THESIS

DEVELOPMENT AND CHARACTERIZATION OF AN *IN VITRO* EQUINE AIRWAY MODEL AS A TOOL FOR THE STUDY OF HOST-PATHOGEN INTERACTIONS AT THE EPITHELIAL CELL BARRIER

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY AYSHEA M. QUINTANA ENTITLED DEVELOPMENT AND CHARACTERIZATION OF AN *IN VITRO* EQUINE AIRWAY MODEL AS A TOOL FOR THE STUDY OF HOST-PATHOGEN INTERACTIONS AT THE EPITHELIAL CELL BARRIER BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

DEVELOPMENT AND CHARACTERIZATION OF AN *IN VITRO* EQUINE AIRWAY MODEL AS A TOOL FOR THE STUDY OF HOST-PATHOGEN INTERACTIONS AT THE EPITHELIAL CELL BARRIER

Equine influenza virus (EIV) and equine herpesvirus-1 (EHV-1) are the main causes of viral respiratory diseases in horses. Despite vaccination efforts, they continue to have serious health and economic impacts on the equine industry. While we have made progress understanding the host adaptive immune response to these viruses, the innate and early immune response remains poorly defined. One reason for this delay in our understanding of this essential component of immunity is the lack of a suitable *in vitro* model. The respiratory epithelium is the primary site of infection and replication for both EIV and EHV-1 and the site where early immune responses occur. As a component of innate immunity, it also functions as a physical barrier, chemical barrier, and microbiological barrier against pathogen invasion through the formation of tight junctions, mucociliary clearance, and the harboring of commensal microorganisms as well as antimicrobial peptides important for facilitating pathogen clearance. Because of the importance of the respiratory epithelium in the pathogenesis of equine respiratory viruses, we developed and characterized, morphologically and immunologically, an

equine respiratory epithelial cell (EREC) culture system grown at the air-fluid interface (AFI). The AFI culture system is unique because the model mimics the natural airway epithelium with the apical surface of the cells exposed to humidified air while the basal surface is submerged in liquid. In addition, respiratory epithelial cell cultures grown at the AFI have been successfully developed for other species and used as a tool in the study of allergy and infectious disease.

To develop an equine airway model, respiratory epithelial tissues were harvested from humanely euthanized horses and epithelial cells were isolated using enzymatic digestion and gentle agitation in calcium and magnesium free minimal essential medium for 48 hours. ERECs were seeded on a collagen-coated membrane and kept at an air-fluid interface until differentiated. Factors that determined the point of differentiation included tight junction formation, mucin production, and cilia development. After four weeks of growth, we have demonstrated cell differentiation in these cultures characterized by the presence of ciliated epithelial cells, secretory cells, and basal cells in addition to tight junction formation and visible mucin production.

While AFI cultures have been shown to undergo successful cell differentiation, little information exists as to how they present immunologically after several weeks of *in vitro* growth conditions. Equine respiratory epithelial tissues, isolated epithelial cells, and four-week old cultured, differentiated airway epithelial cells collected from six locations of the equine respiratory tract were examined for the expression of toll-like receptors (TLRs) and antimicrobial peptides (AMPs) using conventional polymerase chain reaction (PCR). Cultured, differentiated, respiratory epithelial cells and freshly isolated respiratory epithelial cells were also examined for the expression of TLR3, TLR9 and major

histocompatibility complex (MHC) class I and class II using fluorescence-activated cell sorting (FACS) analysis. In addition, cytokine and chemokine profiles from respiratory epithelial tissues, freshly isolated respiratory epithelial cells, and cultured, differentiated, epithelial cells from the upper respiratory tract were examined using real-time PCR. We found that respiratory epithelial tissues and isolated epithelial cells expressed TLRs 1-4 and 6-10 as well as AMPs, MxA, 2'5' OAS, β-defensin-1, and lactoferrin. In contrast, TLRs 8-10 and lactoferrin were no longer detected in epithelial cells cultured at the AFI after four weeks compared to respiratory tissues and freshly isolated epithelial cells. In addition, MHC-I and MHC-II surface expression decreased in epithelial cells cultured at the AFI compared to isolated epithelial cells whereas TLR3 and TLR9 were expressed at similar levels. Lastly, we found that after four weeks of *in vitro* growth conditions, equine respiratory epithelial cells cultured at the AFI expressed granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10, IL-8, TGF-β, TNF-α, and IL-6.

To further define the use of this model, we examined the infectivity and 24 hour endpoint M gene copy numbers in these cultures using canine and equine H3N8 influenza A viruses and compared this to an *in vivo* study using the same influenza isolates in ponies. Our results demonstrated that infectivity and 24 hour endpoint M gene copy numbers in the primary EREC culture system mirrored viral nasal shedding seen in the *in vivo* pony challenge. More specifically, we demonstrated restricted infectivity of a contemporary canine influenza A isolate in the EREC AFI culture and in ponies compared to efficient infection of both *in vitro* and *in vivo* models using a contemporary equine influenza isolate as a positive control.

In summary, we have developed an *in vitro* equine respiratory epithelial cell culture model that is morphologically similar to the equine airway epithelium, retains several key immunological properties, and supports viral infection and replication. In the future this model will be used to study equine respiratory viral pathogenesis and cell-to-cell interactions.

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DEDICATION

To: My family, you know who you are, for all of your sacrifices and support in helping me along this journey

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CHAPTER 1: INTRODUCTION

Equine respiratory disease

Equine influenza virus (EIV) and equine herpesviruses (EHV-1) are the main causes of viral respiratory disease in equids.[1-5] EHV-1 is ubiquitous among horse populations with seasonal epidemics. Incidences of EHV-1 appear to increase during higher foaling periods and in winter through early spring.[6,7] EIV is endemic in horses with epidemics occurring during horse racing events.[8-10] Despite vaccination efforts, EIV and EHV-1 continue to have serious health and economic consequences for the equine industry.[5,11-13] Effective vaccination against EIV and EHV-1 is strongly dependent on the ability of vaccines to induce long-term protective immunity.[14-16] For EIV, protective immunity has been shown to be associated with robust humoral immune responses against influenza hemagglutinin and neuraminidase glycoproteins.[17] Moreover, protection against EIV is correlated with the antigenic relatedness of the vaccine strain to circulating isolates.[18,19] However, antigenic drift has had considerable adverse affects on the efficacy of EIV vaccines.[19] For EHV-1, protection is directly correlated with a cytotoxic T-lymphocyte response.[20-22] There's evidence that EHV-1 evades this response through the down-modulation of major histocompatibility complex (MHC) class I[23] making the development of an effective vaccine a challenging task. While much progress has been made in understanding the adaptive immune response to EIV and EHV-1,[15,24] the innate

and early immune responses at the initial site of viral infection and replication remain poorly characterized. As respiratory epithelium is the primary site of infection and replication for EIV and EHV-1, studying the initial immune response to these viruses at the epithelial cell barrier is critical for a better understanding of host-pathogen interactions at this site, and how this interaction affects further downstream events including adaptive immune responses.

Innate and early immunity

Innate immunity encompasses the earliest forms of defense against pathogen invasion by initiating an immediate response to infection. This involves the recognition of pathogens by endogenous, preformed, pathogen recognition receptors that subsequently lead to the activation of effector molecules and effector mechanisms. These pattern recognition receptors (PRRs) include toll-like receptors, nod-like receptors, and RNA helicases that function to recognize pathogen associated molecular patterns (PAMPs) subsequently mediating pathogen clearance.[25-27] Toll-like receptors (TLRs) are a unique family of PRRs. They are highly conserved type I transmembrane proteins first discovered in *Drosophila melanogaster*.[28] Structurally, TLRs contain an intracytoplasmic domain that is shared with members of the interleukin-1 receptor family.[29] The extracellular domain of TLRs contain amino terminal leucine rich repeats,[29] which are likely involved directly in PAMP recognition. Currently, thirteen TLRs have been characterized in humans, ten in bovines, and as many as sixteen TLRs have been described in certain species of fish.[30] As PRRs are broadly specific, they are able to recognize various PAMPs (Table 1.1).

Table 1.1. PRRs, cellular localization and their known associated pathogen ligands

PRR	Cellular location	PAMP recognition	Reference
TLR 1/2	Plasma membrane	Triacylated lipopeptides	[31,32]
TLR 2/6	Plasma membrane	Diacylated lipopeptides, viral envelope proteins, lipoprotein, peptidoglycan, lipoarabinomannan, zymosan	[31,32]
TLR 3	Endosome	Viral double stranded RNA	[31,32]
TLR 4	Plasma membrane	Lipopolysaccharide, viral envelope protein	[31,32]
TLR 5	Plasma membrane	Bacterial flagellin	[31,32]
TLR 7/8	Endosome	Viral single stranded RNA	[31,32]
TLR 9	Endosome	Double stranded DNA viruses, unmethylated CpG DNA	[31,32]
TLR 10	Plasma membrane	Unknown	[33]
TLR 11	Plasma membrane	Uropathogenic bacteria, profilin	[32]
TLR 12	Plasma membrane	Unknown	
TLR 13	Plasma membrane	Unknown	
Dectin-1	Plasma membrane	Zymosan	[34]
NOD 1 & NOD 2	Cytosol	Peptidoglycan, diaminopimelic acid (NOD1) & muramyl dipeptide (NOD2)	[35,36]
RNA helicases	Cytosol	Viral double stranded RNA	[37]

As key components of innate immunity, TLRs and the complement system can be activated rapidly to act as mediators during the transition from innate to adaptive immunity.[38] Often studied as separate entities, it has become increasingly clear that crosstalk may occur between TLRs and the complement system, in which TLR agonists

activate complement in addition to initiating TLR signaling.[39,40] Several cells of the innate immune response express PRRs including macrophages, dendritic cells, natural killer cells, mast cells, neutrophils, eosinophils and epithelial cells.[41,42]

Recognition of PAMPS by PRRs induces a signaling cascade that activates transcription factors leading to the production and regulation of proinflammatory cytokines, chemokines, and antimicrobial peptides as well as an upregulation of costimulatory molecules, and recruitment of professional immune cells.[43-45] Cytokines and chemokines are regulatory proteins involved in an intricate network that interact with receptors on cell surfaces facilitating cell-to-cell signaling and communication. Cell responses to cytokine and chemokine signaling usually occur in an autocrine (within the cell) and paracrine (on adjacent cells) manner and occasionally in an endocrine (on distant cells) fashion. As a result, cytokine and chemokines are potent effector molecules important in regulating and shaping downstream adaptive immune responses.

In addition to cytokine and chemokine production, signaling through TLRs also results in the expression of antimicrobial peptides classically associated with mucosal surfaces. Antimicrobial peptides (AMPs) are small proteins with demonstrated antimicrobial and antiviral activity and are fundamental components of innate immunity.[48] They inhabit several physiologic locations and are broadly classified according to their structure (Table 1.2). While mechanisms of action vary depending on the AMP, they generally exert inhibitory activity through electrostatic interactions with bacterial cytoplasmic membranes and viral envelope proteins.[56-59] AMPs have also been demonstrated to direct monocyte differentiation toward macrophages through granulocyte- macrophage colony-stimulating factor (GM-CSF) regulation.[43]

Table 1.2. Classes of antimicrobial peptides

AMPs	Structure properties	Known sources	Physiologic location	Reference
Cathelicidins, lactoferrins	Cationic, β-turn	Humans	Blood, saliva, airway epithelium	49,50
α-β-θ-defensin	Cationic, anionic, disulfide bonds, cysteine residues	Mammals, birds, reptiles, plants	Ubiquitous	49
SLPI	Cationic, disulfide bonds, cysteine residues	Humans, mammals	Airway epithelium, intestinal epithelium, skin, liver, kidney, saliva	51,52
MxA, PKR	GTPase, leucine zipper, serine/threonine kinase	Humans, mammals	Ubiquitous	53,54
2'5' OAS/RNaseL	Nucleotidyl-transferase enzymes, polymerase β-sheet domain	Humans, mammals	Ubiquitous	55
Maximin, Dermicidin	Anionic	Humans, amphibians	Airway epithelium	49

In addition they were demonstrated to activate monocyte-derived dendritic cells and modulate IFN-γ production in antigen-presenting cells suggesting they may also play a role in the shaping of adaptive immunity.[60,61] These early innate immune responses involving the recruitment of professional immune cells through cytokine signaling can last up to 4 days before the onset of adaptive immunity.

