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DIFFERENTIAL INNATE IMMUNE RESPONSES CORRELATE WITH THE CONTRASTING PATHOGENICITY OF THE EQUINE H7N7 INFLUENZA VIRUS DEMONSTRATED IN HORSES AND BALB/C MICE

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By

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Lexington, Kentucky

Director: Dr. Thomas Chambers, Associate Professor of Veterinary Science

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Equine influenza virus causes a mild, self-limiting upper respiratory disease in its natural host. In stark contrast, equine influenza viruses of the H7N7 subtype produce lethal infection in BALB/c mice. This dissertation explored the mechanism underlying the differential pathogenicity of the equine H7N7 influenza virus observed in horses and BALB/c mice. Initially, a comparative study of the pathogenesis was conducted in BALB/c mice inoculated intranasally with a representative isolate of either H7N7 or H3N8 subtype equine influenza virus. All H3N8 virus-infected mice survived the infection whereas 100% mortality was documented for the mice receiving the H7N7 virus by day 8 post infection. Both viruses replicated to a similar degree in the lungs at the early stages of infection. However, after day 2 post infection until the death of the mice, the pulmonary viral loads of the H7N7 group were significantly higher than those of the control, whereas the H3N8 virus was eventually eradicated from the mice at day 7 p.i. Correspondingly, a vigorous pro-inflammatory cytokine response in the lung was induced by the H7N7 virus but not the H3N8 virus, which reflected a desperate attempt by the host immune responses to restrain the overwhelming infection. The H7N7 virus was poorly sensitive to the innate immune containment, resulting in a significant higher cumulative mortality rate than that of the control virus in chicken embryos aged 9 days and older. On the contrary, in horses, replication of the paired viruses was completely cleared by the host immune responses at day 7 p.i. and the infections produced an acute yet non-lethal illness, albeit the H3N8 virus induced generally more pronounced clinical manifestations than the H7N7 virus. The clinical severity correlated to the difference in cytokine-inducing capacity between the two viruses in horses, as evidenced by the finding that the H3N8 virus triggered significantly higher levels of gene transcription of multiple key inflammatory cytokines in the circulation than those seen for the H7N7 virus. In addition, equine peripheral monocyte-derived macrophages were found to be a target of equine influenza virus and can support the productive replication of the virus *in vitro*.

KEYWORDS: Equine Influenza, Pathogenicity, Innate Immune, Mice, Cytokines

Liang Zhang

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CHAPTER ONE

Introduction and Literature Review

INTRODUCTION

The *Orthomyxoviridae* are a family of segmented, single-stranded negative sense RNA viruses that consists of the five genera: *Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogatovirus and Isavirus*. Infectious salmon anemia virus is the only known virus of the genus *Isavirus* and appears to cause disease in farmed Atlantic salmon. The genus *Thogotovirus* is currently composed of two species: Thogoto virus and Dhori virus. They are tick-borne viruses that occasionally infect mammals, including humans [1]. Influenza A, B and C viruses are distinguished on the basis of the antigenic differences in their internal nucleocapsid (NP) and matrix (M) proteins. Influenza C virus infects humans and swine, while Influenza B virus can infect humans and seals. Influenza A virus, however, causes highly contagious disease in a wide range of mammalian and avian species, including humans, pigs, horses, cats, dogs, sea mammals, and domestic as well as wild birds [2]. Wild waterfowl play a critical role in the epidemiology of influenza, as these birds function as a vast reservoir of all influenza A viruses in nature, from which viruses can be transmitted to other animals and humans [3]. Influenza A viruses are divided into subtypes based on the antibody response to their surface HA and NA proteins. To date, 16 HA and 9 NA subtypes have been identified among influenza A viruses [4]. Influenza is one of the major respiratory infections of humans, and is responsible for pandemics and annual epidemics in the world. Historically, there have been four devastating human influenza pandemics since the last century. The 1918-1919 pandemic, the famous “Spanish flu”, was the most severe, and killed an estimated 20 to 40 million people globally [5]. Phylogenetic analyses indicated that the 1918 strain was

an H1N1 subtype virus from an avian ancestor [6]. In 1957, the H2N2 subtype emerged causing a less devastating pandemic for humans. The third pandemic of influenza virus occurred during 1968-1969 and involved the H3N2 subtype [7]. The latest pandemic caused by a novel swine-origin influenza A (H1N1) virus emerged in early 2009 [8]. H1N1 and H3N2 and reassortants are currently circulating in the human population causing annual seasonal influenza [9]. Recent outbreaks of avian influenza H5N1, H7N7 and H9N2 virus infections in humans raise concern about the next potential human pandemic. These avian viruses were transmitted directly from domestic poultry to humans without an intermediate host [10]. Fortunately, these viruses have not acquired the ability to spread among humans [11]. To date, only the H7N7 and H3N8 subtype viruses have been responsible for influenza in equine populations. Equine influenza is one of the major infectious respiratory diseases with economic significance in equines. Annual outbreaks have an effect on horse racing, performance and breeding over the world [12]. The general outline of this dissertation begins with a literature review of current knowledge on equine influenza, the biology of influenza A viruses, the pathogenesis of virus in avian and mammals, and the immune responses to the influenza A virus infection, followed by research objectives in the end.

LITERATURE REVIEW

Section I: Equine Influenza

An influenza-like respiratory disease has been recognized in horses for centuries, probably as long as in humans [3, 13]. However, it was not until 1956 that influenza A virus was first officially identified as the etiological agent of equine respiratory disease [14]. The original isolate collected from an infected horse during that outbreak in Central Europe was subtyped as H7N7. Serological evidence suggested that this virus subtype had circulated among horse populations in Europe and the Americas even before 1956 [15-16]. In 1963, a new equine influenza virus of different antigenic subtype (H3N8) appeared during a major epidemic in the USA [13]. Following that isolation, these two antigenically distinct subtypes of equine influenza viruses cocirculated in equine population and in some outbreaks simultaneous isolation of both subtype viruses occurred [17].

Cocirculation of two distinct subtypes provides the opportunity for genetic exchange between both subtypes in nature. Phylogenetic analysis of equine influenza isolates indicated that they probably emerged from avian gene pool [18-19] and the internal genes (NP, PA, PB1, PB2, M and NS) of H7N7 viruses isolated during 1970's derived from H3N8 viruses by genetic reassortment [18, 20-21]. The last confirmed outbreak of equine influenza caused by an H7N7 subtype virus was in 1979 [22]. Since then the H7N7 subtype virus has not been isolated from horses. However, serological evidence indicated that this virus subtype may still have been in circulation as late as 1991 in some parts of the world [23]. In contrast, the H3N8 subtype virus remains prevalent among horses causing annual epidemics worldwide.

The reason(s) that the equine H7N7 subtype virus disappeared from equine populations remains unknown. It is presumed that the H7N7 virus was under negative selection pressure during evolution. Interestingly, this is not a unique scenario. In 1989, an H3N8 influenza virus from birds was introduced into horses in Northeastern China and resulted in up to 20% mortality in some herds [19]. However, this particular strain did not spread beyond or persist in China beyond 1990 [24].

Section II: The Biology of Influenza A Virus

1. Virion Architecture and Genome

Influenza A viruses belong to the family of *Orthomyxoviridae*. The viruses have eight separate single-stranded, negative sense RNA segments enclosed in a lipid envelope [1]. The virion is 80 to 120 nm in diameter and has a regular spherical appearance if propagated in eggs or cell culture. In contrast, influenza A viruses exhibit pleomorphism on initial isolation from humans or animals [25-26]. There are two types of virus encoded surface glycoproteins which protrude like spikes from the lipid envelope: rod-shaped hemagglutinin (HA) and mushroom-shaped neuraminidase (NA). The small quantities of M2 protein function as a membrane-channel protein in the envelope. The M1 protein, the most abundant protein in the virion, underlies the envelope and interacts with the ribonucleoproteins [27]. The eight separate RNPs have the appearance of flexible rods. Each RNP consists of a RNA segment encapsidated by several nucleocapsid protein (NP) molecules. Associated with the end of each RNP are the three viral polymerase proteins PB1, PB2 and PA [28].

2. Viral Proteins and Their Functions

Hemagglutinin (HA)

The surface HA glycoprotein, encoded by the fourth largest RNA segment, is a homotrimeric spike of noncovalently linked monomers that are synthesized in the rough endoplasmic reticulum of infected host cells [29]. The HA molecule is produced as an uncleaved single precursor polypeptide, HA0, which is cotranslationally modified by the addition of oligosaccharide chains. The next processing step is the proteolytic cleavage of the HA0 into two disulfide-linked subunits referred to as HA1 and HA2. The glycine-rich membrane fusion peptide is located at the amino acid terminus of the HA2 subunit [30]. The cleavage step is a prerequisite for the virus to be infectious and is accomplished by host proteases. Depending on the HA structural property of a virus strain, cleavage happens either by ubiquitously distributed proteases such as furin [31-32] or by tissue-specific proteases such as trypsin [33]. Therefore, HA cleavability is a crucial determinant of viral pathogenicity [34]. As a major surface glycoprotein of influenza A virus, HA has two primary functions: (1) it binds to sialic acid containing receptors on cell surfaces during viral attachment, and (2) mediates fusion between the endocytic vesicle and viral membranes during penetration, enabling the viral genome to be released into the cytoplasm. In addition, the HA molecule is the major target for antibody neutralization, and a mutation in HA may allow the virus to escape neutralizing antibodies by antigenic drift [35].

Neuraminidase (NA)

NA is the second surface glycoprotein of the virion. In parallel with HA, NA is also a major antigenic determinant that undergoes antigenic variation in response to host

immune pressure. The function of NA includes the removal of sialic acid residues from viral glycoproteins or glycolipid receptors on the host cell surface, thus allowing the release of progeny virus from the infected cells [36]. NA also promotes virus invasion of the epithelial cells during virus entry [37]. Additionally, NA may play a role in influenza virus pathogenesis such as by induction of apoptosis [38], and activation of TGF-β expression in mice and cell cultures [39].

Matrix (M1) and M2 proteins

The seventh RNA segment encodes two proteins in overlapping reading frames derived by alternative splicing. M1 is a structural protein underlying the lipid envelope and constitutes the most abundant protein of the virion. The M1 interacts with the cytoplasmic tails of HA, NA and M2 proteins as well as RNP structures and is believed to play an important role in assembly and budding of progeny virus [40]. M2 is an integral membrane protein, and functions as a proton ion channel to allow acidification of the interior of the virion during virus entry [41].

Nucleoprotein (NP)

NP is the second most abundant protein of the virion. After synthesis and posttranslational phosphorylation in the cytoplasm, NP is then transported into the nucleus of infected cells, where it binds to newly synthesized viral RNA, forming the ribonucleoprotein (RNP) complexes [42-43]. NP is also the major target of cytotoxic T lymphocytes that non-specifically cross-react with NP of all influenza virus subtypes [44].

Polymerase proteins

The three largest RNA segments encode the PB1, PB2 and PA proteins respectively. The proteins are named based on their properties in isolectric focusing gel, two proteins are basic (PB1 and PB2) and one acidic (PA). These proteins form a complex that provides RNA-dependent RNA polymerase activity for the virus [45-46]. PB2 recognizes and binds the 5' cap structure of host cellular mRNA that is used for the priming of viral mRNA transcription. PB1 serves as the elongation protein for viral mRNA and genomic RNA synthesis [47-48]. The recently identified PB1-F2 protein, which is encoded by an alternative reading frame of PB1, has been found to localize to the inner mitochondria of infected cells. This novel 87-residue peptide disrupts mitochondrial membrane integrity which leads to apoptotic cell death [49-50]. The precise role of PA remains unknown, although there is evidence for its possible role in both viral transcription and replication [51].

Non-structural (NS) proteins

RNA segment eight encodes two non-structural proteins, NS1 and NS2, where the NS2 mRNA is derived from splicing of the NS1 mRNA [52]. Although the NS1 is abundantly present in influenza virus-infected cells, it is not incorporated into the progeny virion [53]. NS1 is a multifunctional protein that plays an important role in the influenza pathogenesis. So far, many functions have been associated with NS1 protein, including inhibiting cellular mRNA splicing [54], inhibiting nuclear export of polyadenylated cellular mRNAs [55], directing preferential translation of viral mRNAs by binding to the 5' UTR of the viral mRNAs [56] and binding to dsRNA to block the

activation of dsRNA-activated protein kinase (PKR) [57]. NS2 has been demonstrated to facilitate the export of viral RNPs from the nucleus to the cytoplasm [58].

3. Replication Cycle of Influenza A Virus

Virus replication consists of three major steps: (1) viral attachment, entry and uncoating (2) viral gene expression and replication and (3) virus assembly, budding and release. (1) Viral attachment involves the binding of HA to sialic acid-containing receptors on the host cell surface [59]. Upon binding, the virus enters the cell by receptor mediated endocytosis. Cellular proton pumps gradually acidify the endosomal compartments and induce a conformational change of the HA, which leads to the exposure of the fusion peptide and ultimately to the fusion of the viral and cellular membranes [60-61]. The M2 ion channel protein allows the flow of ions from the endosome to the virion interior, facilitating the dissociation of the vRNP from M1 and then release into the cytoplasm of the cell as the uncoating process is completed [62]. (2) The RNP segments are then transported into the nucleus where transcription and replication take place [63]. Initiation of transcription of viral mRNA requires priming with capped RNA primers from the 5' end of cellular mRNAs. The process of cap recognition is mediated by the viral PB2 protein, while the PB1 protein functions as the endonuclease and polymerase [64-66]. Transcribed mRNAs are then exported to the cytoplasm for translation by the host translation machinery. Replication of the viral genome is accomplished by copying the viral RNAs into complementary RNA templates which in turn are used for production of progeny viral genome. Both cRNA and vRNA are encapsidated by the NP. Newly synthesized vRNPs are then transported back to the cytoplasm in a process that involves the participation by both M1 and NS2 proteins [67-

69]. (3) Assembly and budding take place at cholesterol-rich lipid raft microdomains in the plasma membrane with RNPs and viral structural proteins [70]. Intrinsic interactions of the viral glycoproteins (HA and NA) with lipid rafts through their transmembrane domains are necessary in preparation for the budding process [71-72]. Outward bending of the plasma membrane caused by the clustering of HA and NA in lipid rafts is likely to initiate bud formation [73-74]. As a result of specific binding with both HA and NA, viral matrix (M1) protein is recruited into the budding assemblage [75], and M1 protein polymerization at the budding site has been postulated to drive the elongation of the emerging bud [76]. Additionally, M1 binding to viral RNPs is believed to direct incorporation of the viral genome into the forming virion [40]. Once formed, the budded virion is released by M2-mediated membrane scission at the boundary between the bud neck and cellular plasma membrane [77]. NA subsequently cleaves the HA-sialic acid bonds on the host cell, which allows for the final release of newly formed virions into the external environment [78].

Section III: The Pathogenesis of Influenza A Virus

1. Birds

The natural reservoirs for all influenza A viruses are wild aquatic birds, in which influenza A viruses have reached an optimal evolutionary equilibrium. Viruses replicate in the gastrointestinal tracts of the hosts, leading to the excretion of large amounts of viruses in their feces usually in the absence of clinical signs [79]. In domestic birds, including chicken and turkeys, most strains cause an asymptomatic or mild infection, with presentation of mild respiratory disease, nasal discharge, fever, lethargy and

declined egg laying. Virus replication is restricted to the respiratory and intestinal tracts. The mortality is generally low and these strains are classified as low pathogenic avian viruses [80]. In contrast, some strains, known as highly pathogenic viruses, cause rapid, fatal disease with extremely high mortality in domestic poultry. To date, outbreaks of highly pathogenic avian influenza viruses are restricted to H5 and H7 subtypes. These viruses spread systemically, including to the brain. Typical clinical symptoms consist of severe depression, cessation of egg production, high fever, respiratory signs, diarrhea, edema of the head and neck, and subcutaneous hemorrhage. These highly pathogenic avian influenza viruses are restricted to the H5N1 and H7N7 subtypes [81].

2. Pigs

Influenza in pigs was first recognized in 1918, at the same time as the devastating human influenza pandemic [82]. Three subtypes H1N1, H3N2 and reassortant H1N2 circulate in swine population [83]. The virus attacks epithelial cells of the respiratory tract and the infection is usually restricted to the respiratory system. Virus isolation from extra-respiratory organs is very rare [84]. Most commonly, swine influenza is an acute respiratory disease characterized by fever, anorexia, lethargy, sneezing, coughing and nasal discharge. The infected pigs usually recover within 7-10 days. Morbidity is very high, but mortality is usually low. Pigs can suffer considerable weight loss during the outbreak, which causes economic impact for the producers. In addition to clinical outbreaks, subclinical infections are also very common [85].

3. Horses

Equine influenza is an acute respiratory disease. Typical clinical presentations by both H7N7 and H3N8 viruses in natural outbreaks include a sudden onset of fever,

anorexia, depression, nasal discharge and coughing, although limited data collected from natural outbreaks show that the H3N8 subtype virus seems to cause more severe clinical symptoms than the H7N7 subtype [13]. Mortality is usually low, though in some cases relatively high mortality can be the result of secondary bacterial infections. Damage to the respiratory epithelial cells by virus replication facilitates bacterial invasion. Equine influenza is usually a self-limiting disease. Uncomplicated recovery happens within 1 to 2 weeks after infection. Nevertheless, the disease has a significant impact on the horse industry because of both performance failure and economic loss associated with the outbreak [12, 86].

4. Humans

The disease severity of human influenza can range from asymptomatic infections to serious illness with fatal outcome. Most commonly, seasonal outbreaks or epidemics are mild. Typical clinical symptoms are fever, headache, sore throat, malaise, anorexia and cough. The virus replicates in the epithelium of both the upper and lower respiratory tracts [2]. People of all ages can be infected; and the severity of illness is greatest in infants, the aged, and those with underlying medical conditions, who are at high risk of developing severe complications, such as viral or secondary bacterial pneumonia [87].

Since 1997, direct natural infections of humans with avian influenza viruses of the H5N1, H7N7 and H9N2 subtypes have been documented. Outbreaks of avian H7N7 and H9N2 influenza viruses in humans were not severe. The predominant clinical manifestation associated with those outbreaks was conjunctivitis. The flu-like symptoms were observed in several patients. Among them, only one person died from pneumonia [88-89]. In contrast, human avian H5N1 influenza virus infection is highly pathogenic,

resulting in high mortality. People of all ages are susceptible to avian H5N1 virus infection. Primary clinical signs include high fever and lower respiratory tract symptoms such as cough and dyspnea. Viral pneumonia is present in most cases. Chest X-ray showed features consistent with pneumonia. Conjunctivitis or upper respiratory symptoms are not common. Gastrointestinal symptoms including diarrhea, vomiting and abdominal pain occurred more frequently. Central nervous system manifestations have been reported in only one patient, and therefore are considered to be rare. Ultimately, patients died from acute respiratory disease syndrome or multi-organ failure. The mortality rate of avian influenza H5N1 infections in human was 60% [90-93]. Avian H5N1 influenza virus replicates in the respiratory system, and no report so far has revealed that virus replication occurs in extra-pulmonary organs [94]. Fatal cases were characterized by highest viral loads, indicating a positive correlation between virus burden and disease severity [95]. High viral loads were suggested to induce hypercytokinemia and hyperchemokinemia. Higher serum levels of proinflammatory cytokines and chemokines have been detected in many patients [95-97]. Pathological findings in the lungs, such as hemophagocytic syndrome, were consistent with an overwhelming inflammatory response [96-98]. In severe cases, lymphopenia and thrombocytopenia were also observed [90-91].

5. Mice

Mice are not a natural host for influenza virus, but can be infected experimentally. The advantages of the mouse model for influenza include the availability of various reagents, defined genetic background, easy handling and sufficient number for statistical power. Intranasal infection of mice with most virus strains, including human influenza

viruses, results in a largely asymptomatic infection, though virus can be isolated from the respiratory tract at a relatively high titer. Viral spread to extrapulmonary organs is extremely rare and clearance of virus in the lung can be completed within 7 days after infection [99-100].

To study influenza pathogenesis and immunity in a mouse model, viruses have to be adapted by a number of serial passages in the lungs of mice with an aim to increase virulence and pathogenicity. Mouse-adapted human influenza A/PR/8/34 (H1N1) virus is one example of such adaptation whereby this adapted virus attacks the respiratory system causing severe viral pneumonia after intranasal inoculation [101]. Another human influenza H1N1 virus, A/WSN/34 strain, which was derived by serial intracerebral passages in mice [102], has been extensively used to study neuropathogenesis of human influenza virus infection in mice [103]. This mouse-adapted neurovirulent strain causes fatal encephalomyelitis when inoculated intracerebrally into adult mice, or in neonates after intranasal inoculation [104], but fails to invade the brain of immunocompetent mice following intranasal infection [105]. In contrast, highly pathogenic avian influenza H5N1 and H7N7 viruses are pathogenic in mice without prior adaptation. Intranasal infection of BALB/c mice with highly pathogenic avian influenza H7N7 viruses leads to viral spread to extrapulmonary organs such as spleen and brain [106]. Highly pathogenic avian influenza H5N1 viruses can display either high or low pathogenicity in mice. Replication of low pathogenic avian viruses was restricted to the respiratory tract and infection was generally nonlethal with virus clearance completed around 7 days. In contrast, lethal strains replicated in multiple organs, including brain, spleen, heart and kidney, in addition to the respiratory system. Most deaths occurred within 9 days. Obvious clinical signs of

infection characterized by ruffled fur, hunched posture, inappetance and labored breathing were usually noticed within 4 days of infection. [107-109]. Unlike influenza in natural hosts, mice infected experimentally with influenza virus fail to display fever during the course of infection [108]. Fatally infected mice had a severe interstitial pneumonia with apparent accompanying infiltration of immune cells and erythrocytes [108]. Neuropathological signs were characterized by neuronal degeneration and inflammatory infiltrates of granulocytes and mononuclear cells in the brain stem and spinal cord [110]. Several studies reported that lethal H5N1 infection also caused depletion of leukocytes and lymphocytes in peripheral blood and lymphoid organs [111-112]. Exaggerated expression of proinflammatory cytokines and chemokines in the lungs, including IL-1β, IL-6, TNF-α and MIP-1α, was another finding in H5N1 virus lethal infection [106-107, 113]. In summary, systemic spread of virus, severe viral pneumonia, aberrant cytokine expression and lymphopenia were associated with the fatal outcome of H5N1 infection in mice.

Section IV: The Immune Responses to Influenza A Virus Infection

The immune responses to the influenza A virus infection have been well studied in humans and in mouse models. The initial infection occurs in the epithelia of the upper respiratory tract, and later, the infection spreads to the lower respiratory tract and leukocytes. In response to infection, infected cells produce interferons, chemokines and many other cytokines [114-117]. IFN-α/β bind to their specific receptors on neighboring cells and subsequently induce the expression of down-stream “antiviral proteins” such as the Mx and dsRNA-dependent protein kinase (PKR) through the JAK-STAT signaling

pathway [118]. Release of proinflammatory cytokines and chemokines mainly by resident alveolar monocytes/macrophages, notably IL-8, MCP-1, MIP-1, IL-1β, IL-6 and TNF-α, leads to recruitment of circulating leukocytes and lymphocytes to the site of infection [119]. Some of these early cytokines activate natural killer cells, which are known to be key players in the early defense against virus infection by lysing virus-infected cells and secreting a variety of cytokines, which in turn regulate adaptive immune response [120-121] . Another important player in the innate immune response is the complement system. Once activated during virus infection, complement components bind to the virus, enabling phagocytes to destroy the pathogens [122]. All of these mechanisms are involved in the antigen non-specific innate immune response, whose task is to limit viral spread until the antigen specific adaptive immune response can be activated for the resolution of infection, which usually takes about 7 days [119]. The adaptive immune response consists of humoral and cellular-mediated immunity. Cellular immune responses are mediated by CD4+ and CD8+ T cells. Activated CD8+ T cells lyse infected cells. Besides the cytolytic function, CD4+ T cells can also facilitate both humoral and cellular immune responses [123]. Antibodies specific for the HA and NA surface proteins of the virus are important for recovery from primary influenza virus infection and in protection from re-infection [124]. In the following review sections, the focus will be on the IFN-induced Mx protein and inflammatory response.

1. Interferons

IFNs belong to a large family of cytokines and can be divided into two major types: type I and type II. Type I IFNs consist primarily of IFN-α and IFN-β, which are synthesized by most cell types including epithelium and monocyte/macrophage in

response to virus infection. These cytokines have essential roles in the innate immune response against viruses [125-126]. The type II IFN consists of a single member, IFN-γ, which is predominantly produced by natural killer cells and T lymphocytes. IFN-γ is an important regulator of cellular immune responses [127].

Once synthesized, IFN-α/β binds to its receptor on the cell surface. This event leads to the activation of tyrosine kinase Jak1 and Tyk2, which then phosphorylate and activate STAT1 and STAT2. Phosphorylated STAT1 and STAT2 form a heterodimer and translocate to the nucleus, where they form the transcription factor complex called the IFN-stimulated gene factor-3 (ISGF-3) with IRF-9. The transcription of interferon-stimulated genes will be initiated when the ISGF-3 complex binds to the promoter region of these genes [128]. Type I IFN is known to induce the synthesis of several hundred cellular proteins [129]. The functions of most IFN-induced proteins remain elusive. A few of them such as Mx, PKR and 2'-5' oligoadenylate synthetase have been studied in some detail. The IFN-induced antiviral Mx protein is discussed further below.

2. Mx

The Mx proteins were discovered as IFN-induced proteins in an inbred mouse strain (A2G) that showed antiviral activity against influenza A viruses [130-131]. The mouse Mx1 protein is localized in the nucleus [132], while the Mx2 protein, which is functional only in feral strains, is localized in the cytoplasm [133]. Rats produce three Mx proteins, one is a nuclear protein while the other two are cytoplasmic proteins [134]. Of note, most strains of inbred mice (e.g. BALB/c) carry defective Mx genes and as a result they are susceptible to influenza A virus infection [135]. In humans, two Mx proteins have been found (MxA and MxB) and both are localized in the cytoplasm [136].

However, human MxB has no antiviral activity [137]. The Mx proteins were also identified in horses and reported to reside in the cytoplasm [138-139], but their functions remain unknown.

Mx proteins are GTPases that belong to the dynamin superfamily of large GTPases [140]. The mouse Mx1 protein inhibits the replication of *Orthomyoviruses*, such as influenza A viruses and *Thogotoviruses*, in the nucleus by blocking the virus transcription [132, 141-142]. The murine Mx2 protein confers resistance to Vesicular Stomatitis Virus and *Bunyaviruses*, which replicate in the cytoplasm [133-134, 143-144]. Irrespective of the virus replication site in host cell, the human MxA protein displays antiviral activity against a wide range of RNA viruses including *Orthomyxoviruses, Bunyaviruses, Paramyxoviruses, Rhabdoviruses, Togaviruses, Picornaviruses*, and even a DNA virus (Hepatitis B virus) [145-148].

3. Inflammatory responses

Influenza A virus can replicate in airway epithelial cells and lung macrophages and dendritic cells [149-151]. Virus-infected epithelial cells release limited amounts of cytokines and chemokines, such as MIP-1, RANTES, IL-8 and IFN-α/β [114-115, 125]. In contrast, pulmonary monocytes/macrophages produce a broader spectrum of inflammatory mediators in response to virus infection, including MIP-1, MCP-1, MCP-3, IP-10, IL-1, IL-6, IL-18, TNF-α, IFN-γ and IFN-α/β [116-117, 152-154]. Dendritic cells, as important mediators of innate and adaptive immune responses to influenza virus infection, have been reported to produce multiple chemokines and cytokines, such as IFN-α/β, IL-6, IL-8, IL-12 and TNF-α [155-157]. It is believed that pulmonary macrophages and DCs are the major contributors of inflammatory cytokine production.

Chemokines are a subfamily of cytokines that function as chemoattractants for leukocytes. They are approximately 70 to 100 amino acids in length and exhibit from 20-90% amino acid homology to each other [158]. The hallmark of pulmonary inflammation following influenza virus infection is the trafficking of effector leukocytes from the peripheral blood flow into inflamed tissue. This process is mainly directed by chemokines [159]. The neutrophils, which are primarily activated by IL-8, dominate the early influx of leukocytes. Activated neutrophils phagocytose virus-infected cells and therefore are recognized to be the first line of defense [160]. Other chemokines such as MCP-1, MIP-1 and RANTES appear to act on other leukocytes and lymphocytes [159]. Large numbers of chemokines and cytokines produced by macrophages and DCs, including the most important cytokines IL-1, IL-6, TNF-α and IFN-γ, will further elaborate the inflammatory response. These cytokines were shown to induce systemic symptoms such as fever, headache and appetite loss by acting on the central nervous system [161-164]. In addition, IL-1, IL-6 and TNF-α are involved in the activation and recruitment of natural killer cells and lymphocytes into the infected lungs [165-166]. Overall, cytokines play a pivotal role in the regulation of inflammatory and immune responses.

Research Objectives

Equine H7N7 influenza viruses share many remarkable pathogenic properties with the highly pathogenic avian influenza viruses, i.e. lethal infection in BALB/c mice [167], the HA gene from an equine H7N7 influenza virus bestows a less pathogenic avian influenza virus with the lethal phenotype for chickens [168] and the equine H7N7 influenza virus HA gene contains multibasic amino acids at the cleavage site, which is therefore theoretically susceptible to cleavage by ubiquitous host proteases [169]. However, the infection by the equine H7N7 influenza virus in its natural host is strikingly different, causing only mild, self-limiting upper respiratory disease [12-13]. Why the equine H7N7 influenza virus displays the differential pathogenicity in BALB/c mice and horses still remains an open question. Thus, the **overall objective** of this research work was to investigate the mechanism(s) underlying the observed pathogenicity difference of the equine H7N7 influenza viruses in these two animal species.

As the first line of inducible defense against invading pathogens, the host innate immune response is immediately mounted upon influenza A virus infection to impede viral spread and to activate the subsequent adaptive immune response, which demands a few days to consolidate and is pivotal for virus elimination and the ultimate recovery from the disease [119]. Therefore, the quality of the innate immune defense against the viral infection is a critical determinant of the magnitude and quality of the following adaptive immune response. During natural and experimental influenza virus infection, it usually takes 5-7 days for the adaptive immunity to be effective for viral clearance and 1-2 weeks for clinical recovery in immunocompetent subjects without complicated infection [2, 12, 170]. In stark contrast, deaths occur as early as day 4 after infection with

equine H7N7 or the highly pathogenic avian H5N1 influenza virus in BALB/c mice [109-111, 167], which indicates the failure of the innate immune response as the only defense weapon for the disease within the early time window. Evidence accumulating from studies in BALB/c mice with the highly pathogenic avian H5N1 influenza virus reveals that sustained high viral load in the lungs early on and an exacerbation of proinflammatory response are central to the unusual pathogenesis of this virus in the mouse model [95, 106, 113, 171]. It was therefore **hypothesized** that the pro-inflammatory responses of horses and BALB/c mice during the equine H7N7 influenza virus experimental infection are different.

To test the experimental hypothesis, **the first specific aim** was to compare the innate immune responses in terms of the intensity of pro-inflammatory cytokine response in BALB/c mice after experimental infection with a representative strain of either equine H7N7 or H3N8 influenza virus, respectively. **The second specific aim** was to determine whether the developmental state of the innate immune system affects the comparative pathogenesis of equine H7N7 and H3N8 viruses in a chicken embryo model. Different-aged chicken embryos, which differ in developmental stages of the interferon system, were inoculated with three different doses of either virus, and cumulative mortality rates following inoculation were recordered to determine and compare the virulence of these two viruses for chicken embryos.

Although limited field observations indicate that equine H7N7 viruses are less pathogenic than H3N8 viruses in the horse [172-173], no studies so far have been reported to investigate the comparative pathogenicity of these two subtypes in horses by use of experimental infections. Moreover, knowledge about equine cytokine responses to

the equine H7N7 virus infection is completely absent. To this end, **the third specific aim** was to compare pathogenesis and inflammatory cytokine responses in horses following exposure to either virus. The result of this experiment will then be used to compare to that of the BALB/c mouse study to test the hypothesis proposed above.

