DEVELOPMENT AND APPLICATION OF A QUANTITATIVE PCR ASSAY TO STUDY THE PATHOGENICITY OF EQUINE HERPESVIRUS 5

By

Lila Marek Zarski

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Comparative Medicine and Integrative Biology – Master of Science

2016

ABSTRACT

DEVELOPMENT AND APPLICATION OF A QUANTITATIVE PCR ASSAY TO STUDY THE PATHOGENICITY OF EQUINE HERPESVIRUS 5

By

Lila Marek Zarski

Equine herpesvirus 5 (EHV-5) infection is associated with pulmonary fibrosis in horses, but further studies on EHV-5 persistence in equine cells are needed to fully understand viral and host contributions to disease pathogenesis. We developed a quantitative PCR (qPCR) assay to measure EHV-5 viral copy number in equine cell culture, blood lymphocytes, and nasal swabs of horses. The PCR primers and a probe were designed to target gene E11 of the EHV-5 genome. Specificity was verified by testing multiple isolates of EHV-5, as well as DNA from other equine herpesviruses. Four-week old, fully differentiated (mature) and newly seeded (immature) primary equine respiratory epithelial cell (ERECs) cultures were inoculated with EHV-5 and the cells and supernatants collected daily for 12-14 days. Blood lymphocytes and nasal swabs were collected from horses experimentally infected with EHV-1. The qPCR assay detected EHV-5 at concentrations around 10^4 intracellular genomes per cell culture in experimentally inoculated mature ERECs, and these values remained stable throughout 12 days. Intracellular EHV-5 copies detected in the immature cultures increased over 14 days and reached levels greater than 10^6 genomes per culture. EHV-5 was detected in the lymphocytes of 97% of horses and in the nasal swabs of 88% of horses both pre and post EHV-1 infection. In conclusion, qPCR was a reliable technique to investigate viral load in *in vivo* and *in vitro* samples, and EHV-5 replication in equine epithelial cells may be influenced by cellular stages of differentiation.

This thesis is dedicated to the memory of my sister, Anna.
Thank you for always looking out for me; I love you!

ACKNOWLEDGEMENTS

This work would not have been possible without the mentorship and guidance of each of my committee members. First, I would like to thank my primary advisor, Dr. Gisela Soboll Hussey. I have grown so much under her guidance, and with her mentorship and support I have developed a passion for research that I will carry with me throughout my life. I would like to thank Dr. Steven Bolin for his patient and thorough teaching and always encouraging me to think about how the techniques relate to the "bigger picture." I also would like to thank Dr. Philip Pellett for his many trips to East Lansing, his ability to simplify complex topics, and his eagerness for me to learn molecular and cellular fundamentals so that I could more thoroughly understand the scope of my research. Furthermore, I must thank Dr. Kurt Williams for introducing me to the field of comparative medicine, and prompting my interest in this project as a way to investigate complex disease processes using veterinary species as models.

This work would not have been possible without the help of my lab mates, Dr. Rahul Nelli, Dr. Carine Holz, Dr. Eilidh Wilson, Dr. Yao Lee, Julie Dau, and Rachel Baumgardner. I thank each one of them for their unique contribution to this project. Additionally, I would like to thank other members of the College of Veterinary Medicine or the Diagnositic Center of Population and Animal Health, in particular, Dr. Patty Weber, Dr. Ailam Lim, Janet Hengesbach, and Shannon Griffin. I also would like to acknowledge all of the horses and ponies that have helped me in my research.

I also would like to thank Dr. Vilma Yuzbasiyan-Gurkan, Dr. Linda Mansfield, Dimity Palazzola, and all other members of the Comparative Medicine and Integrative Biology program

for welcoming me into their community and providing constant advice, support, and encouragement.

Finally, I must mention the incredible contribution of my family and friends. Without their love, I could not have done this. I especially would like to thank Stephen Hughey – I would not be where I am without him by my side.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

αSMA	alpha smooth muscle actin
AEC	alveolar epithelial cell
AEC I	type I alveolar epithelial cell
AEC II	type II alveolar epithelial cell
C_q	quantification cycle
DPBS	Dulbecco's phosphate buffered saline
EBV	Epstein-Barr virus
ECM	extracellular matrix
EHV-1	equine herpesvirus 1
EHV-2	equine herpesvirus 2
EHV-4	equine herpesvirus 4
EHV-5	equine herpesvirus 5
EMPF	equine multinodular pulmonary fibrosis
EMT	epithelial mesenchymal transition
ER	endoplasmic reticulum
EREC	equine respiratory epithelial cell
FBS	fetal bovine serum
gHV	gamma herpesvirus
IFNγ	interferon gamma
IIP	idiopathic interstitial pneumonia