Pathogen recognition by epithelial cells

For EIV and EHV-1, early immunity occurs at the respiratory epithelium, the initial site of host-pathogen interactions for respiratory viruses. Therefore, epithelial cells play a central role in the induction of early immunity. In addition to pathogen recognition, PRRs along the epithelial cell barrier are essential to maintaining homeostasis through the sampling of the external environment, which consists of pathogens as well as non-pathogenic commensal microorganisms.[62,63] These interactions prime and condition the immune response and facilitate epithelial cell crosstalk with underlying immune cells including antigen-sampling dendritic cells.[62] As professional immune cells are known to secrete regulatory cytokines and chemokines (Table 1.3), epithelial cells have also been demonstrated to produce IL-1, IL-3, IL-6, IL-8, IL-33, GM-CSF, MCP-1, TGF-α, TGF-β, and TNF-α supporting their role in the regulation of host immunity.[64-66] It has become clear that airway epithelial cells are able to regulate the recruitment and function of professional immune cells through the secretion of cytokines.[67] Conventional cultures of rat trachea epithelial cells were demonstrated to produce the cytokine, GM-CSF, a potent activator of macrophages, neutrophils and eosinophils.[67] Moreover, it was shown that secretion of GM-CSF by rat trachea epithelial cells triggered a proliferation of macrophages indicating that GM-CSF secreted by epithelial cells can regulate professional immune cells.[67] In addition, previous studies have demonstrated the expression of TNF-α, TGF-β, IL-8, IL-10 and IL-6, by airway epithelial cells.[72-74] Lastly, as AMPs primarily inhabit mucosal surfaces, the expression of cathelicidins, lactoferrin, β-defensins, secretory leukocyte protease inhibitor (SLPI),

maximin, dermicidin, and MxA have been demonstrated in airway epithelial cells (Table 1.2).[50,75-78]

Table 1.3. Cytokine and chemokine production in epithelial cells and other immune cells.[64-71]

Cell source	Cytokines		Others
	Type 1	Type 2	
Epithelial cell	IL-1, IL-8	IL-6, IL-10	IL-33, TGF-α, TGF-β, TNF-α, GM-CSF, MCP-1
CD4 T-cell	IFN-γ, IL-12, TNF-β	IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-21,	IL-17, GM-CSF
CD8 T cell	IL-2, IFN-γ	IL-4, IL-5, IL-10	IL-17
NK cell	IFN-γ, TNF-β		
Monocyte/macrophage	IL-12, IL-27	IL-3	GM-CSF, MCP-1
B cell	IL-12, TNF-β		
Dendritic cell	IL-12, IL-27, IFN-α, IFN-β		
Neutrophil	IL-12		
Mast cell		IL-4, IL-5, IL-6	
Eosinophil	IFN-γ	IL-4, IL-5, IL-6, IL-13, IL-10	

Antigen-presentation by epithelial cells

Major histocompatibility complexes (MHC) are essential components in facilitating pathogen clearance and function by binding and presenting short antigenic peptides on the surface of immune cells to T-cell receptors. The MHC genes are divided into three groups: MHC class I (MHC-I), MHC class II (MHC-II), and MHC class III

though only MHC-I and MHC-II are capable of antigen processing and presentation. MHC-III genes encode other immune components such as complement and cytokine components but do not encode antigen-processing molecules. MHC-I and MHC-II differ not only in antigen processing, but also in the type of T-cell for which they present peptide-MHC complexes. For example, CD8 T-cells recognize peptides processed in the cytosol, transported to the endoplasmic reticulum for additional modification and loaded onto MHC-I complexes,[79] while CD4 T-cells recognize exogenous antigenic peptides processed in intracellular vesicles and loaded onto MHC-II complexes.[80]

While all nucleated cells express MHC-I, only professional antigen-presenting cells such as dendritic cells, macrophages, and B-cells are known to express MHC-II. In addition, certain types of epithelial cells are known to process and present antigen through MHC-II presentation, namely thymic epithelial cells,[81,82] intestinal epithelial cells,[83,84] and renal tubular epithelial cells.[85-87] However, little information exists on whether airway epithelial cells express MHC-II and are capable of processing and presenting antigen to professional immune cells. In fact, dendritic cells have been regarded as the antigen-presenting cell of the airways. Recently, a study demonstrated increased surface expression of MHC-II in immortalized human bronchial epithelial cells in response to stimulation with organic particulate matter[88] suggesting natural airway epithelial cells may also function as professional antigen-presenting cells providing an opportunity for investigation into this novel area of research.

Epithelial cell barrier

The mucosal defense at the respiratory epithelium comprises physical, chemical, and microbiological barriers to evade pathogens. Examples of physical barriers include tight junctions, adherens junctions, and mucociliary clearance. Tight junctions and adherens junctions function to regulate solute transport and cell permeability while forming a barrier from the external environment.[89] Respiratory epithelium is a unique environment comprised of ciliated pseudostratified columnar epithelial cells, secretory cells such as Goblet cells, and basal cells that function to maintain airway homeostasis while providing protection from antigens brought in from the external environment through respiration.[26]

As resident structural cells, airway epithelial cells lie at an interface that separates the external and internal environments of the respiratory tract.[26] Mucociliary clearance is an important physical barrier in airway epithelia that involves the coating of pathogen and debris with mucin proteins preventing them from adhering to the epithelium and moving them out of the airway through the movement of cilia.[26] In addition, respiratory mucus is a complex array of highly charged mucin glycoproteins produced by secretory cells that function in preventing dehydration of the apical surface of the airway epithelial cell, as well as harboring host protective proteins.[26]

Fatty acids, enzymes, and antimicrobial peptides are examples of chemical barriers that are important components of innate immunity and can exhibit direct activity on pathogens resulting in limited growth, reduced pathogen entry into host cells, and a decrease in viral replication.[90-92] Microbiological barriers include commensal

microorganisms of the normal flora lining epithelial surfaces that compete with invading pathogens for space and nutrients.

Models of airway epithelium

Our lack of understanding of innate and early immunity at the primary site of infection and replication or airway pathogens is partly the result of a lack of a suitable *in vitro* model. Earlier conventional models of respiratory epithelial cell cultures were established by fully immersing cells in media.[96-98] One limitation to this method is the loss of respiratory epithelial cell differentiation over time.[99,100] In contrast, the development of a biphasic hamster airway epithelial cell culture model provided a powerful new technique to culture airway epithelial cells that re-established and maintained characteristics of cell differentiation.[101] Airway epithelial cell culture models grown at the air-fluid interface (AFI) have since been developed for other species including human, rat, mouse, bovine, and swine as a tool to study allergy and infectious disease.[102-107] Recently, successful culture of differentiated equine bronchial epithelial cells cultured at the AFI has been described.[108] Morphologically, respiratory epithelial cells cultured at the AFI appear to mimic the natural airway epithelium as a heterogeneous population of ciliated cells, basal cells, and secretory cells generating a pseudo-stratified mucociliary epithelium contrary to what is seen with respiratory epithelial cells cultured submerged in liquid.[109,110] In addition, secretion of mucin proteins has been characterized in human, rat, and equine differentiated respiratory epithelial cell cultures grown at the AFI.[108,109,111] Moreover, tight junction formation characteristic to epithelial cells and responsible for creating a physical barrier has also

been demonstrated through transepithelial electrical resistance (TEER) measurements.[109,112] Finally, respiratory epithelial cells cultured at the AFI have been demonstrated to support viral infection and replication supporting their use as a tool to study host-pathogen interactions at the respiratory epithelial barrier.[113,114] While respiratory epithelial cells cultured at the AFI are known to mimic the natural airway epithelium and support viral replication, no information exists on how these culture compare immunologically to the equine airway epithelium after several weeks of *in vitro* growth conditions.

Research goal

The goal of this project was to establish and characterize a primary equine respiratory epithelial cell culture system in terms of morphological and immunological properties, to study host-pathogen interactions at the epithelial cell barrier to equine viral respiratory diseases. The first paper outlines the morphological features of ERECs cultured at the AFI after four weeks with respect to tight junction formation, production of mucus, and development of ciliated epithelial cells. Moreover, since EREC AFI cultures are grown *in vitro*, immunological properties including expression of toll-like receptors and antimicrobial peptides are described and compared to natural airway epithelium and isolated uncultured epithelial cells. MHC-I and MHC-II surface expression in ERECs cultured at the AFI is compared to isolated uncultured ERECs. In the second paper, ERECs cultured at the AFI were infected with H3N8 canine and equine influenza A viruses and compared to an *in vivo* experimental challenge using these same influenza isolates as a way to support the use of this model. This study demonstrated

successful infection of ERECs cultured at the AFI and viral infection and replication characteristics that mirrored the *in vivo* challenge study.

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CHAPTER 2

IMMUNOLOGICAL CHARACTERIZATION OF THE EQUINE RESPIRATORY TRACT AND OF A PRIMARY EQUINE AIRWAY EPITHELIAL CELL CULTURE MODEL

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ABSTRACT

Currently, our understanding of innate immunity within the equine respiratory tract is limited. As the first interface to undergo pathogen invasion, the respiratory epithelium plays a key role in the immediate defense as well as the shaping of the adaptive immune response. For this reason, we examined the innate immune characteristics of the equine respiratory tract and compared them to an *in vitro* equine respiratory epithelial cell model cultured at the air-fluid interface (AFI). Respiratory epithelial tissues, isolated epithelial

cells, and four-week old cultured, differentiated airway epithelial cells collected from six locations of the equine respiratory tract were examined for the expression of toll-like receptors (TLRs) and antimicrobial peptides (AMPs) using conventional polymerase chain reaction (PCR). Cultured, differentiated, respiratory epithelial cells and freshly isolated respiratory epithelial cells were also examined for the expression of TLR3, TLR9 and major histocompatibility complex (MHC) class I and class II using fluorescence-activated cell sorting (FACS) analysis. In addition, cytokine and chemokine profiles from respiratory epithelial tissues, freshly isolated respiratory epithelial cells, and cultured, differentiated, epithelial cells from the upper respiratory tract were examined using real-time PCR. We found that respiratory epithelial tissues and isolated epithelial cells expressed TLRs 1-4 and 6-10 as well as AMPs, MxA, 2'5' OAS, β-defensin-1, and lactoferrin. In contrast, TLRs 8-10 and lactoferrin were no longer detected in epithelial cells cultured at the AFI after four weeks compared to respiratory tissues and freshly isolated epithelial cells. In addition, MHC-I and MHC-II surface expression decreased in epithelial cells cultured at the AFI compared to isolated epithelial cells, whereas TLR3 and TLR9 were expressed at similar levels. Lastly, we found that after four weeks of *in vitro* growth conditions, equine respiratory epithelial cells cultured at the AFI expressed granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-10, IL-8, TGF-β, TNF-α, and IL-6. In summary, we have developed an *in vitro* equine respiratory epithelial cell culture model that is morphologically similar to the equine airway epithelium and retains several key immunological properties. In the future this model will be used to study equine respiratory viral pathogenesis and cell-to-cell interactions.

ABBREVIATIONS

AFI	Air-fluid interface
FACS	Fluorescence-activated cell sorting
TLR	Toll-like receptor
AMP	Antimicrobial peptide
EIV	Equine influenza virus
EHV-1	Equine herpesvirus-1
EREC	Equine respiratory epithelial cell
PRR	Pattern recognition receptor
PAMP	Pathogen-associated molecular pattern
GM-CSF	Granulocyte-macrophage colony-stimulating factor
MCP-1	Monocyte chemoattractant protein-1
TEER	Transepithelial electrical resistance

INTRODUCTION

Respiratory pathogens such as equine influenza (EIV) and equine herpesvirus-1 (EHV-1) continue to have serious health and economic impacts on the equine industry,[1,2] despite vaccination. While in recent years we have made progress understanding the adaptive immune response to these common respiratory pathogens,[2,3] the innate immune response remains poorly characterized.

Elucidating the innate immune response to these viruses is important, as the early events following infection not only determine whether the virus can establish infection but also set the stage for downstream adaptive immune responses. Initiation of innate immunity relies on the activation of germline encoded pattern recognition receptors (PRRs) through the recognition of pathogen-associated molecular patterns (PAMPs).[4] Toll-like receptors (TLRs) are classic PRRs that are found on immune cells as well as mucosal epithelia exposed to invading pathogens. Thirteen mammalian TLRs have been identified of which TLRs 2-4 and 7-9 have been identified in horses.[4-6] These TLRs function to recognize various pathogen ligands including lipopolysaccharides, diacylated and triacylated lipopeptides, flagellin, single-stranded and double stranded RNA, and CpG motifs among others.[4,5] Recognition of invading pathogens by PRRs results in a signaling cascade that leads to the production of cytokines, chemokines, and antimicrobial peptides (AMPs). Cytokines and chemokines are key mediators that initiate immediate immune responses and ultimately shape the adaptive immune response via chemoattraction, activation of immune cells, and up-regulation of co-stimulatory molecules for antigen presentation.[4] Antimicrobial peptides are small proteins with

demonstrated antimicrobial and antiviral activity.[7,8] Moreover, AMPs have been shown to activate monocyte-derived dendritic cells and modulate IFN-γ production in antigen-presenting cells suggesting they may also play a role in the shaping of adaptive immunity.[9,10]

For respiratory pathogens, including EHV-1 and EIV, the respiratory epithelium is the first to encounter and interact with invading pathogens and initiate early immune responses. In recent years it has become evident that as this first line of defense, epithelial cells are multifunctional, playing an important role in immunity in addition to providing a physical barrier.[11] Epithelial cells have been shown to express TLRs, secrete antimicrobial peptides, cytokines and chemokines, and even present antigen.[11-13] In addition, innate immune recognition by the epithelial cell barrier largely determines the functional properties of resident tissue macrophages and dendritic cells, thus driving the outcome of antigen-specific immunity.[11,14] While respiratory epithelia of many mammalian species have been studied in recent years and have been found to express TLRs, AMPs, cytokines and MHC class I and II molecules,[11,13,15] equine immunity at the respiratory epithelial cell barrier remains poorly described.

Investigation of this important area has been hindered by a lack of a suitable *in vitro* model. Conventional models of respiratory epithelial cell cultures were established by fully immersing cells in media.[16,17] One limitation to this culture method is the loss of respiratory epithelial cell differentiation over time.[18,19] To address this limitation, a biphasic hamster airway epithelial cell culture model provided a powerful new technique to culture airway epithelial cells that maintained characteristics of cell differentiation.[20] Airway epithelial cell culture models grown at the air-fluid interface (AFI) have since

been developed for other species as a tool to study allergy and infectious disease.[21-23] More recently, successful culture of differentiated equine bronchial epithelial cells cultured at the AFI has been described.[24] Morphologically, respiratory epithelial cells cultured at the AFI appear to mimic the equine airway epithelium as a heterogeneous population of ciliated cells, basal cells, and secretory cells generating a pseudo-stratified mucociliary epithelium contrary to what is seen with respiratory epithelial cells cultured submerged.[25] In addition, secretion of mucin proteins has been characterized in differentiated respiratory epithelial cell cultures grown at the AFI.[24,25] Tight junction formation, characteristic of epithelial cells and responsible for creating a physical barrier, can be demonstrated by transepithelial electrical resistance (TEER) measurement.[25] Finally, these cultures have been demonstrated to support viral infection and replication.[26,27]

Despite evidence that respiratory epithelial cells grown at the AFI mimic the equine airway epithelium morphologically, little information exists as to how these cultures perform immunologically. So far, toll-like receptor and antimicrobial peptide expression has only been described in human airway epithelial cells cultured at the AFI.[28] While constitutive cytokine expression has been demonstrated in respiratory epithelial tissues and conventional primary respiratory epithelial cell cultures,[11] no such information is available for differentiated airway epithelial cells cultured at the AFI. The goals of this study were to establish baseline immunological characteristics in equine respiratory epithelial cells cultured at the AFI and compare them to the equine airway epithelium. In the future this model will be used study host immunity to respiratory

pathogens including EIV and EHV-1 and may open pivotal new avenues for the development of novel preventative and therapeutic strategies.