It has been demonstrated that influenza virus can infect alveolar macrophages *in vivo* after intranasal challenge of ponies [174]. However, to date there are no published reports addressing whether *ex vivo*-cultured equine peripheral blood mononuclear cells (PBMCs) or monocyte-derived macrophages support the growth of influenza virus. If the highly permissive equine immune cell culture can be established, this *in vitro* research model could be exploited to investigate the equine cytokine response following influenza virus infection and to extend the current *in vivo* study in the future. **The fourth specific aim** of this dissertation was to determine whether equine PBMCs or blood-derived macrophages support the prolific replication of equine influenza virus.

CHAPTER TWO

Materials and Methods

Viruses

Influenza A/equine/New York/49/73 (H7N7) and influenza A/equine/Kentucky/5/2002 (H3N8) were used throughout all experiments in this dissertation. Both virus strains were grown and propagated in 10-day-old chicken embryonated eggs. To avoid generation of von Magnus-type defective interfering (DI) particles [175] during virus preparation, which are non-infectious due to internal deletion of genomic RNA segments [176], seed virus was 10-fold serially diluted in phosphate buffered saline (PBS) supplemented with 1% PSA antibiotics (100 U/ml penicillin, 100 µg/streptomycin, 0.25 µg/ml fungizone; BioWhittaker), and 100 µl from each dilution was inoculated into the allantoic cavity of each egg. All eggs were incubated for 48 hours at 37°C in an incubator with humidity. After incubation eggs were removed to 4°C for 24 hours. Allantoic fluids were then harvested, following by clarification by centrifugation at 1000 ×g for 10 minutes. Virus titers were determined by hemagglutination (HA) and EID50 assays (to be described below). Allantoic fluids of the eggs with the largest dilution number combined with the highest HA titer were collected and pooled to make stock virus. The ratios of EID50 titer/HA titer for the H7N7 and H3N8 virus stocks were 1.06×10^6 and 3.4×10^5, respectively, which is indicative of a highly replication-competent virus preparation and a very low incidence of DI particles. The virus-containing egg fluids were then aliquoted and stored at -70°C for later use.

Preparation of 0.5% chicken red blood cells

Chicken red blood cells used for the HA assay were collected weekly from healthy male non-vaccinated leghorn chickens. Approximately 5ml of chicken blood was

collected from the wing vein into a vacutainer blood collection tube supplemented with sodium heparin (BD, Franklin Lakes, NJ). Blood was washed with physiological saline solution two times and centrifuged at 500 × g for 5 minutes at 4 ℃. After the supernatant was removed, the cell pellet was washed again with PBS and centrifugated at 500 × g for 20 minutes at 4 ℃ to pack the cell pellet, from which 0.25 ml was pipetted and mixed with 50 ml cold PBS to make 0.5% chicken red blood cells.

Hemagglutination Assay (HA)

The HA test was used to determine the titer of influenza virus based on the fact that influenza virus can agglutinate erythrocytes through the interaction between the hemagglutinin protein and sialic acid residues on the cell surface. Briefly, samples (50 µl) were serially diluted 2-fold in PBS in a u-bottom microtiter plate. A negative control using PBS was included in the test. Each well was mixed with equal volume of 0.5% chicken red blood cells and incubated at room temperature for 30 minutes. Negative was indicated by the presence of a red button in the well. The HA titer was expressed as the reciprocal of the highest virus dilution exhibiting complete hemagglutination.

EID50 Assay

The 50% egg infectious dose (EID50) titers for the H7N7 and H3N8 viruses were calculated by the method of Reed and Muench (1938). To perform an EID50 assay a series of 10-fold dilutions of each virus was made in PBS with 1% PSA antibiotics. 0.1 ml of each dilution was injected with syringe and needle into the allantoic cavity of 10-day-old chicken egg (4 eggs/dilution). The eggs were sealed with melted paraffin and incubated at 37℃ in a humidified incubator. At 72 hours post injection the eggs were transferred to 4℃ for 24 hours. Allantoic fluids were harvested and then clarified to

remove any egg debris by centrifugation. HA assay was employed as described above to identify the eggs in which virus grew. The virus titer was determined by the Reed and Muench method, and expressed as EID50/ml.

Horses and experimental challenge

6 seronegative mares, aged at least 19 months (Table 2.1), were placed within a closed tent where they were challenged with 1×10^7 EID50 units of Influenza A/equine/New York/73 (H7N7) virus/m^3 of tent (21.5 m^3) for 45 minutes using a nebulizer. Another group of 4 seronegative horses of mixed sex, aged 11-12 months (Table 2.1), were infected experimentally with 1×10^7 EID50 units of Influenza A/equine/Kentucky/5/2002 (H3N8) virus/m^3 of tent (33 m^3) in nebulized aerosols for 45 minutes. The challenge fluids consisted of virus-containing allantoic fluids and PBS. The care, maintenance and use of all horses followed the Guidelines for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture.

Virus isolation from the horses

Nasal swabs were collected daily up to day 8 post challenge. From selected days, titers of excreted virus in nasal secretions were quantified by inoculation into 10-day-old chicken embroynated eggs. Viral titers were calculated by the EID50 method.

Clinical examinations of the horses

Rectal temperatures were monitored daily during the study, and subjects with rectal temperature more than 38.9°C were defined as pyrexic. Each horse was examined daily with the assistance of licensed veterinarian for clinical signs associated with equine influenza, including coughing, nasal discharge, depression and anorexia. Clinical signs of

horse depression may include loss of appetite, lack of motivation and head drooping. A system which was adapted from [177] (Table 2.2) was used to score clinical signs.

Equine peripheral cytokine mRNA response

Peripheral blood was collected using PAXgene collection tubes (Qiagen, Hilden, Germany). The tubes were centrifuged for 10 minutes at 3000 × *g*, and then the supernatant was decanted. The lysed cell pellet was resuspended in 5 ml of RNase-free water. The tubes were re-centrifuged and the supernatant was removed. The resultant cell pellet was resuspended in 350 µl PAXgene Buffer BR1, 300 µl Buffer BR2 and 40 µl Proteinase K, and then was incubated for 10 minutes at 55 ℃. After centrifugation, the supernatant was removed and the lysed cell pellet was mixed with 350 µl of 100% ethanol and then applied to the PAXgene spin column and centrifuged for 1 minute at 10,000 × *g*. The centrifugation was repeated again with the Buffer 3, 4, and 5, respectively. The isolated RNA was denatured by incubation at 65℃ for 5 minutes before cDNA synthesis.

The cDNA was synthesized from DNase-treated total RNA using an oligo(dT) primer and the stratascript® first-strand synthesis system (Stratagene, La Jolla, CA). Primer/probe sets specific for detection of equine β-Gus, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α and Mx (Table 2.3) were designed to cross the exon-exon junction for discrimination between genomic DNA and cDNA derived from mRNA (Assays-by-Design, Applied Biosytems). mRNA expression was quantified by Applied Biosystems 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). PCR cycling conditions comprised an initial enzyme activation step at 95℃ for 20s, and 40 cycles of 95°C for 3s (denaturation) and 60°C for 30s (annealing/extension). Duplicate wells were

run for each 10µl PCR reaction containing 1 µl 10× primer/probe set for the gene tested, 4 µl cDNA template and 5 µl 2 × Taqman® fast universal PCR master mix (No AmpErase® UNG).

The RT-PCR raw data were exported to the LinReg PCR software [178] to calculate the amplification efficiency of each primer pair. Primer efficiency calculations were based on the slope of a linear regression line containing 4–6 data points, and only values with an R^2 (squared correlation coefficient) greater than 0.97 and an efficiency ranging between 1.7 and 2.2 for each amplicon were considered. The value of each PCR cycle threshold (C_T), which is defined as the cycle number at which the fluorescent signal passes the threshold, was then corrected by the corresponding PCR efficiency for individual primer set. The relative quantification of each target mRNA was calculated by the method $2^{-\Delta\Delta CT}$ [179] , where $\Delta\Delta C_T = \Delta C_T$ samples for target gene at a certain time point − ΔC_T the calibrator (averaged ΔC_T of all horses within the same group for each individual gene at day 0) for the target gene, and $\Delta C_T = C_T$ target gene − C_T endogenous control gene. The β-glucuronidase (β-GUS) was used as an internal endogenous control gene [180]. Results were expressed as the mean fold change in individual mRNA expression by the H7N7 or H3N8 groups.

Inoculation of mice

Female, 7-week-old BALB/c mice (Harlan Sprague-Dawley, Indianapolis, IN) were inoculated intranasally with 40 µl of saline containing 10^6 EID50 units of either the H7N7 or H3N8 virus. The viruses were purified by the method of sucrose density gradient centrifugation. Briefly, a 15 ml of continuous sucrose density gradients ranging from 25% to 70% (w/v) sucrose was added into Beckman ultracentrifuge tubes and then

overlayed with virus-containing allantoic fluid without disturbing the interfaces. All tubes were carefully balanced before ultracentrifugation at 26,000 rpm (in a Beckman SW-28 rotor) for 90 minutes at 4 °C. The visible virus-containing band was collected through puncture at the very bottom of the tube using a needle and subsequently mixed with cold PBS for a second centrifugation. The resultant pelleted virus was resuspended and aliquoted in PBS for storage at -70°C. A group of 6 mice for each virus were monitored daily for morbidity and mortality, and also measured for body weight up to day 8 post inoculation. Other groups of 5 mice per virus per day were euthanized daily until day 7 post inoculation. Brain, spleen, heart, liver, kidney and bronchioalveolar Lavage (BAL) samples were collected and immediately stored at -70°C for later determination of virus replication as described below. Cytokine protein assay was measured from BAL samples as described below. All experiments using BALB/c mice were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Virus titration in the mouse tissues

Tissues of brain, spleen, heart, liver and kidney were homogenized in 1ml of cold PBS, and clarified homogenates were titrated in 10-day-old eggs from an initial 1:10 dilution in PBS. BAL fluids were used for pulmonary virus titration. Virus titers were calculated by EID50 using the method of Reed and Muench (1938) and expressed as mean ± SD.

Quantification of cytokines and chemokine in the mouse lungs

BAL fluids from day 2 to day 7 p.i. were assayed for protein levels of IL-6, IFN-γ, TNF-α, IL-10, MCP-1 and IL-12 by use of a Cytometric Bead Array kit (BD, Franklin Lakes, NJ) according to the manufacturer's manual. Briefly, the capture antibody-coated

beads, PE-conjugated detection antibodies, and antigen standards or test samples were incubated together for two hours to form sandwich complexes. Flow cytometry data acquisition was performed on a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). CellQuest software was used for data analysis (BD Biosciences). The detection limit of the assay is 5 pg/ml (IL-6), 17.5 pg/ml (IL-10), 52.7 pg/ml (MCP-1), 2.5 pg/ml (IFN-γ), 7.3 pg/ml (TNF), and 10.7 pg/ml (IL-12p70).

Inoculation of chicken embryos

7, 9 and 11-day-old white leghorn chicken embryonated eggs from the same flock were used. A candling light was used to determine the position of the embryo eyes, which was then marked on the shell with a pencil. The shell was disinfected with 75% alcohol and a hole was drilled above the pencil mark along the line of air sac. A 25-gauge needle was inserted vertically into the hole and 0.1ml inoculum (virus-containing allantoic fluid), which was diluted in PBS supplied with 1% PSA antibiotics, was injected into the chorioallantoic sac of the embryo using a tuberculin syringe. The opening was sealed with melted paraffin, and the eggs with blunt end up were returned to a 37°C humid incubator. Eight eggs in each age group were inoculated with 3 different doses (10^2, 10^4 and 10^6 EID50 units/egg) of the H7N7 or H3N8 virus. A group of 8 eggs was inoculated with each dose. Inoculated eggs were candled daily to determine death for the calculation of cumulative mortality. Death was ascertained by absence of embryonic movement.

Gene sequencing of the H7N7 virus HA cleavage site

Viral RNA isolation was carried out by QIAamp® viral RNA mini kit (Qiagen, 52904) according to the manufacturer's manual. cDNA was synthesized with a 12-base oligonucleotide primer (5' AGCAAAGCAGG), which can form base pairs with the

widely conserved nucleotide sequences at the 3' termini of influenza A virus RNAs, by using superscript™ first-strand synthesis system (Invitrogen, 11904-018). The sequencing reactions for the H7N7 HA gene were performed with the primers listed below (Table 2.4) using the Bigdye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). The HA cleavage site sequence of the H3N8 virus was derived from GeneBank (Accession ID, AY855341). 3M sodium acetate and 100% ethanol were used to precipitate and purify the sequencing products. After washing twice with 75% ethanol, the pellet was dried under a vacuum and resuspended in 20 µl of Hi-Di™ Formamide for loading on an ABI 310 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The data were analyzed with the Vector NTI suite 9 software package (InforMax Inc, Frederick, MD).

Isolation, culture and infection of equine peripheral blood mononuclear cells (PBMCs)

Blood was collected from healthy, adult horses by venipuncture into heparinized tubes. Upon returning to the lab, blood was diluted with phosphate buffed saline (PBS) at 1:1 ratio. 4 ml histopaque®-1077 (Sigma, St. Lous, MO) was added into a 15 ml conical centrifuge tube, and prediluted blood was gently layered onto histopaque®-1077. Tubes were balanced and centrifuged at 400 x g for 30 minutes at room temperature without braking (Beckman GS-6KR Centrifuge; Rotor type GH-3.7). After centrifugation, a 1-ml sterile pipette was used to penetrate the top plasma layer and aspirate the white cell ring containing mononuclear cells at the interface between plasma and Histopaque. PBMCs were transferred into a clean conical centrifuge tube and mixed with 10 ml PBS by gentle inversion. Tubes were centrifuged again at 250 x g for 10 minutes at room temperature with braking. Supernatant was aspirated, and pellet was resuspended with 10 ml PBS.

Centrifugation was repeated at 250 x g for 10 minutes at room temperature with braking. After removing the supernatant, the cell pellet was resuspended with complete RPMI-1640 medium supplied with 10% autologous serum, 100 U/ml Penicilin G, 100 µg/ml Streptomycin, and 0.25 µg/ml amphotericin B, and then was plated in a 24-well plate. For influenza virus infection, culture medium was replaced with serum-free RPMI-1640 medium supplied with 1 µg/ml TPCK-treated trypsin, then cells, which were plated in a 24-well plate (in a volume of 100 µl of media per well), were infected at a multiplicity of infection of 5 EID50 units per cell with either of the two viruses in allantoic fluid. After absorption of virus for 1h at 37 °C in 5% CO2, cells were washed five times with warm PBS then incubated in serum-free RPMI-1640 medium supplied with trypsin. At 0 and 24 hours post infection, supernatants of infected cell culture were collected for extracellular virus titration by EID50 in embryonated chicken eggs, and cells were collected and processed for indirect immunofluorescence assay**.**

Monocyte-derived macrophage isolation, culture and infection

Macrophages were derived from blood monocytes, which were isolated from PBMCs. The isolation and culture of PBMCs were the same as descried above. To isolate monocytes from PBMCs, the cells were allowed to adhere onto 12 or 24-well plates for 2h at 37 °C in 5% CO2. After monocyte binding, non-adherent cells (most are lymphocytes) were removed and adherent cells were then allowed to differentiate for 7 days with the medium being changed every other day before infection [181]. The purity of macrophages was measured by performing cytochemical assay for the non-specific esterase activity using the α-naphthyl-acetate esterase kit (Sigma) [182]. To infect macrophages with influenza virus, cells were washed twice in warm PBS and then culture

medium was replaced with serum-free RPMI-1640 medium supplied with 1 μg/ml TPCK-treated trypsin. Macrophages were exposed to infection at a multiplicity of infection of 5 EID50 units per cell with either of the two viruses indicated above. After virus absorption for 1h at 37 °C in 5% CO2, cells were washed five times with warm PBS then incubated in serum-free RPMI-1640 medium supplied with trypsin. Supernatants of cell culture were collected for extracellular virus titration by EID50 on chicken eggs and for TNF-α protein analysis by using the equine TNF-α screening set (Endogen, Rockford, IL). Also, macrophages were collected and processed at designated times post infection for indirect immunofluorescence assay**.**

Indirect Immunofluorescence Assay

Cells were rinsed twice with cold PBS, and then fixed with 3% paraformaldehyde for 10 min at room temperature. After washing once with cold PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Non specific antibody-binding sites were blocked by incubation of cells for 1h with 10% normal horse serum in PBS (Sigma). Monoclonal primary antibody (Fitzgerald, Concord, MA) against influenza virus nucleoprotein (NP) was diluted in blocking solution (1:100 dilution), and then reacted with cells for 1h at room temperature. After washing three times, cells were incubated at room temperature for 1h with FITC-conjugated goat antimouse IgG antibody (Pierce, Rockford, IL) (1:400 dilution in blocking buffer), which was diluted with 0.02% Evans Blue in blocking solution. Cells were mounted with coverslips before examination using Axioplan 2 fluorescent microscope (Zeiss) equipped with cytovision©/Genus™ Application software version 2.7 (Applied Imaging).

Statistical analysis

A repeated measures, two-way analysis of variance (RM-ANOVA) was used to determine differences in clinical scores and daily rectal temperatures between the two viruses over time for the horse challenge study. Since data of horse cytokine mRNA expressions was not normally distributed, the values of IFN-γ, TNF-α, IL-1β and IL-18 relative quantifications had to be $\log_{10}$-transformed first before running the two-way RM-ANOVA to determine differences in each of the cytokines between the two viruses over time. The statistical analysis of horse IL-6, IL-8, IL-10 and Mx data was performed using Friedman repeated measures ANOVA on Ranks and Mann-Whitney Rank Sum Test. The samples that were identified as outliers by the LinReg PCR analysis were treated as missing data in the statistical model mentioned above, and were not considered when computing the mean of the respective measurement. The analysis of Spearman Rank Order Correlation was used to evaluate the correlation among virus shedding, febrile response, clinical severity and cytokine expressions. For mouse challenge study, the significant differences in viral titers and mouse body weight loss after the virus infections between the two viruses over time were determined using a two-way ANOVA and two-way repeated measures ANOVA, respectively. Non-parametric analysis (ANOVA on RANKS and Mann-Whitney Rank Sum Test) were used to evaluate differences in cytokine levels within the H7N7 group mice and between the two groups (IL-10 only) in the course of viral infections. Any mouse cytokine with undetectable expression level by the assay was valued as 0 in the statistical model. The degree of correlation among the expression of individual cytokines, and between the levels of virus replication and cytokines in the lung within the H7N7 group were assessed using Spearman Rank Order

Correlation. Differences in cumulative chicken embryo mortalities induced by the two viruses over the period of observation were analyzed by a Student's t-test, which was also used to determine the significant differences of TNF-α protein levels in cell supernatants from the H7N7 virus-infected and mock-infected macrophages. $P<0.05$ was considered to be statistically significant for all statistical analyses performed with SigmaPlot (V11.0) (Systat Inc., Richmond, CA).

Table 2.1 Age of each individual horse in the study

Treatment (Group)	Horse ID	Age (month)	Sex
H7N7 virus	85 160 C011 D001 F017 F019	>35 >35 55 44 19 19	F F F F F F
H3N8 virus	G001 G002 G005 G009	12 12 12 11	M F F M

Table 2.2 Scoring system for clinical signs

Clinical Sign	Degree	Score
Coughing	No Mild=once during 20 minutes Moderate=two or more times during 20 minutes	0 1 2
Nasal discharge	No Mild=transient, slight serous Moderate=readily observed mucopurulent Severe=copious discharge	0 1 2 3
Depression	No Present=loss of appetite, lethargy, head down	0 1
Anorexia	No Present	0 1
Labored breathing	Normal Mild Moderate	0 1 2

Table 2.3 Sequences of primers and probes used in real-time PCR

Gene	Primer/Probe	Sequence
IL-1β	Forward	5'-CCGACACCAGTGACATGATGA-3'
	Reverse	5'-ATCCTCCTCAAAGAACAGGTCATTC-3'
	Probe	5'-ATTGCCGCTGCAGTAAG-3'
Il-6	Forward	5'- GGATGCTTCCAATCTGGGTTCAAT-3'
	Reverse	5'- TCCGAAAGACCAGTGGTGATTTT-3'
	Probe	5'- ATCAGGCAGGTCTCCTG-3'
IL-8	Forward	5'- GCCGTCTTCCTGCTTTCTG-3'
	Reverse	5'- CCGAAGCTCTGCAGTAATTCTTGAT-3'
	Probe	5'- CAACCGCAGCTTCAC -3'
IL-10	Forward	5'- AGGACCAGCTGGACAACATG -3'
	Reverse	5'-GGTAAAACTGGATCATCTCCGACAA -3'
	Probe	5'-CCAGGTAACCCTTAAAGTC-3'
IL-18	Forward	5'- CCTGTGTTTGAGGATATGCCTGATT-3'
	Reverse	5'- GCTAGACCTCTAGTGAGGCTATCTT-3'
	Probe	5'- ATTGTACAGACAACGCACCC -3'
IFN-γ	Forward	5'-AGCAGCACCAGCAAGCT-3'
	Reverse	5'-TTTGCGCTGGACCTTCAGA-3'
	Probe	5'-ATTCAGATTCCGGTAAATGA-3'
TNF-α	Forward	5'- TTACCGAATGCCTTCCAGTCAAT-3'
	Reverse	5'- GGGCTACAGGCTTGTCACTT-3'
	Probe	5'- CCAGACACTCAGATCAT-3'
Mx	Forward	5'- CCGACAGGAGTTCCAGAAATGG-3'
	Reverse	5'- CTGCCACGATACTGATTTTCAAATGT-3'
	Probe	5'- CTTCGCCACCTTTTCG-3'
β-glucuronidase	Forward	5'- GCTCATCTGGAACTTTGCTGATTTT-3'
	Reverse	5'- CTGACGAGTGAAGATCCCCTTTT-3'
	Probe	5'- CTCTCTGCGGTGACTGG-3'

Table 2.4 Primers used for sequencing of the H7N7 virus HA cleavage site

Primer	Sequence 5'-3'
HA-697 Forward	CCTGGACCAAGACCGCAAAT
HA-1377 Reverse	CTCCACTGCTACGAGGAATTCT

CHAPTER THREE

Results

Characterization of the H7N7 and H3N8 viruses in BALB/c mice

As addressed above in the section of research objectives, the aim of experimental infection of BALB/c mice with the two subtype viruses was to compare their pathogenesis in the mouse model. Mice were infected as described in Materials and Methods. Mice inoculated with the H7N7 virus began to lose weight at day 3 post infection ($p<0.001$). By day 7 p.i., there was an approximately 25% loss of their initial weight at inoculation ($p<0.001$) (Figure 3.1A). Pronounced clinical signs of disease characterized by ruffled fur, lethargy, hunched posture and labored breathing were noticed on day 4 and afterward. By day 8 p.i., all the H7N7-infected mice succumbed to death (Figure 3.1B). In contrast to the lethal outcome of the H7N7 virus infection, neither weight loss nor clinical symptoms were demonstrated in the H3N8-infected mice during 8 days of observations, and all mice survived the infection.

Although the disease progression differed strikingly for the two viruses in BALB/c mice, both viruses replicated to a similar degree during the first 2 days of infection in the lungs without prior mouse adaptation. However, the H3N8 virus had significantly lower lung virus titers than the H7N7 virus after day 2 p.i. ($p<0.05$) and was cleared from the mice at day 7 p.i. (Figure 3.2A), indicating that the H3N8 virus exhibits normal sensitivity to the innate immune control of BALB/c mice. In contrast, the H7N7 virus demonstrated the extraordinary ability to escape the innate host defense and multiplied uniformly to high titers in the lung until the death of these mice. Furthermore, large amounts of erythrocytes in lung lavage fluids were noticed in four mice out of five

within the H7N7-infected group on days 6 and 7 only post infection, which implies the occurring of pulmonary hemorrhage. Such a phenomenon was not observed in the H3N8 group of mice. Virus replication in the brain, heart, spleen, kidney and liver were also examined in mice infected with either the H7N7 or H3N8 virus over the course of infection. The H7N7 virus displayed efficient replication in the brain in which virus titers increased gradually from very early infection until these mice died (day 4 vs day 1, $p<0.05$; day 7 vs day 1, $p<0.001$) (Figure 3.2B). No virus was isolated from any other organs tested. The H3N8 virus failed to spread to organs outside of the respiratory system, except for days 2 and 5 on which virus was recovered from the brain of one mouse in these groups.

Cytokine and chemokine protein levels in the lungs of infected mice

Since the two viruses exhibited a clear disparity of pathogenicity in BALB/c mice, the extent of protein expression of inflammatory cytokines induced by each virus was examined in the lungs over time (Figure 3.3). In the H3N8 virus-infected mice, the presence of IL-6, IFN-gamma, MCP-1 and TNF-α was undetectable by the assay at the times of observation except for TNF-α, which was detected at a low level in three mice on day 3 (14, 17 and 43 pg/ml, respectively) and one mouse (25 pg/ml) on day 2 p.i., respectively. In contrast, the H7N7 virus infection elicited expression of IL-6 in most or all mice at each time point examined except on days 2, 3 and 7 p.i. when only a small number of animals exhibited the presence of it in the lungs. The expression of IFN-γ in mice infected with the H7N7 virus followed a similar pattern as IL-6, but on day 7 p.i., IFN-γ was detected in 3 mice. Following exposure to the H7N7 virus, the majority of mice showed detectable presence of TNF-α in the lung during the examination period

except for days 2 and 7 p.i., on which the assay failed to detect the cytokine in 4 and 3 animals, respectively. The production of MCP-1 in the H7N7 group was detected in only two mice between days 2 and 5 p.i. In contrast, the number of mice with detectable expression of MCP-1 increased to five on both days 6 and 7 p.i. The expression of anti-inflammatory cytokine IL-10 was detected in the lungs of most mice infected with either of the two viruses. As for statistical analysis, any samples with undetectable levels of the respective cytokines were treated as zero. One-Way ANOVA on Ranks analysis showed that neither the H7N7 nor H3N8 virus infection was able to induce significant increases in the expression of all cytokines studied here in the lungs of mice during the examination period. However, the H7N7 virus induced greater levels of IL-10 expression than the H3N8 virus with significant differences on days 2, 6 and 7 p.i. ($p<0.05$, Mann-Whitney Rank Sum Test). Neither the H7N7 nor H3N8 virus infection triggered detectable IL-12 expression since the assay applied in the present study failed to reveal the presence of IL-12 in the lungs. Following the H7N7 virus infection in mice the pulmonary expression of TNF-α correlated with that of IFN-γ (correlation coefficient=0.56, $p<0.05$) and IL-6 (correlation coefficient=0.64, $p<0.05$). However, such a relationship between IFN-γ and IL-6 could not be established. Furthermore, the expression of IFN-γ also correlated relatively weakly with that of IL-10 (correlation coefficient=0.47, $p<0.05$). The relationship between the virus replication and cytokine expression in the mouse lung was also evaluated by Spearman Rank Order Correlation analysis. This analysis revealed that the viral replication correlated with the expression of TNF-α (correlation coefficient=0.49, $p<0.05$) and anti-inflammatory cytokine IL-10 as well, but in an inverse pattern (correlation coefficient=0.5, $p<0.05$) (Figure 3.4). In other

words, the H7N7 virus replication had a negative correlation with the expression of pulmonary IL-10 in BALB/c mice. Near the end of this dissertation, a dot plot graph of the expression levels of different pulmonary cytokines in individual mice from the H7N7 group is also attached (Appendix B).

The cDNA-derived amino acid sequences of the HA cleavage site of both viruses

To examnine the composition of amino acids at the HA cleavage site of the two viruses, genetic sequences of the two HA genes were obtained and used to deduce the corresponding amino acid sequences. This analysis showed that the H7N7 virus used in this study contains a series of basic amino acid residues at the HA cleavage site, whereas the HA of H3N8 virus has a single basic amino acid residue at the cleavage site (Table 3.1). Therefore, the HA of H7N7 virus can be cleaved by ubiquitous host proteases whereas the H3N8 virus HA can only be activated by trypsin-like proteases distributed specifically in the intestinal and respiratory organs.

Differential cumulative mortality produced by the H7N7 and H3N8 viruses

The innate immune system develops functionally as the chicken embryo grows up. To compare the abilities of both viruses to evade the innate immune control of the developing chicken embryo, embryos of different ages were used for virus inoculation in this study. As seen in Figure 3.5A, when inoculated into 7-day-old eggs, the H7N7 virus at higher doses (10^4 and 10^6 EID50 units per egg) induced 100% cumulative mortality by day 3 p.i., and more than 80% for the low dose of 10^2. Similarly, more than 80% mortality was produced by the H3N8 virus infection with all 3 different doses. In 9-day-old eggs (Figure 3.5B), at each of the three doses used, the H7N7 virus infection resulted in significantly higher cumulative mortality than that by the H3N8 virus by day 4 p.i.

Strikingly, in 11-day-old eggs (Figure 3.5C), the H3N8 infection at all doses tested caused less than 13% mortality by day 5 p.i., which was significantly lower than that of the H7N7 virus. In conclusion, the present study demonstrates that the H7N7 virus is significantly more resistant to the action of the developing innate immune system than the H3N8 virus in chicken embryos aged 9 days and older.

Viral shedding in the horse challenge study

The results of the comparative study on the pathogenesis of the two viruses in the BALB/c mouse model presented above demonstrated that the H7N7 virus was more pathogenic than the H3N8 virus in the mice. Contrary to this, based on field observations it is hypothesized here that the H7N7 virus is less virulent than the H3N8 virus in its natural host, the horse, during experimental infection. To this end, influenza virus infection was documented in all the horses by recovery of virus from nasal swab extracts tested in 10-day-old eggs. Viral shedding persisted in individual animals through day 6 p.i. and no viruses were isolated from them from day 7 post challenge onwards. On day 2 p.i., which is expected to be the day of peak virus shedding, the H7N7 virus titer was 4.49 ± 1.69 log10 EID50/ml while at the same day the H3N8 virus titer was 3 ± 1.35 log10 EID50/ml (mean ± SD). The H3N8 virus titers of day 1 and from day 3 to day 6 p.i. were not determined. There was no significant difference between the two viruses in the duration and amount of virus excreted on day 2 p.i. (Figure 3.6).

Clinical outcome of the horses

Both groups displayed increases in rectal temperature after challenge infection with a peak on day 2 p.i. ($p<0.05$ for the H7N7, $p\leq0.001$ for the H3N8), which then subsided after that day (Figure 3.7). But, rectal temperatures induced by the H3N8 virus

were significantly higher than those by the H7N7 virus on days 2 (p<0.001), 3 (p<0.05) and 5 post infection (p<0.05). Specifically, 4 out of 6 horses infected with the H7N7 virus had pyrexia (>38.9 °C) on day 2 p.i. But after that, fever was absent in all except on days 7 and 8 p.i. At those times the four horses had increased rectal temperature as the consequence of hyperventilation caused by the reaction to the DORMOSEDAN® (detomidine hydrochloride, Pfizer Animal Health, West Chester, PA), which was administered intravenously to horses as a sedative before nasal tampon sampling. Therefore, statistical analysis was not applied for both groups since day 7 p.i. onwards. All animals in the H3N8 group developed fever on day 2 p.i. (40.65 ± 0.66 °C). Pyrexia was still present in some horses until day 8 p.i. There was a clear difference between the two groups regarding clinical signs, with statistical significance reached on days 4, 5 and 6 p.i. (p<0.001) (four horses in the H7N7 group were administered medication from days 7-14 affecting their clinical signs, therefore statistical analysis was not run for that period of time) (Figure 3.8). Mild clinical signs observed after day 3 p.i. among only two horses in the H7N7 group. In striking contrast, obvious illness persisted in all horses infected with the H3N8 virus from day 4 to day 9 p.i.