ILC	innate lymphoid cell
IPF	idiopathic pulmonary fibrosis
MEM	minimum essential media
MHV68	murine herpesvirus 68
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
p.i.	post inoculation
qPCR	quantitative polymerase chain reaction
RK-13	rabbit kidney 13
ROS	reactive oxygen species
SFTPC	surfactant protein C
TGF-β1	transforming growth factor beta 1
T_H	T helper
T_H2	T helper type 2
UIP	usual interstitial pneumonia
UPR	unfolded protein response
VCA	viral capsid antigen

CHAPTER 1.

General Introduction

GENERAL INTRODUCTION

Equine Herpesvirus 5 (EHV-5) infection is ubiquitous in the equine population. Scientific investigation of this gamma herpesvirus has lagged due to the inability to implicate it in clinical disease. This changed in 2007 when it was linked to a newly described progressive and fatal fibrotic lung disease of the horse termed equine multinodular pulmonary fibrosis (EMPF).[1] A few years later, it was found that *in vivo* inoculation of the lung with EHV-5 induced the development of pulmonary fibrosis with features similar to EMPF.[2] The importance of this discovery extends beyond veterinary medicine. The human disease idiopathic pulmonary fibrosis (IPF) has been associated with gamma herpesvirus infection of the lung, however, to what degree the infection contributes to disease pathogenesis remains unknown. Due to the practical limitations of human research and the imperfection of murine models, certain key mechanisms involved in the progression of gamma herpesvirus related IPF remain to be evaluated in the natural host.

Because it is a naturally occurring disease, EHV-5 associated EMPF in the horse could prove to be a valuable model by which to study potential mechanisms by which gamma herpesviruses contribute to the pathogenesis of IPF in humans. In order to justify the costs and labor involved in *in vivo* equine research, more information about EHV-5 must be obtained and used to develop efficient and useful *in vivo* protocols. The following work represents the first use of primary equine cell cultures to our knowledge to investigate the interaction of EHV-5 with its host cells.

This thesis contains three chapters. The first chapter will review the existing literature describing the cellular mechanisms involved in the development of IPF, the possible mechanisms by which gamma herpesvirus infection of the lung can influence these processes, and a summary of the current knowledge of EHV-5. The second chapter describes the research performed for this thesis. This work includes the development of a quantitative assay to measure viral load and the use of this assay to characterize viral growth in equine cell culture. The third chapter includes conclusions drawn from this work as well as the future directions of this study.

CHAPTER 2.

Review of the Literature

IDIOPATHIC PULMONARY FIBROSIS

Interstitial lung diseases are diseases of the lung parenchyma (versus disease of the airway) and can be classified based on whether the cause is known or unknown. Interstitial lung diseases in human medicine of unknown etiology are classified as idiopathic interstitial pneumonias (IIP) (Figure 1). Of the several distinct IIPs, the most common and severe form is idiopathic pulmonary fibrosis (IPF). [3,4] IPF is distinguished from the other IIPs based on radiographic and histologic evidence of a usual interstitial pneumonia (UIP). UIP is characterized by lesions of excessive and dense deposits of collagen along with accumulation of activated myofibroblasts (referred to as fibroblast foci) and hyperplasia of type II alveolar epithelial cells (AECs). The lesions are bordered by seemingly unaffected parenchyma. Importantly, the lesions are spatially and temporally heterogeneous, indicating the progressive and chronic nature of the diseases as opposed to the results of a single insult. [5] The median survival after diagnosis is 3-5 years in humans regardless of treatment course. [6]

Historically, the view of IPF pathogenesis was that an unknown inciting event triggered a chronic inflammatory response in the lung that continued to injure the tissue throughout the course of the disease. Inflammatory cells have been identified in IPF diseased lungs, and though not a conclusive criterion, presence of inflammatory cells in the bronchoalveolar lavage can support a diagnosis of IPF.[7–11] Although it is evident that inflammation plays a role in creating a pro-fibrotic environment, it is likely that this view may not explain the complete pathogenesis of fibrotic lung disease seen in IPF. Immunosuppressive and corticosteroid treatments have little to no effect on the progression and expected outcome of the disease, indicating that the

classical hypothesis that chronic inflammation is the primary mediator of lung damage needs revision.[12]