METHODS

Animals, epithelial tissue collection, and processing

Respiratory epithelium was collected from three horses with no apparent signs of respiratory disease. The horses consisted of a 2 year old mixed breed stallion, a 5 year old Morgan gelding, and an 8 year old Quarter Horse mare. Horses were humanely euthanized intravenously by administration of an overdose of 390 mg/mL of pentobarbital (Buthanasia) for medical reasons unrelated to any respiratory condition. For each horse, a large section of epithelium was collected from the larynx and two tracheal rings were collected from the upper, middle and lower trachea. Bronchial rings from the left and right primary bronchi were collected distal to the carina of the trachea. A large section of lung was pulled from the center of the right or left lobe. For RNA isolation, respiratory tissues were collected in cold phosphate buffered saline (PBS; Cellgro, Mediatech Inc., Manassas, VA) and 200 mg tissue sections were excised and snap-frozen in liquid nitrogen. For epithelial cell isolation, respiratory tissues were collected from the same locations in cold Dulbecco's Modified Eagle Medium supplemented with Ham's F12 nutrient mixture (DMEM/F12) (Gibco, Invitrogen, Carlsbad, CA) for further processing. Procedures and experimental protocols followed the animal care guidelines of the Animal Care and Use Committee, Colorado State University.

Isolation and culture of primary equine respiratory epithelial cells

Isolation and culture of primary respiratory epithelial cells were performed as previously described.[25] Briefly, equine epithelial tissues were washed in PBS to remove red blood cells. Following enzymatic digestion in 1.4% pronase (Roche Applied Science, Indianapolis, IN) and 0.1% deoxyribonuclease I (Sigma-Aldrich, St. Louis, MO), epithelial cells from each location were harvested using gentle agitation in calcium and magnesium free minimal essential medium for 48 hours. Epithelial cells were then incubated in a plastic uncoated petri dish for two hours to reduce fibroblast contamination by adherence. Isolated ERECs were stored in liquid nitrogen at a density of 2 million cells per cryovial until further use. For culture at the air-fluid interface, primary equine respiratory epithelial cells (ERECs) were seeded into Type IV collagen (Sigma-Aldrich) coated transwell cell culture wells (Costar, Corning, Fisher Scientific, Fair Lawn, NJ) in DMEM/F12, containing 2% Ultroser G (Pall Life Sciences, Pall Corp., Cergy, France), 1% penicillin-streptomycin (Gibco, Invitrogen, Carlsbad, CA) and 0.5% amphotericin B (Biowhittaker, Walkersville, MD). ERECs were incubated in a 37°C, 5% CO_2, humidified incubator until differentiated. Factors that determined the point of differentiation included tight junction formation, mucin production, and cilia development. A transepithelial electrical resistance (TEER) voltometer (EVOM, World Precision Instruments, Sarasota, FL) was used to determine the presence of tight junctions. A positive control for TEER measurements provided by the manufacturer was used for comparison. The presence of ciliated epithelial cells was assessed microscopically following formalin-fixation, paraffin-embedding, and routine hematoxylin and eosin staining.

RNA isolation and Polymerase Chain Reaction

Total RNA was extracted from tissues through homogenization on ice using an Omni™ hand-held tissue homogenizer (Omni international, Kennesaw, GA) in TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was isolated from fresh, uncultured ERECs or ERECs cultured at the AFI by adding 1mL TRIzol to a tube containing 1 x 10^6 cells per location, or directly onto the transwell insert, respectively. Total RNA was extracted from homogenized airway tissues, spleen, mesenteric lymph node, isolated, uncultured ERECs, and four-week epithelial cell AFI cultures using a Qiagen RNeasy Minikit (Qiagen, Hilden, Germany) following the manufacturers instructions and treated with deoxyribonuclease (Sigma-Aldrich, St. Louis, MO) to eliminate genomic DNA contamination. RNA isolated from spleen and mesenteric lymph node was used as positive control tissues for the mRNA expression of TLR9. RNA quality and quantity was determined using spectrophotometry. Reverse transcriptase PCR (RT-PCR) of 1μg of RNA was performed using the iScript ™ cDNA synthesis kit (Bio Rad, Hercules, CA) in a total volume of 20μl following the manufacturers recommended incubation conditions (5 min at 25°C, 30 min at 42°C, 5 min at 85°C). Controls consisting of reactions without the addition of reverse transcriptase were included for each sample to detect genomic contamination. All cDNA samples were stored at -20°C until further use.

For detection of TLR and AMP expression, sequence-specific primers were designed based on published and predicted equine DNA sequences from the NCBI database using Primer 3 Input software version 4.0 (http://frodo.wi.mit.edu/primer3/) (Table 2.1). For each reaction, 3 μl of cDNA was amplified in a 25 μl standard reaction using 20 μl Platinum PCR SuperMix (Invitrogen, Carlsbad, CA) and 2 μl of forward and

reverse primers at the following cycling conditions: 35 cycles of 94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 1 min. All reactions included β-actin as a housekeeping gene. PCR products were stained using EZvision Three™ fluorescent dye (Amresco, Solon, Ohio) and analyzed on a 2% agarose gel. PCR reaction products were purified for sequencing using Phenol:Chloroform (Fisher Scientific, Fair Lawn, NJ) followed by standard ethanol precipitation and sequenced at the Proteomics and Metabolomics Core Facility at Colorado State University using an ABI 3130 Genetic Analyzer. All sequenced PCR products were confirmed using DNASTAR Lasergene software (DNASTAR, Inc, Madison, WI).

Table 2.1. Primer design (5' to 3') for respective equine toll-like receptors and antimicrobial peptides.

Name	Forward	Reverse	Product (bp)	Reference
TLR1	actttgcccaccacaatctc	ccaaaagcagcaacagtgaa	275	
TLR2	acggcagctgtgaaaagtct	cctgaaccaggaggacgata	213	
TLR3	acctcccagcaaacataacg	ctggaggtccaaaatttcca	179	
TLR4	gacgactcaggaaagccttg	cacaatgcctggtatgttgc	194	
TLR6	tcagaccccattagacagcc	caagtaccttgaccctggga	640	
TLR7	atcttgacgcctctcatgct	ggaatgtccgtcaaatgctt	249	
TLR8	ggaatctgacacggcttgat	agaaggcaggtgggaaatct	272	
TLR9	gtgactggctacctggcaagac	tggttatagaagtggcggttgtcc	373	[29]
TLR10	gcttgccccaaagtattcaa	aagtggaggcagcagaaagt	484	
β-actin	ggcatcctgaccctcaagta	ggggtgttgaaggtctcaaa	203	
β-defensin 1	ttaagctcaccagccatcag	ctgtcacagcagtttctccg	243	
MxA	agagtcctcgatggcagaaa	tgagacagagagcccgattt	298	[30]
2'5' OAS	agacagcgaggacgacactt	cttctcaccaggcacacaga	444	[31]
Lactoferrin	caaagactctgccctggt	ggagcctgtctggttgaaga	541	

Flow cytometry

Epithelial cell purity in both freshly isolated ERECs and 4 week ERECs cultured at the AFI were estimated by permeabilizing with 1% Saponin (Fluka, Biochemika, Sigma-Aldrich, St. Louis, MO) and staining the cells for two hours at room temperature with a pan-anticytokeratin antibody (Zymed, Invitrogen, Carlsbad, CA; 5 μg/ml) followed by a secondary fragment goat anti-mouse IgG antibody conjugated with fluorescein (3 μg/mL) (Jackson Laboratories, West Grove, PA) and analyzed using FACS. Expression of TLRs 3 and 9 in freshly isolated ERECs and 4 week ERECs cultured at the AFI were determined by incubating 1 x 10^6 cells for two hours at room temperature in 5μg/ml of either mouse anti-human TLR 3 or TLR 9 conjugated with Alexa-Fluor 647 (Imgenex, San Diego, CA). MHC-I and MHC-II expression was examined using anti equine MHC-I and MHC-II (CVS 22 and CVS 10) monoclonal antibodies.[32] One million cells were incubated with either CVS10 or CVS22 for 45 minutes at 4°C followed by incubation with a secondary fragment goat anti-mouse IgG antibody (Jackson Laboratories, West Grove, PA) conjugated with allophycocyanin (0.1μg/mL) for 30 minutes at room temperature and analyzed by flow cytometry.

Cytokine/Chemokine Analysis

Cytokines to be examined were selected based on what has been reported for epithelial cytokine panels in the literature[12,33] in addition to those important in innate homeostatic regulation and anti-viral defense. These cytokines included IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, TGF-β, IFN-α, IFN-β, GM-CSF and MCP-1. Cytokine and chemokine expression was compared between epithelial tissues, freshly isolated,

uncultured ERECs, and ERECs grown at AFI. RNA was isolated and 1µg of RNA was reverse transcribed as described above. Samples of cDNA and their respective no-RT controls were analyzed using previously established real-time PCR assays (http://www.ca.uky.edu/Gluck/HorohovDW_EIRClonedCytokines.asp). A standard housekeeping gene (β-gus) was used as an internal control. Relative expression of each gene was determined by calculating the 2(-Delta Delta C(T)) values using average delta CT values of equine airway tissue samples as calibrators.[34]

Statistical Analyses

All values for the expression MHC-I, MHC-II, TLR3, and TLR9 determined by FACS staining and analyses are expressed as mean percent ± standard errors of the mean (SEM). Mann-Whitney tests were used to determine differences in the mean percent expression of MHC-1, MHC-II, TLR3, and TLR9 between isolated ERECs, and ERECs cultured at the AFI. Kruskal-Wallis rank sum tests were used to determine differences in the mean percent expression of MHC-I, MHC-II, TLR3, and TLR9 from FACS staining between five different locations of the equine respiratory tract in isolated ERECs, and EREC AFI cultures. Means were considered significantly different at $P \leq 0.05$.

Kruskal-Wallis rank sum tests were also used to evaluate differences in the mRNA cytokine and chemokine expression from real-time PCR between equine airway tissue and ERECs cultured at the AFI after four weeks. Differences in mean relative expression of cytokines and chemokines between EREC AFI cultures and equine airway tissues were considered significantly different when $P \leq 0.05$.

RESULTS

Equine respiratory epithelial cell differentiation

Differentiation of respiratory epithelial cells was defined as cultures exhibiting ciliated epithelial cells, mucin production, and tight junction formation. AFI cultures of epithelial cells from locations throughout the respiratory tract of three horses displayed consistent differentiation by week four. Representative images from hematoxylin and eosin stains of the larynx, upper trachea, lower trachea and bronchus are shown in figure 2.1. Cultures featured ciliated epithelial cells, non-ciliated epithelial cells and basal cells. Additionally, mucin production was clearly visible on the apical surface of the cells and tight-junction formation was demonstrated by mean TEER measurements of 267 Ω-cm^2 when compared to a positive control (167 Ω-cm^2).

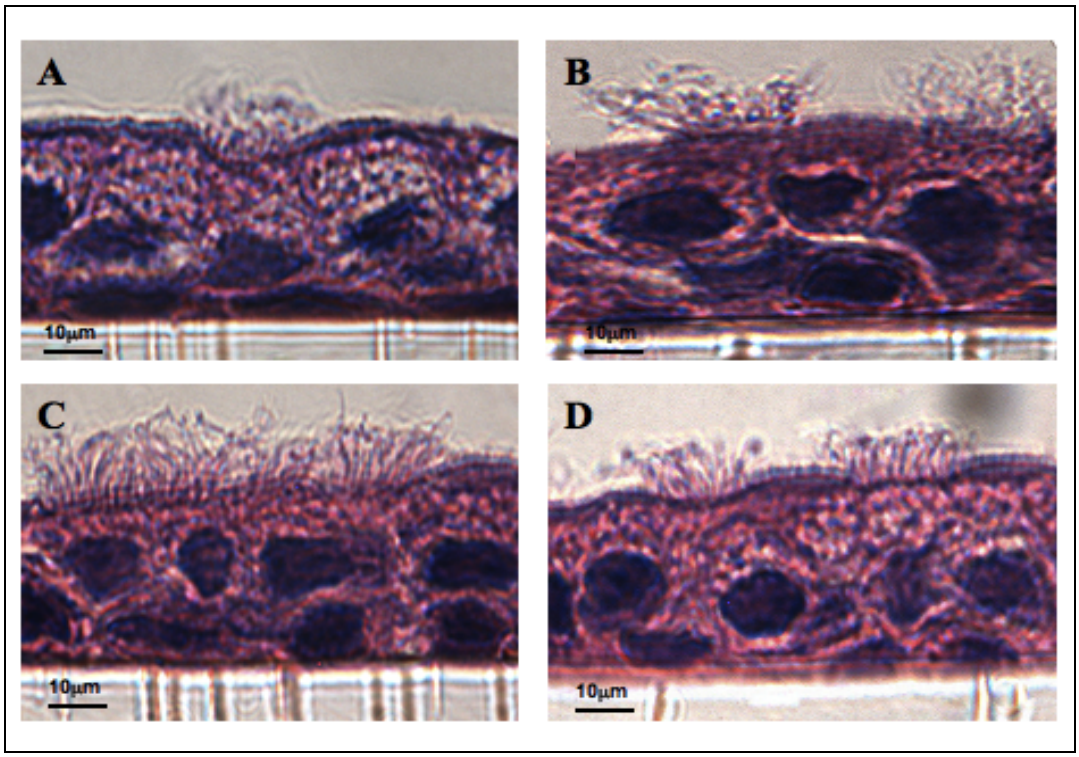

Figure 2.1. Hematoxylin and eosin staining showing morphological appearance of ciliated pseudostratified ERECs cultured at the AFI from the (A) larynx, (B) upper trachea, (C) lower trachea, and (D) bronchus. Images from middle trachea are not shown but mirrored images from the other locations.

Expression of TLRs and AMPs in equine airway tissues

Expression mRNA specific for TLRs 1-4, 6-8 and 10 and the antimicrobial peptides beta-defensin 1, MxA, the 2'5' oligoadenylate synthase-like (2'5' OAS) molecule and lactoferrin was detected in homogenized airway tissues of the larynx, upper, middle, and lower trachea as well as the bronchus of three different horses. A representative gel for these results is shown in figure 2.2A. In addition, expression of equine TLR9 mRNA was detected in airway tissue epithelium from all locations of the respiratory tract, as well as in spleen and mesenteric lymph node (Figure 2.2B). However, the band intensity for TLR9 was low for all locations of the airway epithelium as well as the spleen tissue compared to mesenteric lymph node tissue. Sequencing of PCR products

confirmed the identities of all respective TLRs and AMPs through comparison of each respective sequence to those published in the database of the National Center for Biotechnology Information.