Cytokine transcription response in the horses

The mRNA expression levels of IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α and Mx genes in individuals during the course of infection were quantified relative to the average level of each respective gene for all subjects within the same group on day 0 p.i. (Figure 3.9). Because the values of squared correlation coefficient and/or PCR amplification efficiency for the cytokine IFN-γ didn't fall within the range set by LinReg analysis, many samples from the H7N7 group animals were excluded from further

analysis and the resulting underrepresented data from this group were not subject to statistical evaluation. Specifically, 4 samples were identified as outliers on day 1 p.i. After that day, the number of outliers decreased to one on day 2 p.i. Between days 3-5 p.i., no more than 3 samples were excluded from the statistical evaluation. In contrast, only two samples from the H3N8 group were identified as outliers (1 sample on day 0 and day 1 p.i., respectively). However, statistical analysis revealed that the H3N8 virus infection failed to induce robust gene transcription of IFN-γ in the blood. Similarily, TNF-α gene expression was not significantly enhanced in either group following the infections, although 1 horse in the H7N7 group and 2 horses in the other group showed more than 3-fold increase over the basal level. Both viral infections induced increases in the production of IL-1β mRNA transcripts as the level on day 2 was significantly higher than that on days 0 and 1 p.i. in the H7N7 group, and on days 2, 3 and 5 compared to the basal level in the H3N8 group ($p<0.05$, Two-Way RM ANOVA). Overall, however, the IL-1β mRNA expressions in the H7N7 group were not significantly different from the levels in the H3N8 group. The transcription of proinflammatory IL-6 gene was significantly induced by H7N7 virus infection not H3N8 virus infection except in one horse, and the increase in the mRNA expression occurred on day 2 p.i. ($p<0.05$, Friedman RM ANOVA on Ranks) and then subsided thereafter ($p<0.05$, day 2 compared to days 4 and 5 p.i.). However, on that day, no significant difference in IL-6 mRNA relative expression levels between the two viruses was observed (Mann-Whitney Rank Sum Test). Specifically, 5 out of 6 horses in the H7N7 group demonstrated more than a 10-fold increase in IL-6 mRNA expression on day 2 p.i. In contrast, only one animal had a similar increase in IL-6 mRNA abundance at that time point in the H3N8 group. As a major chemoattractant for

neutrophils, chemokine IL-8 mRNA peripheral expression levels in both groups were virtually unchanged during the course of infection, except that one horse in the H3N8 group demonstrated remarkable augmentation in the transcription level of this gene on day 2 p.i. compared to the calibrator. Neither the H3N8 nor H7N7 virus infection induced a significant enhancement in the transcription of anti-inflammatory cytokine IL-10 gene in the blood throughout the experiment (Friedman RM ANOVA on Ranks), although two out of four animals in the H3N8 group showed a large increase in this gene's mRNA expression on day 2 p.i. over the basal level. On the same day, however, the Mann-Whitney rank sum test revealed that the median of IL-10 levels in the H3N8 group was significantly greater than that in the H7N7 horse population ($p<0.05$). The peripheral blood mRNA levels of IL-18 in the H7N7 group remained consistently low for the duration of the study. In contrast, the H3N8 virus infection induced significant upregulation of this gene's transcription, as 2 out of 4 animals in the group showed more than 10-fold increase in the mRNA level over the baseline level on days 2, 3 and 5 p.i. The most pronounced differences in the IL-18 transcription levels between the two groups also emerged at these time points ($p<0.05$, Two-Way RM ANOVA). As for a quick reference, another dot plot was created to represent coordinated expression of different cytokines in individual horses in temporal sequence (Appendix C). Both viruses induced a dramatic increase in the anti-viral Mx gene transcription on day 2 p.i. ($p<0.05$, Friedman RM ANOVA on Ranks). However, it needs to be pointed out that the Mx mRNA production of one animal from the H7N7 group was virtually unchanged during the course of infection. In the group of the H7N7-infected horses, the expression of IFN-stimulated Mx gene correlated with the virus replication (correlation coefficient=0.48,

$p<0.05$), which was also correlated with the febrile response (correlation coefficient=0.35, $p<0.05$) (Figure 3.10). Among all cytokines examined here, only the IL-6 expression profile was found to correlate with the febrile response in the H7N7 group (correlation coefficient=0.42, $p<0.05$). In contrast, all inflammatory mediators except IFN-γ demonstrated a close correlation with the febrile development in the H3N8 group (for instance, correlation coefficient for IL-1β and IL-10 was 0.82 and 0.73, respectively.)

Equine PBMCs failed to produce progeny virus after exposure to influenza virus in vitro

The permissiveness of equine PBMCs to influenza virus infection was first tested since PBMCs can easily be obtained compared to macrophages and they play an important role in host immune defense against various pathogen infections. Exposure of equine PBMCs from a blood donor to equine influenza viruses of the H7N7 and H3N8 subtypes did not result in an increase in progeny virus titers, which indicates that equine PBMCs cannot support the productive replication of influenza virus (Figure 3.11). Similar results were obtained when the experiment was repeated in PBMCs from three more different horses (data not shown). To confirm the results above, the immunofluorescent staining technique was subsequently employed to examine whether the translation of viral proteins occurred inside the host cells. The viral nucleoprotein (NP) protein was chosen as the target of monoclonal antibody used in this technique because it is the major structural protein and highly conserved among influenza A viruses. Nearly the entire cell population was negative for the expression of NP protein except for only a couple of positive signals in the whole slide (Figure 3.12). The occurrence of sporadic signals may be due to the presence of myeloid dendritic cells in

exceedingly small quantities in blood. Primary blood myeloid dendritic cells from humans are found to be permissive to influenza virus infection [183-184].

Equine blood monocyte-derived macrophages are highly susceptible to influenza virus infection

On day 0, blood monocytes were homogeneously small with round shape (Figure 3.13A). Over 7 days in culture the monocytes differentiated into mature macrophages, which were enlarged heterogeneous cells with cytoplasmic projections (Figure 3.13B). Cytochemical analysis revealed that virtually the whole population of cells was positive for non-specific esterase staining (Figure 3.14). The esterase enzyme is primarily present in monocytes and macrophages but absent in granulocytes [182]. To determine whether *ex vivo*-differentiated macrophages were permissive for influenza virus infection, 7-day-cultured equine macrophages were infected with either the H7N7 or H3N8 virus at a multiplicity of infection of 5 EID50 units per cell. Both viruses exhibited similar replication kinetics from 0-36 hours p.i. (Figure 3.15). Viral nucleoprotein was detected by indirect fluorescence staining. As shown in Figure 3.16, the vast majority of cells were infected with either virus by 12 h.p.i., thus indicating that equine primary macrophages are highly efficient in supporting influenza virus infection. Productive viral infection of macrophages resulted in cytopathic effect (CPE) and morphological changes, such as cell rounding and detachment, were visually evident by light microscopy as early as 12 h.p.i. At 18 h.p.i. about 50% of cells detached from culture flask and complete cell detachment happened by 24 h.p.i.

No CPE or morphological alterations were observed in macrophages after treatment with UV-inactivated viruses, suggesting that virus replication is necessary for

CPE induction. The effect of monocyte differentiation stage on virus replication was also investigated. Infection of day-0-monocytes with equine influenza virus showed no detectable increase in virus titer over 24 hours incubation. In contrast, productive infection was noticed from day-3-cultured cells with day-7-cultured cells being highly permissive to the infection as the cells differentiated from precursor monocyte to mature macrophage (Figure 3.17).

Infection of macrophages by equine H7N7 influenza virus resulted in TNF-α protein expression

TNF-α is a major inflammatory cytokine in the pathogenesis of influenza. Levels of soluble TNF-α protein in culture supernatants from either the H7N7 virus-infected or mock-infected macrophages were analyzed by ELISA. Infection with influenza virus resulted in activation of equine primary macrophages as evidenced by the significantly enhanced expression of TNF-α protein compared with the mock infected cells at 18 hours p.i. ($p<0.05$) (Figure 3.18).

Table 3.1 The cDNA-deduced amino acid sequence of the HA cleavage site

Virus	Cleavage site
Influenza A/equine/New York/49/73 (H7N7)	Q L T H H M R K K R * G L F G
Influenza A/equine/Kentucky/5/2002 (H3N8)[a]	M R N V P E K Q I R * G I T G

[a] : The HA cleavage site amino acid sequence of the H3N8 virus was obtained from Gene Bank (Accession ID: AY855341)

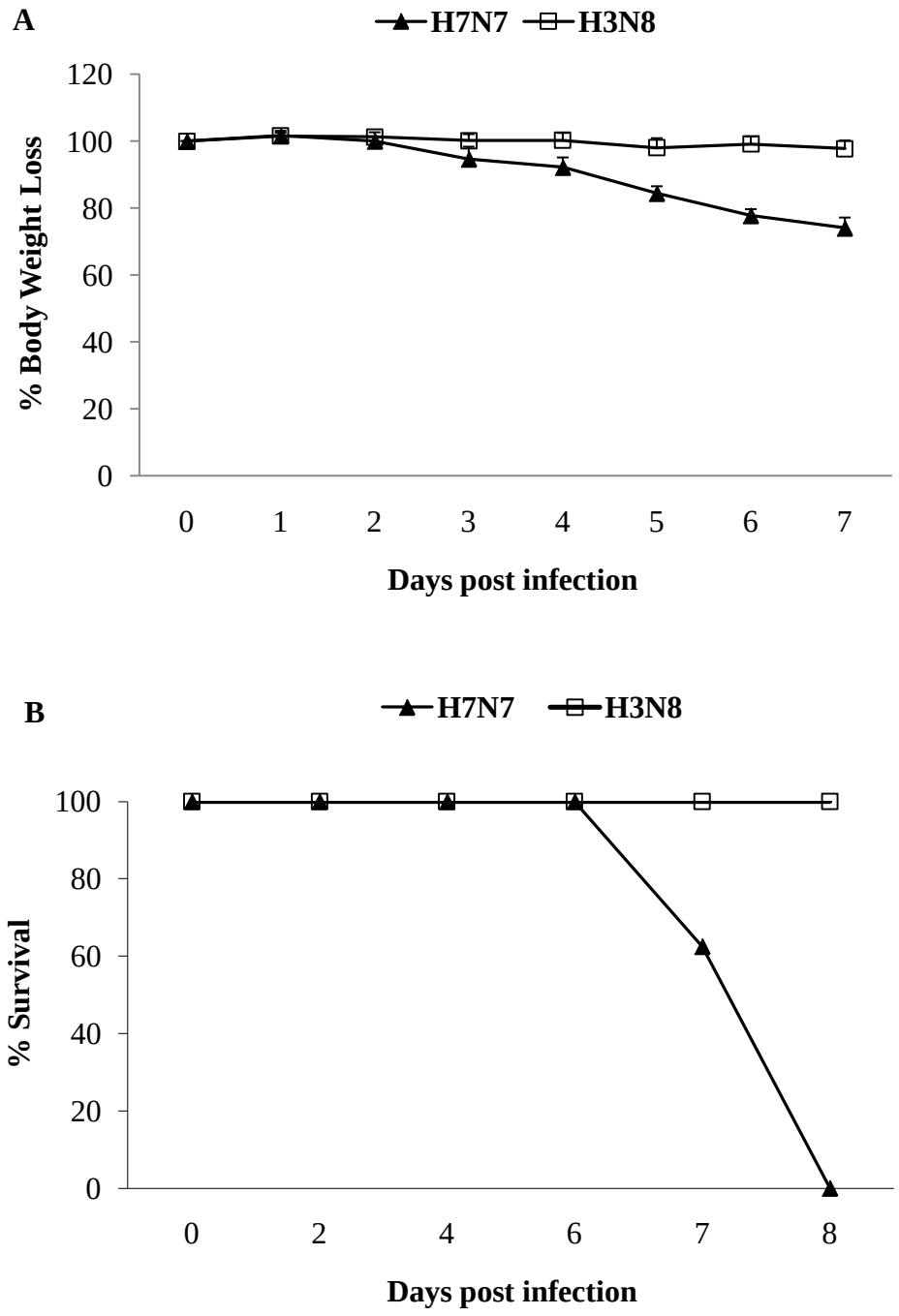


Figure 3.1 Body weight loss rate and survival rate of BALB/c mice. Groups of 6 mice were inoculated intanasally with 10^6 EID50 units of each virus. (A) Mice were weighed daily, and the percentage of body weight loss was calculated relative to the starting weight at day 0 before virus inoculation. Data are expressed as mean ± SD. (B) survival rate in each group over time.

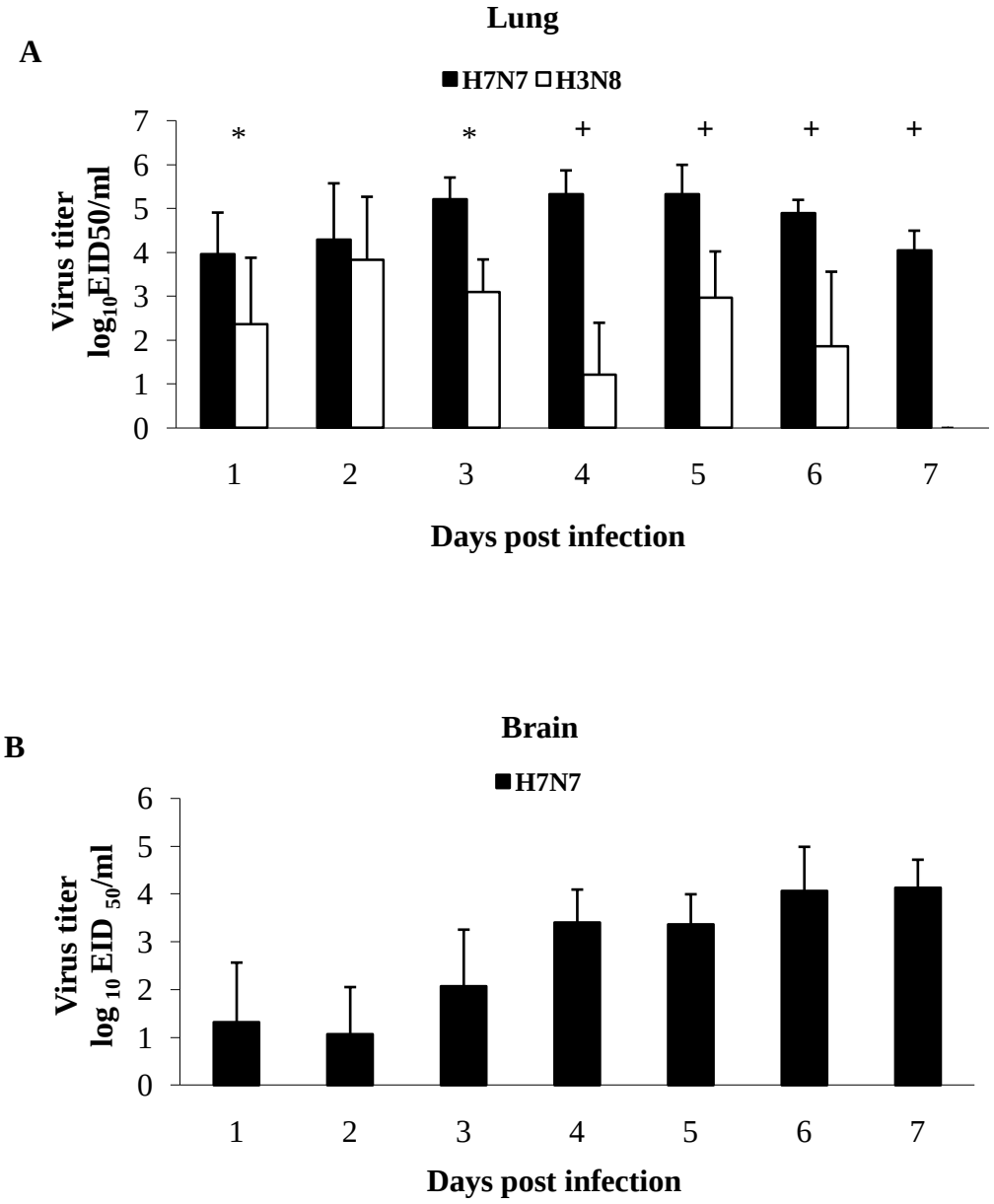
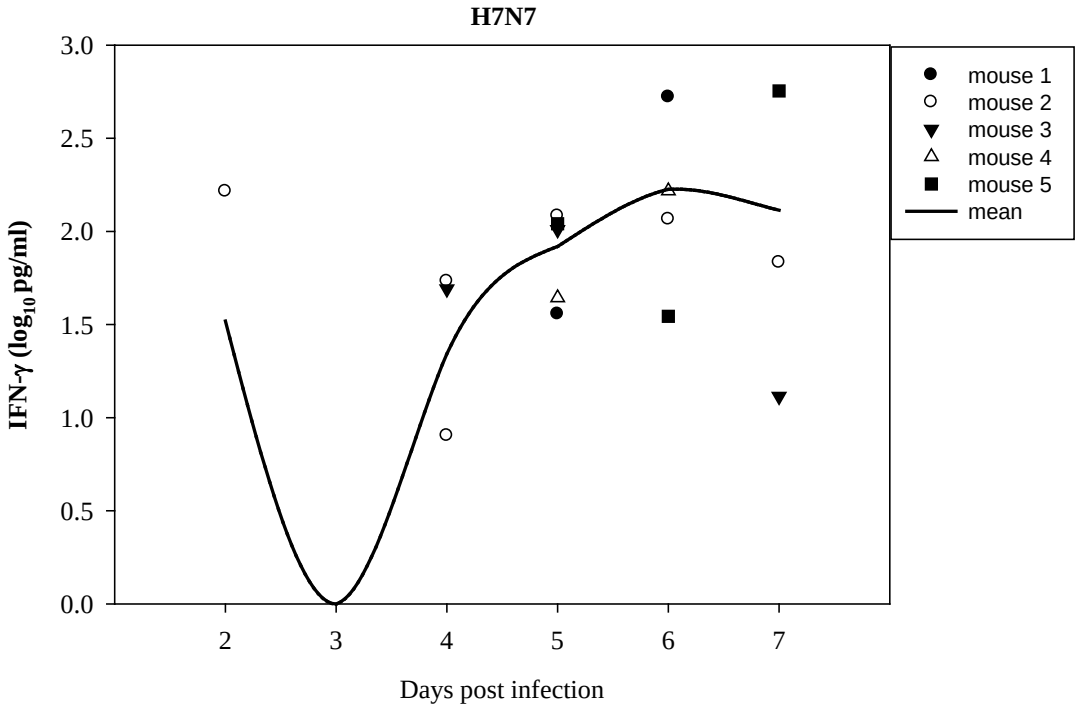
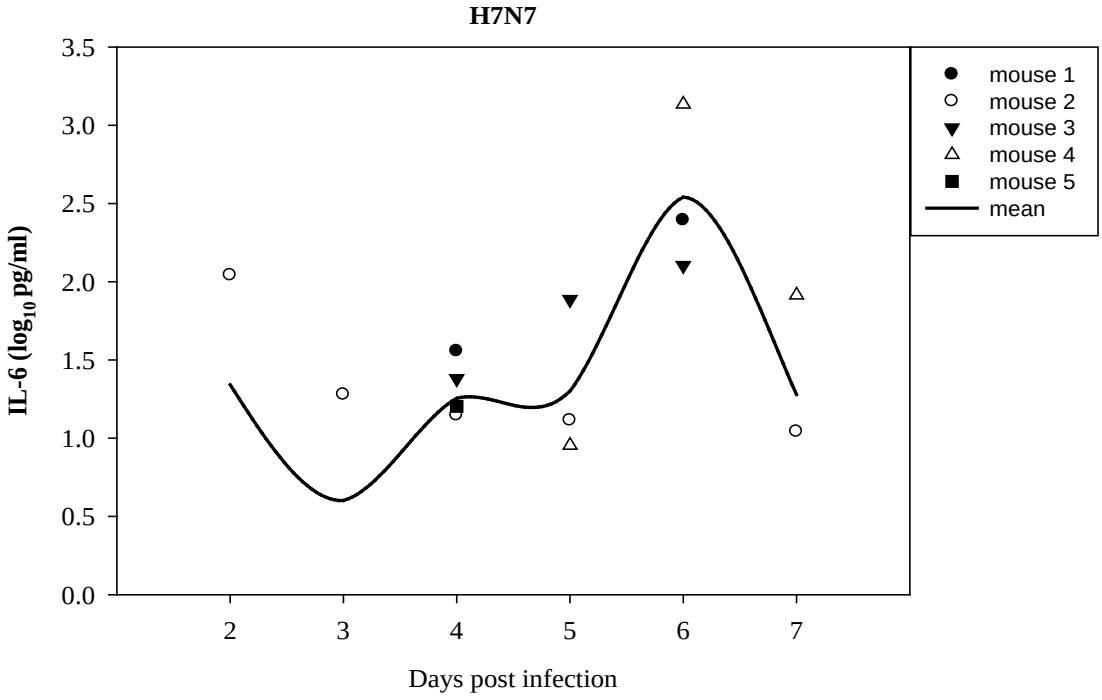
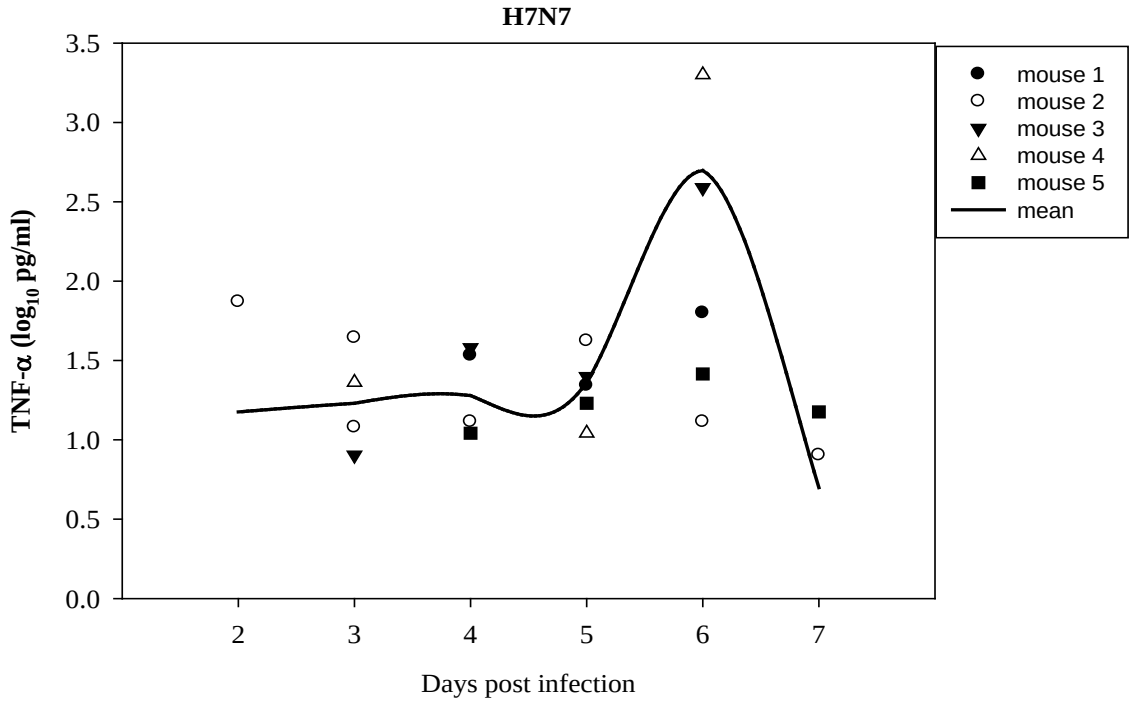
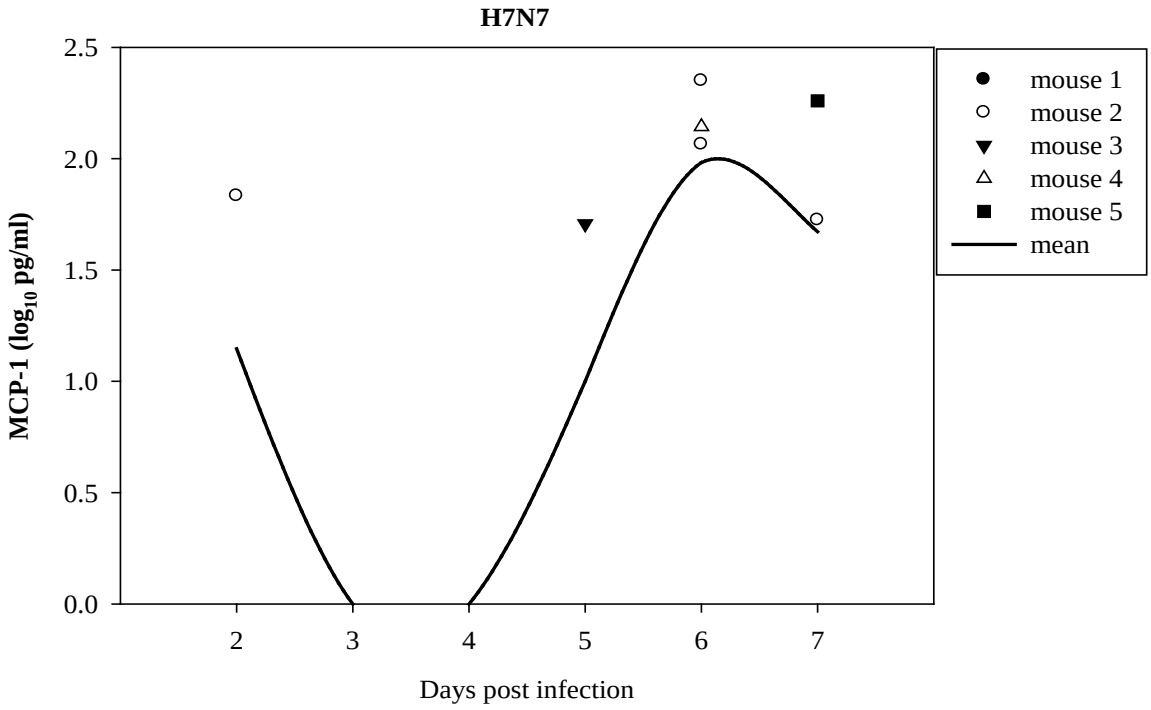


Figure 3.2 Virus titers in mouse lungs and brains. Mice were inoculated intranasally with 10^6 EID50 units of either H7N7 or H3N8 virus. 5 mice per virus were euthanized daily, and brains and BALs were collected. Virus titers in tissues were quantified in 10-day-old eggs by EID50 assay. (A) lung. (B) brain (H3N8 virus was not detected in brain tissues except in one mouse on day 2 and day 5 p.i., respectively). Results are expressed as mean ± SD. * indicates $P< 0.05$, + indicates $P< 0.001$


H7N7
IFN-γ (log10 pg/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
3
4
5
6
7
Days post infection
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5
mean


H7N7
IL-6 (log10 pg/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
3
4
5
6
7
Days post infection
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5
mean


H7N7
TNF-α (log10 pg/ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
3
4
5
6
7
Days post infection
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5
mean


H7N7
MCP-1 (log10 pg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
2
3
4
5
6
7
Days post infection
mouse 1
mouse 2
mouse 3
mouse 4
mouse 5
mean

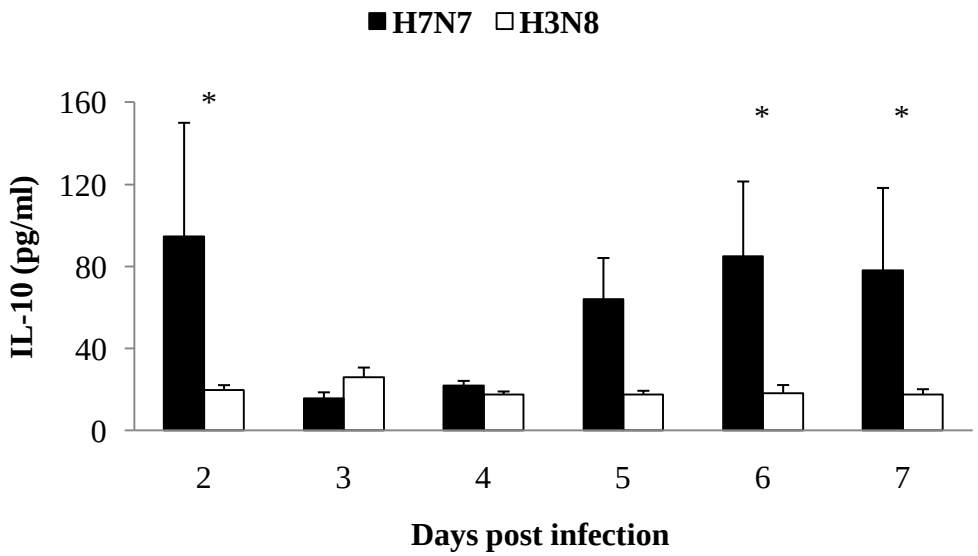


Figure 3.3 Quantification of cytokine and chemokine protein levels in the BALs. Mice were inoculated intranasally with 10^6 EID50 units of either the H7N7 or H3N8 equine influenza virus. BALs of 5 mice per group were used to determine the levels of cytokine and chemokine by Cytometric Bead Array as described in the methods. The expression levels of IFN-γ, IL-6, MCP-1 and TNF-α in the H3N8 group were below the detection limit of the assay. Samples with undetectable levels of the respective cytokines were valued as 0. Data of IL-10 are presented as mean ± SEM. * indicates $P< 0.05$ (Mann-Whitney Rank Sum Test).

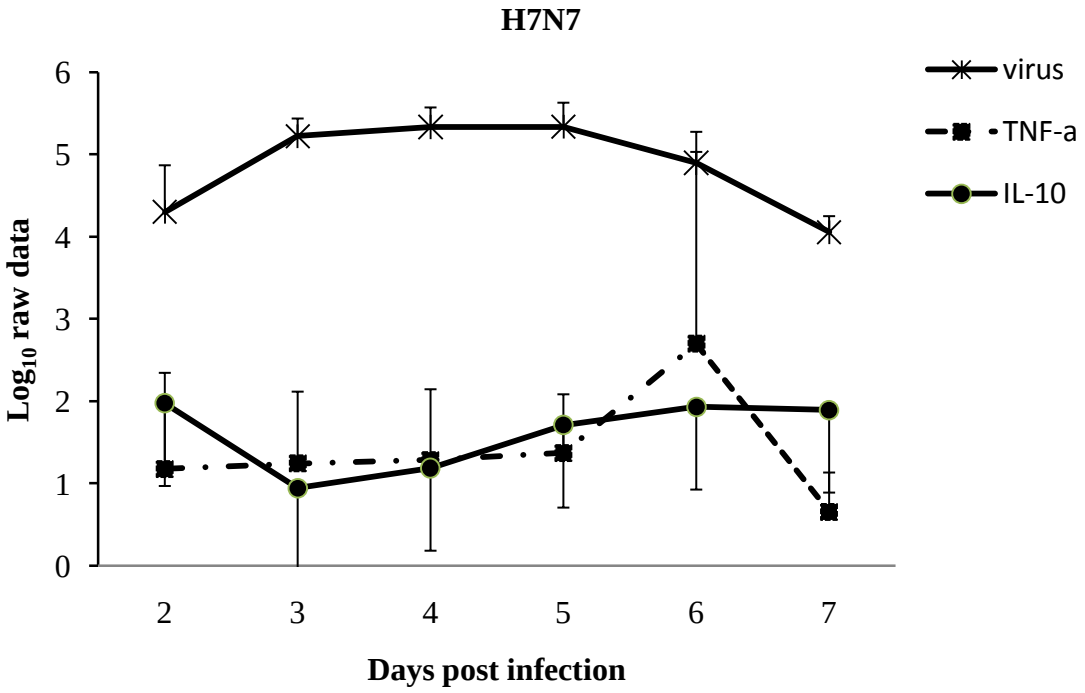


Figure 3.4 Correlation of virus replication and the expression of cytokines in the H7N7 mice. Mice were inoculated intranasally with 10^6 EID50 units of the H7N7 equine influenza virus. BALs of 5 mice per group were used to determine the levels of TNF-α and IL-10 by Cytometric Bead Array as described in the methods. Virus titers in the BALs were quantified in 10-day-old eggs by EID50 assay. All values were log10-transformed and presented as mean ± SEM. Virus titer (log_{10} EID50/ml). Cytokine level (log_{10} pg/ml). Correlation analysis was performed by Spearman Rank Order Correlation. Virus replication/TNF-α (correlation coefficient=0.49, $p<0.05$). Virus replication/IL-10 (correlation coefficient=0.5, $p<0.05$).