The current working hypothesis considers IPF a disease that results from dysregulated wound repair in the lung, rather than chronic inflammation. In the normal lung, over 90% of the alveolar surface area is covered by large flat terminally differentiated type I alveolar epithelial cells (AEC I), with 7% of the surface area consisting of the more plastic and cuboidal shaped type II cells (AEC II).[13] After damage occurs to the epithelium of the alveoli, several mechanisms occur in order to promote effective wound healing. Local fibroblasts are activated to become myofibroblasts, which function to produce collagen. Furthermore, the AEC II migrates to the area of injury, where these cells eventually differentiate into and replace the damaged AEC I.[14,15] This re-epithelialization following injury allows the alveoli to regain its gas-exchange function. However, IPF lesions are characterized by hyperplasia of AEC II along with excessive collagen deposition, which indicates dysfunctional wound healing: the failure to re-epithelialize and the aberrant accumulation of myofibroblasts. Fibrotic disease is ultimately the result of an increase in collagen deposition, and a decrease in collagen degradation.[16] The activation and accumulation of myofibroblasts occurs as a result of signals from the epithelium, and can be perpetuated by inflammatory events that contribute to the pro-fibrotic environment (Figure 2). Though there is no known cause for IPF, several risk factors are associated with the disease, such as old age, cigarette smoking, inhaling occupational particles, gastro-esophageal reflux, viral infections, and diabetes. [5,17–21] These factors likely initiate one or multiple cascades of events that promote alveolar injury and inflammation and ultimately lead to the development

and progression of fibrosis. The following sections review the crucial roles that epithelial damage and pulmonary inflammation have in the activation of myofibroblasts.

Role of epithelial cells in IPF – activators of myofibroblasts

Apoptosis

Prolonged apoptosis of epithelial cells perpetuates the dysfunctional wound healing signals characteristic of IPF and contribute to the failure to re-epithelialize. Epithelial apoptosis is a critical event in animal models of pulmonary fibrosis, and inhibition of apoptosis leads to attenuation of the disease. [22,23] Targeted destruction of AEC II in a mouse model has shown to be enough to incite a fibrotic response. [24] However, it is important to note that in laboratory animal models, unlike the natural human disease, the inciting event of the disease is a known and controlled agent. However, given the results of these studies along with the evidence of apoptotic events in the IPF lung, it is likely that epithelial injury is an important and critical factor to disease development and progression. Lung biopsies taken from IPF patients show an increase in epithelial apoptosis in areas of fibroblast foci of collagen producing myofibroblasts. [25] Although the inciting factor involved in apoptosis in IPF is not known, it is likely that a combination of environmental and genetic factors are responsible for the perpetuation of epithelial damage.

Increased oxidative stress in the lungs of IPF patients may contribute to AEC cell damage and perpetuate activation of apoptotic pathways.[26] These damaging oxidants can be produced from neutrophils or macrophages recruited to the region.[27] However, because anti-inflammatory treatments do not affect the outcome of IPF patients, it is likely that immune cell mediated epithelial damage is not solely responsible for the progression of the disease.[12] A

human cell culture system has identified that myofibroblasts isolated from the lungs of IPF patients are capable of producing hyrdrogen peroxide, which can contribute to cell death of nearby AECs. [28]

Genetic studies have provided important clues to other contributors to epithelial apoptosis and the pathogenesis of IPF. A mutation of the surfactant protein C (*SFTPC*) gene is associated a familial form of the disease. [29,30] As a result of this mutation, the surfactant C protein is misfolded as it is being processed by AEC II cells. The aggregation of misfolded proteins within the cell results in endoplasmic reticulum (ER) stress, and contributes to a cellular process known as the unfolded protein response (UPR). Mutation of the surfactant protein results in ER stress, the UPR, and subsequently cell death by apoptosis in human epithelial cells. [31] It does not appear that sporadic IPF patients have mutations in *SFTPC*, however, there are other mechanisms that could generate a similar cellular response. [32] Markers of ER stress and UPR have been found in the AECs of IPF patients. [33] Chronic ER stress and UPR may be responsible for the apoptosis of AEC II in IPF. [34]

Telomere shortening is a feature of cell senescence and aging, and is also accelerated in smokers when compared to non-smokers. [35] Both smoking and ageing are co-factors that can be involved with the development of the disease and have been associated with IPF. [18] Genetic evaluation of familial IPF have also highlighted the association between the disease and a mutation of the telomerase gene, *TERT*. [36,37] This gene encodes the polymerase responsible for lengthening the telomeres following chromosome replication. A defect in *TERT* results in shortened telomeres, cell senescence and eventually apoptosis.[38] Though the *TERT* mutation is not necessary in order to develop all cases of IPF, it indicates that other mechanisms of

abnormal shortening of telomeres may be responsible for the pathogenesis of the disease, by inducing apoptosis of epithelial cells.