Immunological characteristics of isolated ERECs

TLR and AMP expression was evaluated in epithelial cells harvested from tissue epithelium to evaluate the effects of enzymatic digestion and processing on their immunological properties. There were no differences in TLR and AMP expression between equine airway epithelium and isolated ERECs, therefore, PCR gels from both equine airway epithelial tissue and isolated ERECs can be depicted in Figure 2.2. In addition to mRNA expression, TLR3 and TLR9 protein expression was also demonstrated throughout several locations of the respiratory tract in isolated epithelial cells using fluorescence-activated cell sorting (FACS) staining and analysis (Table 2.2).

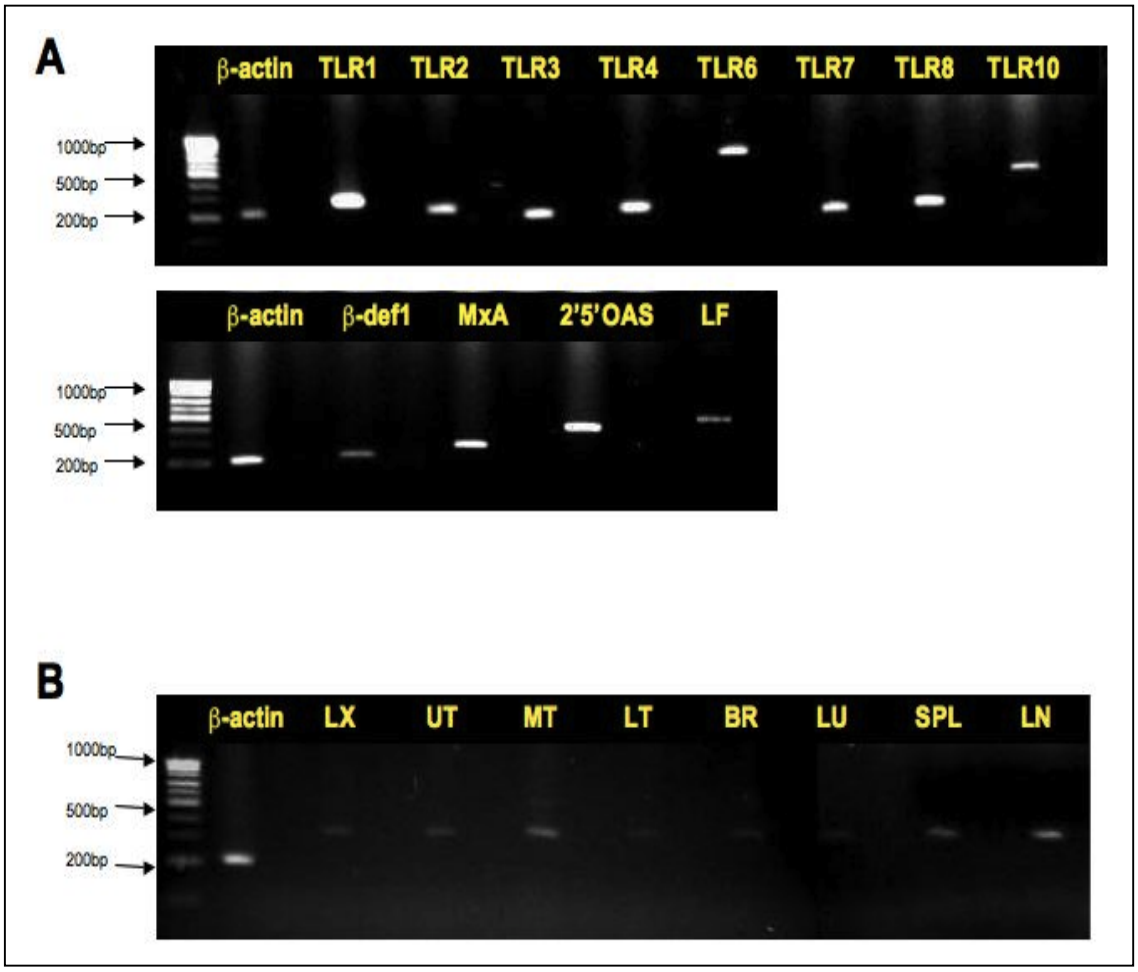

Figure 2.2. A) Top: mRNA expression of TLRs 1-4, TLRs 6-8 and TLR 10 in equine bronchus epithelium; bottom: mRNA expression of beta-defensin 1, MxA , 2'5' OAS and lactoferrin. Beta-actin was the housekeeping gene used as an internal control. Results were consistent for all locations of the respiratory tract in three horses; therefore, the gels depicted are representative. B) mRNA expression of TLR 9 in the epithelial tissue of the larynx, upper, middle, and lower trachea, as well as the bronchus of one horse. Spleen and mesenteric lymph node were incorporated as controls. Results were consistent for all three horses examined.

Table 2.2. Comparison of TLR3 and TLR9 between isolated ERECs and ERECs cultured at the AFI throughout the respiratory tract using FACS staining and analysis. Data is shown as mean percent of cells (± SEM) that stain positive for TLR3 and TLR9.

	TLR9		TLR3	
	Isolated ERECs	EREC AFI	Isolated ERECs	EREC AFI
Larynx[ab] (n=3)	28.2 ± 9.3	37.9 ± 6.9	11.4 ± 5.7	39.9 ± 8.8
Upper trachea[b] (n=3)	15.3 ± 4.4	37.2 ± 7.6	2.0 ± 1.2	35.3 ± 15.6
Middle trachea (n=4)	24.7 ± 4.8	34.6 ± 7.5	5.0 ± 1.7	26.9 ± 11.9
Lower trachea (n=4)	28.1 ± 8.4	32.7 ± 6.4	7.0 ± 2.3	32.2 ± 10.0
Bronchus[a] (n=3)	17.6 ± 14.7	40.2 ± 0.6	5.9 ± 4.3	35.1 ± 6.0

[a]$P<0.05$ is where significant differences were observed in the expression of TLR3 between isolated ERECs and EREC AFI cultures.
[b]$P<0.05$ is where significant differences were observed in the expression of TLR9 between isolated ERECs and EREC AFI cultures.
No differences were observed in the expression of TLR3 and TLR9 between different locations of the equine respiratory tract.

Lastly, MHC-I and MHC-II expression in isolated ERECs from throughout the equine respiratory tract is shown in Table 4. MHC-I was expressed in the majority of ERECs isolated from the respiratory tract from all locations evaluated. This ranged, on average, from 86.7% ± 0.7% MHC-I expression in isolated epithelial cells of the bronchus to 96.7% ± 0.6% MHC-I expression in isolated epithelial cells of the larynx (Table 2.3). A similar pattern was observed in the expression of MHC-II where, on average, MHC-II expression in freshly isolated, uncultured ERECs ranged from 59.6% ± 6.8% in the bronchus to 81.0% ± 5.3% in the larynx (Table 2.3).

Table 2.3. Comparison of MHC-I and MHC-II between isolated epithelial cells and epithelial cells cultured at the AFI throughout the respiratory tract (n=3) using FACS staining and analysis. Data is shown as mean percent of cells (± SEM) that stain positive for MHC-I and MHC-II.

	MHC-I		MHC-II	
	Isolated ERECs	EREC AFI	Isolated ERECs	EREC AFI
Larynx[ab]	96.7 ± 0.6	56.6 ± 13.2	81.0 ± 5.3	20.6 ± 2.9
Upper trachea[ab]	97.4 ± 0.8	49.4 ± 4.8	72.8 ± 16.3	9.2 ± 4.3
Middle trachea[ab]	92.2 ± 2.8	57.8 ± 2.8	76.8 ± 6.1	17.4 ± 7.5
Lower trachea[ab]	88.0 ± 1.4	63.2 ± 3.5	64.9 ± 3.1	18.4 ± 9.5
Bronchus[ab]	86.9 ± 0.7	53.6 ± 6.8	59.6 ± 6.8	19.4 ± 12.8

[a]$P<0.05$ is where significant differences were observed in the expression of MHC-I between isolated ERECs and EREC AFI cultures.
[b]$P<0.05$ is where significant differences were observed in the expression of MHC-II between isolated ERECs and EREC AFI cultures.
No differences were observed in the expression of MHC-I and MHC-II between different locations of the equine respiratory tract.

Immunological characteristics of ERECs cultured at the AFI

EREC AFI cultures retained several of the same immunological properties characterized in equine airway tissues and isolated epithelial cells. Consistent mRNA expression was demonstrated for TLRs 1-4, TLR6 and TLR7 of three different horses (Figure 2.3A) and is summarized in Table 2.4. Interestingly, mRNA expression of TLR8, TLR9 and TLR10 was not detected despite the fact that moderate band intensity of TLR8 and TLR10 was found in the airway epithelial tissues. All antimicrobial peptides characteristic to airway tissues were detected in the AFI cultures after four weeks with the exception of lactoferrin (Figure 2.3B).

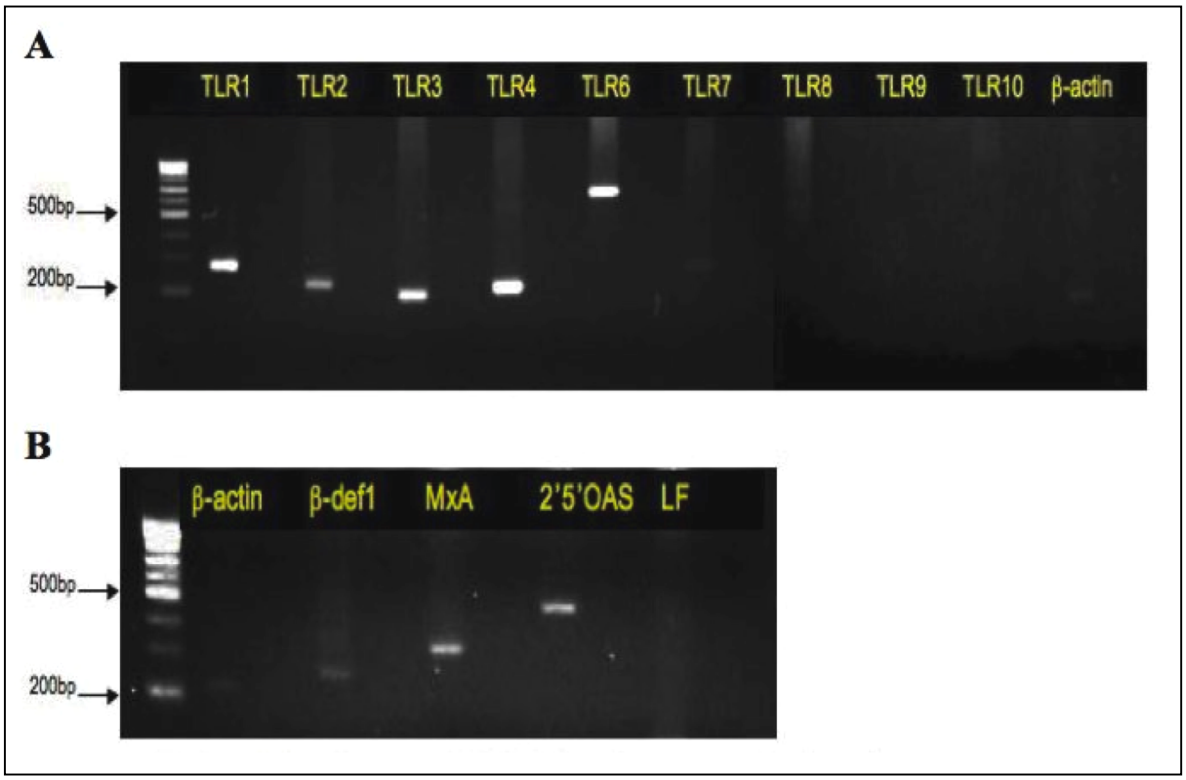

Figure 2.3. A) mRNA expression of TLRs 1-4, 6 and 7 in bronchus epithelial cells cultures at the air-fluid interface after 4 weeks and B) mRNA expression of β-defensin, MxA, and 2'5'OAS in bronchus epithelial cells cultures at the air-fluid interface after 4 weeks. Gel picture is representative for other locations of the respiratory tract cultured at the air-fluid interface from three different horses, as no differences between locations were seen.

Table 2.4. Comparison of mRNA expression of TLRs and AMPs in equine epithelial tissue, isolated ERECs, and EREC AFI cultures using conventional PCR where n=3.

	Epithelial tissues	Isolated ERECs	EREC AFI cultures
TLRs	TLRs 1-4, 6-10	TLRs 1-4, 6-10	TLRs 1-4, 6, 7
AMPs	β-def 1, MxA, 2'5' OAS, lactoferrin	β-def 1, MxA, 2'5' OAS, lactoferrin	β-def 1, MxA, 2'5' OAS

Expression of TLR3 and TLR9 protein was also demonstrated in epithelial cells cultured at the AFI throughout the respiratory tract using FACS (Table 2.2). Interestingly, TLR9 protein was detected using FACS staining and analysis in EREC AFI cultures throughout the respiratory tract despite the loss of detection for TLR9 mRNA (Figure 2.3A). While no differences were observed in the expression of TLR9 between isolated, uncultured ERECs and ERECs cultured at the AFI after four weeks, significant differences were found in the expression of TLR3 between isolated, uncultured ERECs, and EREC AFI cultures from the upper trachea (P=0.023) and the bronchus (P=0.034).

Differences were also observed in the expression of MHC-I and MHC-II between isolated, uncultured ERECs, and ERECs grown at the AFI from different locations of the equine respiratory tract with the exception of the upper trachea (Table 2.3). After four weeks of *in vitro* growth conditions, MHC-I expression in EREC AFI cultures ranged, on average, from 50.0% ± 8.3% in the upper trachea to 63.2% ± 3.5% in the lower trachea (Table 2.3). Differences in MHC-II expression between isolated ERECs and EREC AFI cultures were even more pronounced. MHC-II expression in EREC AFI cultures ranged from 6.4% ± 5.6% in the upper trachea to 20.6% ± 2.9% in the larynx (Table 2.3).

Equine airway cytokine and chemokine expression

We found that after four weeks of *in vitro* growth conditions, ERECs cultured at the AFI expressed IL-8, IL-10, TNF-α, TGF-β, GM-CSF, and IL-6, but not IL-1, IL-12, IFN-α, IFN-β, or MCP-1 (Figure 2.4A). While constitutive cytokine expression was evaluated in EREC AFI cultures from three different horses, TNF-α and IL-10 were

detected in only two of the three cultures examined, however, the culture not secreting TNF-α and the culture not secreting IL-10 were not from the same horse. No significant differences were observed in the expression of GM-CSF, IL-10, and TGF-β, between equine airway tissue, and ERECs cultured at the AFI after four weeks. In contrast, significant differences ($P \leq 0.05$) were observed in the mRNA expression of IFN-α, IFN-β, IL-1, IL-12, IL-6, IL-8, MCP-1, and TNF-α.