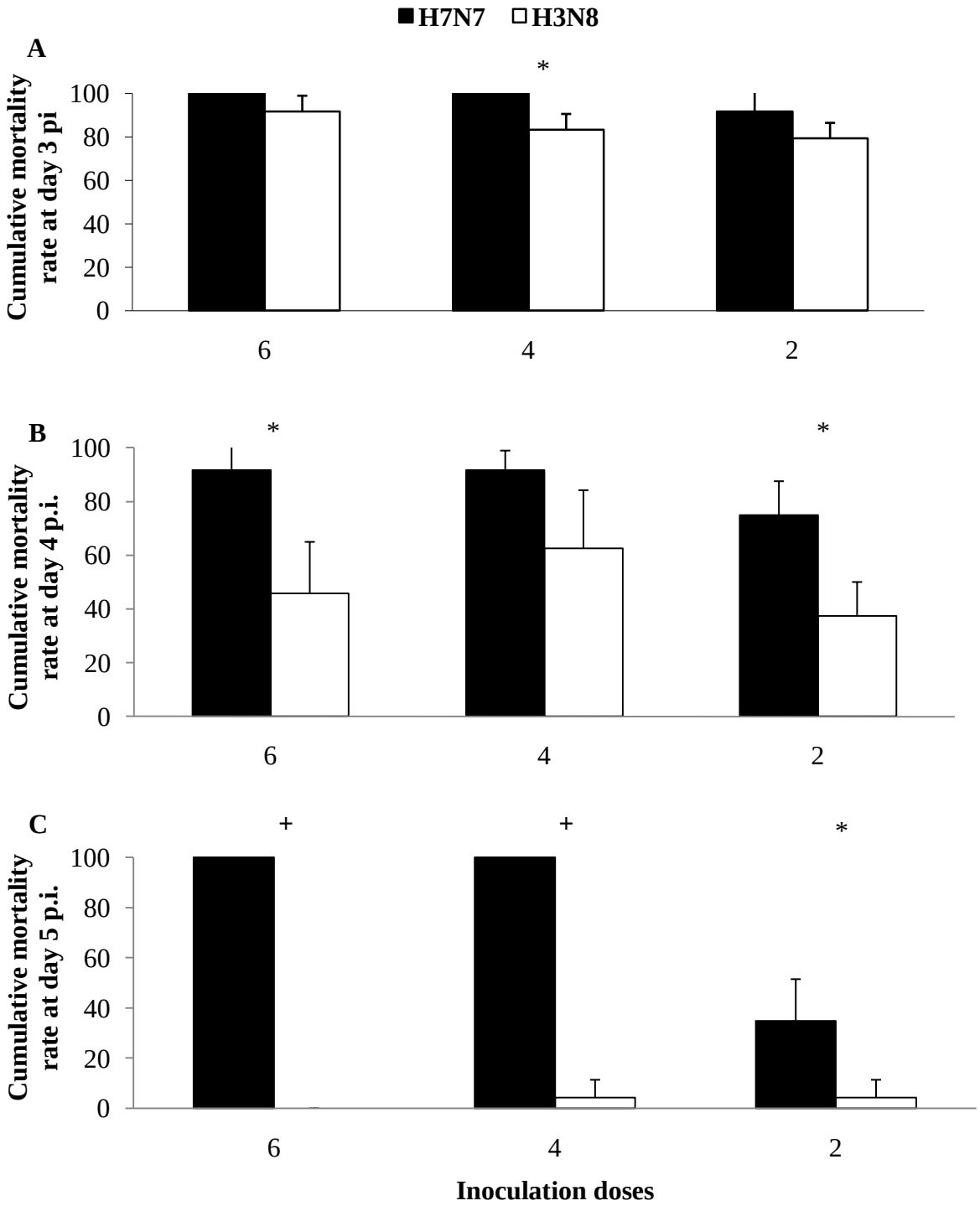


Figure 3.5 Cumulative mortality rates in different-aged chicken embryos. 7 (A), 9 (B) and 11-day-old (C) eggs were inoculated with either the H7N7 or H3N8 equine influenza virus at three different $\log_{10}$ virus doses. Cumulative mortality rates at indicated days post infection were recorded. Experiment was repeated three times, and data were expressed as mean ± SD. * indicates $P< 0.05$, + indicates $P< 0.001$

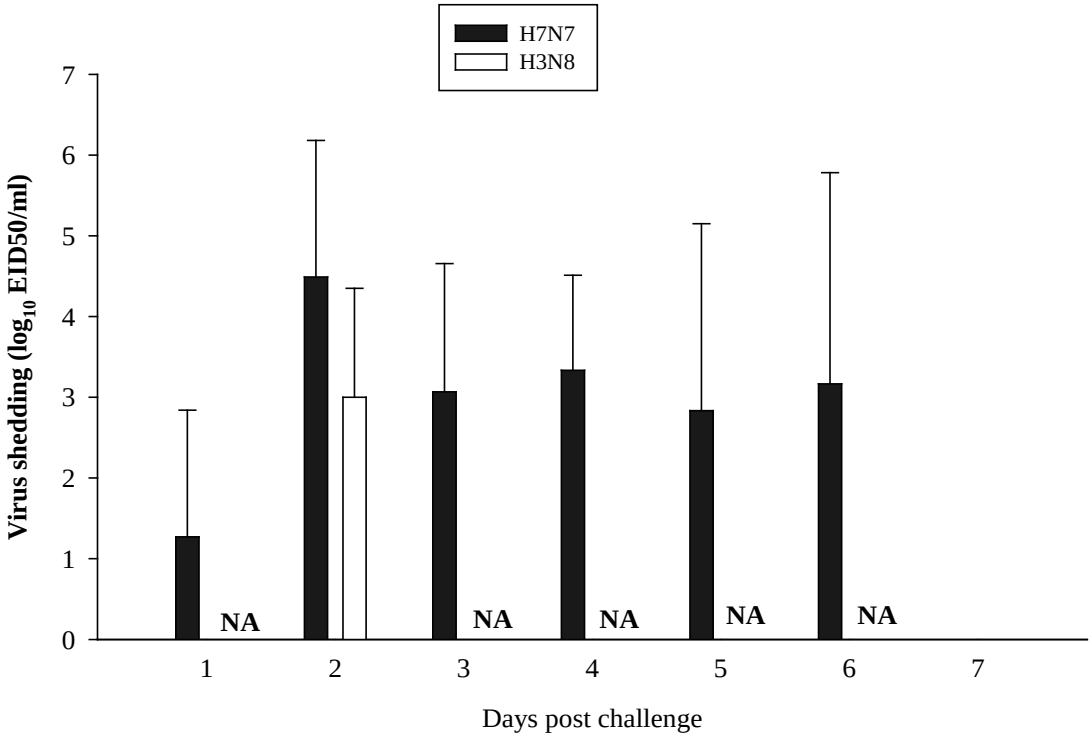


Figure 3.6 Viral shedding in horses challenged with equine influenza virus. 6 horses were challenged with the H7N7 virus, and 4 horses were challenged with the H3N8 virus. Nasal swabs were collected daily post challenge. Virus isolation was performed in 10-day-old chicken eggs. Results are expressed as Mean ± SD. (virus shedding of day 1 and from day 3 to day 6 for H3N8 group were not determined).

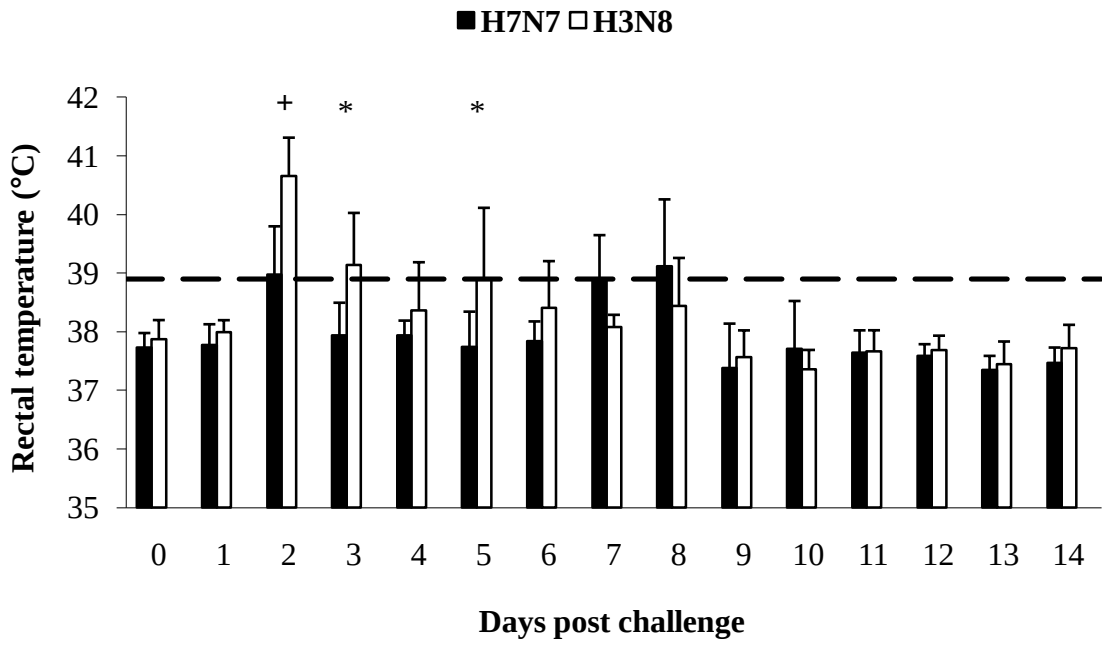


Figure 3.7 Daily rectal temperatures in horses. Mean ± SD. Temperature > 38.9 ^{0}C was regarded as pyrexia. At days 7 and 8 p.i. four horses from the H7N7 group had increased rectal temperature as the consequence of hyperventilation caused by the reaction to the DORMOSEDAN® (detomidine hydrochloride). Therefore, statistical analysis was not applied for both groups since day 7 p.i. onwards. * indicates $P< 0.05$, + indicates $P< 0.001$

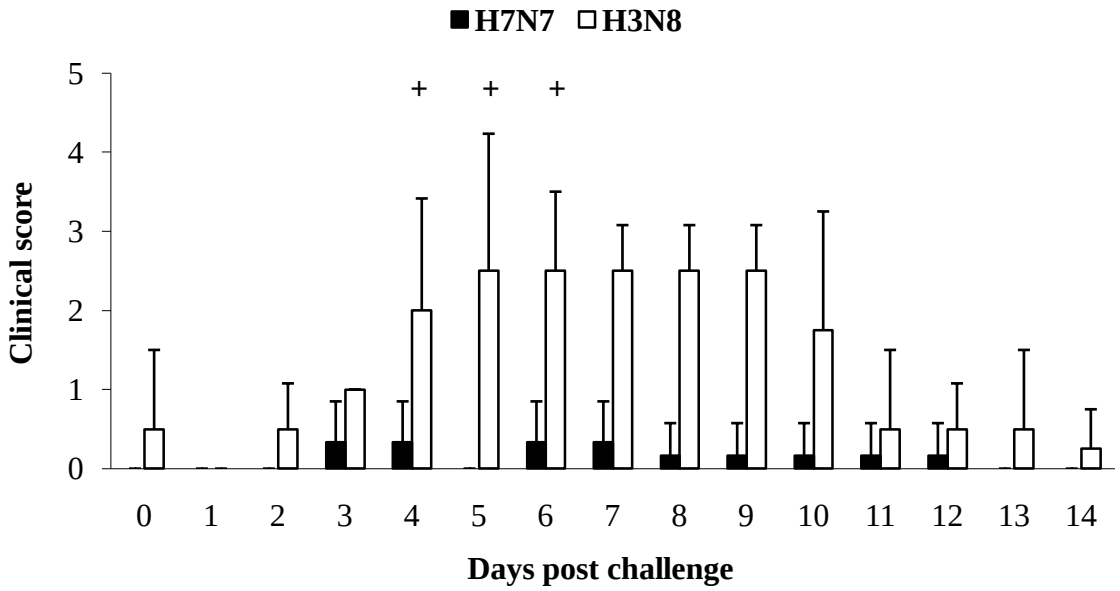
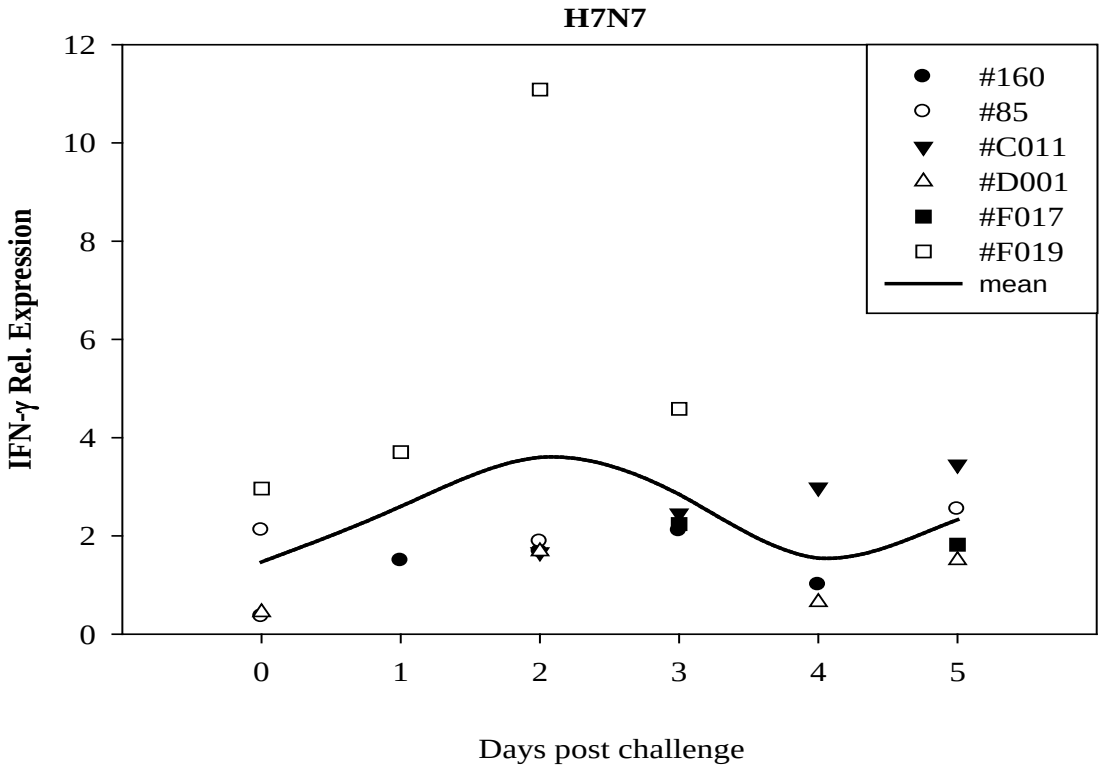
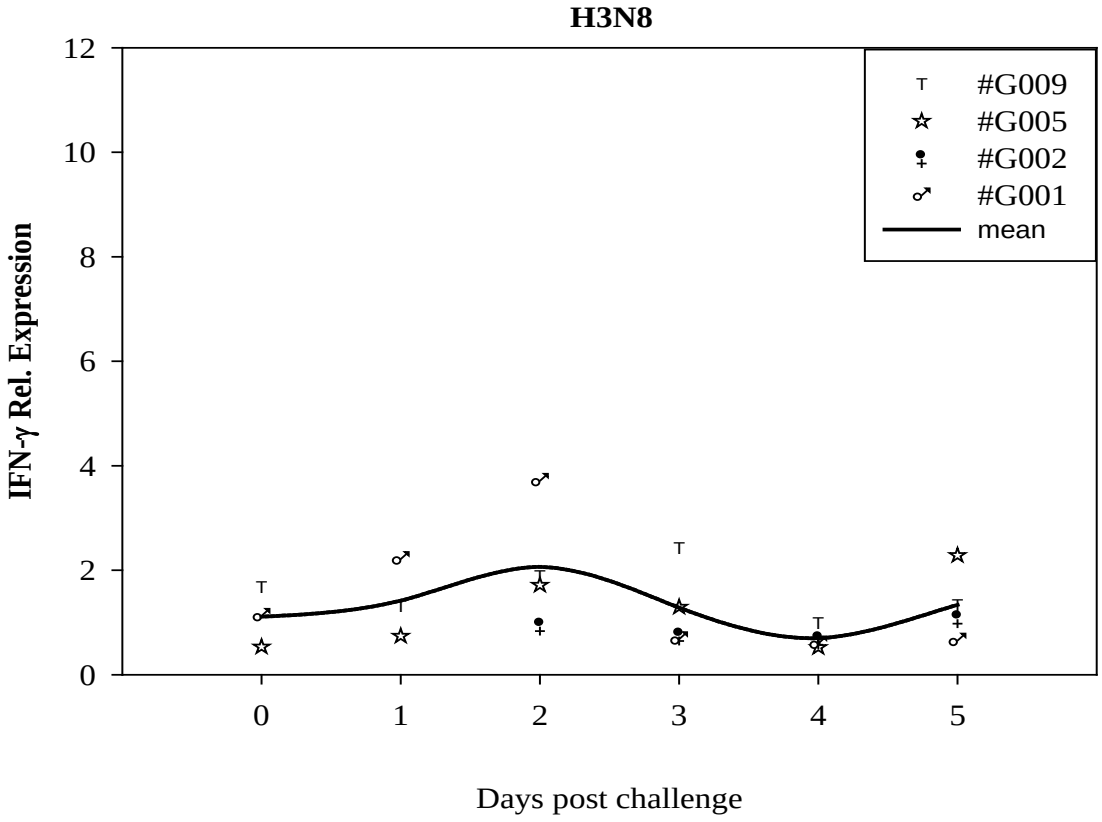
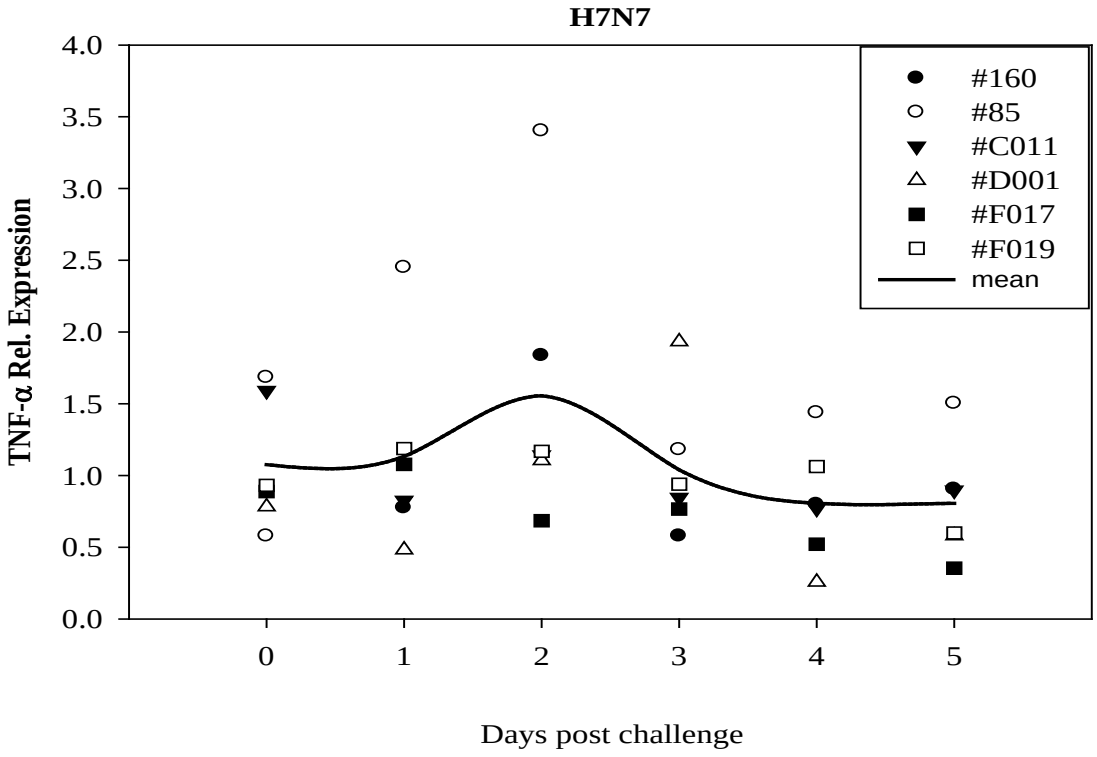
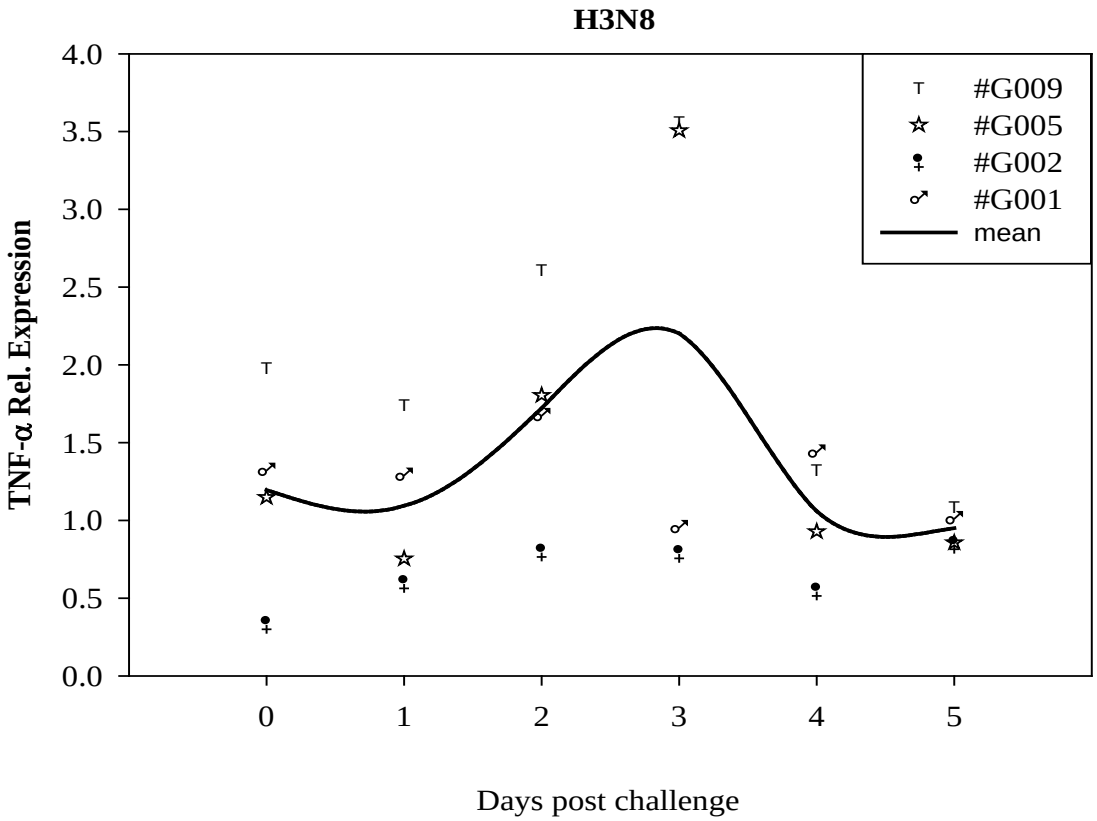


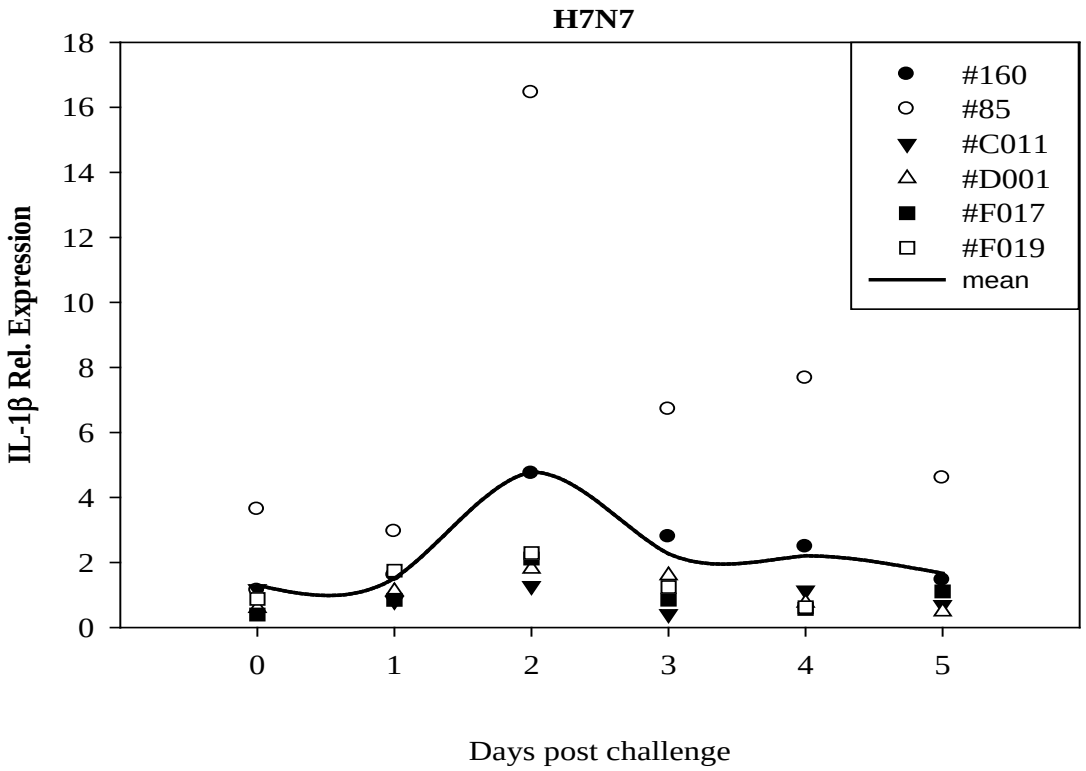
Figure 3.8 Mean clinical scores in horses challenged with equine influenza virus. Each horse was examined daily for clinical signs associated with equine influenza, including coughing, nasal discharge, depression and anorexia.Severity of clinical symptoms was assessed according to a clinical scoring system (Table 2.2). Total clinical scores were then calculated for each animal. Values were expressed as Mean ± SD. Statistical analysis was not run for days 7-14. + indicates $P< 0.001$


H7N7
IFN-γ Rel. Expression
Days post challenge
#160
#85
#C011
#D001
#F017
#F019
mean
0
2
4
6
8
10
12
0
1
2
3
4
5


H3N8
IFN-γ Rel. Expression
Days post challenge
#G009
#G005
#G002
#G001
mean
0
2
4
6
8
10
12
0
1
2
3
4
5


H7N7
TNF-α Rel. Expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Days post challenge
#160
#85
#C011
#D001
#F017
#F019
mean


H3N8
TNF-α Rel. Expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Days post challenge
#G009
#G005
#G002
#G001
mean


H7N7
IL-1β Rel. Expression
Days post challenge
#160
#85
#C011
#D001
#F017
#F019
mean
0
2
4
6
8
10
12
14
16
18
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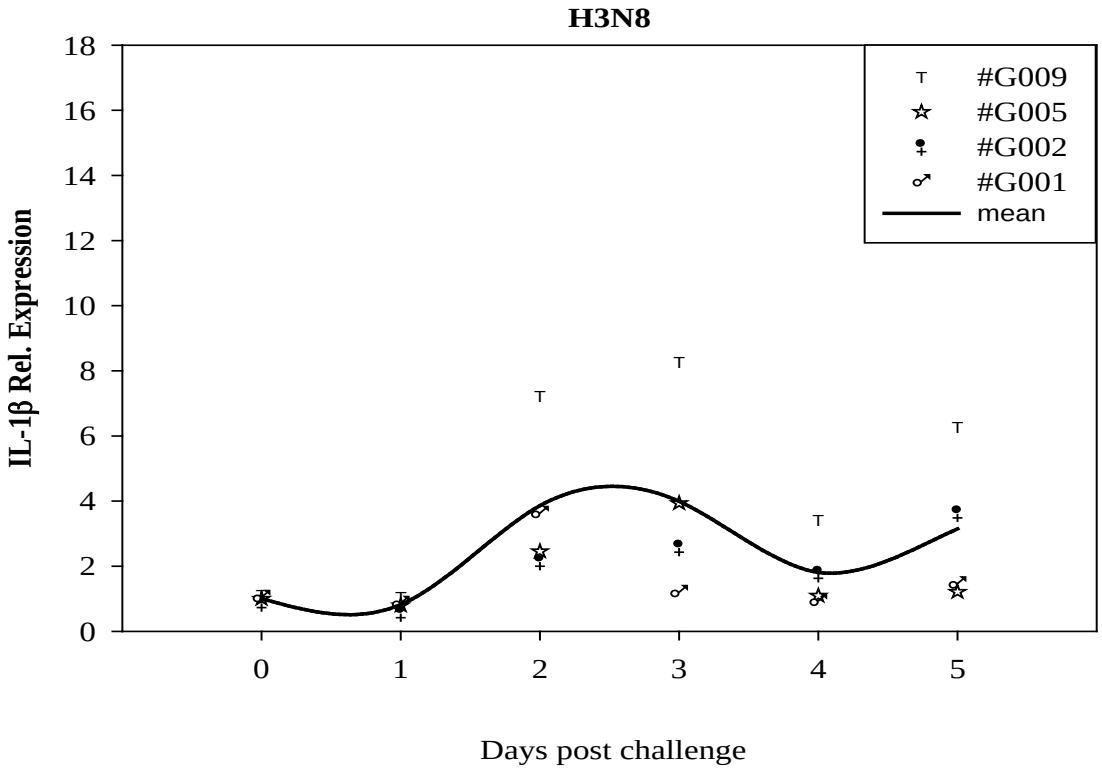
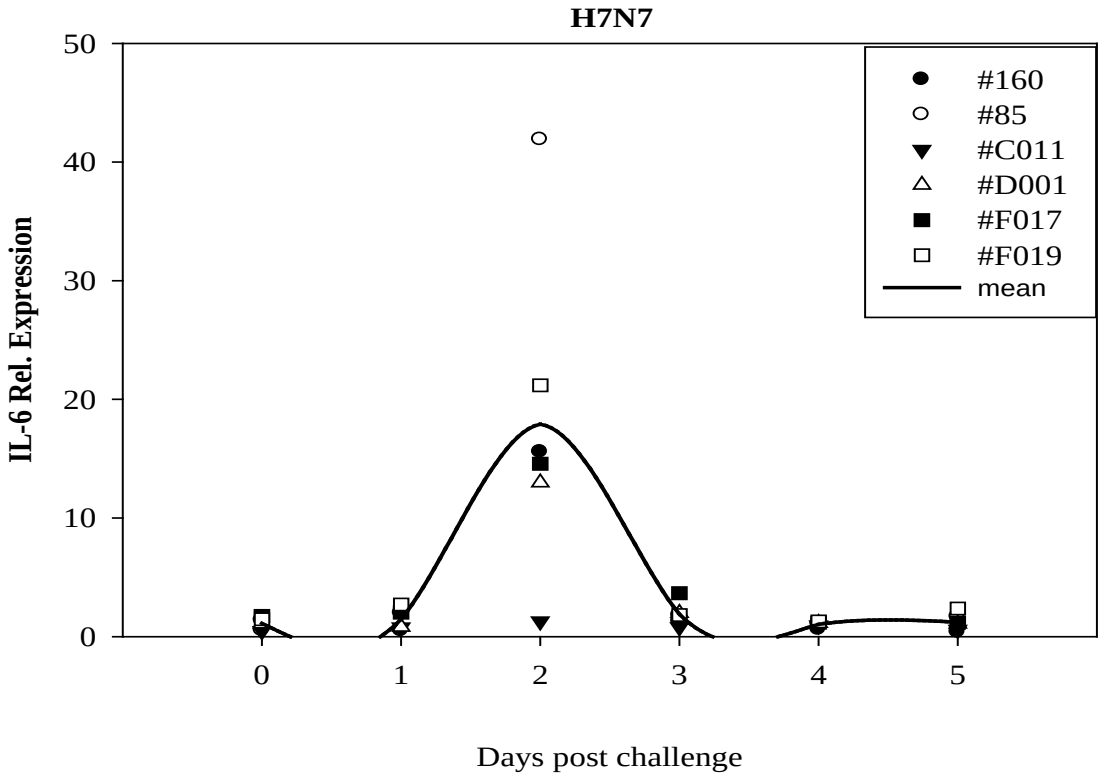
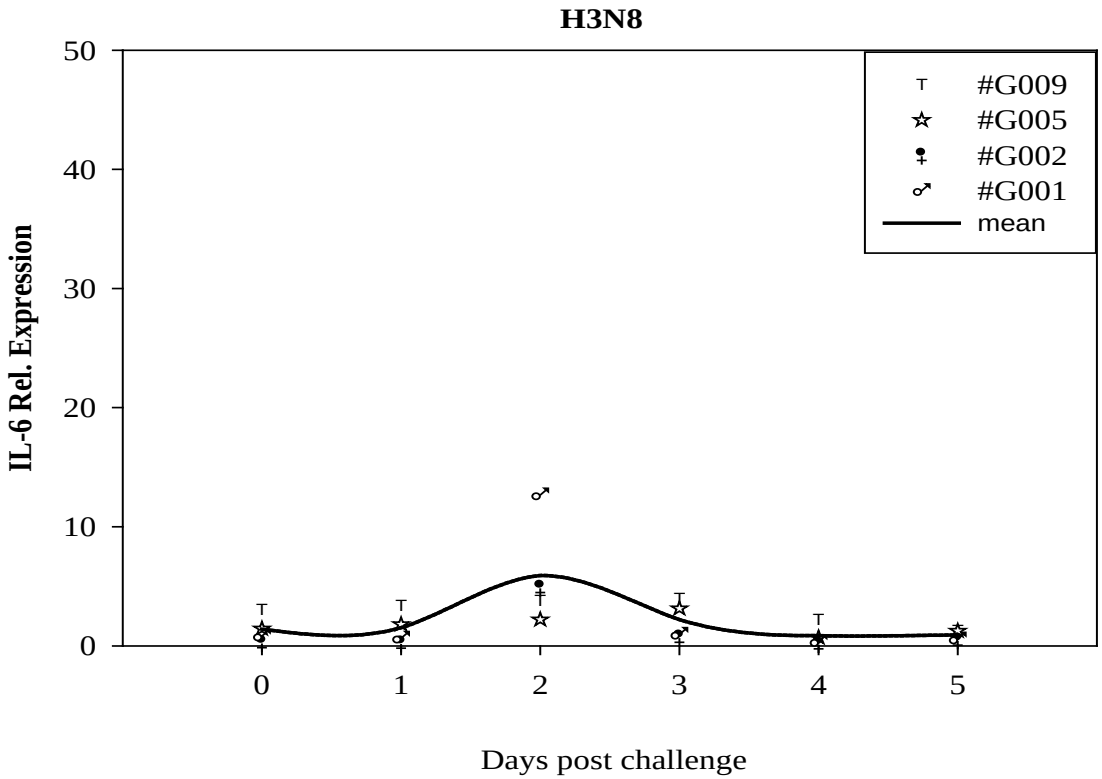
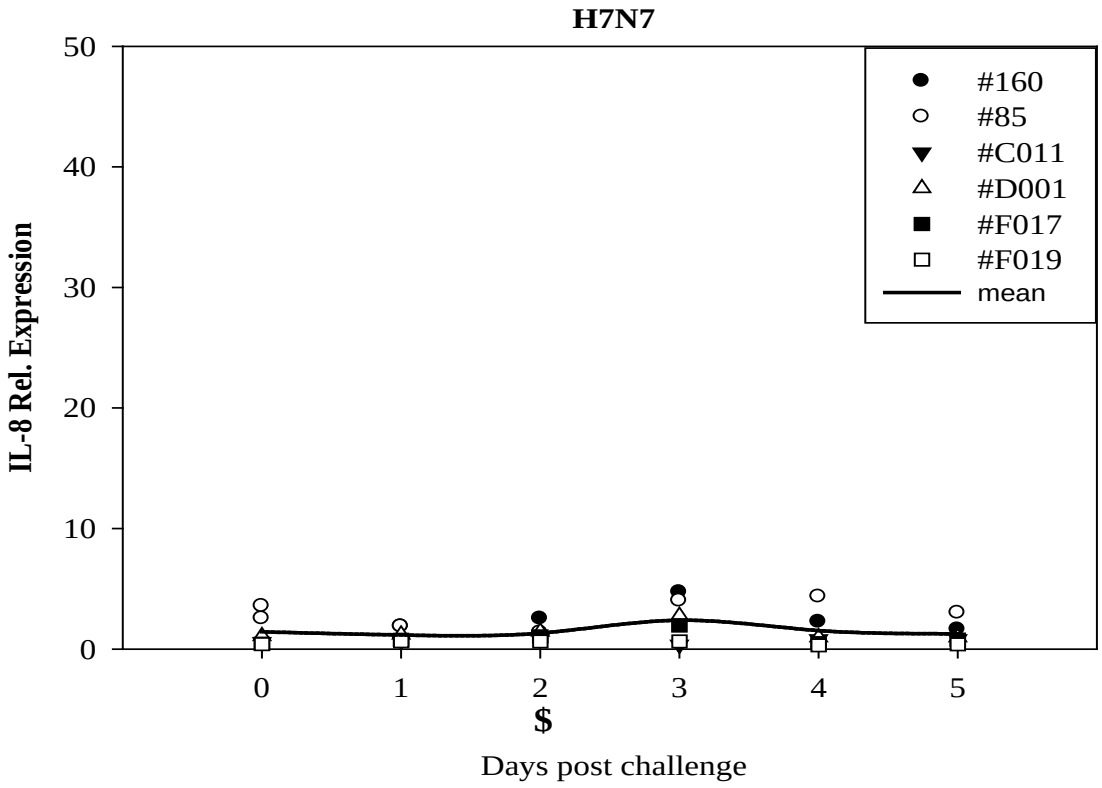
4
5


