	1125	2.97
244	Neu5Aca2-6(Neu5Aca2-3Galb1-3)GalNAca-Sp8	6449	5754	6589	0.87
245	Neu5Aca2-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	5376	7129	5770	1.24
238	Neu5Aca2-3GalNAcb1-4GlcNAcb-Sp0	6084	3243	300	10.82
246	Neu5Aca2-3Galb-Sp8	3403	2270	1917	1.18
261	Neu5Aca2-3Galb1-4GlcNAcb-Sp8	4079	2513	4225	0.59
260	Neu5Aca2-3Galb1-4GlcNAcb-Sp0	2527	2713	4428	0.61
480	Neu5Aca2-3Galb1-4GlcNAcb1-6GalNAca-Sp14	4619	4604	6681	0.69
472	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	1763	4697	7628	0.62
463	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	8569	8197	10239	0.80
462	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	6672	6715	9736	0.69
318	Neu5Aca2-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	8057	8292	10132	0.82
289	Neu5Aca2-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	2064	4650	8330	0.56
461	Neu5Aca2-3Galb1-4GlcNAcb1-4Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-4(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	7759	6735	9029	0.75
377	Neu5Aca2-3Galb1-4GlcNAcb1-3GalNAc-Sp14	3185	2142	764	2.81
442	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb-Sp8	4372	3431	2935	1.17
262	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	1581	3250	6737	0.48
597	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3)GalNAca-Sp14	1749	3905	8143	0.48

601	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-6(Galb1-3)GalNAca-Sp14	4132	4877	5538	0.88
593	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3GalNAca-Sp14	3230	1822	1515	1.20
259	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb-Sp0	6145	5570	8851	0.63
604	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	2674	2879	3376	0.85
296	Neu5Aca2-3Galb1-4GlcNAcb1-3Galb1-3GlcNAcb-Sp0	2618	2237	1618	1.38
327	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-6Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	3710	3822	2923	1.31
326	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp12	8171	8103	8491	0.95
484	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24	5063	3758	6379	0.59
460	Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-6(GlcNAcb1-4)(Neu5Aca2-3Galb1-4GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp21	4148	3836	5633	0.68
265	Neu5Aca2-3Galb1-4Glcb-Sp8	2122	4113	4484	0.92
264	Neu5Aca2-3Galb1-4Glcb-Sp0	697	1733	3552	0.49
242	Neu5Aca2-3Galb1-4(Neu5Aca2-3Galb1-3)GlcNAcb-Sp8	5371	6932	8411	0.82
256	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp8	10128	6566	617	10.64
255	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	3981	3744	1131	3.31
257	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb-Sp8	5737	6235	2514	2.48
258	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4GlcNAcb-Sp8	8271	10128	1916	5.28
254	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	7059	7615	2576	2.96
535	Neu5Aca2-3Galb1-4(Fuca1-3)GlcNAcb1-2Mana-Sp0	10377	8680	2642	3.28
253	Neu5Aca2-3Galb1-4(Fuca1-3)(6S)GlcNAcb-Sp8	8764	9783	3495	2.80
252	Neu5Aca2-3Galb1-4(6S)GlcNAcb-Sp8	4063	3318	4147	0.80
251	Neu5Aca2-3Galb1-3GlcNAcb-Sp8	5659	4849	6400	0.76

250	Neu5Aca2-3Galb1-3GlcNAcb-Sp0	5870	3982	3456	1.15
492	Neu5Aca2-3Galb1-3GlcNAcb1-6GalNAca-Sp14	721	2500	3476	0.72
475	Neu5Aca2-3Galb1-3GlcNAcb1-6(Neu5Aca2-3Galb1-4GlcNAcb1-2)Mana1-6(Neu5Aca2-3Galb1-3GlcNAcb1-2Mana1-3)Manb1-4GlcNAcb1-4GlcNAcb-Sp19	7631	10317	10802	0.96
400	Neu5Aca2-3Galb1-3GlcNAcb1-3GalNAca-Sp14	6264	5845	6450	0.91
248	Neu5Aca2-3Galb1-3GlcNAcb1-3Galb1-4GlcNAcb-Sp0	6943	6800	9189	0.74
295	Neu5Aca2-3Galb1-3GlcNAcb1-3Galb1-3GlcNAcb-Sp0	6422	4649	7167	0.65
527	Neu5Aca2-3Galb1-3GlcNAcb1-2Mana-Sp0	7006	6269	5060	1.24
235	Neu5Aca2-3Galb1-3GalNAcb1-4(Neu5Aca2-3)Galb1-4Glcb-Sp0	5858	6952	8144	0.85
247	Neu5Aca2-3Galb1-3GalNAcb1-3Gala1-4Galb1-4Glcb-Sp0	7200	7245	8047	0.90
224	Neu5Aca2-3Galb1-3GalNAca-Sp8	8059	5380	5011	1.07
225	Neu5Aca2-3Galb1-3GalNAca-Sp14	5914	5829	6330	0.92
240	Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb-Sp8	10500	6808	1338	5.09
241	Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-4(Fuca1-3)GlcNAcb-Sp0	4929	7092	6188	1.15
332	Neu5Aca2-3Galb1-3(Fuca1-4)GlcNAcb1-3Galb1-3(Fuca1-4)GlcNAcb-Sp0	10291	9714	5430	1.79
239	Neu5Aca2-3Galb1-3(6S)GlcNAc-Sp8	1952	3755	8089	0.46
243	Neu5Aca2-3Galb1-3(6S)GalNAca-Sp8	4887	7815	6222	1.26
46	Neu5Aca2-3(6S)Galb1-4GlcNAcb-Sp8	4309	1047	133	7.89
14	Manb-Sp8	1213	1542	1982	0.78
605	GlcNAcb1-6(Neu5Aca2-3Galb1-3)GalNAca-Sp14	7749	8570	10258	0.84
13	Glcb-Sp8	759	1549	1169	1.33
2	Glca-Sp8	1468	1901	2279	0.83
494	Galb1-4(Fuca1-3)GlcNAcb1-6(Neu5Aca2-6(Neu5Aca2-3Galb1-3)GlcNAcb1-3)Galb1-4Glc-Sp21	679	1359	1941	0.70
1	Gala-Sp8	1203	1487	1722	0.86
115	Gala1-3Galb1-3GlcNAcb-Sp0	705	1322	1389	0.95