Intriguingly, mRNA cytokine and chemokine expression in isolated, uncultured ERECs was higher than both the equine respiratory epithelial tissue and EREC AFI cultures for all cytokines and chemokines evaluated with the exception of IFN-α, IL-12, and MCP-1 (Figure 2.4B).

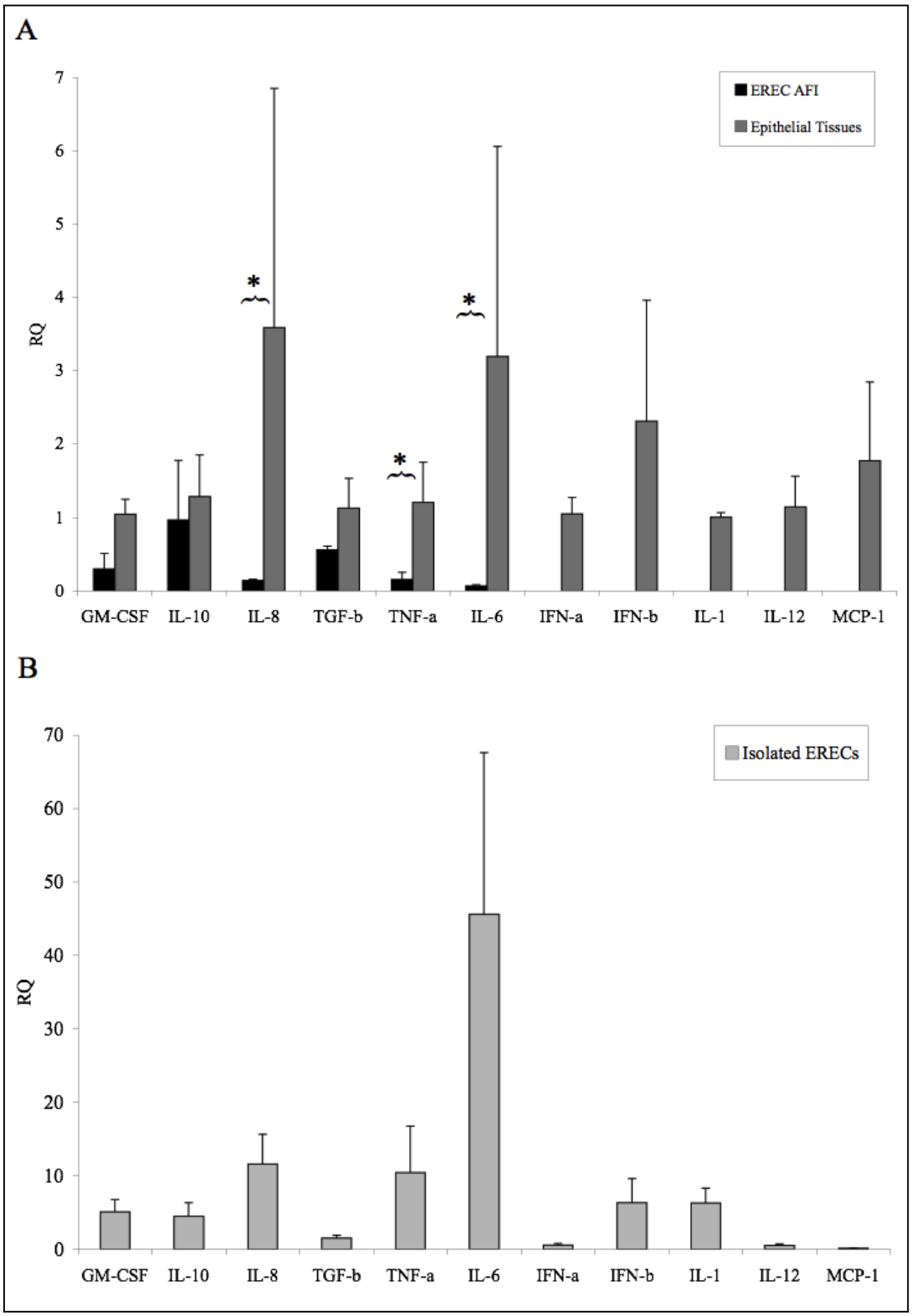

Figure 2.4. mRNA cytokine and chemokine expression from real-time PCR in A) ERECs cultured at the AFI compared to equine respiratory tissues and B) isolated ERECs where n=3 for all groups; *P ≤ 0.05.

DISCUSSION

Our study is the first to describe the development of a novel equine respiratory epithelial cell culture AFI system that is morphologically similar to the equine airway epithelium, and retains several immunological characteristics important in host immune defense after four weeks of *in vitro* growth conditions. We examined the same immunological characteristics in equine airway epithelium, freshly isolated epithelial cells and 4 week old equine respiratory epithelial cell cultures (ERECs) grown at AFI as a basis for comparison. The results of our study demonstrate that equine respiratory epithelial cell cultures grown at AFI retain mRNA expression of TLRs 1-4, and 6-10 and express TLR3 and TLR9 protein at similar levels seen in freshly isolated epithelial cells from the same location. In addition, we could demonstrate expression of several antimicrobials including β-defensin, MxA and 2'5'OAS that have the potential to serve as a defense mechanism from viral invasion. Lastly, we were able to show expression of both MHC-I and MHC-II, molecules critical for antigen presentation.

While the loss of TLR9 mRNA was not unexpected considering the low band intensity seen in the equine respiratory epithelium tissue samples and the isolated epithelial cells, it was interesting that TLR9 expression could still be demonstrated in EREC AFI cultures using FACS. In human respiratory epithelial cell lines and fully differentiated primary respiratory epithelial cells cultured at the AFI, TLR9 expression is demonstrated at low levels using quantitative real-time PCR.[35] It is possible that the

differences between our mRNA expression results and protein expression results are due to differences in assay sensitivity.

The loss of TLR8, TLR10 and lactoferrin mRNA expression is interesting and similar observations were made in a study in human lung epithelial cells that showed absence of TLR7 or TLR8.[36] A later study in human fallopian tube epithelial cells demonstrated expression of TLRs 1-9 but not 10.[37] As ERECs cultured at the AFI are grown *in vitro* for four weeks, a reduction or loss of antigenic stimulation could account for the loss of expression of these molecules. Previous studies have demonstrated the activation and up-regulation of TLRs using synthetic and non-synthetic immune modifiers.[36,38] Whether the introduction of TLR agonists restore TLR8 or TLR10 expression in the EREC AFI model needs to be examined.

Intriguingly, we also found a reduction of both MHC-I and MHC-II in ERECs cultured at the AFI after four weeks when compared to freshly isolated epithelial cells. This is an interesting observation, particularly for MHC-I, which is normally expressed on all nucleated cells. In the airway epithelium, the surface expression of MHC-I and MHC-II molecules is tightly regulated by cytokines, particularly interferons (IFN). Additionally, cytosolic peptidases are known to regulate MHC-I peptide production and a number of studies have demonstrated that over expression of the endopeptidase EP24.15 is primarily responsible for degrading MHC-I peptides resulting in the suppression of surface MHC-I expression.[39] However, this study demonstrated that IFN-γ treatments (10ng/ml) can restore MHC-I surface expression in EP24.15-overexpressing cells.[39] In the equine airway epithelium, major sources of IFN-γ include natural killer cells and T-cells, whereas fibroblasts, dendritic cells and leukocytes are major sources of IFN-α and

IFN-β. While IFN-γ production by EREC AFI cultures was not measured, IFN-α and IFN-β were examined using quantitative real-time PCR and were not detected in these cultures after four weeks of *in vitro* growth conditions suggesting that the lack of underlying immune cells and IFNs in the AFI model may account for the reduction of MHC-I, MHC-II or both.

As cytokines and chemokines are paramount in the regulation of host immunity, cytokine and chemokine mRNA expression was examined using real-time PCR in ERECs cultured at the AFI, isolated ERECs, and equine airway tissue. While constitutive cytokine expression in EREC AFI cultures was reduced when compared to whole tissues, constitutive expression of several cytokines was still demonstrated in the AFI model including those important in maintaining airway inflammation homeostasis (IL-10 and TGF-β).[40] The production of TNF-α, IL-8, IL-6, and GM-CSF was not unexpected, as previous literature has demonstrated secretion of these cytokines by airway epithelial cells.[11,41,42] Secondly, IL-12, IFN-α, and IFN-β were not detected in EREC AFI cultures after four weeks of *in vitro* growth conditions. However, epithelial cells have not been implicated as a major source for these cytokines. In contrast, the loss of IL-1 and MCP-1 in the EREC AFI cultures is remarkable as epithelial cells are robust producers of IL-1 and have been also known to produce MCP-1.[43] TLRs 1, 2, 4, and 6 are known to signal the production of IL-1 through NF-κB[4] and these TLRs were detected in our EREC AFI cultures. However, an earlier study found that unstimulated thymic epithelial cells produced negligible amounts of IL-1α and IL-β[44] suggesting ERECs cultured at the AFI might produce IL-1 upon stimulation. Therefore, a lack of antigenic stimulation and

underlying immune cells would likely account for the low expression levels of cytokines and chemokines in the EREC AFI model.

The finding that isolated, uncultured ERECs consistently produced higher levels of cytokines and chemokines with the exception of IFN-α, IL-12 and MCP-1 compared to EREC AFI cultures and equine airway tissues is interesting and suggests that these cells may have been stimulated during processing through enzymatic tissue digestion but may decrease following cell culture *in vitro*.

In summary, we have demonstrated, through the comparison of six different locations of the respiratory tract, consistent patterns in the expression of toll-like receptors, antimicrobial peptides, MHC-I, and MHC-II for ERECs cultured at the AFI, isolated ERECs, and equine airway epithelial tissue. Furthermore, we have confirmed that ERECs cultured at the AFI are morphologically similar to the equine airway epithelia and retain a number of key immunological properties after four weeks of standard laboratory growth conditions. While we can demonstrate that ERECs cultured at the AFI are immunologically competent, it appears that some of the immunological properties are altered, most likely as a consequence of sterile in vitro growth conditions and presumably as a temporary state that could be remedied by adding antigenic stimuli and/or underlying immune cells. In the future, this EREC-AFI model will be used as a tool to study equine infectious respiratory disease and cell-to-cell interactions during host-pathogen interactions.

ACKNOWLEDGEMENTS

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CHAPTER 3

RESTRICTED INFECTIVITY OF A CANINE LINEAGE H3N8 INFLUENZA A VIRUS IN PONIES AND IN PRIMARY EQUINE RESPIRATORY EPITHELIAL CELLS

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ABSTRACT

The purpose of this study was to evaluate whether an equine-derived contemporary canine H3N8 influenza A virus remained capable of infecting and transmitting among ponies. Twenty influenza virus seronegative ponies aged 12 to 24 months were used in this study. Two groups of 5 ponies were inoculated by aerosol exposure with 10^7 $TCID_{50}$ dose per pony of A/Canine/Wyoming/86033/07 (Ca/WY) for one group and, serving as a positive control, a contemporary A/Eq/CO/10/07 (Eq/CO) for the second group. As a

negative control, four ponies were mock-inoculated. To evaluate the potential for virus transmission to in-contact horses, three ponies were introduced two days after aerosol exposure and housed with the inoculated animals as sentinels. Clinical signs, nasal virus shedding, and serological responses to inoculation were monitored in all ponies for up to 21 days after virus challenge. Growth and infection characteristics of challenge isolates were examined using MDCK cells and primary equine and canine respiratory epithelial cells. Ponies inoculated with Ca/WY demonstrated mild changes in clinical appearance compared to the Eq/CO inoculated ponies. Additionally, Ca/WY produced significantly lower M gene copy numbers in nasal secretions as well as significantly lower systemic antibody responses in ponies than Eq/CO. Lastly, the Ca/WY isolate did not transmit to the sentinel ponies. Compared to the equine H3N8 influenza isolate, inoculation of ponies with the contemporary canine H3N8 isolate resulted in mild clinical disease, minimal nasal virus shedding and weak systemic antibody responses. Taken together, these results suggest that Ca/WY has not maintained infectivity for horses.

ABBREVIATIONS

$TCID_{50}$	50% tissue culture infectious dose
EIV	Equine influenza virus
CIV	Canine influenza virus
HA	Hemagglutinin
NA	Neuraminidase
M	Matrix
Ca/WY	A/Canine/Wyoming/86033/07
Eq/CO	A/Equine/Colorado/10/07
REC	Respiratory epithelial cell
CREC	Canine respiratory epithelial cell
EREC	Equine respiratory epithelial cell
HI	Hemagglutination inhibition
MDCK	Madin-Darby canine kidney
MEM	Minimal essential medium
RT-PCR	Reverse transcriptase polymerase chain reaction
MOI	Multiplicity of infection
BSA	Bovine serum albumin
ICC	Immunocytochemical staining

INTRODUCTION

Infections with equine influenza virus have remained a serious health and economic problem in horses worldwide.[1] The immune response induced by influenza virus infection protects against re-infection with the same or antigenically similar virus strain. However, as influenza virus undergoes frequent antigenic change ("antigenic drift"), protection provided by the host's immunity may be reduced as the virus becomes more antigenically distinct.[2] The occasional introduction of gene segments or entire viruses from other host species also adds to the vast genetic and antigenic diversity of influenza viruses.[3] Although horses have often been regarded as isolated or "dead-end" hosts for influenza cross-species transmission,[3,4] the transmission and subsequent maintenance of an equine-lineage H3N8 virus to dogs in the U. S. highlights the fact that the barrier for influenza viruses emerging from horses is not absolute.[5]

Since their first isolation in 2004, the canine influenza viruses have continued to evolve genetically. Phylogenetic analyses of the HA and NA genes of contemporary canine isolates indicate that the canine viruses since have segregated from the equine H3 "Florida lineage" as a distinct sub-lineage.[5,6] Five amino acid residues at positions 54, 83, 222, 328 and 483 of the H3 HA appear to differentiate the canine isolates from contemporary equine H3N8 viruses.[5,6] Although the biological significance of these amino acid substitutions remains unclear, from a host immunological perspective, the asparagine to lysine mutation at position 54 and possibly the serine to asparagine substitution at position 83 are of particular interest as they are located in antibody binding

regions of the HA protein.[7,8] The potential importance of these amino acid residues is highlighted by the finding that residue 83 has been shown to be involved in antigenic drift in human H3 viruses.[9,10] Similarly, the N54K substitution occurred in the center of an *N*-linked glycosylation motive and as a consequence, the posttranslational glycosylation of the protein may be altered,[11] thereby resulting in the reduced accessibility of the epitope.[12,13] In light of these findings, the mutations at residues 54 and 83 have been hypothesized to facilitate viral escape from neutralization by preexisting antibodies.[8] As asparagine at position 54 is highly conserved in equine and other non-canine H3N8 influenza viruses, including the strains used for production of equine vaccines currently marketed in the U.S., we hypothesized that the canine isolates could represent an emerging disease threat to horses provided that they have maintained the ability to infect horses. Moreover, the finding that dogs could represent a source of infection for horses would have importance for the development and implementation of biosecurity protocols on equine farms.