H3N8
IL-1β Rel. Expression
Days post challenge
#G009
#G005
#G002
#G001
mean
0
2
4
6
8
10
12
14
16
18
0
1
2
3
4
5


H7N7
IL-6 Rel. Expression
50
40
30
20
10
0
0
1
2
3
4
5
Days post challenge
#160
#85
#C011
#D001
#F017
#F019
mean


H3N8
IL-6 Rel. Expression
50
40
30
20
10
0
0
1
2
3
4
5
Days post challenge
#G009
#G005
#G002
#G001
mean


H7N7
IL-8 Rel. Expression
50
40
30
20
10
0
0
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2
3
4
5
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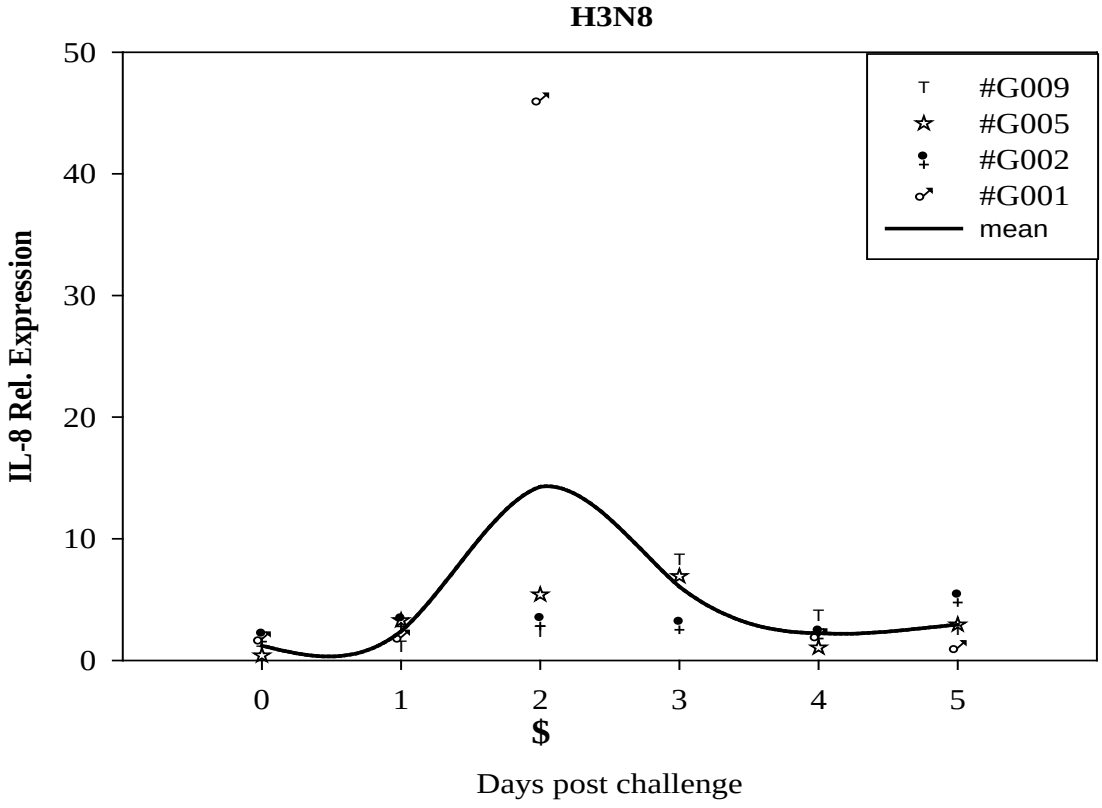
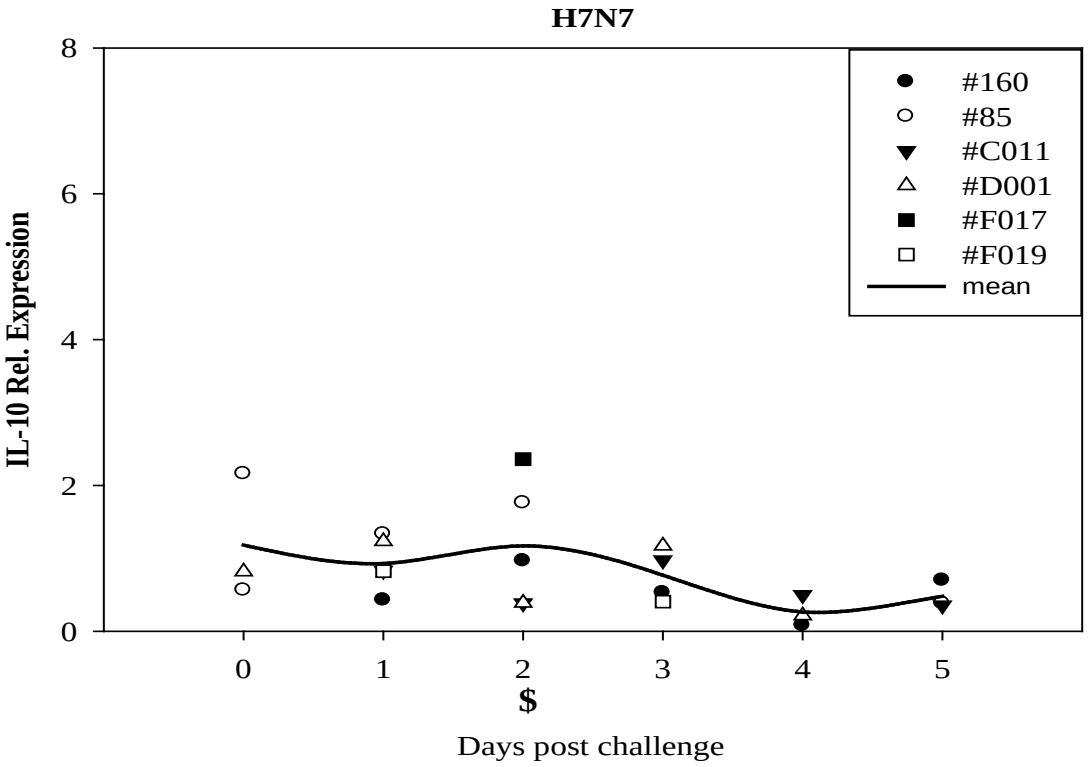
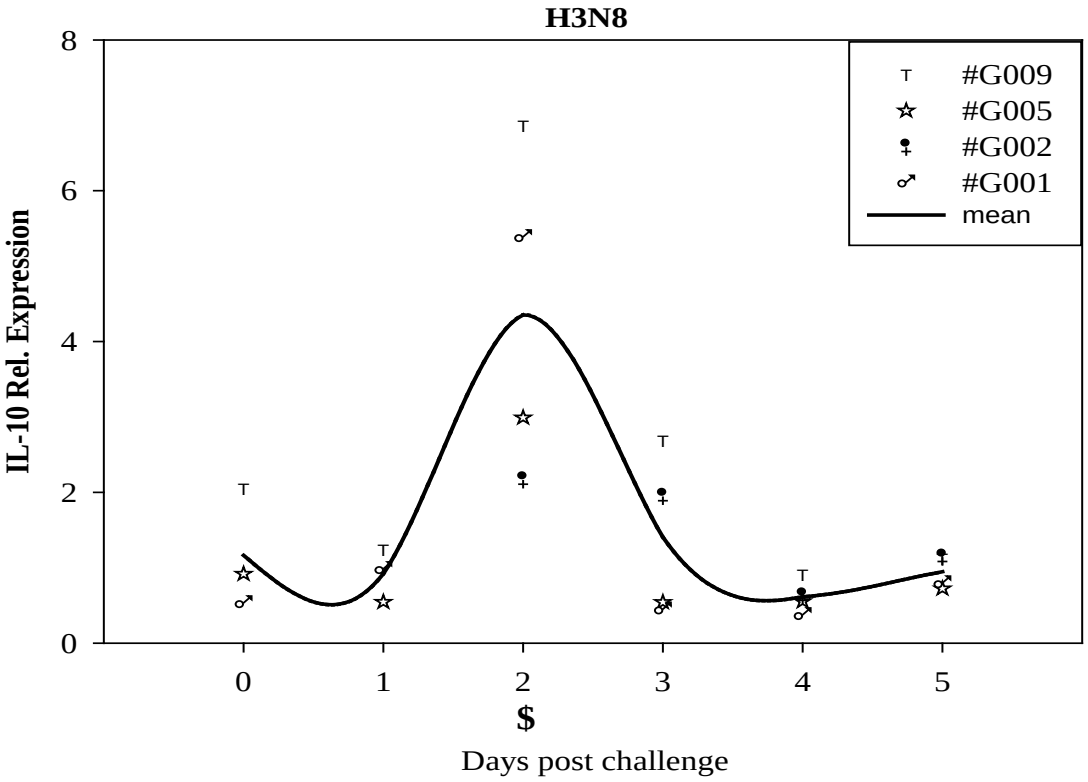
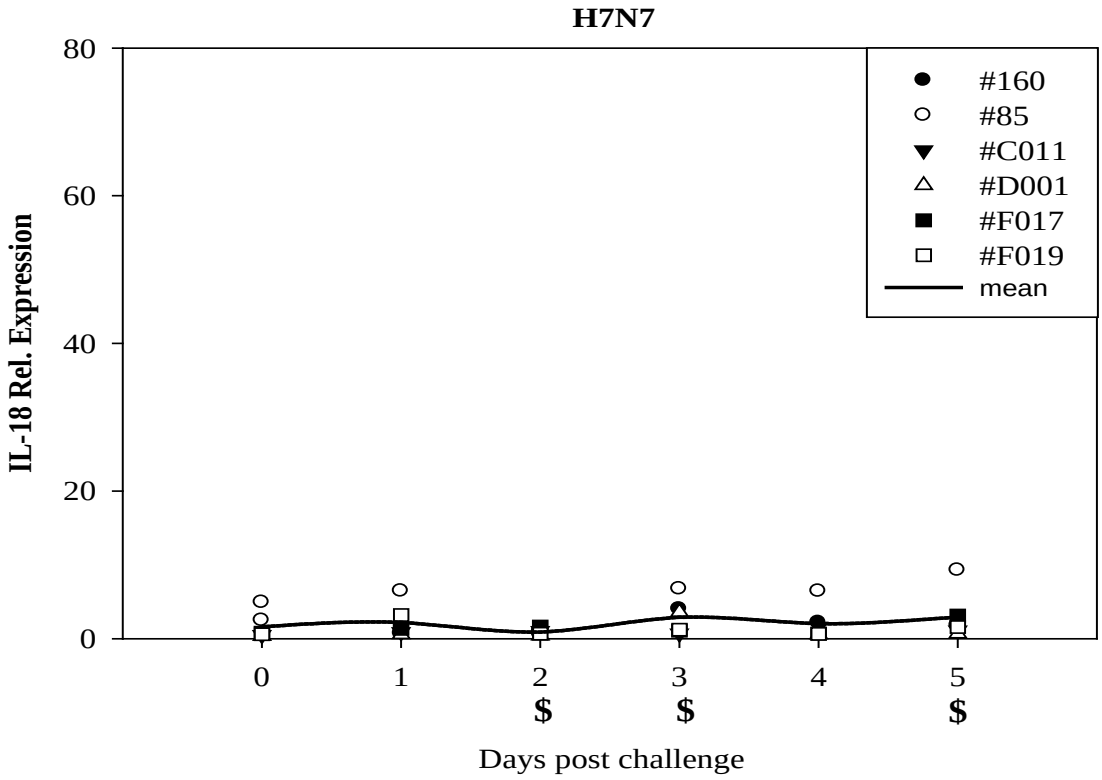
Days post challenge
#160
#85
#C011
#D001
#F017
#F019
mean


H3N8
IL-8 Rel. Expression
50
40
30
20
10
0
0
1
2
3
4
5
$
Days post challenge
#G009
#G005
#G002
#G001
mean


H7N7
IL-10 Rel. Expression
8
6
4
2
0
0
1
2
3
4
5
$
Days post challenge
#160
#85
#C011
#D001
#F017
#F019
mean


H3N8
IL-10 Rel. Expression
8
6
4
2
0
0
1
2
3
4
5
$
Days post challenge
#G009
#G005
#G002
#G001
mean


H7N7
80
60
40
20
0
IL-18 Rel. Expression
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#85
#C011
#D001
#F017
#F019
mean
0
1
2
3
4
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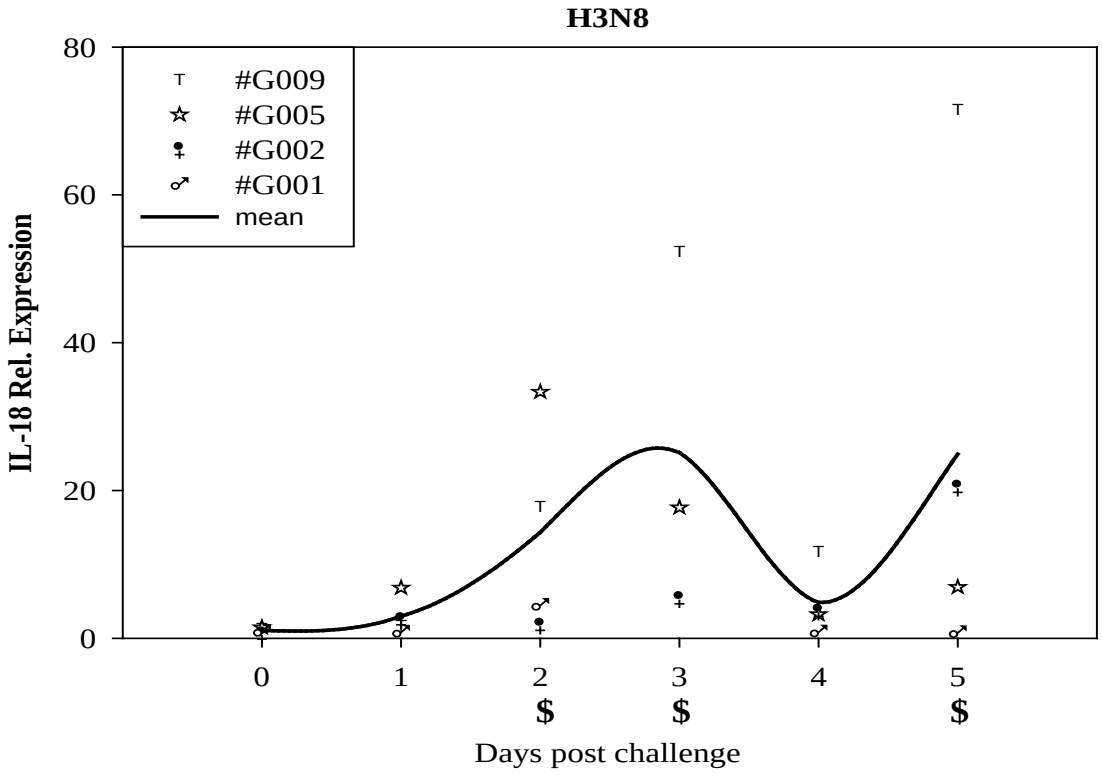
$
$
Days post challenge


H3N8
80
60
40
20
0
IL-18 Rel. Expression
#G009
#G005
#G002
#G001
mean
0
1
2
3
4
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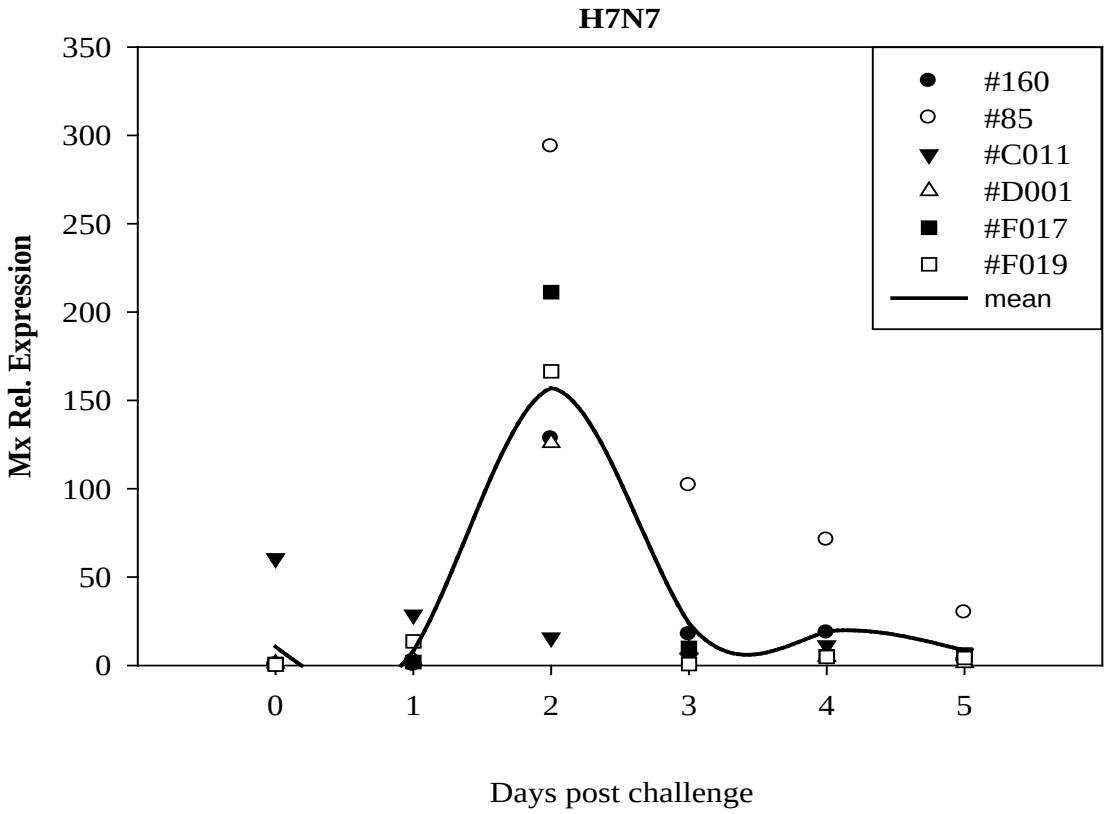
Days post challenge


H7N7
Mx Rel. Expression
350
300
250
200
150
100
50
0
0
1
2
3
4
5
#160
#85
#C011
#D001
#F017
#F019
mean
Days post challenge

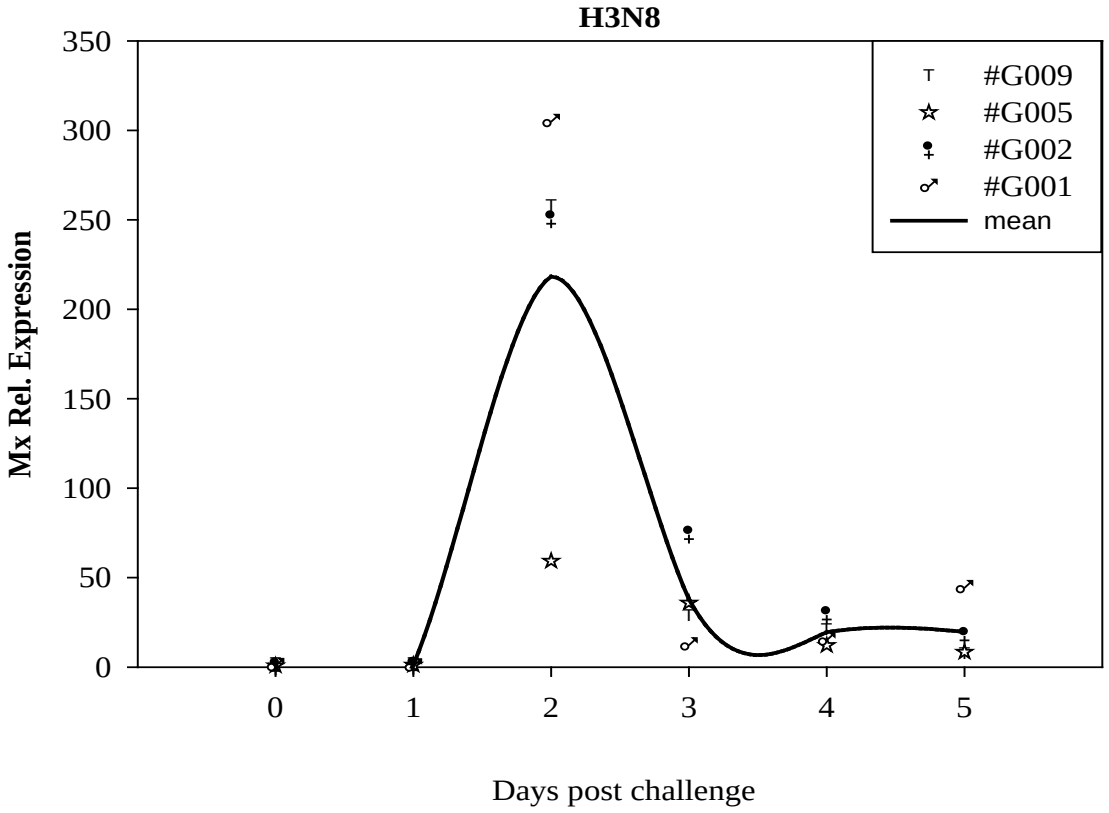

H3N8
Mx Rel. Expression
350
300
250
200
150
100
50
0
0
1
2
3
4
5
#G009
#G005
#G002
#G001
mean
Days post challenge

Figure 3.9 Relative mRNA expressions of cytokine and anti-viral Mx genes in peripheral blood cells. 6 horses were challenged with the H7N7 virus, and 4 horses were challenged with the H3N8 virus. Peripheral blood was collected and processed for RNA isolation and cDNA synthesis. Relative quantification of the respective mRNA transcripts to the calibrator was performed by real time PCR. Samples marked as the outliers for PCR efficiency and/or correlation coefficient by LinReg program were excluded from further statistical analysis. $ indicates that significant difference in the respective cytokine was observed between the two viruses on that time point ($p< 0.05$).

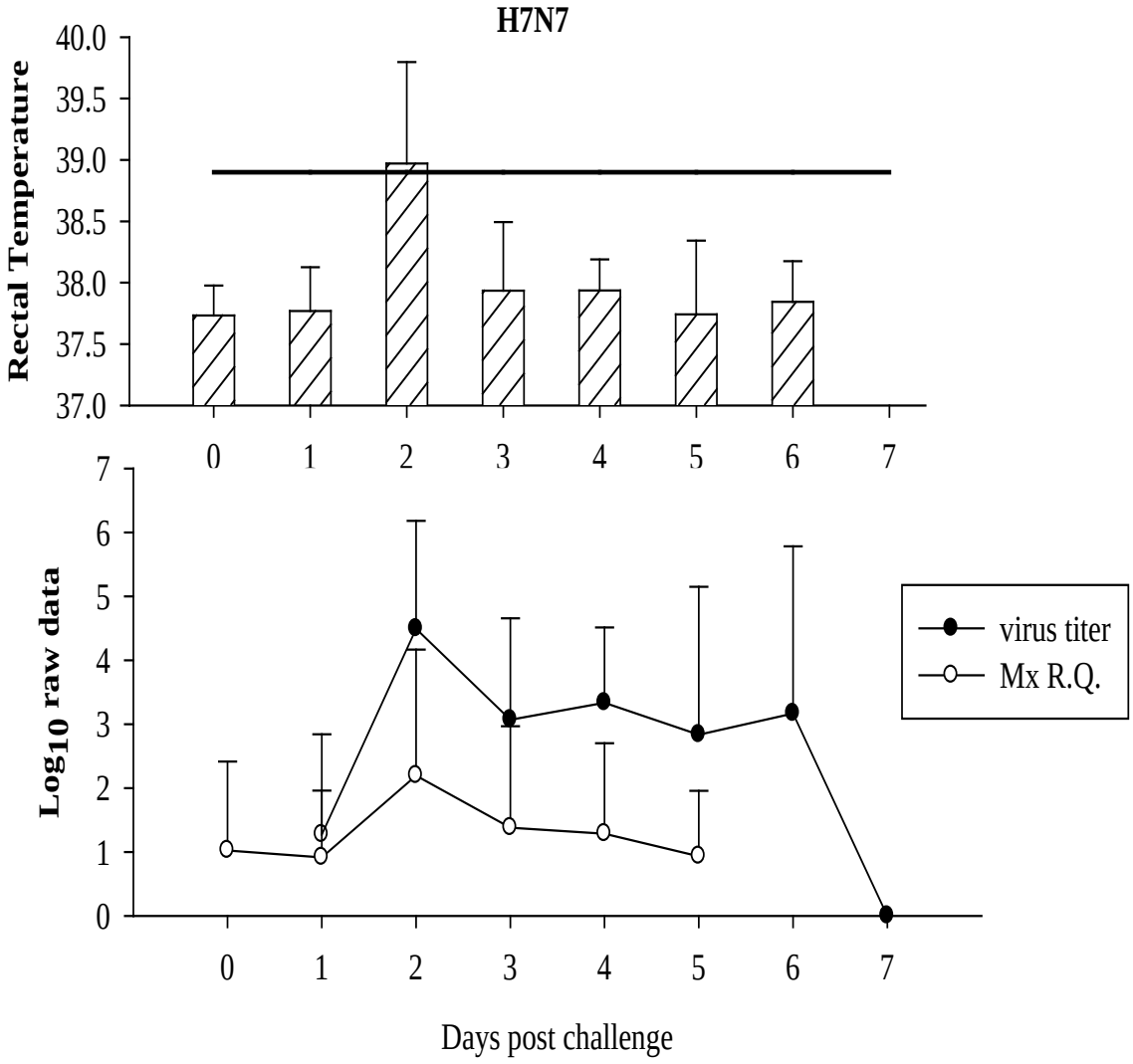


Figure 3.10 Correlation of virus replication, febrile response and relative quantification of Mx mRNA in the blood in horses following the H7N7 virus challenge. Horse experimental infection with the H7N7 virus, quantification of virus shedding and real-time PCR analysis of Mx gene transcription were carried out as described in the methods. Values of virus titers and Mx gene mRNA levels were log10-transformed. All data was presented as mean ± SD. Temperature > 38.9 ^{0}C was regarded as pyrexia.