The above literature strongly indicates that epithelial apoptosis is an important contributor to the activation of myofibroblasts. Epithelial apoptosis can occur as a result of many factors, including direct oxidative damage, ER stress, and cell senescence. These factors can be incited by many genetic and environmental stimuli. In addition to apoptosis, epithelial cells likely play another role in the activation of fibroblasts , which is transitioning into fibroblasts themselves.

Epithelial mesenchymal transition

Damage to the alveolar epithelium is not the only pro-fibrotic fate of the AEC II in IPF. It is likely that a process known as epithelial mesenchymal transition (EMT) contributes to the unwanted accumulation of fibroblasts in the fibrotic lung. Though likely not the only source of fibroblast accumulation, epithelial cells have the ability to lose key epithelial characteristics and acquire a fibroblast phenotype. One study found that primary rat AECs as well as a rat AEC II cell line lost epithelial markers and gained fibroblast markers after exposure to transforming growth factor β1 (TGF-β1), a known pro-fibrotic factor. [39] The epithelial cells of the lungs of a mouse model developed mesenchymal cell markers after 3 weeks of exposure to TGF-β1. In this study, the authors found that nearly all of the fibroblasts in the diseased lung were of epithelial origin. [40] Similarly, TGF-β1 also can induce EMT in a human lung cell line. [41] TGF-β1 and pro-fibrotic exogenous factors are not the only mechanisms by which AECs may be stimulated towards EMT. There is evidence that ER stress is capable of inducing EMT as well.[42,43] This phenomenon is likely to play a role in the natural disease of IPF, as lung biopsy samples from

patients revealed cells with markers for both epithelial cells and fibroblasts, indicating that this transition occurs in the natural disease. [39] Tanjore and colleagues observed EMT *in vivo* in the lungs of their mouse model of pulmonary fibrosis. However, contrary to some other findings by another laboratory, in this study only a small percentage of fibroblasts of epithelial origin had differentiated into activated myofibroblasts. [40,44,45] In the natural disease, it is difficult to determine whether a fully differentiated myofibroblast is of epithelial or another origin. So although EMT may occur, the extent to which epithelial derived fibroblasts are responsible for collagen deposition in IPF is still unknown.

Contribution of the immune system to IPF – perpetuation of a pro-fibrotic environment

Though the importance of chronic inflammation to the development and progression of IPF has recently been challenged, there is evidence that certain immune processes are present within diseased lungs. The adaptive immune response likely plays an important role in the chronic damage that perpetuates progressive fibrosis. CD4+ T cells, or T helper (T_H) cells, can be divided into subtypes based on the cytokines they produce. Type 2 T_H (T_H2) cells are known to produce high levels of type 2 cytokines, namely IL-4, IL-5, and IL-13. In addition to the T_H cells of the adaptive immunity, recent work has revealed an important role for innate lymphoid cells (ILC) that secrete cytokines in response to a non-specific antigen. The subset that produces type 2 cytokines upon activation are known as ILC2. Epithelial derived factors, IL-25, IL-33, and TSLP are currently thought to be the crucial activating stimuli for ILC2.[46–49] Both T_H2 and ILC2 may be critical sources of profibrotic type 2 cytokines necessary for the development or progression of fibrosis. Investigations using transgenic laboratory mice or neutralizing factors have indicated a critical role for type 2 cytokines in the development of fibrotic disease.[50–53] Analysis of

bronchoalveolar lavage fluid of IPF patients has revealed elevated type 2 cytokines, specifically IL-13.[54] Furthermore, type 2 cytokines appear to be up-regulated in the tissues of lungs from patients with IPF.[55] These results indicate that a type 2 environment is an important contributor to the development of IPF.

Cell culture work has shown that type 2 cytokines contribute by directly stimulating fibroblasts to differentiate in to the ECM producing myofibroblast phenotype.[56] Type 2 cytokines are also responsible for activating macrophages into their alternative phenotype.[57] Alveolar macrophages isolated from patients with pulmonary fibrosis express markers indicated an alternative activation state.[58] Alternatively activated macrophages, as well as type 2 lymphocytes, are important producers of pro-fibrotic factors that not only can perpetuate the type 2 inflammatory response, but also directly activate fibroblasts to produce collagen. [59,60] Perhaps one of the most crucial factor directly affecting the activity of fibroblasts, and the subsequent development of tissue fibrosis is TGF-β1.