Despite the fact that cross-species transmission of influenza A viruses occurs relatively frequently, such newly introduced viruses are only rarely maintained in the new host species.[3] While it is not known what properties are necessary to allow a virus to form a stable lineage, the viral HA is considered to play a key role in influenza species-specificity.[14,15] Given the importance of the HA protein in limiting transmission of influenza A viruses among species, it is unclear whether the genetic divergence of the canine and equine H3 viruses, including the five amino acid mutations in the HA protein, has resulted in a reduction of infectivity of canine-lineage influenza in horses. To examine whether an equine-derived canine H3N8 influenza A virus maintained its

infectivity in equines, we inoculated ponies by aerosol exposure with a canine influenza isolate (Ca/WY)**.** Serving as positive controls, a second group of ponies was inoculated with a contemporary equine H3N8 virus (Eq/CO). Lastly, as efficient horse-to-horse transmission is an important requirement for virus maintenance, we also studied the horizontal spread of the virus to influenza naïve sentinel ponies housed in direct contact with the inoculated animals. Growth and infection characteristics of Ca/WY and Eq/CO were also examined in MDCK cells and primary canine and equine respiratory epithelial cells, which previously have been used as a tool to study infection with influenza viruses.[16-19]

METHODS

Animals

Twenty ponies aged 12 through 24 months were purchased from a commercial source and shown to be serologically negative for EIV by enzyme-linked immunosorbent assay[20] and hemagglutination inhibition assay as previously described.[21] The animals were clinically healthy and in good body condition and were maintained in accordance with guidelines of the Colorado State University Research and Animal Resources Committee. They were fed a diet of hay and a pelleted vitamin and mineral concentrate, and group-housed outdoors in pens in three geographically separate locations with access to water and shelter. Prior to initiation, this study was reviewed and approved for conduct by the Colorado State University Institutional Animal Care and Use Committee.

Influenza viruses

The contemporary H3N8 canine influenza virus Ca/WY and the contemporary H3N8 equine influenza virus Eq/CO were isolated during field outbreaks of EIV and CIV respectively. EIV infection was confirmed at an equine boarding facility housing 42 horses in July 2007. Clinical signs of equine influenza consisting of fever, coughing, and nasal discharge had first been observed among two horses that had returned from a show three days earlier. Subsequently, clinical disease spread rapidly among the remaining horses on the property. Nasal shedding of influenza virus was diagnosed in four of 32 horses tested by real-time RT-PCR. EIV was isolated on first passage in embryonated chicken eggs from two of the four real-time RT-PCR positive nasal swab specimens and designated as Eq/CO. After the positive diagnoses, serum samples were collected from 32 of 42 horses. A convalescent serum sample was taken from all 32 horses 14 days later. Testing of the paired serum samples for influenza-specific antibodies by HI assay demonstrated seroconversion in 20 of 32 (62.5%) horses tested. The contemporary canine virus was isolated that same year. In mid February 2007, an outbreak of respiratory disease occurred in dogs at a humane shelter. The outbreak involved all 27 dogs housed at the shelter at the time. Clinical signs observed included fever, lethargy, cough, and nasal discharge. Nasal swab and serum samples were collected from 18 of 27 dogs. Paired acute- and convalescent-phase serum samples were available for collection from 13 dogs and were tested for CIV-specific antibodies by HI assay. Twelve of 18 nasal swab samples tested positive for CIV by real-time RT-PCR and virus was isolated from three dogs on first passage in embryonated chicken eggs and designated as Ca/WY. Of the paired serum samples tested, 13 (100%) dogs showed seroconversion.

Genetic characterization and growth characteristics of viruses

Both Ca/WY and Eq/CO were passaged three times in MDCK (American Type Culture Company, Manassas, VA) cells grown in MEM (Gibco, Invitrogen, Carlsbad, CA) as previously described.[21] To rule out introduction of spurious mutations during cell culture passage, the full-length protein coding regions of all eight gene segments of Eq/CO and Ca/WY from allantoic fluid (pre cell culture passage) and 3rd passage MDCK stocks (used to inoculate ponies) were amplified by RT-PCR. The sequences of the amplified genes were determined by direct cycle sequencing (BigDye Terminator Cycle Sequencing Ready Reaction Kit, Perkin-Elmer Applied Biosystems). Sequence comparisons at the nucleotide and deduced amino acid levels were made using clustal analysis (http://align.genome.jp) and commercially available software (Lasergene, DNASTAR, Inc. Madison, WI). The phylogenetic relationships among the HA and NA genes of the virus isolates and selected reference strains were estimated from their nucleotide sequences by maximum parsimony with bootstrap analysis with a commercially available software program (PAUP 4.0 Macintosh beta version 10; Sinauer Associates, Inc., Sunderland, MA). Phylogenetic analyses of the HA and NA genes confirmed that Ca/WY clustered with the canine isolates and Eq/CO clustered with the contemporary equine viruses, placing them into the previously described canine and equine sub-lineages of the equine H3 "Florida lineage".[5,6] Moreover, amino acid sequence analysis of the HA genes of Ca/WY and Eq/CO verified the presence of five amino acid substitutions that differentiate the equine from the canine H3 consensus sequence.[5,6]

To characterize virus growth, one-step growth curves were performed in MDCK cells as previously described[22] as well as in primary CRECs and ERECs grown at an air-fluid interface. Briefly, MDCK cells were grown in 35 x10 mm tissue culture plates and infected at an MOI of 10 $TCID_{50}$/cell with either Ca/WY or Eq/CO. After 1 hr of adsorption, the inoculum was removed and 2 mLs of MEM containing 0.5% BSA (Fisher Scientific, Fair Lawn, NJ), penicillin-streptomycin (Gibco, Invitrogen, Carlsbad, CA), amphotericin B (Biowhittaker, Cambrex Bioscience, Walkersville, MD) and tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (1 μg/mL) (Worthington Biochemical Corp, Lakewood, NJ) was added. Supernatants were harvested at 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 20, and 24 hrs after infection and frozen at -80ºC until analysis by quantitative real-time RT-PCR. Isolation, culture and estimation of purity of primary RECs were performed as previously described.[16,23] Briefly, RECs were isolated from fresh canine and equine trachea by enzymatic digestion using 1.4% pronase (Roche Applied Science, Indianapolis, IN) and 0.1% deoxyribonuclease I (Sigma-Aldrich Chemical Co., St. Louis, MO) in calcium and magnesium free MEM for 48 hours. After digestion, epithelial cells were harvested and incubated on an uncoated petri dish for two hours to reduce fibroblast contamination. Epithelial cell purity was estimated by ICC using a pan-anticytokeratin antibody (5 μg/ml) (Zymed, Invitrogen, Carlsbad, CA) and analyzed by flow cytometry. Primary CRECs and ERECs were seeded into Type IV collagen (Sigma-Aldrich Chemical Co., St. Louis, MO) coated transwell cell culture wells (Costar, Corning, Fisher Scientific, Fair Lawn, NJ) and cultured at the air-fluid interface in DMEM/F12 (Gibco, Invitrogen, Carlsbad, CA), 2% Ultroser G (Pall Life Sciences, Pall Corp., Cergy, France), penicillin-streptomycin and amphotericin B until

confluent. Primary cells were washed with DMEM/F12 to remove mucin and wells were infected in duplicate at an MOI of 10 with either Ca/WY or Eq/CO. After 2 hrs of adsorption, the inoculum was removed and replaced with fresh maintenance media. Alliquots of media were collected at 3, 4, 5, 6, 8, 12, 16 and 24 hours after infection and stored at -80 until real-time PCR analysis. Influenza M gene copy numbers in the cell culture media were determined by use of a quantitative real-time RT-PCR assay, as previously described.[24,25] In addition, the presence of virus antigen was determined by ICC using a mouse anti-nucleoprotein monoclonal antibody (68D2) (courtesy of M. McGregor and Y. Kawaoka, University of Wisconsin, Madison, WI) as previously described.[21]

Experimental design

Ponies were randomly assigned to two groups of eight (primary inoculated ponies [n=5] and sentinel ponies [n=3]) and one group of four ponies (controls), respectively. For the duration of the experiment, each group was housed separately to prevent cross-contamination. Physical examination and clinical scoring was conducted on all ponies throughout the course of the experiment at the same time as collection of serum and nasal swab samples daily from 2 days prior to inoculation and 14 days after inoculation (primary inoculated and control ponies), and daily from 2 days prior to 21 days after group introduction (sentinel animals). Clinical scoring was performed as previously described.[26] Briefly, animals were observed for 20 min each day to assess their general appearance (attitude, food intake, and respiration were quantified on a scale of 0 to 1, with 0 indicating a clinically normal animal and 1 indicating an abnormal finding),

coughing (with 0 indicating that no cough is present, 1 indicating coughing once during observation, and 2 indicating coughing twice or more during observation), nasal discharge (with 0 indicating no discharge, 1 indicating serous discharge, 2 indicating mucopurulent discharge, and 3 indicating profuse mucopurulent nasal discharge), and rectal temperatures. Two days prior to inoculation, pre-inoculation nasal swab (sterile Dacron polyester tipped applicators, Hardwood Products Company LLC, Guilford, ME) samples and serum samples were collected. On day 0 of the experiment five ponies in the challenge groups were sedated by intravenous administration of 5 μg/kg of body weight of detomidine (Dormosedan® (10mg/mL), Pfizer, Kalamazoo, MI) and 0.05 mg/kg of body weight of butorphanol (Torbugesic® (10mg/mL), Fort Dodge, Fort Dodge, IA) and inoculated with either Eq/CO or Ca/WY at 10^7 $TCID_{50}$ by aerosol inhalation as previously described.[27] Four ponies were mock inoculated to serve as uninfected controls. On day 2 post inoculation, three influenza seronegative ponies were introduced to each of the virus inoculated groups and housed together with the inoculated ponies as sentinels throughout the remainder of the experiment. At daily intervals after inoculation, nasal swabs were obtained from each animal. The swabs were placed in 1 ml of viral transport medium containing phosphate-buffered saline, 0.5% BSA, and the antimicrobials penicillin-streptomycin, nystatin (Sigma-Aldrich Chemical Co, St Louis, MO), and gentamicin (Gibco, Invitrogen, Carlsbad, CA) and stored at -80ºC until further analysis. Additional blood samples for serology were collected from all animals on days 7, 14, and 21.

Evaluation of virus shedding

Matrix gene copy numbers in nasal swab specimens were determined by use of a previously established quantitative real-time RT-PCR assay.[24,25] Briefly, from each nasal swab sample, RNA was extracted from 140 μl of viral transport medium using a commercial RNA extraction kit (QIAamp® Viral RNA Mini Kit, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. One-tube real-time RT-PCR assay was performed using the following cycling conditions: 10 min 52°C, 5 min at 95°C, 45 cycles of 10 sec at 95°C and 45 sec at 68.4°C. Each nasal swab sample was run in duplicates. Negative controls included with each assay consisted of water and neat transport medium. The positive control consisted of 10^1 $TCID_{50}$ of A/Ca/Fort Collins/224986/06 in viral transport medium. Purified full-length M gene RNA was used as a standard for quantification of influenza virus M gene copy number. Matrix gene RNA was transcribed with a large scale RNA production kit (RiboMAX® Large Scale RNA production systems kit, Promega, Madison, WI) from the T7 promoter according to the manufacturer's instructions. The RNA generated was treated with RNase-free DNase I and tested for purity by both gel electrophoresis and PCR prior to use in real-time RT-PCR. The purified RNA was suspended in RNase-free water, quantified by spectrophotometer and stored at -80°C in 10 μL aliquots. To determine the minimum detection level for real-time RT-PCR, the *in vitro* transcribed RNA was serially diluted in RNase-free water to produce dilutions ranging from 10^7 to 10^0 copies/mL of M gene RNA. To evaluate inter-assay variation, the threshold cycle (C_t) values of 10 RNA standard curves, run on different days were determined. The mean, standard error of the mean, and coefficient of variation were calculated. The minimum detection level of RNA

was determined to be 10^3 M gene copies/reaction for real-time RT-PCR. Amplification of dilutions of the RNA transcripts showed linearity over a range of 6 orders of magnitude. The average C_t values corresponding to each dilution of M gene RNA was 15.9 (± 0.13) at 10^8 copies, 19.5 (± 0.14) at 10^7 copies, 22.9 (± 0.1) at 10^6 copies, 26.3 (± 0.11) at 10^5 copies, 29.9 (± 0.13) at 10^4 copies, and 33.8 (± 0.3) at 10^3 copies. Matrix gene RNA at 10^2, 10^1 and 10^0 copies was either undetectable or detectable at higher C_t (above 36). Coefficients of variation were between 1.5 and 4.7% for the C_t values.

Hemagglutination inhibition assay

Hemagglutination inhibition assays were performed as previously described.[21] Briefly, sera were pretreated with receptor destroying enzyme (Denko Seiken Co., Tokyo, Japan) and incubated overnight at 37°C. Following enzyme inactivation, two-fold serial dilutions of sera were mixed with 4 hemagglutination units of Eq/CO and Ca/WY, respectively. The assays were developed by adding 0.5% (vol/vol) chicken red blood cells and the HI antibody titers were interpreted as the reciprocal of the highest dilution causing complete inhibition of agglutination.

Statistical analyses

Generalized estimating equations were used to analyze the overall mean differences in the levels of M gene copy numbers in nasal swab specimens, HI antibody titers, clinical scores, and body temperatures between the Ca/WY and Eq/CO infected groups and between the infected groups and the mock-inoculated controls. Mean differences were adjusted for days and clustered on repeated measures for each outcome in the analysis.