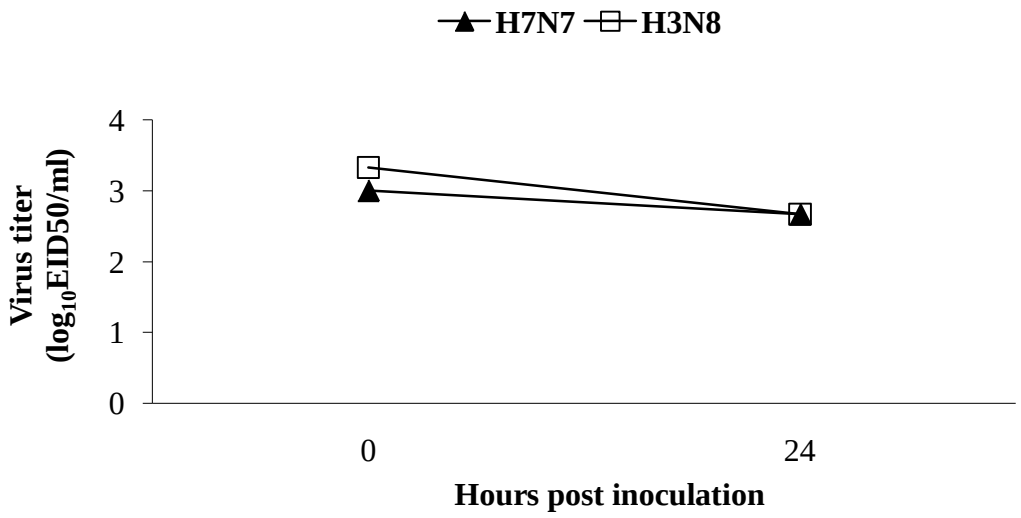
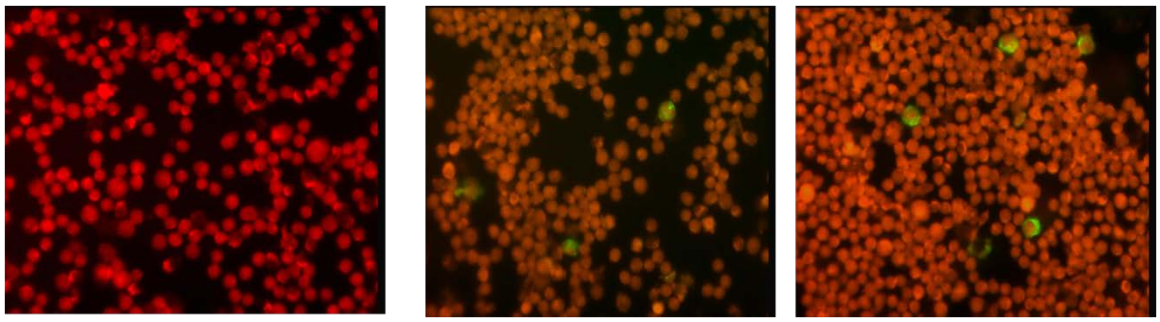


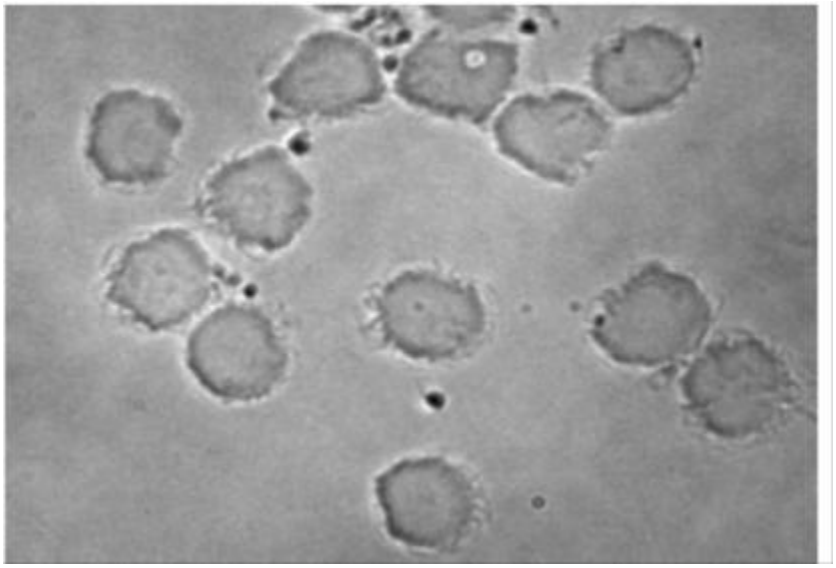
Figure 3.11 Nonproductive replication of equine influenza virus in primary equine PBMCs. Freshly isolated, unstimulated equine PBMCs were inoculated with either the H7N7 or H3N8 virus at a multiplicity of infection of 5 EID50 units per cell. At 0 and 24 hours post inoculation, cell supernatants were collected for infectious virus titration on 10-day-old chicken eggs. The results were from the same horse blood donor and are representative of three different horses with similar results.



Mock H7N7 H3N8

Figure 3.12 Detection of viral nucleoprotein (NP) in equine PBMCs by immunofluorescence microscopy. Fresh, unstimulated equine PBMCs were inoculated with either the H7N7 or H3N8 virus at a multiplicity of 5 EID50 units per cell. At 12 h.p.i. cells were collected and stained for viral NP protein (green) with Evan's blue (red) as a counterstain for cytoplasm. Magnification 40 ×.

A



B

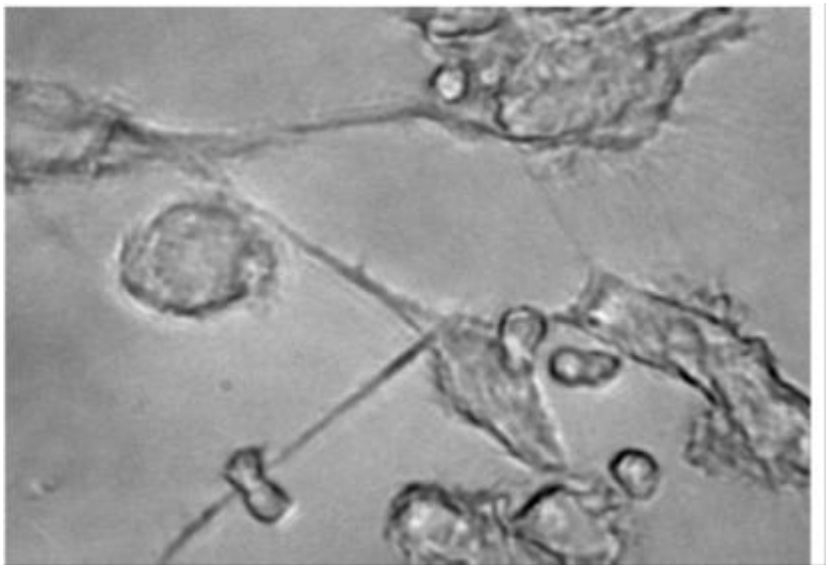


Figure 3.13 Morphology of monocytes and differentiated macrophages. Phase-contrast light microscopy. Magnification 100 ×. (A) day 0 culture, homogeneous population of small round monocytes. (B) day 7 culture, heterogeneous larger cells with cytoplasmic projections.

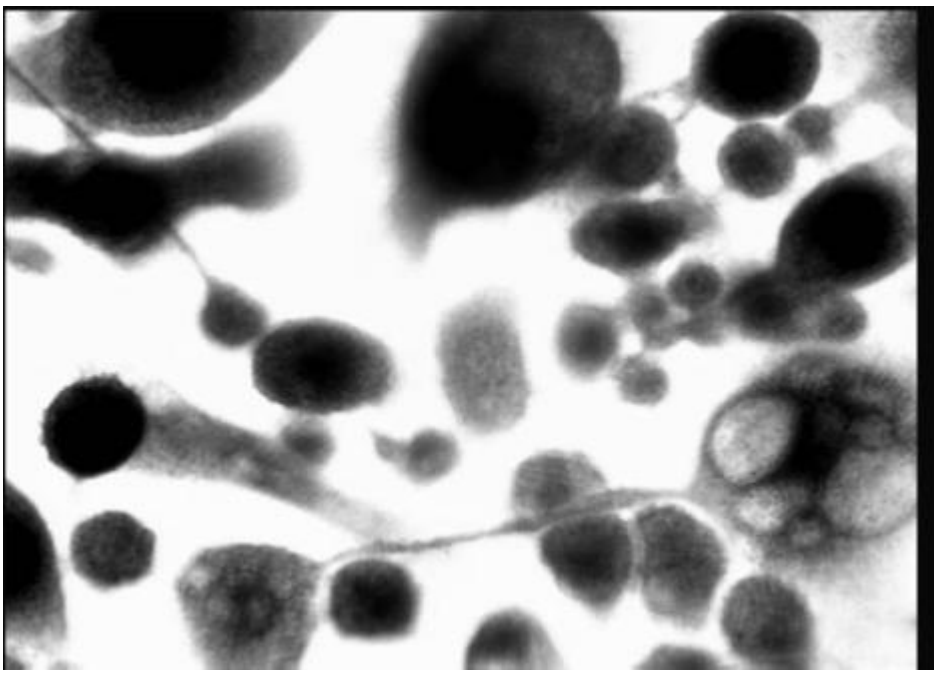


Figure 3.14 Cytologic demonstration of non-specific esterase activity in 7-day cultured macrophges. Cells were processed for cytochemical staining of α-naphthyl-acetate esterase, which is present in monocyte/macrophage lineage. Esterase-positive macrophages were identified due to their black granulation. Magnification 40 ×

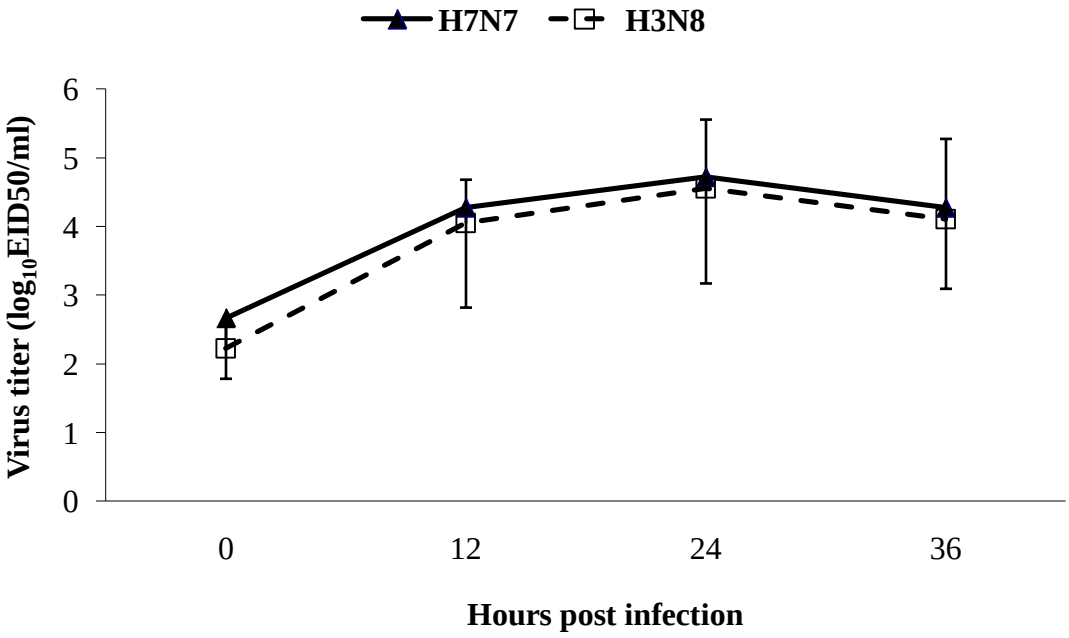
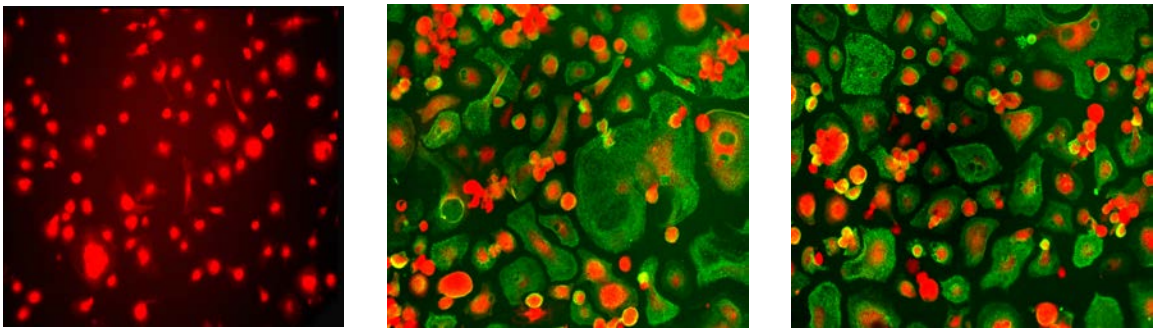


Figure 3.15 Productive replication of equine influenza virus in 7-day-cultured macrophages. Cells were infected with either the H7N7 or H3N8 virus at a multiplicity of infection of 5 EID50 units per cell. Titration of infectious virus was performed on 10-day-old chicken eggs. Mean ± SD of blood from 3 different horses.



Mock H7N7 H3N8

Figure 3.16 Detection of viral nucleoprotein (NP) in macrophages by immunofluorescence microscopy. 7-day-cultured macrophages were infected with either the H7N7 or H3N8 virus at a multiplicity of 5 EID50 units per cell. At 12 h.p.i. cells were collected and stained for viral NP protein (green) with Evan's blue (red) as a counterstain for cytoplasm. Magnification 40 ×.

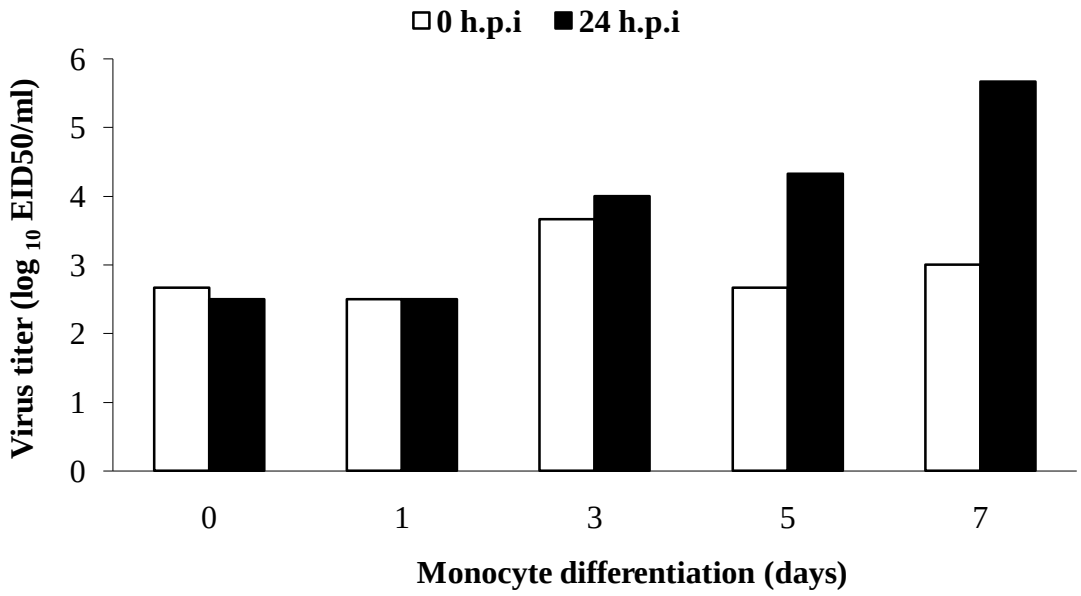


Figure 3.17 Replication of equine influenza virus in different-aged monocyte-derived macrophages. Day-0, 1, 3, 5, 7 cultured cells from the same horse were infected with the H7N7 virus at a multiplicity of infection of 5 EID50 units per cell. Cell supernatants were collected at 0 and 24 hours p.i. for virus titration on 10-day-old embryonated chicken eggs.

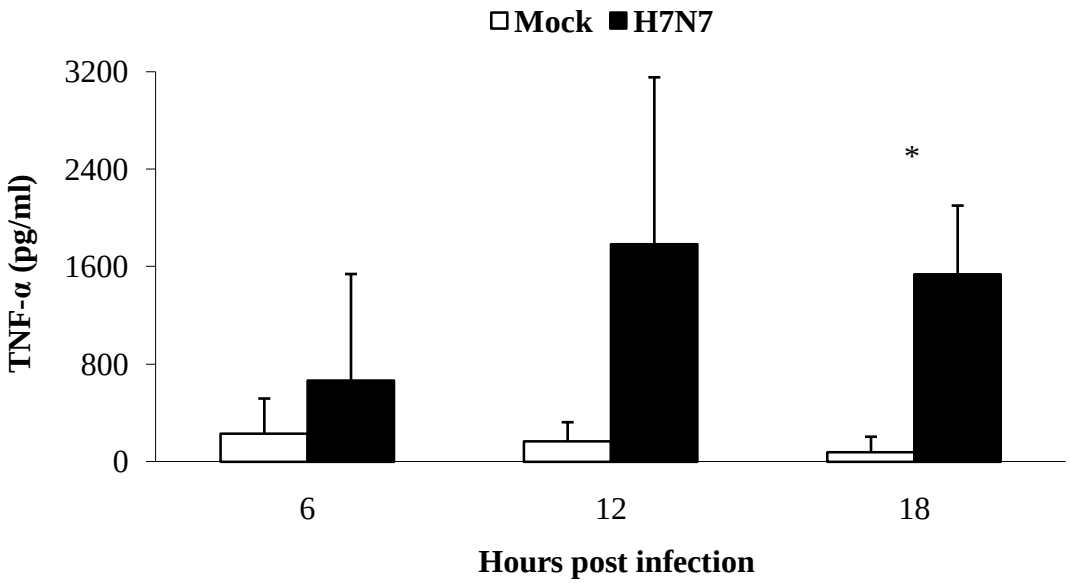


Figure 3.18 TNF-α production by equine primary macrophages in response to influenza virus infection. 7-day-cultured peripheral monocyte-derived macrophages were either mock infected or infected with the H7N7 virus. Cell supernatants were collected at indicated times for TNF-α protein quantification by ELISA. The results are the means ± SD of cells from three different horses. * indicates $P<0.05$

CHAPTER FOUR

Discussion

The primary interest of this dissertation was to decipher the determinant(s) for the differential pathogenicity of the equine H7N7 virus in its natural host and the BALB/c mouse model, and to address whether inflammatory cytokine responses are involved in this characteristic of this virus. A prerequisite of influenza virus infection of airway epithelial cells is the interaction between the HA protein and the corresponding cell surface receptor containing a terminal sialic acid residue (sialyoligosaccharides) [185]. These residues are bound to glycans through α 2,3 or α 2,6 linkage that are distributed in a cell- and species-specific manner [186]. For example, human tracheal epithelial cells present mostly α 2,6 linkage [187], while equine and mouse respiratory tracts express predominantly α 2,3 linkage [188-189]. Influenza viruses differ in their preference for recognition of sialic acid-galactose linkages. Studies show that the avian and equine influenza strains bind preferentially the α 2,3 linkage [188, 190], while the human viruses favor the α 2,6 linkage [190]. Surprisingly, human influenza viruses can still efficiently infect knock-out mice that lack α 2,6 linkages in the respiratory tract [191]. Taken all together, mouse airway epithelial cells are potentially susceptible to all strains of influenza A virus.

Tissue tropism of the two viruses in BALB/c mice

In concert with this, the current mouse challenge study showed that both equine H7N7 and H3N8 viruses exhibited a high degree of replication fitness ($>10^{3.5}$ EID50/ml at day 2 p.i.) in the lungs of mice without prior adaptation after intranasal inoculation, but produced contradictory clinical outcomes. This observation is in good agreement with a

previous study [167]. The equine H3N8 virus infection in BALB/c mice was asymptomatic and the virus was isolated almost exclusively from the lungs among various organs tested. In contrast, the equine H7N7 virus was isolated from the brains as well as the lungs of infected mice. This demonstration is clearly distinguishable from a previous study [167] in which a different isolate of equine H7N7 influenza virus was recovered from the brain on rare occasions. However, this is not surprising, considering that the heterogeneity of organ tropism has also been documented for various isolates of avian H5N1 virus as well as H7N7 virus [97, 107, 109].

Neurotropism has also been observed in mice intranasally infected with some isolates of highly pathogenic avian H5 and H7 influenza viruses [106, 171, 192]. These viruses reached the brain along the vagus and/or trigeminal nerves following intranasal instillation [171, 192]. In addition, a mouse-adapted equine H7N7 isolate was shown to invade the brain by a similar route [193]. The proteolytic cleavage of the HA protein is a prerequisite for virus infectivity and spread, and hence is a primary determinant for the pathogenicity of influenza viruses [34]. The HA cleavage sites of avian H5 and H7 highly pathogenic viruses contain multibasic amino acids, which are therefore theoretically susceptible to cleavage by ubiquitous host subtilisin-like proteases, resulting in systemic infection in the host. In contrast, avirulent strains with monobasic cleavage sites can only be activated by proteases such as trypsin and plasmin, which are found specifically in cells from the respiratory or intestinal tracts, or both, and thus cause non-systemic infections [31, 194]. The present study confirmed the presence of multiple basic residues at the cleavage site in the H7N7 virus used here, whereas the H3N8 virus lacks this property (Table 3.1).

Increasing evidence indicates, however, that the highly cleavable HA is necessary, but not sufficient, to confer these viruses with neurovirulence in mice. It is generally accepted that neurotropism in mice should be attributed to a constellation of genes although numerous studies point to an important role played by polymerase genes [110, 171, 195-196]. In particular, some amino acids at specific positions (e.g. the lysine residue at position of 627 in PB2) have been demonstrated to be critical in controlling viral polymerase activity. Therefore, this molecular feature is believed to provide a growth advantage to the virus and to be responsible for tissue tropism [197-199]. To this end, a survey of the amino acid composition at residue 627 in PB2 protein was carried out for two representative strains of the equine H7N7 subtype and the H3N8 virus used in this dissertation. As a result of the comparison, a glutamate rather than lysine residue was found to be present at this specific position in PB2 protein of the equine H7N7 and H3N8 viruses examined here (Appendix D). It has been recognized that a single substitution of glutamate for lysine at position of 627 in PB2 is a critical determinant for neurotropism in mice [196, 200]. Moreover, as for PB2 protein sequence homology, each of the two H7N7 viruses shares 99% amino acid identity with the H3N8 virus. As a matter of fact, the H7N7 viruses had acquired the internal gene segments (i.e. NP, NS, PA, PB1 and PB2) from the H3N8 viruses as the consequence of genetic reassortment between the two subtype viruses while they co-circulated circa 1970 [18, 20-21]. Taken together, the findings from this study suggest that multiple factors are involved in the neurovirulence of the equine H7N7 virus in BALB/c mice, and that PB2 is not a critical determinant.

The current observation justifies an important role played by the internal genes of influenza virus for the mouse brain invasion since the two equine H7N7 virus strains used

in the present investigation and that previous study [167] are the same for the HA/NA serotype and HA cleavability. Neurotropism has been proposed to account for higher lethality in mice infected with highly pathogenic avian H5N1 influenza viruses [171, 201]. However, this may not be necessarily applied to equine H7N7 viruses. The two isolates of equine H7N7 viruses used in the current study and the previous one [167] induced 100% mortality rates despite having distinct organ tropisms. It is therefore concluded based on the present findings that invasion of the brain may not contribute significantly to the disease outcome of mice infected with equine H7N7 viruses.

Inflammatory responses induced by the viruses in BALB/c mice

The work reported here demonstrated indirectly that the two virus subtypes had differential capacities to affect the innate immunity of the BALB/c mouse. This distinction between the two viruses suggests that a comparable difference might be seen in the pulmonary cytokine expression levels since cytokines are known to be the integral mediators of the immune responses. To this end, quantification of temporal changes in cytokine abundances in a sequential manner during the course of infection was performed. The production levels of pulmonary cytokines and chemokine studied here in the H7N7-infected mice appear to reach maximum in the late phase of infection (on day 6 p.i.) (Figure 3.3). However, statistical evaluation didn't provide solid support for that trend. Nevertheless, a previous study showed that expression levels of pulmonary cytokines/chemokines peaked on day 5 after infection with the highly pathogenic avian H5N1 influenza virus in mice [111]. A great deal of variation in the absolute quantity of cytokine produced was noticed among individual mice of the H7N7 group, which resulted in the large standard deviations in Figure 3.3. Despite the high genetic homogeneity of inbred mice, mouse-to-mouse variation in response to stimulus or

treatment within the same strain has been commonly observed [202-204]. In this study, such substantial intersubject variation was unlikely to be due to the degree of virus replication (Figure 3.2), and instead may be attributed to the intrinsic interanimal variability in the immune response dynamics [202] to the virus or to considerable individual differences in the response of inbred mice to social stress arising among cage mates during group housing. Social and psychological stress are known to have a direct impact on immune responses to infection in humans as well as various animals [205]. And moreover, the small number of animals (n=5) employed here could make that mouse-to-mouse variation more visible [204].

Among the cytokines studied here, IL-6, as one of the principal proinflammatory cytokines in the pathogenesis of influenza [206], does not contribute significantly to the avian H5N1 virus lethal infection in mice [113, 207]. Another critical mediator in the inflammation, TNF-α, has been shown to have deleterious effect on the disease progression [113, 208]. Gene-knockout analysis targeting the TNF-α receptor in a mouse model provided the latest evidence that TNF-α signaling is more important in regulating the pathogenesis of the highly pathogenic influenza virus infection [209]. In fact, the present investigation showed that among several proinflammatory cytokines, only the TNF-α response correlated with the H7N7 virus replication in the mouse lung.

An array of chemotactic cytokines, such as monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein (MIP-1α/β) and IL-8, are secreted rapidly by respiratory epithelial cells and resident tissue macrophages underneath the infected epithelial layers during the acute phase response to primary influenza virus infection. These chemokines promote massive leukocyte trafficking from the circulation to sites of

inflammation, which are crucial requirements for the subsequent cell-mediated viral clearance [210]. MCP-1 is a predominant chemotactic factor responsible for the recruitment of macrophages and neutrophils, and blockade of MCP-1 function by antibody treatment dramatically reduced leukocyte influx into the lungs after influenza virus infection in mice, and consequently resulted in enhanced lethality [211]. However, the present findings showed that the expression of pulmonary MCP-1 was below the detection limit in almost all mice infected with the H7N7 virus before day 6 p.i. This might be due to the relatively high detection limit of the assay applied here (52.7 pg/ml for MCP-1). Nevertheless, a previous investigation has already demonstrated that the quantity of MCP-1 present in mouse lungs was directly proportional to the numbers of pulmonary cellular infiltrates following intranasal inoculation of mice with the highly pathogenic avian H5N1 influenza virus infection [212]. The increased presence of these immune cells in the mouse lung assists in limiting the virus replication [213]. Meanwhile, however, the excessive accumulation of infiltrated inflammatory immune cells has been demonstrated to contribute to pulmonary inflammatory pathology [213-214].

IFN-γ is a proinflammatory cytokine with well-characterized antiviral activity and many other functions involved in the adaptive immunity including augmentation of major histocompatibility complex (MHC) expression and stimulation of T and natural killer (NK) cells [206]. However, efficient viral clearance or humoral and cellular responses during primary influenza A virus infection in mice is not dependent on IFN-γ, and it rather acts as a typical proinflammatory mediator by regulating leukocyte recruitment into the inflamed site during the acute phage of infection [215-218]. Indeed, in this study the detectable expression of this cytokine was recorded in the lungs of most mice infected

with the H7N7 virus whereas the expression of IFN-γ was beyond the detection limit of the assay in all mice from the H3N8 group.

IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of TNF-α, IL-1, and IL-6 by monocytes and macrophages [219-220]. For the fatal avian H5N1 virus infection, high levels of IL-10 expression were detected in humans [95] and mice [221]. Consistent with previous studies, higher IL-10 protein concentrations in the H7N7 virus-infected mice compared to those in the H3N8 virus group were observed in the present study. As an endogenous antagonist to the pro-inflammatory cytokines [222], the production of IL-10 is believed to offer feedback inhibition in limiting uncontrolled inflammatory responses in mice with lethal avian H5N1 infection [221].

IL-12 is primarily produced by monocyte-macrophages and has a role in the early innate immune defense against primary influenza virus infection in BALB/c mice [166]. During influenza virus infection in mice, the level of its pulmonary production was reported to be low [166, 223]. Here, neither of the two viruses induced a detectable IL-12 expression in the lungs of mice at any time point tested in the present report.

Although an exaggerated cytokine response is widely believed to be one of major contributors to the severe pathogenesis of avian H5N1 influenza and 1918 pandemic influenza, debate continues as to whether the unusual severity of the disease associated with avian H5N1 viruses is the consequence of dysregulated immune response or instead is caused by the intrinsic pathogenicity of the virus itself [113, 207]. The later argument is exemplified by the extraordinary replication efficiency of a particular influenza A virus strain (generated by serial lung passages of A/PR/8/34 (H1N1) virus in Mx-competent mice), which has been shown to overrun the innate immune defense response and thereby

endow it with high virulence in mice [224]. Specifically, high viral loads have been reported in the avian H5N1 virus infection in humans and mouse models as well [95, 107, 111]. The present study showed that the equine H7N7 viral loads in the lungs of mice were significantly greater than the H3N8 viral loads after day 2 p.i. until the death of those mice. It is therefore tempting to speculate that the equine H7N7 influenza virus was resistant to the initial host immune control, thus leading to a remarkable viral burden, which in turn drove an overwhelming inflammatory response, and all of these characteristics contributed to the lethal outcome of the equine H7N7 influenza virus infection in BALB/c mice. We can derive from this that the key of successful therapeutic treatment during highly pathogenic influenza virus infection is the blockage of uncontrolled virus replication by timely administration of antiviral medication first, and combined with alleviation of over-exuberant immune responses if necessary.

Pulmonary hemorrhage in the mice caused by the virus infection

Large amounts of erythrocytes in the lung lavage fluids were noticed in the later stages of the equine H7N7 virus infection in mice, which implied the the occurrence of lung hemorrhage. Pulmonary hemorrhage was also observed by histopathological examination in some cases of avian H5N1 infection in humans [94, 98] and in a mouse model [225]. Hemorrhage is the consequence of destruction of vascular integrity and subsequent increase in permeability. Two contributory mechanisms are currently known to be responsible for vascular damage. The first one is the direct cytotoxic effect of virus replication within vascular endothelial cells. Human influenza virus was shown to be able to infect human endothelial cells *in vitro* [226]. In some severe cases of influenza in children, viral antigen was detected in endothelial cells *in vivo* [227]. Moreover, certain isolates of highly pathogenic H5N1 avian influenza virus were found to target vascular

endothelial cells of domestic chickens [228-229] and wild birds [230-231]. A particular highly pathogenic strain of the H7N1 subtype predominantly attacked endothelial cells in all organs of chicken embryos [232]. In this regard, it is desirable to investigate whether equine H7N7 influenza virus can infect vascular endothelial cells of the chicken and mouse in the future studies.

Second, prominent dysregulation of host inflammatory responses may be a mechanism for vascular damage. Inflammation is an orchestrated series of events that must be elaborately regulated to facilitate host recovery from infection. Any event that disrupts this carefully balanced process may have a deleterious impact on the host [11, 233-235]. Data from this dissertation showed that the equine H7N7 influenza virus infection triggered a heightened potent expression of pulmonary inflammatory cytokines compared to the H3N8 virus in BALB/c mice. As discussed above, each of these cytokines has its specific function yet also overlapping activity with other cytokines. Compelling evidence has demonstrated that exacerbation of inflammatory cytokine responses may impose injury to endothelial function [236]. In particular, TNF-α is a major mediator of the endothelial system, causing vasodilation and increased vascular permeability [162]. Therefore, intense expression of inflammatory cytokines observed in this study may contribute to the development of pulmonary hemorrhage in BALB/c mice infected by the equine H7N7 influenza virus.

Chicken embryo innate defense against the viral infections

Embryo age at the time of inoculation has been known to influence the non-specific innate immunity against virus infection [237]. The innate immune system develops functionally during embryogenesis, as does the interferon system, which may account in part for the age-dependent mortality [237-238]. Interferons are well known for

their antiviral activities. Once induced after virus infections, they mount powerful antiviral responses through the action of downstream IFN-induced effector genes [129]. Previous studies, however, showed that highly pathogenic avian H5N1 influenza viruses compared with the less virulent ones possess the outstanding capacity to counteract the interferon system probably by suppressing the production of IFNs [239-240], or by downregulating the signaling pathway required for the induction of IFN-stimulated genes [241], or most likely even both *in vivo*. The present investigation demonstrated that the action of chicken embryo innate immunity, which was probably led by the interferon-induced antiviral system, failed to restrain cytopathic effect of the equine H7N7 virus. To test this possibility, future work including quantification of IFN production and virus replication in IFN-pretreated and non-treated cell lines as well as in embryos following inoculation is warranted.