Clinical scores were ranked prior to analysis. All other outcome variables were log transformed, if necessary, to meet the major assumptions, including linearity and normality. HI antibody titers with values of zero were converted to 1 in the challenge groups for data transformation and easier statistical analyses. Comparison of M gene copy numbers of Ca/WY in ERECs and CRECs at 24 hours post inoculation was determined using a one-way ANOVA. M gene copy numbers of Ca/WY in CRECs and Eq/CO in ERECs were compared using a student's T-test. M gene copy numbers were log transformed prior to both analyses. Significant differences were determined when $P<0.05$. All statistical analyses were performed using commercially available software (STATA statistical software, Macintosh version 10.1, StataCorp LP, College Station, TX).

RESULTS

In vitro growth characteristics

In MDCK cell cultures, Ca/WY and Eq/CO demonstrated no differences in growth kinetics and M gene copy numbers at 24 hours post inoculation (data not shown). Similarly, no significant differences were found when comparing the growth and M gene copy numbers of Eq/CO in ERECs and Ca/WY in CRECs ($P>0.5$). Correspondingly, visual inspection of the ERECs and CRECs infected with Eq/CO and Ca/WY, respectively, and stained with ICC revealed that the viruses were able to infect virtually every available cell derived from their respective host species (Figure 3.1). In contrast, Ca/WY demonstrated a low infectivity phenotype in ERECs (Figure 3.1), which was

paralleled by significantly lower end-point M gene copy numbers (data not shown) reached compared to M gene copy numbers achieved in CRECs (P<0.005).

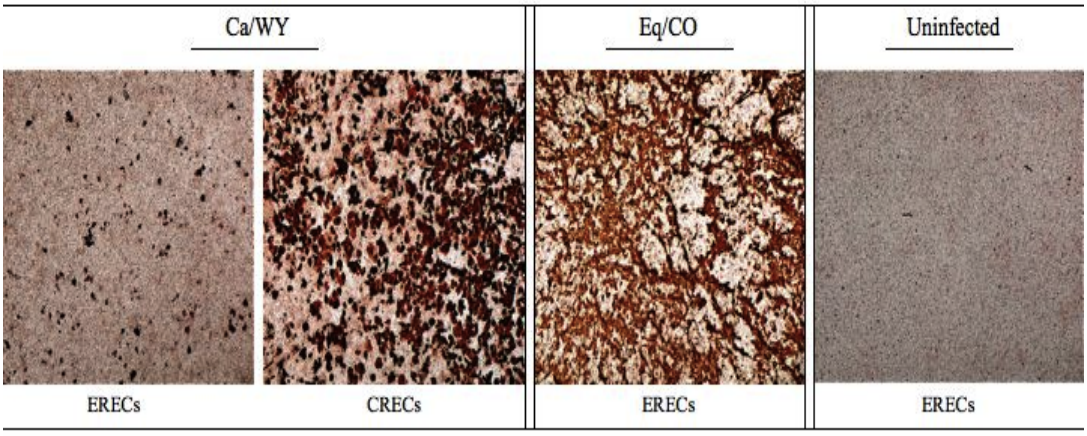

Figure 3.1. ICC staining of virus-infected ERECs and CRECs. From left to right: Ca/WY in ERECs and Ca/WY in CRECs; Eq/CO in ERECs and uninfected control ERECs. These are representative images. Similar patterns of infection were seen with repeated experiments and with cells from multiple animal donors. Magnification is shown at 4X.

Clinical responses to inoculation

The clinical responses to inoculation in ponies were remarkably different for the two viruses. More specifically, after inoculation, all ponies inoculated with Eq/CO developed severe mucopurulent nasal discharge of 7-11 days duration, and spontaneous coughing for 12-14 days (Figure 3.2). In addition, all five ponies were pyrexic (T > 101.5ºF) for 2-7 days (Figure 3.3). In contrast, in the Ca/WY inoculated group, only one of five ponies developed mild to moderate mucopurulent nasal discharge for 2 days duration. Moreover, none of the ponies in the CIV inoculated group developed a cough or pyrexia. Statistically, the Eq/CO inoculated ponies showed significantly (P<0.001) more disease, as assessed by clinical scores and rectal temperatures, than either the Ca/WY or mock-

inoculated ponies. No statistical differences (P>0.05) in the clinical scores or rectal temperatures were found between the Ca/WY and the mock-inoculated ponies.

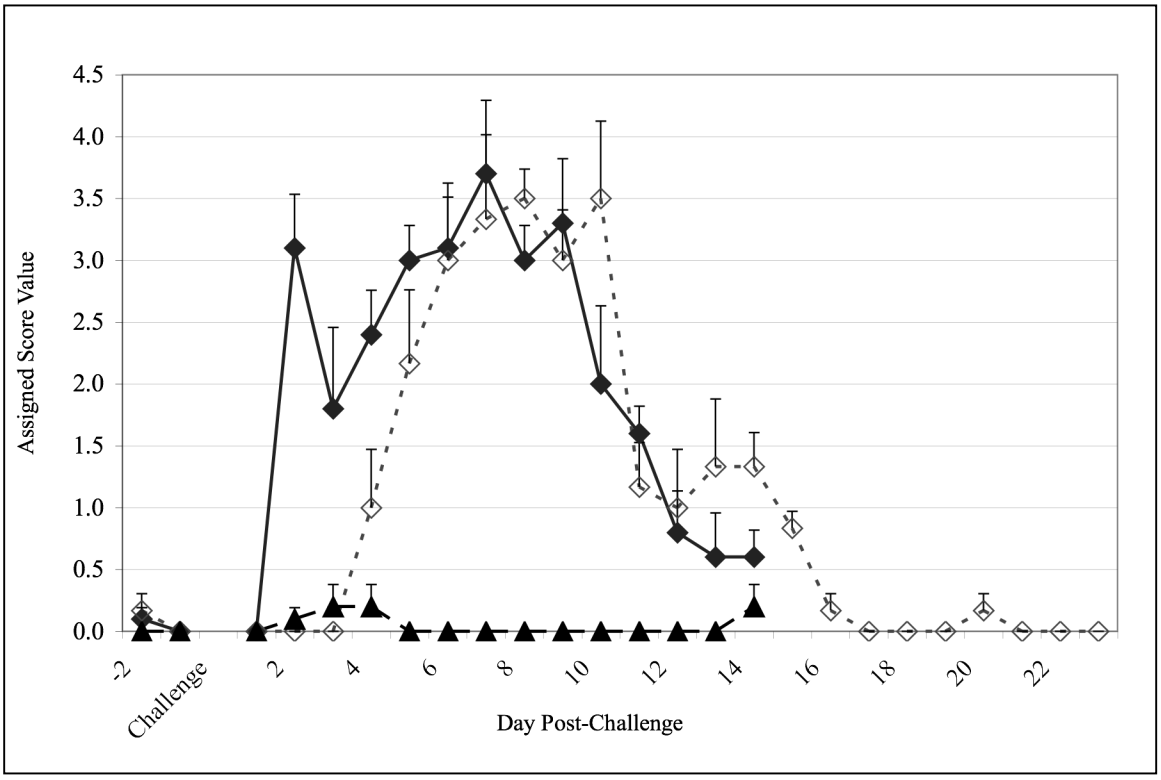

Figure 3.2. Group mean scores of clinical responses by day for ponies inoculated with Eq/CO (◆) along with Eq/CO sentinels (◇) and Ca/WY inoculated ponies (▲). Group mean scores of Ca/WY sentinel animals and mock inoculated ponies remained =0 throughout the experiment and therefore they are not represented in the graph.

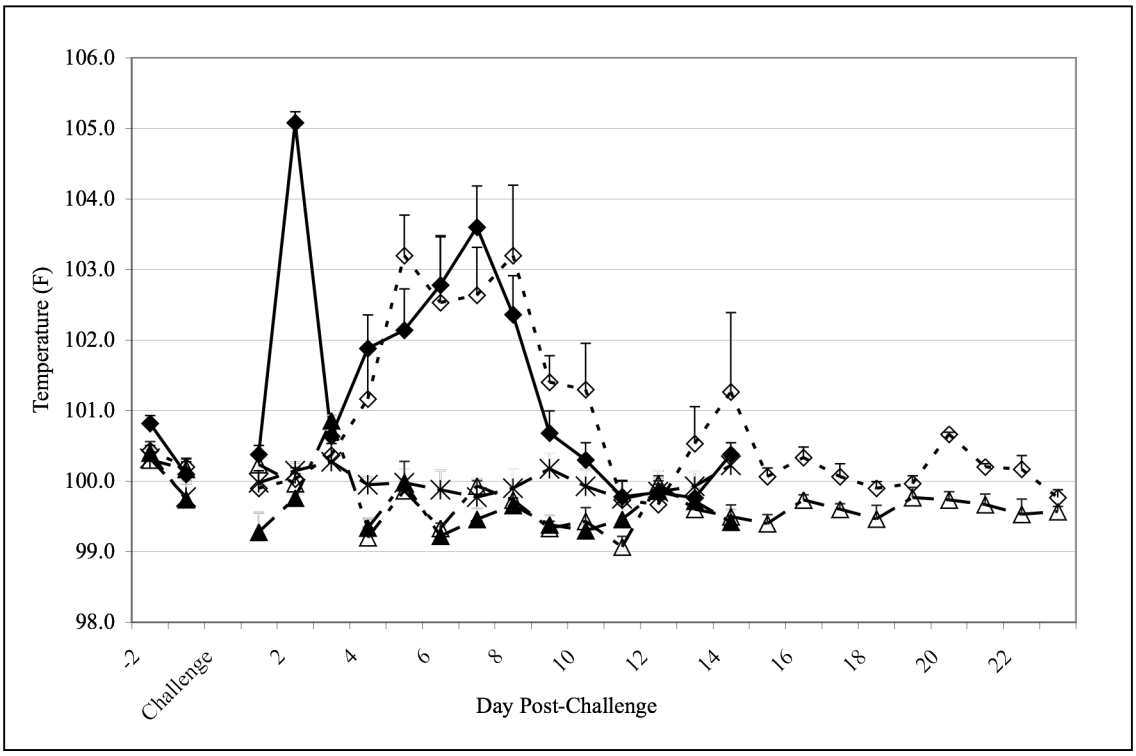

Figure 3.3. Group means ± standard error (SEMs) of rectal temperatures by day for ponies inoculated with either Eq/CO (◆) or Ca/WY (▲), along with Eq/CO sentinels (◇), Ca/WY sentinels (△), and mock inoculated controls (✻).

Virus shedding

The duration and levels of M gene copies detected in nasal secretions were distinctly different between the canine and equine H3N8 virus (Figure 3.4). In the Eq/CO infected ponies, the influenza virus M gene was detected in nasal secretions in all five ponies starting as early as 2 days after inoculation. Nasal virus shedding was detectable for up to 8 days with M gene copy numbers of $\geq 10^4$ for at least 5 days in all five ponies. In contrast, among animals infected with Ca/WY, in 4 ponies M-gene copies were never detected in the nasal swab samples. Only one pony demonstrated real-time RT-PCR

positive nasal swab specimens at $\leq 10^4$ M gene copies for two consecutive days (days 5 and 6) after inoculation. Overall, the mean M gene copy numbers in the nasal swab specimens was significantly higher in ponies inoculated with Eq/CO compared to ponies inoculated with Ca/WY. In contrast, there were no significant differences in the numbers of M gene copies detected in nasal secretions of ponies challenged with Ca/WY and the mock-inoculated control animals.

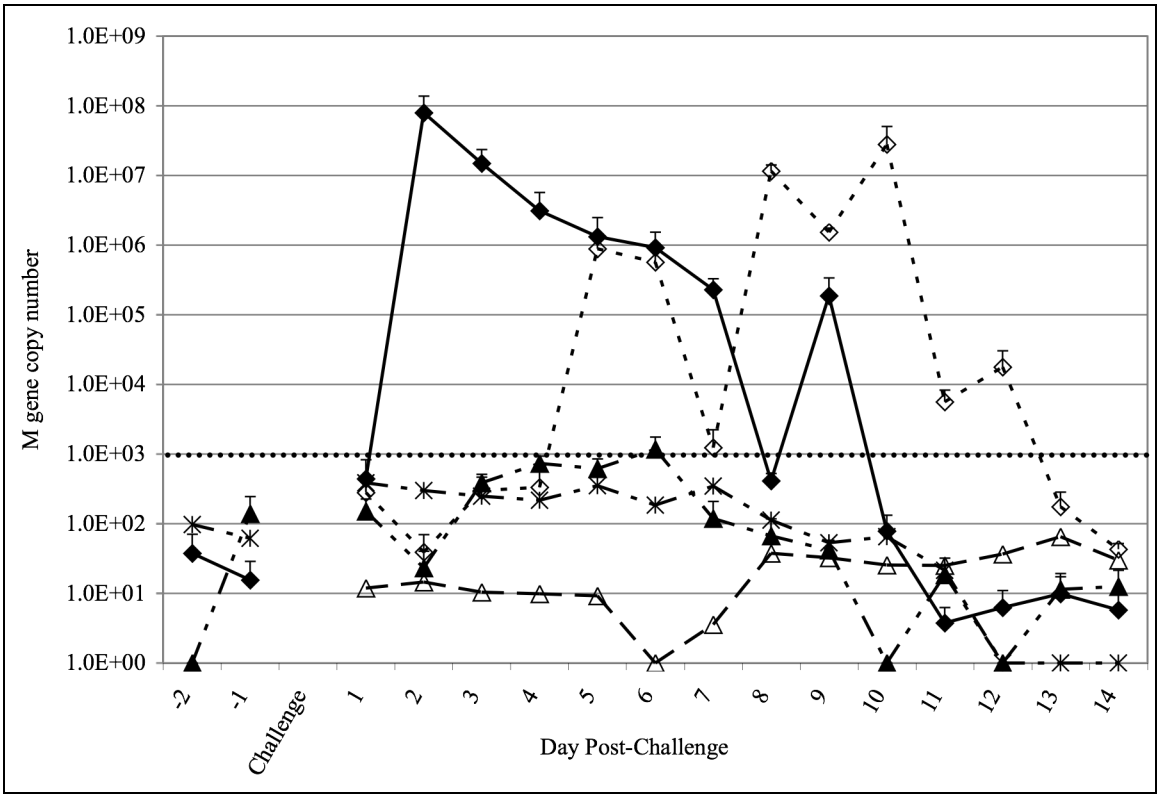

Figure 3.4. Group means ± SEMs of virus shed in the nasal passages by day for ponies inoculated with either Eq/CO (◆) or Ca/WY (▲), along with Eq/CO sentinels (◇). The minimum detection level of the real-time RT-PCR was 1000 M gene copies per reaction corresponding to 10^0 $TCID_{50}$ of A/Ca/Fort Collins/224986/06. The Ca/WY sentinels and mock-inoculated control animals did not shed detectable levels of virus at any point during the experiment and are not represented in the graph.