Inoculation of the allantoic cavity of embryonated chicken eggs aged 10 or 11 days old is the primary route for propagation of influenza virus in laboratory settings. In 11-day-old eggs, cytopathic effect of the H7N7 virus but not the H3N8 virus likely spread beyond the allantoic compartment, which, therefore, could explain the highly virulent phenotype of the H7N7 virus in chicken embryos observed in the present study. Certainly, high cleavability of the HA protein enables the H7N7 virus to acquire extended tissue tropism. Furthermore, a previous study clearly demonstrated that a highly pathogenic avian influenza virus of the H7 subtype with highly cleavable HA induced systemic infection in 11-day-old chicken embryos [232]. It is worthy to note that the maximal cumulative mortality rates recorded for 11-day-old eggs occurred at day 5 after infection with high doses of the H7N7 virus (Figure 3.5C). Therefore, cytopathic effect of

the H7N7 virus in chicken eggs cannot affect the process of the virus amplification, which, as described in the Materials and Methods, needs 2 days of incubation following inoculation with the seed virus.

Horse inflammatory responses induced by the viral infections

Both viral infections in horses remained well controlled by the host immune defensive responses and detectable viral clearance was successfully accomplished by day 7 p.i., yet the H3N8 virus was more pathogenic in terms of clinical signs than the H7N7 virus in horses. This observation fits well with previous reports [172-173]. The clinical severity correlated with the transcription levels of inflammatory cytokines in the peripheral blood, which complements a previous study that documented differential clinical responses in ponies challenged with H3N8 influenza virus strains of different pathogenicity [242].

To study immune responses to influenza virus in the horse, cytokine quantification could be carried out in nasal washing fluid or lung lavage fluid to identify local cytokine responses to the infection, or could be measured in plasma and serum reflecting systemic immune response. However, a paucity of immunological reagents for the horse constitutes a major obstacle to better understanding of the immune system function in equine influenza. An alternative way to quantify the cytokine expression is the measurement of cytokine transcription levels by real time PCR. Some studies, however, have demonstrated that the processes of collection, transport and processing of samples could not only result in mRNA degradation [243] but also be responsible for *ex vivo* stimulation of mRNA expression [244]. The PAXgene blood RNA collection system was used in the present study for RNA isolation from whole blood because it offers several technical superiorities including immediate RNA stabilization and ease of use

[245]. This system was successful applied before for equine cytokine mRNA quantification [246].

IL-1β, Il-6 and TNF-α have been recognized as the hallmark proinflammatory cytokines readily produced by macrophages/monocytes in response to influenza virus infection and they act in a synergistic fashion to contribute to the induction and development of the disease symptoms [210, 247]. Among them, only IL-6 was found to be directly correlated to some extent with the clinical severity of the disease, as extensively demonstrated in humans [248-250], pigs [251-252], horses [242, 246] and ferrets [253]. The present study revealed that equine influenza virus of the H3N8 subtype was more pathogenic than the H7N7 subtype during experimental infection in horses with respect to clinical manifestations and febrile reactions induced. However, it was unexpected that the H7N7 virus induced greater, although not significant, IL-6 mRNA expression in peripheral blood on day 2 post infection than the H3N8 virus did. The results definitely warrant further investigations of local and systemic production of IL-6 at the protein level in the future to verify the current observation. By comparison, both viruses triggered a similar magnitude of transcriptional changes of TNF-α and IL-1β genes in whole blood despite apparent difference regarding clinical outcome. Therefore, the findings from the present study do not support a significant correlation between clinical signs and the extent of TNF-α or IL-1β released following equine influenza infection in horses. In a previous horse challenge study with equine H3N8 influenza virus [246], the transcription of the TNF-α gene was not significantly elevated in the peripheral blood after experimental infection. Bioassay also failed to detect the presence of TNF-α protein locally or systemically in ponies challenged with equine influenza virus [242]. In

accordance with these observations, neither of the two viruses in the present study induced a pronounced increase in the circulating levels of TNF-α mRNA.

IL-8, a potent neutrophil chemotactic cytokine [254], increases significantly in nasal fluid and lung lavage fluid in response to influenza virus infection in humans [250, 255] and pigs [256]. But, it is not detected at the protein level in human plasma in seasonal human influenza cases [250]. Likewise, neither the H7N7 nor H3N8 virus induced significant changes in IL-8 mRNA expression in horse peripheral blood. Notably, rather high levels of IL-8 in serum were observed in the fatal cases of human infection with highly pathogenic avian influenza viruses [95].

IL-18, a member of the IL-1 family [257], is induced mainly in monocyte/macrophage cells after influenza virus infection [154]. The current data show that the H3N8 virus induced significantly greater IL-18 gene transcription in horse peripheral blood than the H7N7 virus. IL-18 was originally named as IFN-γ-inducing factor because of its ability to induce IFN-γ production in an antigen-independent manner by natural killer cells and T lymphocytes synergistically with the cofactor IL-12 [258-259]. It has proinflammatory properties, such as induction of TNF-α, IL-1β and several chemokines [260]. Besides its function in regulation of inflammatory responses, IL-18 plays an important role in directing the immune response against viral infection toward cell-mediated immunity. This point has been highlighted in several *in vivo* studies using mouse models, in which IL-18 was found to facilitate the host defense against influenza virus infection through activation of natural killer [261] and T cells [262]. The exact role played by IL-18 during equine influenza infection of horses merits further investigations.

IFN-γ is a product of activated T lymphocytes and natural killer cells after induction by macrophage-derived cytokines such as IL-18, IL-12 and IL-1β or pathogen specific stimulation via contact with an antigen-presenting cell. As a "late" mediator in the cytokine cascade produced in response to influenza virus infection [210], IFN-γ is well known for its critical role in the development and regulation of antigen-specific cellular immune responses to the viral infection. Enhancement of IFN-γ mRNA expression in equine PBMCs following *in vitro* re-stimulation with influenza virus was observed 2 weeks or even longer after experimental infection of donor equids by equine influenza virus [263-264]. Therefore, it is not unexpected that equine influenza viruses of both H7N7 and H3N8 subtypes failed to upregulate the IFN-γ gene transcription in the peripheral blood during the early stages of experimental infection in horses.

In this study, compared to the H7N7 virus, the H3N8 virus infection induced significantly higher expression of IL-10 mRNA in the peripheral blood on day 2 post infection. As discussed above in the section of mouse study, IL-10 is a major anti-inflammatory cytokine. However, information about the function of IL-10 in equine influenza is extremely rare. It still remains unresolved from the current investigation whether IL-10 actually antagonizes over-exuberant inflammatory responses during equine influenza virus infection in horses.

The present horse challenge study has a major limitation in that it did not investigate the transcriptional levels of these cytokine genes in the lung. It is well known that influenza virus attacks the airway epithelial cell layer and resident tissue macrophages and dendritic cells situated in most proximity with it. Damage to the respiratory tract tissue caused by virus infection elicits a subsequent enormous migration

of leukocytes from the peripheral blood to this inflamed micro-anatomical compartment, which is therefore the prime site of cytokine secretion, and systemic spread of inflammatory mediators probably represents the “spill-over” effects [252]. Several previous experimental influenza studies have provided evidence to support this notion by showing that cytokine protein levels in the circulation are generally lower than those in the pulmonary compartment or are undetectable [250, 252]. In this regard, significance may be underestimated when interpreting the role of a particular cytokine in the pathogenesis of influenza based solely on the magnitude of its presence in the circulation. Furthermore, a caveat arises, especially when comparing the results of the present horse experimental infection study to other investigations on the translational expression of cytokines during influenza infection, which is that mRNA transcript abundance does not necessarily correlate with the level of encoded protein. In eukaryotes, synthesized mRNAs have to go through post-trancriptional processes such as mRNA splicing and levels are also affected by mRNA stability before producing encoded proteins [265].

Species-specific differences in proteases

As discussed above, the HA protein of the H7N7 virus examined in this study carries a multibasic motif at the cleavage site that renders the HA capable of proteolytic activation by ubiquitous host subtilisin-type proteases such as furin and PC6 [266]. These proteases display similar substrate specificities for the HA with furin being identified to-date as the primary one in respect of cleavage efficiency in murine species [266-268]. Since the proteases responsible for processing of HA of equine influenza viruses in the horse have not been characterized, the study presented here cannot rule out the possibility that the differential pathogenicity of the H7N7 virus in horses and BALB/c mice is attributable to the intrinsic difference in host HA-activating protease profiles between the

two species, specifically, for instance, their predominant distribution at tissue or cellular levels [269] and cleavage potency [266-267] of the respective leading candidate proteases. Furthermore, such a difference may be further augmented during the process of the viral infection *in vivo*, as supported by the findings that influenza virus is able to facilitate its spread in mice by upregulating the local expression of multiple trypsin-like proteases in various organs tested, including the lung [270-272]. Notably, host genetic background has a significant influence on the differential expression of these enzymes in laboratory mouse strains [270]. In addition to their requirement for processing of many viral glycoproteins [267, 272], proteases and their target cell surface receptors are also an integral element of the host innate immune system against microbial infections including influenza virus [273-274]. This fact, in one respect, simply highlights the indispensable role of the immune defense system, an intricate and dynamic network of components, as the body's safeguard against invading threats no matter whatever form or scale. Finally, the outcome of any pathological process is shaped by the overall force balance between the two adversaries engaged in such a war.

The role of antiviral Mx protein in the innate immunities

The mouse innate immunity was unsuccessful in limiting the H7N7 virus spread, which, however, was conquered by the horse innate immune defense. The inherent species-specific differences in innate immunity might be due to the absence of a functional Mx gene in the strain of BALB/c mouse but not in the horse [135, 138-139]. Nine out of ten horses tested here demonstrated inducible expression of Mx mRNA after influenza virus infection. A previous investigation also identified Mx protein production in peripheral blood mononuclear cells from some but not all horses examined [139]. However, it is currently unclear whether equine Mx protein has antiviral activity. Many

standard laboratory strains of mice (including the BALB/c mouse) are highly inbred lines as the consequence of systematic artificial inbreeding for specific desirable traits within the context of scientific research. As a double-edged sword, the deliberate inbreeding, leads to reduced genetic diversity, enhanced risk for certain genetic defects and corresponding diseases. One case for such deleterious byproducts of inbreeding is that BALB/c mice carrying a defective Mx gene in the genome are susceptible to influenza virus infection [135]. As one of IFN-stimulated genes with antiviral activity, Mx protein has been recognized to be the major antiviral effector against influenza virus infection in mice. Transgenic expression of mouse Mx protein rendered Mx-deficient mice resistant to not only mouse-adapted strains [275-276] but also to the pandemic 1918 and highly pathogenic avian H5N1 influenza viruses [277-278]. Yet, it is noteworthy to mention that such protection was inversely correlated with the inoculation dose. When the Mx-competent mice were inoculated with a highly pathogenic H5N1 virus isolate at a dose of 10 LD_{50} (Lethal Dose, 50%), all animals survived whereas six out of eight mice died when the challenge dose was increased to 100 LD_{50} [278]. In other words, the protective effect of Mx could be overcome by a higher dose of virus.

Nevertheless, the hypothesis proposed above that Mx gene is responsible for the observed species-specific differences in innate immune response between the BALB/c mouse and the horse may not be extended to explain the similar differences between the horse and the chicken embryo model studied here. In spite of its presence and induction during influenza virus infection, Mx protein was found to be devoid of antiviral activity against influenza virus in the wild duck [279] and most chicken breeds tested [280-281]. Therefore, Mx protein is not likely to be a significant contributing factor to the innate

defense of the chicken embryo. However, the absence of an Mx-mediated antiviral element does not compromise fully immunocompetent chickens against most influenza virus strains except for the highly pathogenic avian influenza viruses of the H5 and H7 subtypes. Comparably, although the BALB/c mouse strain lacks a functional Mx gene, the innate immunity of these mice was strong enough to defeat the equine H3N8 virus infection as clearly demonstrated by the present study. This innate defense could be compensated by the function of other IFN-induced antiviral components, such as the dsRNA-dependent protein kinase (PKR), the oligoadenylate synthetase (OAS)/RNase L system or additional still-to-be-dentified protectors. A knock-out mouse study featuring the triple deficiencies of PKR, RNaseL and Mx clearly revealed the existence of additional antiviral pathways against influenza virus [282]. No matter what molecular executors have been involved in the innate resistance to influenza virus in the BALB/c mouse and the chicken embryo, the current study has provided indirect, but compelling evidence that the equine H7N7 virus had an extraordinary ability to escape the host innate immune control, a characteristic that is reminiscent of highly pathogenic avian H5N1 influenza viruses, which are known to circumvent the innate immune system by various strategies from suppressing IFN production and its JAK/STAT signaling pathway [240-241] to directly blocking the action of antiviral effector proteins, such as PKR and RNaseL [283].

Equine primary cell cultures following exposure to the viruses

PBMCs are a heterogeneous population, composed predominantly of lymphocytes and monocytes. Monocytes comprise about 10% of the cell population and are adherent, while lymphocytes comprise about 75% and are non-adherent [284]. The current study showed that fresh, unstimulated equine PBMC are not a permissive cell culture for

influenza virus. This finding is in agreement with previous studies [285], in which influenza virus infection of human PBMC was abortive. Absence of viral translation activity demonstrated by immunofluoresent assay indicates that an early block in the replication cycle of virus could account for the failure of equine influenza virus replication. The stage of interruption does not likely occur during virus entry into cells, as previous studies on human PBMCs showed that lymphocytes possess surface receptors for influenza virus [286] and PBMC can be infected by this virus [285, 287].

Typical characteristics of the mature macrophage phenotype were observed during monocyte *in vitro* differentiation in this study, which agree with a previous demonstration [288]. It has been well documented that the status of monocyte-macrophage differentiation influences the susceptibility of macrophage to virus infections [289-290]. Present data show that freshly isolated equine blood monocytes were refractory to influenza virus infection, whereas fully differentiated macrophages were highly susceptible. Perhaps it is not surprising that equine influenza virus does not display monocyte/lymphocyte tropism since the infection by this virus is restricted to the respiratory epithelium and does not spread systemically [12]. *In vitro* monocyte differentiation is accompanied by upregulation of cell surface maker expression and phagocytic capacity in addition to the morphological changes [291], which may account for their increased permissiveness to equine influenza virus infection. Productive replication of equine influenza virus turned macrophages from inertness into activated status, as represented by the potent expression of cytokine TNF-α in virus-infected cells compared to mock-infected controls.

Rapid progeny virus production in mature macrophages eventually caused progressive cell destruction. Previous studies reported that CPE induction in cells infected with poxvirus or avian hemangioma retrovirus occurs regardless of viral replication [292-293]. To rule out the possibility that CPE may be the result of cell surface receptor-virus interaction, macrophages were infected with UV-inactivated viruses. The current results indicate the necessity of active viral replication for cell pathology. This is also consistent with a previous demonstration in a murine macrophage cell line infected with human influenza virus [294].

In conclusion, this dissertation demonstrated for the first time that a representative isolate of the equine influenza virus H3N8 subtype is more pathogenic in respect of clinical signs than one of the equine influenza virus H7N7 subtype in horses during experimental infection, and the observed difference in clinical severity correlated with the transcription levels of inflammatory cytokines in the peripheral blood. However, both viral infections produced mild, non-lethal disease for their natural host, the horse. In contrast, the equine H7N7 virus infection is lethal for chicken embryos and BALB/c mice, a characteristic that is vividly reminiscent of highly pathogenic avian H5 and H7 influenza viruses. This equine H7N7 virus had the outstanding capacity to escape the host innate immune control of BALB/c mice and chicken embryos, and consequently induced high viral loads and elevated expression of inflammatory cytokines in the lungs of BALB/c mice. Now there is increasing concern about the potential pandemic of avian influenza. The present mouse study with the equine H7N7 virus may offer a safer surrogate research model for dissection of the molecular basis for the unusual pathogenesis of highly pathogenic avian influenza viruses. Moreover, in view of the

striking dichotomy of virulence demonstrated by the equine H7N7 virus infection in BALB/c mice and horses, a thorough understanding of the viral and host determinants that interact to dictate the disease outcome of cross-species infections may throw light on the development of potential preventive and therapeutic strategies for treating humans threatened by highly pathogenic avian influenza viruses and by the recent global outbreaks of swine-origin influenza viruses.

Future Directions

This dissertation clearly demonstrated that transcription of the equine Mx gene was greatly induced early in the course of infection. Therefore, there is an urgent need to ascertain whether the equine Mx protein actually exhibits inhibitory activity against influenza virus. If it does then the degree of sensitivity of equine H7N7 influenza virus to the action of equine Mx protein versus the mouse counterpart can be measured by *in vitro* test first, and a comparative pathogenesis study could be conducted subsequently by using a transgenic mouse model expressing an introduced mouse or equine Mx gene during equine H7N7 influenza virus experimental infection if the results found in the *in vitro* study show promise. This will provide a chance to test the hypothesis obtained from this dissertation that the discrepancy in pathogenicity of equine H7N7 influenza virus in two disparate mammalian species is attributable to the presence of Mx antiviral function in the horse but not in the BALB/c mouse. Considering that the innate immune system is a complicated and tightly orchestrated network of molecular and cellular components that interplay across various levels within the host, and equine H7N7 influenza virus has the ability to evade the host innate immune defense as revealed by the present study, it would be useful to further perform a comparative analysis of global gene expression profiling

between the horse and the BALB/c mouse or the chicken in the context of equine H7N7 virus infection through a microarray platform. By virtue of such an approach, intriguing findings and ultimately a conclusive explanation for the observed differential pathogenicity of equine H7N7 influenza virus in different species may be obtained.

APPENDIX A:

ABBREVIATIONS

ANOVA = analysis of variance

β-Gus = beta-glucuronidase

BAL = bronchoalveolar lavage

BALB/c = a laboratory-bred strain of the house mouse

CPE = cytopathic effect

C_T = cycle threshold

DCs = dentritic cells

DNA = deoxyribonucleic acid

EID50 = 50% egg infectious dose

ELISA = enzyme-linked immunosorbent assay

FITC = fluorescein isothiocyanate

GTPases = a large group of enzymes that bind and hydrolyze guanosine triphosphate

HA = hemagglutination test

HA = influenza A virus hemagglutinase

IFN-α/β = interferon alpha/beta

IFN-γ = interferon gamma

IL-1β =interleukin-1 beta

IL-6 = interleukin-6

IL-8 = interleukin-8

IL-10 = interleukin-10

IL-12 = interleukin-12

IL-18 = interleukin-18

IP-10 = interferon gamma-induced protein 10

IRF-9 = interferon regulatory factor 9

ISGF-3 = interferon-stimulated gene factor 3

Jak1 = janus kinase 1

JAK-STAT = janus kinase-signal transducer and activator of transcription pathway

LD_{50} = lethal dose, 50%

LinReg = linear regression analysis

M = influenza A virus matrix protein

MCP-1 = monocyte chemotactic protein-1

MHC = major histocompatibility complex

MIP-1α = macrophage inflammatory protein-1 alpha

Mx = myxovirus resistance gene/protein

NA = influenza A virus neuraminidase

NK = natural killer cells

NP = influenza A virus nucleoprotein

NS = influenza A virus nonstructural protein

OAS = oligoadenylate synthetase

PA = influenza A virus polymerase complex subunit A

PB1 = influenza A virus polymerase complex subunit B1

PB1-F2 = a protein encoded by an alternative reading frame within the PB1 gene

PB2 = influenza A virus polymerase complex subunit B2

PBMCs = peripheral blood mononuclear cells

PBS = phosphate buffered saline

PC6 = prpprotein convertase-6

PCR = polymerase chain reaction

p.i. = post infection

PKR = protein kinase R

R^2 = squared correlation coefficient

RANTES = regulated upon activation, normal T cell expressed and secreted

RM-ANOVA = repeated measures analysis of variance

RNA = ribonucleic acid

RNase L = an interferon-induced ribonuclease (Latent)

RNP = influenza A virus ribonucleic acid-protein complex

RT-PCR = real-time polymerase chain reaction

SD = standard deviation

SEM = standard error of the mean

STAT 1/2 = signal transducer and activator of transcription 1/2

TGF-β = transforming growth factor beta

TNF-α = tumor necrosis factor-alpha

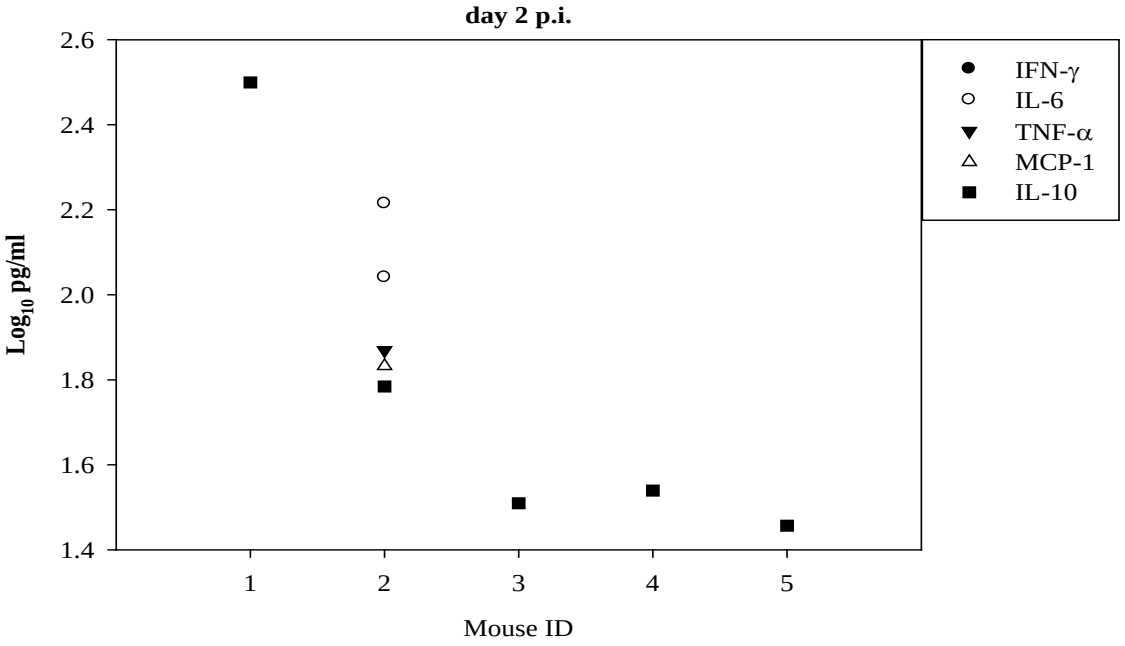
Tyk2 = tyrosine kinase 2

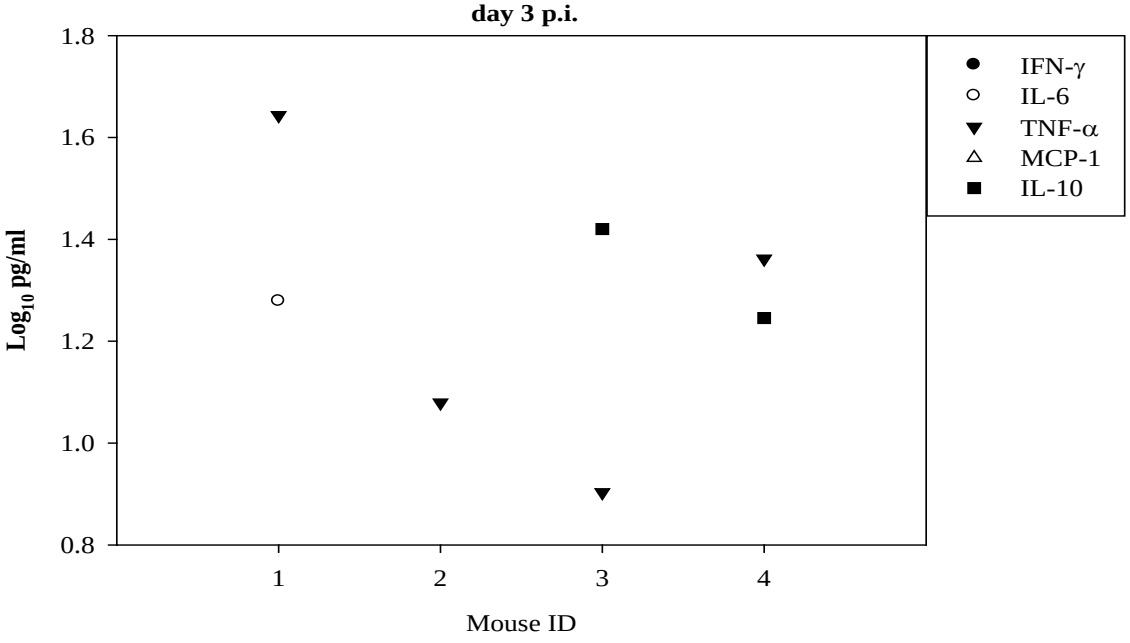
UTR = untranslated region in ribonucleic acid

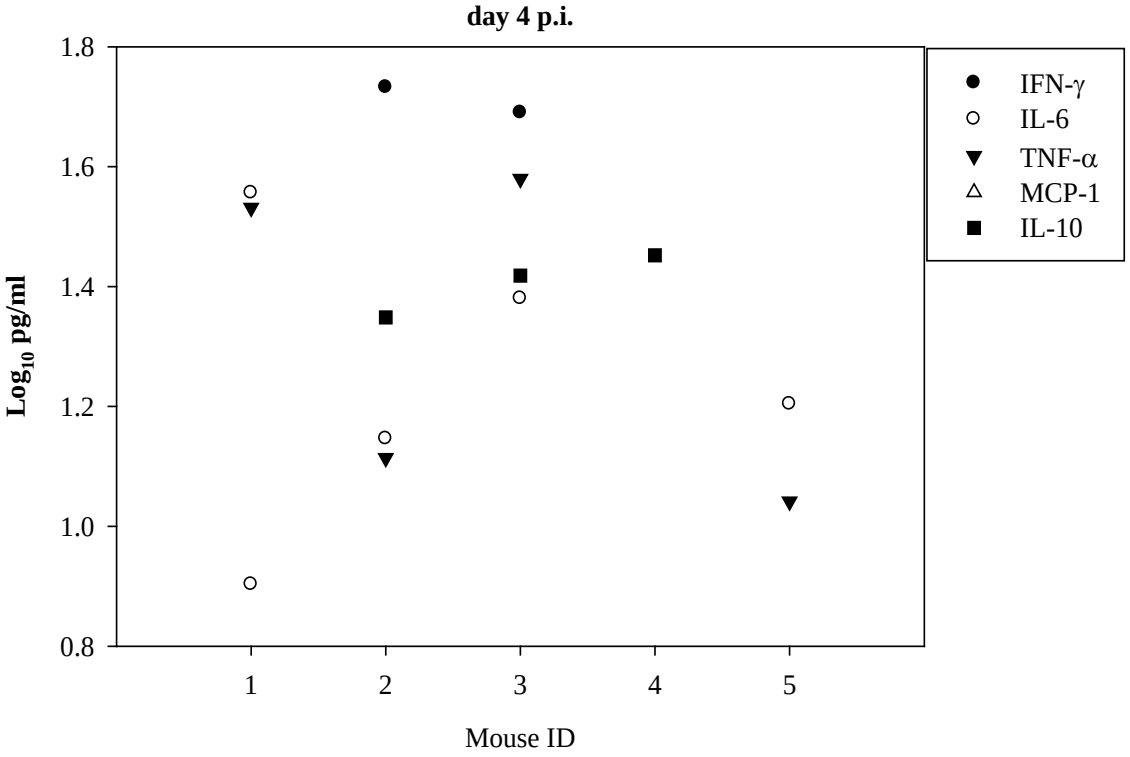
APPENDIX B:

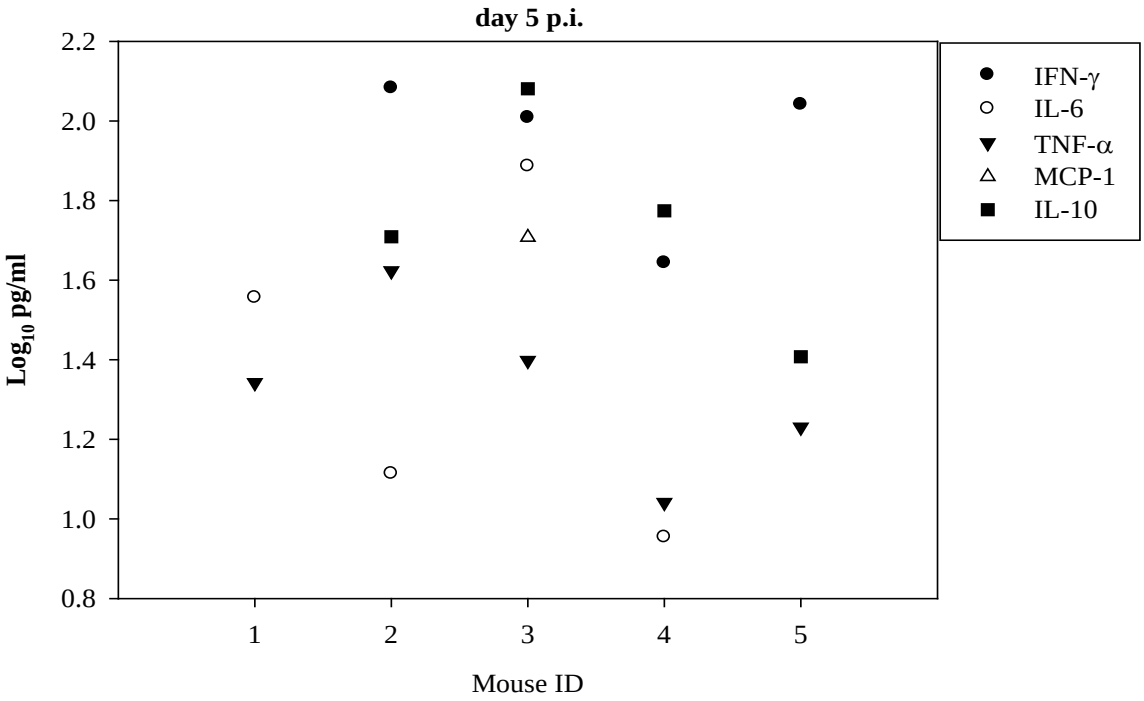
The expression levels of multiple pulmonary cytokines in each H7N7-infected mouse during the assay period

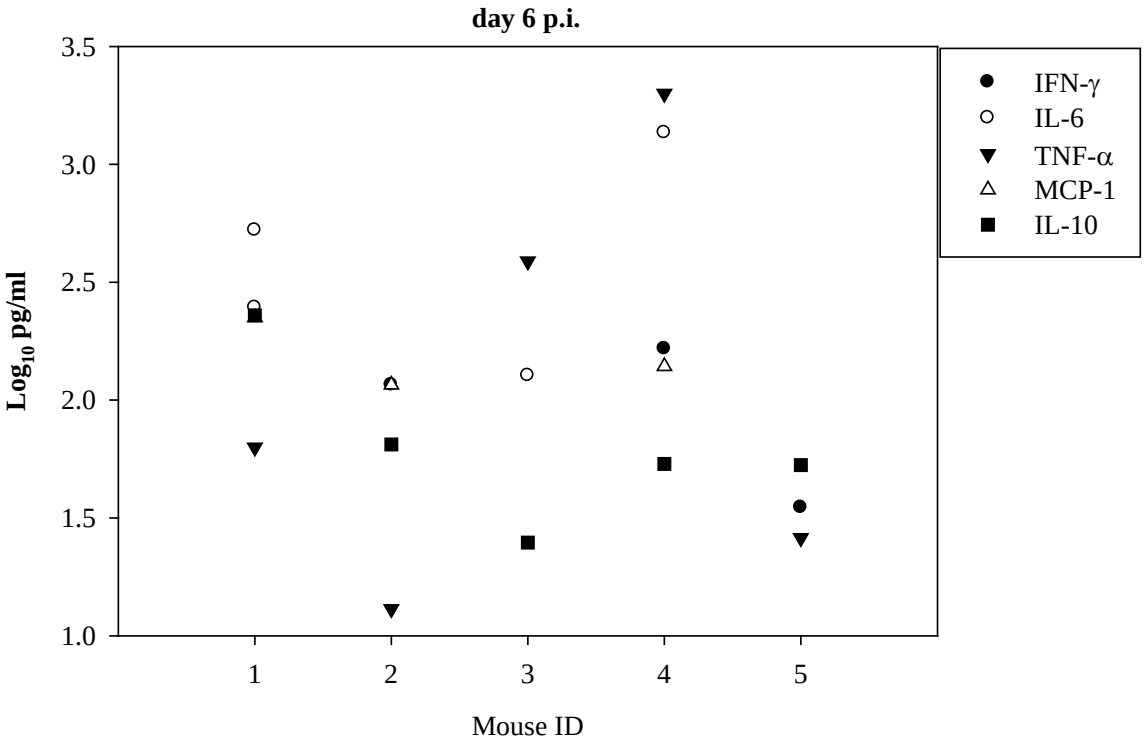
Notes: Since mice had to be euthanized to collect lung lavages for cytokine assay daily, therefore the mice used at each time point were not the same. All data was expressed as $\log_{10}$ pg/ml.