HI antibody responses

The pattern of systemic antibody responses generally mimicked the differences in the severity of clinical signs and extent of nasal virus shedding found between the challenge groups. Prior to inoculation, all animals were serologically negative for the equine and canine influenza virus (Table 3.1). Seven days post infection, all five ponies inoculated with Eq/CO had detectable, low-level virus-specific antibody titers and by day 14 and day 21 these titers had increased substantially (≥ 1:1024). In contrast, none of the ponies inoculated with Ca/WY developed detectable antibody titers by day 7. Moreover, only two of five ponies developed a low-titered antibody response by day 14 (1:64 and 1:16) and day 21 (1:64 and 1:4). Statistically, the overall mean HI antibody responses in ponies inoculated with the contemporary equine isolate were significantly higher compared to the ponies inoculated with the canine isolate.

Table 3.1. Serum antibody response (titer) to Ca/WY measured by hemagglutination inhibition[1].

Pony challenge group	Pre-challenge[a]	Day 7	Day 14	Day 21
Controls[b]	0	0	0	0
Eq/CO[c, e]	0	32.0 ± 11.0	2560.0 ± 494.6	1356.8 ± 656.7
Eq/CO sentinels[d, f, e]	0	2.7 ± 3.8	768.0 ± 161.9	853.3 ± 358.0
Ca/WY[c]	0	0	31.2 ± 35.8	10.4 ± 9.5
Ca/WY sentinels[d, f]	0	0	0	0

[1]Serum antibody responses to Eq/CO did not differ significantly from Ca/WY and therefore are not represented in this table.
[a] Data are means ± SEMs.
[b, c, d] Sample sizes for controls, challenge groups and sentinels are n = 4, n = 5, and n = 3, respectively; [e] $P < 0.05$.
[f] Serum was collected two days after primary challenge group for Days 7, 14 and 21.

Virus transmission to sentinels

To examine the potential for horizontal transmission of virus, three influenza seronegative ponies were introduced to the EIV and CIV inoculated groups to serve as sentinels. The clinical signs, virus shedding, and antibody responses of the Eq/CO sentinels closely mirrored the responses observed in the inoculated animals. More specifically, all three ponies exposed to the Eq/CO inoculated animals developed mucopurulent nasal discharge, spontaneous coughing, and pyrexia as early as 2 days post group introduction (Figures 3.2 and 3.3). In addition, nasal virus shedding was detected in all three Eq/CO sentinels on day 3 after introduction and persisted for up to 7 days (Figure 3.4). All three sentinels showed influenza-specific seroconversion by day 14 (Table 3.1). In contrast, none of the Ca/WY sentinels developed any clinical abnormalities or shed detectable levels of virus in nasal secretions. Lack of biologically relevant virus transmission was further confirmed by the absence of an immune response in these ponies (Table 3.1).

DISCUSSION

The aims of this study were to evaluate whether a contemporary CIV isolate could infect, replicate, cause clinical disease, and spread among ponies and in primary respiratory epithelial cells grown in cell culture. While CIV was first identified subsequent to the interspecies transfer of an equine-lineage H3N8 virus to dogs,[5] the canine viruses have since evolved genetically resulting in segregation of the canine genes from the equine H3 "Florida lineage".[5,6] Based on this genetic diversion, we hypothesized that CIV could

represent a disease threat to horses provided the viruses had maintained the ability to infect horses. The results of this study indicate that the contemporary canine H3N8 influenza virus Ca/WY infected and replicated poorly in ponies. Moreover, virus inoculation did not result in clinical disease or spread of virus to naïve in-contact ponies. By comparison, the contemporary Eq/CO isolate, infected, replicated, and transmitted efficiently between ponies. Although the latter finding was not unexpected, based on the epidemiology of the outbreak during which Eq/CO was isolated and results of similar equine influenza challenge experiments conducted by our group and others,[20,26,27] the near complete lack of infectivity of the canine isolate in horses is interesting. Ca/WY is clearly not an inherently replication-defective virus. This notion is supported by the rapid spread of virus among dogs at an animal shelter as well as the efficient growth of the virus in MDCK cells and primary canine RECs. In mammals, the primary targets of influenza viruses are the airway epithelial cells and these cells are being used with increasing frequency to study influenza virus-host interactions. In fact, recent studies have demonstrated that primary RECs represent a suitable *in vitro* system to investigate species-specific infection characteristics and host range of influenza A viruses.[16-19] In the present study, we extended our findings of the *in vivo* infection characteristics of Ca/WY and Eq/CO to an *in vitro* primary respiratory cell culture system. Although both viruses were able to infect and replicate to comparable levels in primary airway epithelial cells derived from their respective host species, the viruses displayed distinctly different levels of infection and replication efficiencies in primary ERECs. These results closely paralleled the infection characteristics observed in ponies, in particular the nasal virus shedding data, suggesting that primary ERECs and CRECs could represent a feasible

model system to dissect virus and host factors controlling species-specific replication characteristics of influenza in horses. Studies are currently being conducted in our laboratory to further validate this *in vitro* system.

The first step of influenza virus infection is dependent on the interaction of the viral HA with cellular sialic acid residues. As such the viral HA is thought to be a major contributor of influenza virus host range.[15,28] Previous work has shown that CIV isolates (including Ca/WY) contain five amino acid differences in their HA protein that are not present in the most closely related equine H3 HAs (including Eq/CO) and it has been hypothesized that these amino acid substitutions have occurred as a result of mutational adaptation of the virus to its new host species.[5,6] It is therefore possible that the amino acid differences in the HA protein could account for at least part of the observed differences in infectivity and replication efficiency of Ca/WY and Eq/CO. For example, the isoleucine (I) to threonine (T) substitution at position 328 occurred near the cleavage site of the H3 HA protein.[7] As the peptide structure connecting the HA_1 and HA_2 subunit has been found to determine tissue tropism of avian influenza virus,[29,30] it is possible that diminished cleavability of the CIV HA by equine cellular proteases resulted in the reduced infectivity of Ca/WY in ponies. Similarly, the N483T substitution results in the loss of a glycosylation site in the HA_2 subunit,[5,6] which has been found to affect the HA's interaction with the host cell receptor and the release of progeny virus from the host cell.[31] Lastly, the substitution of leucine for tryptophan at position 222 is remarkable because it represents a non-conservative change adjacent to the receptor binding pocket.[5,6] As species-specificity of influenza is partly determined by the binding preference of the HA protein to cellular sialic acid species,[15,28] modulation of receptor

binding specificity as a result of the W222L substitution could provide an attractive explanation for the inability of Ca/WY to infect and to replicate in ponies. Currently, studies are underway in our laboratory to determine the sialic acid receptor binding specificities of canine and equine influenza virus isolates. Finally, while the HA is recognized as a key factor controlling the species-specificity of influenza A viruses, it is clear that the other seven influenza virus gene segments also contribute to influenza virus host range. It is therefore possible that the critical viral factors that impact infection and replication of Ca/WY in ponies may include equine to canine amino acid substitutions that are present in other gene segments than the HA.

In summary, our findings indicate that Ca/WY, a contemporary equine-derived canine H3N8 influenza virus, has virtually lost the ability to infect, replicate, cause clinical disease, and spread among ponies. This was further supported by the significantly lower levels of infection and M gene copy numbers of Ca/WY in ERECs compared to the Eq/CO isolate. However, while these results appear to support the existence of a barrier to CIV infection in horses, it remains to be determined whether other canine isolates demonstrate similar restricted infectivity in equids.

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CHAPTER 4: CONCLUSION

The primary goal of this Master's project has been the development and characterization of a primary equine respiratory epithelial cell culture model grown at the air-fluid interface. Data presented in both papers show that these cultures are morphologically similar to equine airway epithelium, retain several immunological characteristics important in the host immune defense, and present similar patterns of infection, replication, and pathogenesis with influenza virus infection both *in vitro* and *in vivo.* The experiments performed as part of this project clearly show that we have developed an equine airway model that can be used as tool to study host-pathogen interactions. The usefulness of this model is considerable. In addition to examining host-pathogen interactions including mechanisms of viral immune evasion, pathogen binding and host cell entry, this model might also be used to investigate the effects of novel preventatives and immunotherapeutics against equine respiratory pathogens.

Despite vaccination, both EIV and EHV-1 continue to have detrimental effects on equine health.[1-4] As mentioned previously, antigenic drift represents a significant challenge in the continuing development of effective vaccines against EIV. Conversely, EHV-1 modulation of host immunity remains an obstacle for EHV-1 vaccine development. Novel therapeutics against EIV and EHV-1 might be further investigated as we come to learn more about innate and early immunity to equine respiratory viruses at

the epithelial cell barrier using the EREC AFI model. For example, one study demonstrated that mice pretreated with a TLR3 agonist (poly I:C) had significant increases in survival rates after they were challenged with an encephalitic strain of herpes simplex virus.[5] While this study supports the usefulness of TLR agonists as therapeutics, it can often be difficult to apply results of studies in mice to an equine model.

When *in vivo* studies of equine respiratory disease using equine models may prove costly and logistically difficult, the EREC AFI model would be a simple, practical, and less costly alternative. This is especially true when performing experiments involving sequential sampling or repeated measures. As experiments done *in vitro* are often performed in a more controlled environment, this would also limit the possibility of confounding effects on experimental studies. For example, EREC AFI cultures might be more useful than an *in vivo* model when studying cell-to-cell interactions or when examining the immune responses of specific cell types. In addition, we have demonstrated in this project that patterns of infection using H3N8 canine and equine influenza A isolates in ponies and in primary RECs cultured at the AFI were similar. Therefore, RECs cultured at the AFI could be useful for assessing viral infectivity.

Secondly, preliminary experiments in our lab have demonstrated that infection of the EREC AFI model with EHV-1 down-modulates surface expression of MHC-I and MHC-II (data not shown). As EHV-1 has demonstrated immune modulation similar to other alpha herpesviruses,[6,7] we are currently examining the effects of host immunomodulatory genes using deletion mutants of EHV-1 at the epithelial cell barrier in the EREC AFI model. In addition, herpesviruses are known to alter the host cytokine network.[8] We have demonstrated that EREC AFI cultures still express low levels of GM-

CSF, IL-10, IL-8, TGF-β, TNF-α, and IL-6 after four weeks of *in vitro* growth conditions. Even though IFN-α, IFN-β, IL-1, IL-12, and MCP-1 were not detected in the EREC AFI cultures, it would be interesting to see how infection with EHV-1 affects the expression in both of these groups of cytokines. Immune modulation by EHV-1 has been demonstrated in an *in vitro* study using peripheral blood mononuclear cells in as early as six hours post-infection[8]. As immune modulation by EHV-1 at the respiratory epithelial cell barrier also likely occurs within the first several hours after infection, the EREC AFI model could be a suitable alternative to an *in vivo* study when examining immune modulation at the early stages of EHV-1 infection.

However, while there are several advantages in using this model, some challenges do exist. It has been made clear by this project that after four weeks of *in vitro* growth conditions epithelial cells undergo differentiation. However, contaminating fibroblasts represent a challenge in cultures of respiratory epithelial cells where *in vitro* growth conditions are suitable for fibroblast cell lines. An essential requirement for *in vitro* epithelial cell culture is the presence of an extracellular matrix. In the case of the EREC AFI model, 5% collagen is utilized as this matrix, however, a fibroblast feeder layer is probably a more natural form of extracellular matrix secretion.[9] While the EREC AFI system is a model to study host-pathogen interactions at the epithelial cell barrier, the presence of other cells such as contaminating fibroblasts in this model should be taken into consideration. While we know that the EREC AFI model is not a pure culture of epithelial cells but rather predominately epithelial (65%) in origin, we are currently investigating the presence of other cell types in the model.

In addition, while the natural airway epithelium and isolated, uncultured, epithelial cells expressed TLRs 1-4 and 6-10, after four weeks of *in vitro* growth conditions, TLRs 8 and 10 could no longer be detected in the EREC AFI model using conventional PCR techniques. However, antigenic stimulation may restore these properties. Previous studies have implicated that TLRs 2, 3, 7, 8, and 9 as well as RNA helicases plays a role in the recognition of viruses including influenza and herpesviruses among other pathogens.[10-15] For example, evidence supports that type I interferon (IFN-α and IFN-β) production critical to controlling herpesvirus infection is primarily mediated through the toll-like receptor 9 pathway.[11,16,17] In addition, TLR2 has been implicated as a secondary pathway in the recognition of herpesviruses where TLR2 expressed on the surface of antigen-presenting cells recognizes extracellular virions.[10,18] Even though TLRs 3, 7, and 8 have often been implicated in the recognition of influenza viruses,[12,19,20] some research has indicated that TLR3 serves a role in the recognition of herpesviruses as well[5]. Future studies using this model might investigate how EIV and EHV-1 affect the expression of toll-like receptors in airway epithelial cells using real-time PCR for which assays are currently being developed.

Lastly, mRNA expression of β-defensin1, MxA, 2'5' OAS, and lactoferrin were detected in the equine natural airway epithelium and in the isolated epithelial cells, however, lactoferrin was no longer detected in the EREC AFI cultures. In addition, MHC-I and MHC-II expression was greatly reduced in the EREC AFI cultures compared to isolated, uncultured, epithelial cells. As the lack of antigenic stimulation and underlying immune cells might account for the reduction in these immunological

properties, the loss of these characteristics could possibly be restored after infection with EHV-1 or EIV but merits further examination.

In conclusion the development of the EREC AFI model presents exciting new opportunities as a tool to understand host-pathogen interactions at the epithelial cell barrier to equine respiratory diseases. For years, our understanding of the innate and early immune responses to equine respiratory diseases has been hindered by a lack of a suitable *in vitro* model. The development and characterization of this model will undoubtedly advance our understanding of host-pathogen interactions at this site.

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