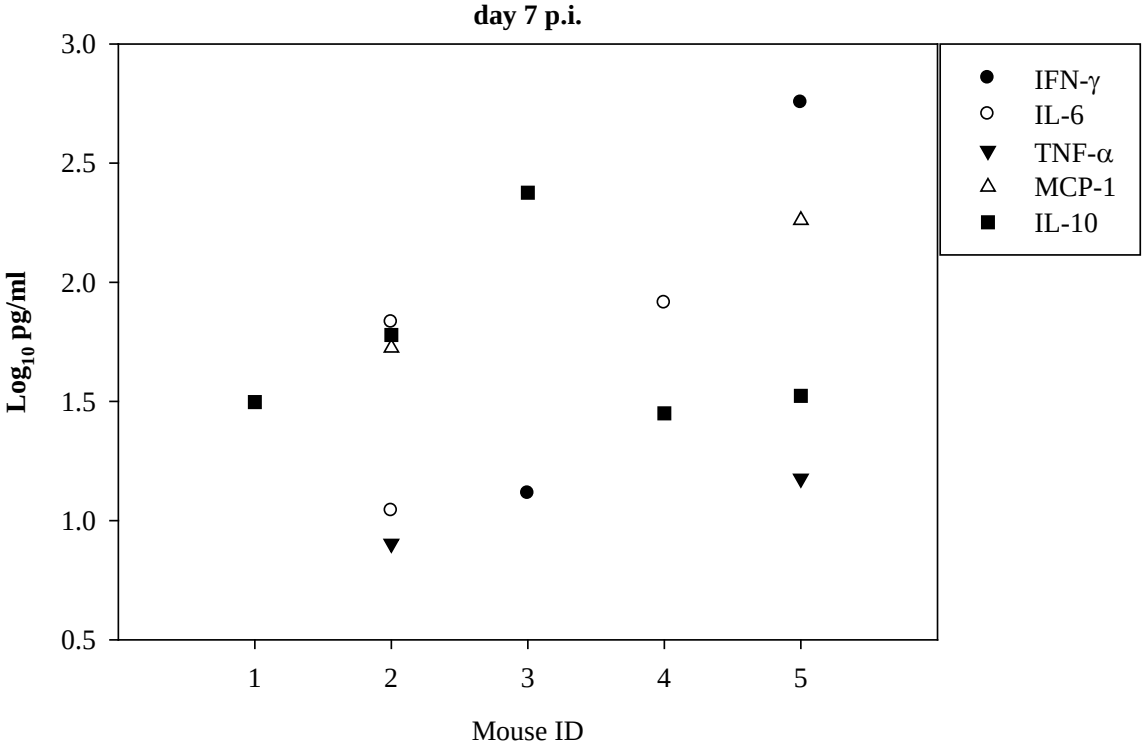





day 4 p.i.
1.8
1.6
1.4
1.2
1.0
0.8
Log10 pg/ml
1
2
3
4
5
Mouse ID
IFN-γ
IL-6
TNF-α
MCP-1
IL-10


day 5 p.i.
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
Log10 pg/ml
1
2
3
4
5
Mouse ID
IFN-γ
IL-6
TNF-α
MCP-1
IL-10

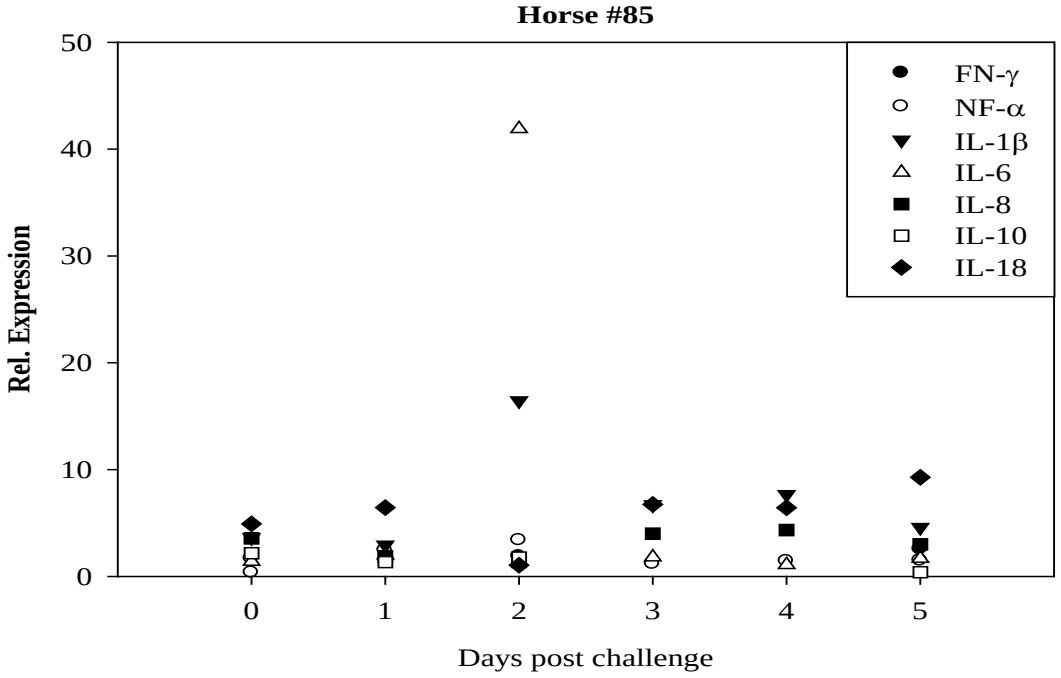

day 6 p.i.
3.5
3.0
2.5
2.0
1.5
1.0
Log10 pg/ml
1
2
3
4
5
Mouse ID
IFN-γ
IL-6
TNF-α
MCP-1
IL-10

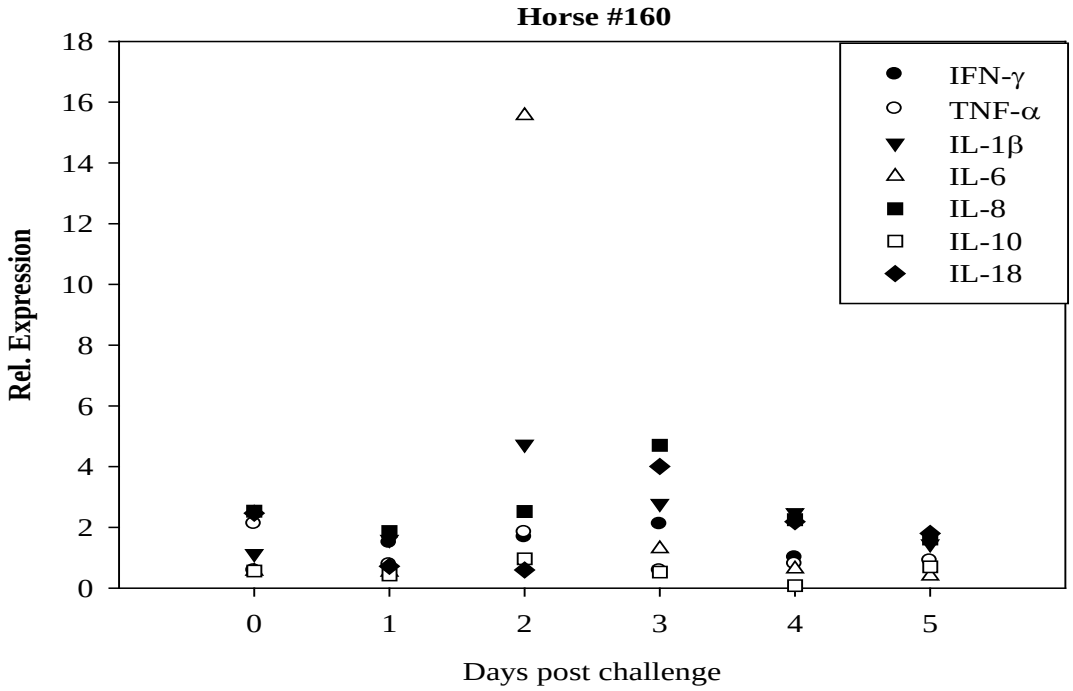

day 7 p.i.
3.0
2.5
2.0
1.5
1.0
0.5
Log10 pg/ml
1
2
3
4
5
Mouse ID
IFN-γ
IL-6
TNF-α
MCP-1
IL-10

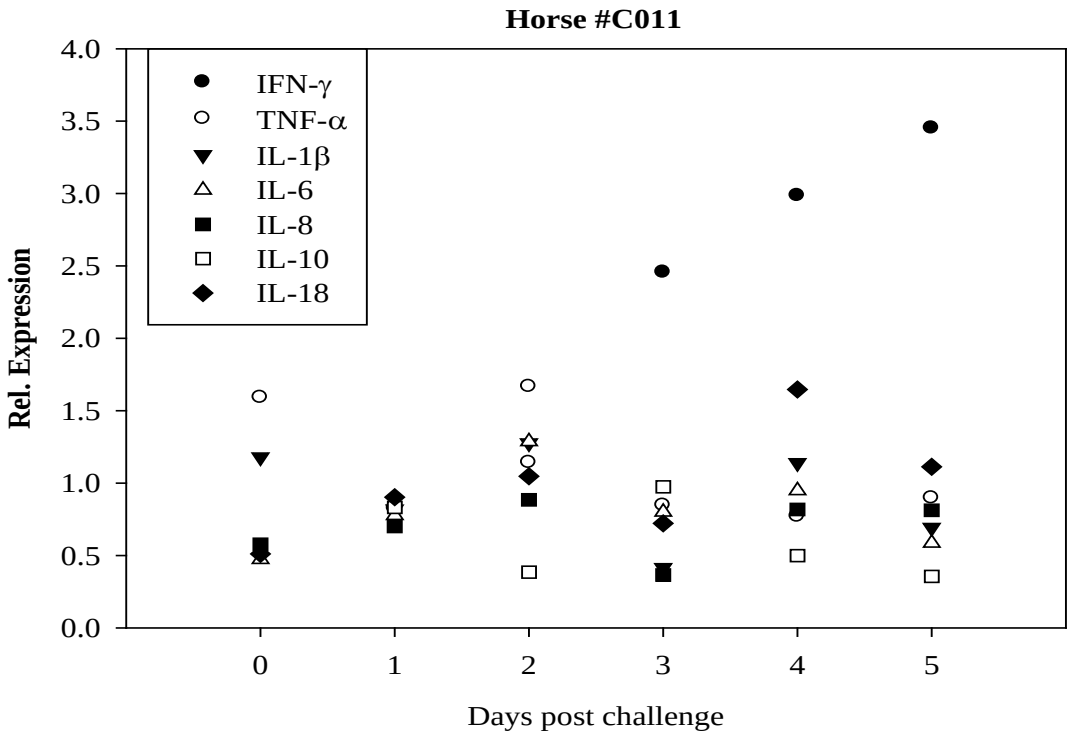
APPENDIX C:

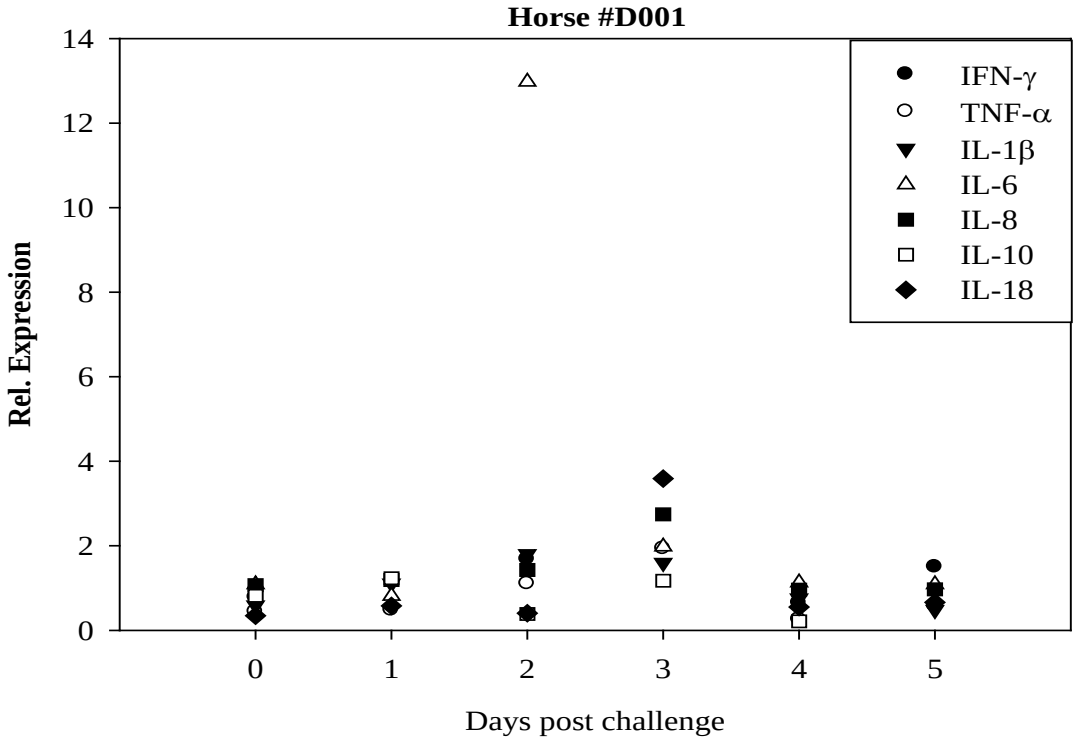
Relative quantification of several cytokine mRNAs in individual horses in the course of infection

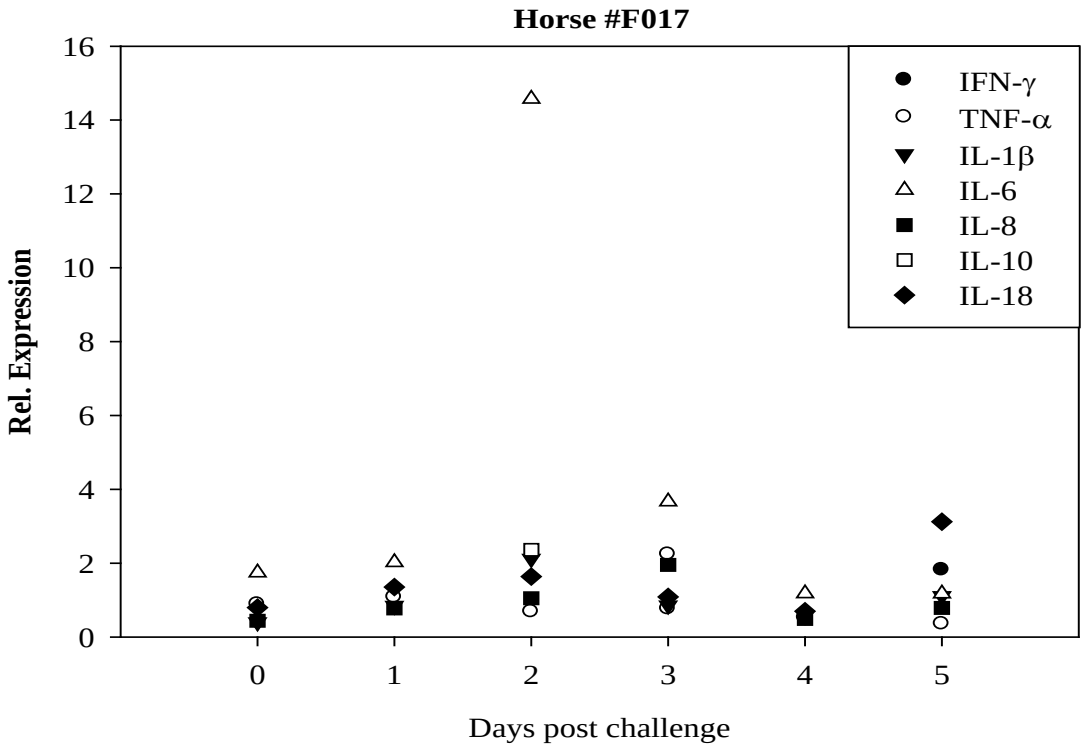
Note: Horses challenged with the H7N7 virus were (#85, #160, #C011, #D001, #F017, #F019). Horses challenged with the H3N8 virus were (#G001, #G002, #G005, #G009)

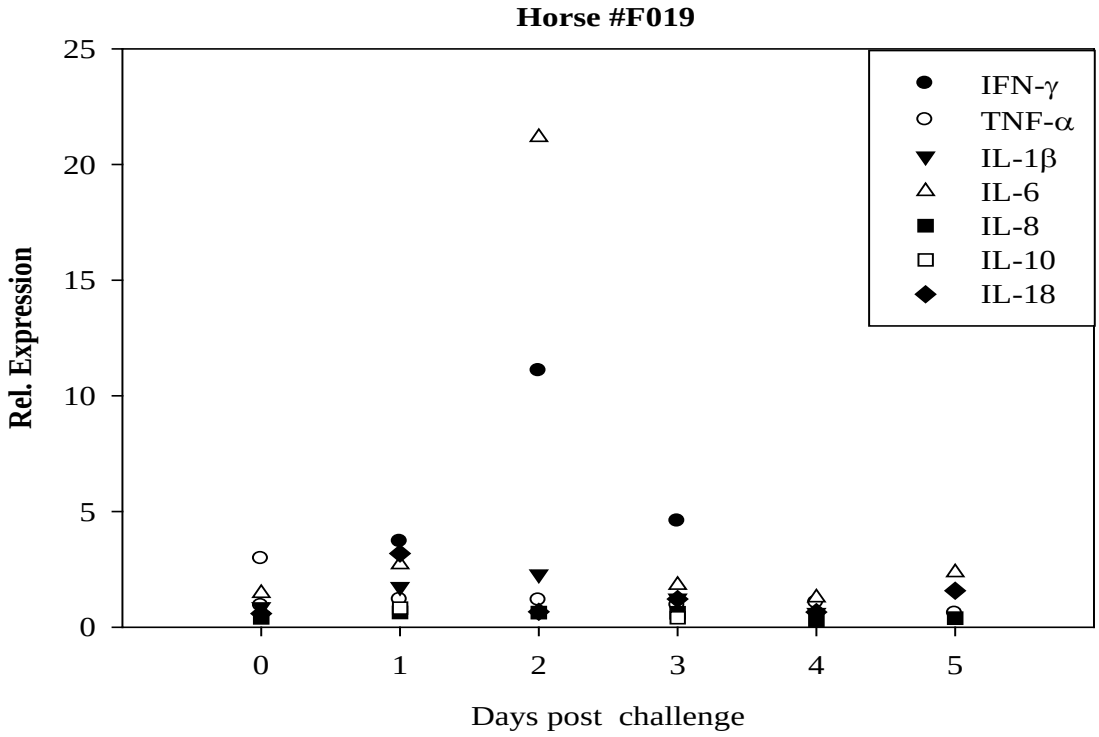





Horse #C011
Rel. Expression
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IFN-γ
TNF-α
IL-1β
IL-6
IL-8
IL-10
IL-18
0
1
2
3
4
5
Days post challenge


Horse #D001
Rel. Expression
14
12
10
8
6
4
2
0
IFN-γ
TNF-α
IL-1β
IL-6
IL-8
IL-10
IL-18
0
1
2
3
4
5
Days post challenge


Horse #F017
Rel. Expression
Days post challenge
IFN-γ
TNF-α
IL-1β
IL-6
IL-8
IL-10
IL-18
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5


Horse #F019
Rel. Expression
Days post challenge
IFN-γ
TNF-α
IL-1β
IL-6
IL-8
IL-10
IL-18
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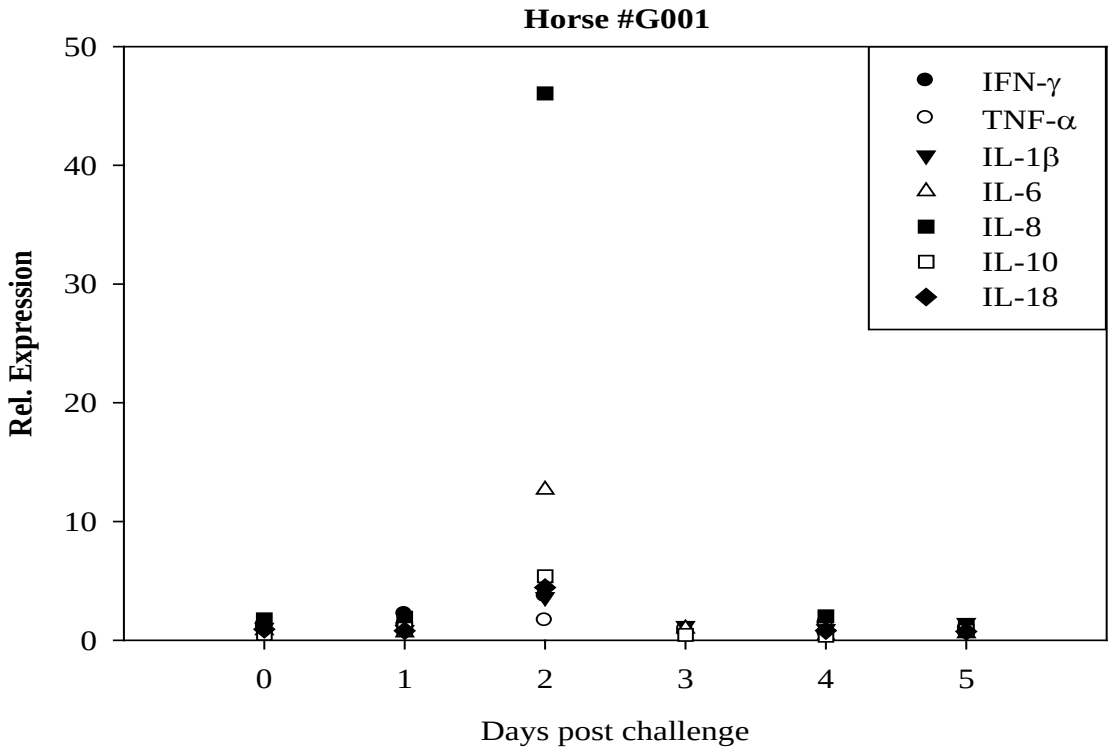
5
10
15
20
25
0
1
2
3
4
5


Horse #G001
Rel. Expression
Days post challenge
IFN-γ
TNF-α
IL-1β
IL-6
IL-8
IL-10
IL-18
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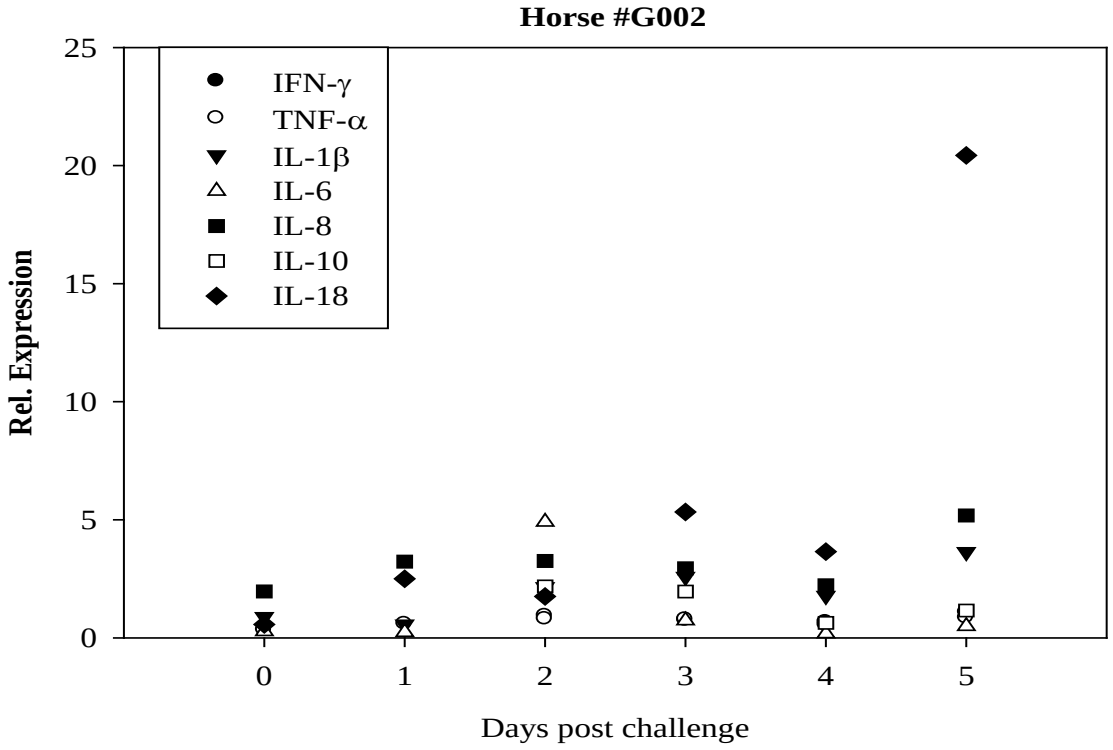
10
20
30
40
50
0
1
2
3
4
5


Horse #G002
Rel. Expression
Days post challenge
IFN-γ
TNF-α
IL-1β
IL-6
IL-8
IL-10
IL-18
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15
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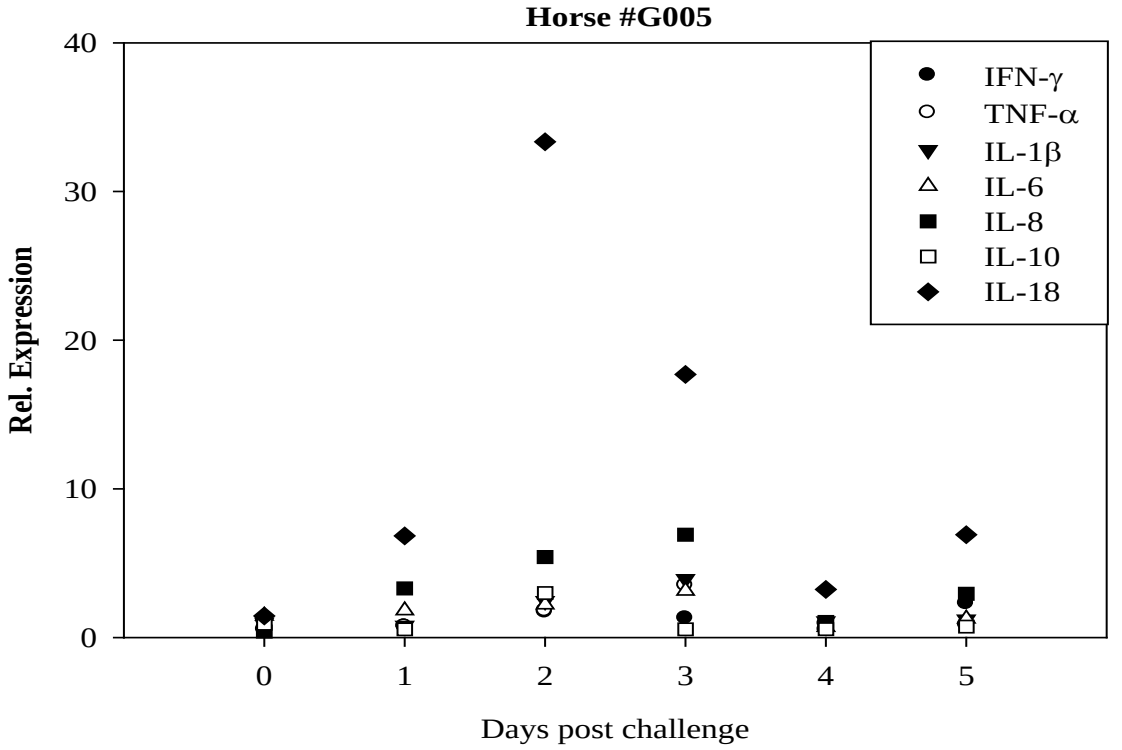
25
0
1
2
3
4
5


Horse #G005
Rel. Expression
Days post challenge
IFN-γ
TNF-α
IL-1β
IL-6
IL-8
IL-10
IL-18
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30
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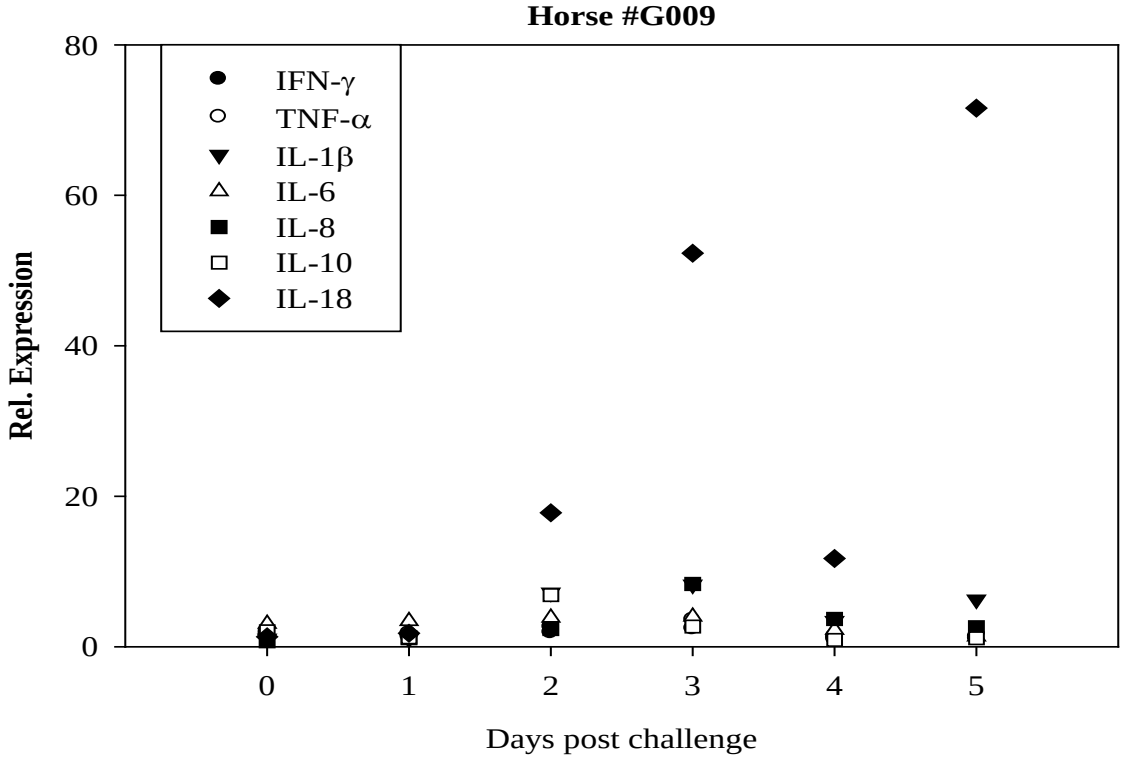
1
2
3
4
5


Horse #G009
Rel. Expression
Days post challenge
IFN-γ
TNF-α
IL-1β
IL-6
IL-8
IL-10
IL-18
0
20
40
60
80
0
1
2
3
4
5

APPENDIX D:

Comparison of PB2 amino acid sequences at position of 627 of the equine influenza viruses

virus	Amino acid residue at position of 627 in PB2
Influenza A virus A/equine/Cornell/16/74 (H7N7)	------F-A-A-A-P-P-**E**-Q-S-R-M-Q-F-----
Influenza A virus A/Equine/London/1416/73 (H7N7)	------F-A-A-A-P-P-**E**-Q-S-R-M-Q-F-----
Influenza A virus A/equine/Kentucky/5/02 (H3N8)	------F-A-A-A-P-P-**E**-Q-S-R-M-Q-F-----

Notes:
(1) The individual amino acid sequence of PB2 examined here was obtained from Gene Bank.
(2) Influenza A virus A/equine/Cornell/16/74 (GeneBank Accession No. ACB30161.1)
(3) Influenza A virus A/Equine/London/1416/73 (GeneBank Accession No. AAA43141.1)
(4) Influenza A virus A/equine/Kentucky/5/02 (GeneBank Accession No. AAX23572.1)

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