TGF-β1

In fibrotic lung disease, TGF-β1 can be secreted by inflammatory cells as well as AEC II, and is a paracrine or autocrine signal for activating mechanisms involved in the fibrotic pathway. It is secreted as an inactivated precursor protein. Interestingly, this latent form of TGF- β1 can accumulate within the ECM for later activation [61] Interaction with epithelial and fibroblast derived factors are necessary to activate TGF- β1 into its functional form. [62,63] Activated TGF- β1 has long been known to contribute to the expression and deposition of ECM, perhaps by promoting the differentiation of fibroblasts into activated myofibroblasts [64,65] TGF-β1 is also capable of promoting differentiation of epithelial cells into fibroblasts, through the

process of EMT. [39,41] Because of its role in ECM production, TGF- β1 has been investigated in IPF as a potential contributor to the activation of fibroblasts and ECM deposition in the disease. Using immunoshistochemical staining techniques, Khalil and colleagues found heavy localization of TGF- β1 to hyperplastic type II AEC in fibrotic lesions. [66] This observation indicates that AEC II driven secretion or activation of TGF- β1 may contribute to the disease and severity of lesions. As a follow up study, this group found that in lungs of those with early disease and less severe lesions, TGF- β1 was limited to alveolar macrophages. However, in the lungs of those with more advanced disease and severe fibrosis, TGF- β1 was predominantly located in the AEC II. [67] Interestingly, although TGF- β1 is considered an important molecule for normal wound healing, there is evidence that heavy local expression can reduce the re-epithelialization of wounds. [68,69] These observations indicate that epithelial derived or activated TGF- β1 is involved in the progression of the disease, perhaps in a feed-forward cycle. This could explain treatment with steroids is ineffective in cases of advanced disease. It has been demonstrated that TGF- β1 expression is not reduced in response to corticosteroid treatment, and in fact corticosteroids may up-regulate activation of the latent TGF- β1. [70,71]

The myofibroblast – effector of disease

It is the accumulation of excessive extracellular matrix (ECM) proteins, such as collagen, that leads to the damage of the alveolar architecture and consequentially impaired gas exchange and organ dysfunction. The effector cells responsible for normal and abnormal deposition of ECM are activated fibroblasts, known as myofibroblasts. Myofibroblasts are mesenchymal cells distinguished by the *de novo* production of α-smooth muscle actin (αSMA), which has become the marker to identify effector cells in normal and abnormal ECM

deposition.[72,73] In IPF lesions, the fibroblast foci are characterized by an accumulation of myofibroblasts and therefore an increase in production of ECM proteins. [74] There are many possible origins of precursor cells. Myofibroblasts can differentiate from tissue resident fibroblasts, bone marrow derived circulating fibrocytes, or from epithelial cells in a process known as epithelial mesenchymal transition (EMT).[39,65,75] In order to understand the progressive pathology of IPF it is important to understand the factors that can contribute to the aberrant accumulation and activation of myofibroblasts. As reviewed above, this can occur through various mechanisms. Cellular damage to the AEC by multiple exogenous and endogenous signals contributes to the activation of myofibroblasts. Additionally, pro-fibtrotic factors such as TGF- β1 can induce AECs to transition into myofibroblasts themselves. The profibrotic environment is exacerbated and perpetuated by the accumulation of type 2 inflammatory cell recruitment. However, a direct insult to initiate these mechanisms in IPF remains unknown. The development of the disease is likely due to a “perfect storm” of co-factors that trigger the multiple pathways reviewed above. However, another important co-factor to the development of this complex disease is a topic of current investigation. Evidence has shown that viral infection of the lung is associated with IPF, and it may be involved in the perpetuation of unwanted collagen accumulation.

GAMMAHERPESVIRUSES AND PULMONARY FIBROSIS

Herpesviridae are a family of viruses in the order herpesvirales that infects mammals, birds, and reptiles. The gammaherpesvirinae are one of three subfamilies, and includes the genera Lymphocryptovirus, Macavirus, Rhadinovirus, and Percavirus. [76,77] A hallmark of all herpesviruses is their ability to establish lifelong latency in their host. The latent genome packages itself within the host cell nucleus as a circular extra-chromosomal episome of viral DNA.[78] Unlike other subfamilies of herpesviruses, infection with gamma herpesviruses usually produce only a mild clinical disease (if any) upon primary infection, and quickly establish latency in the B lymphocytes of the host.

Of the gamma herpesviruses (gHVs), Epstein Barr virus (EBV) is perhaps the most well studied. EBV was first detected in the 1960's by electron microscopy in the lymphocytes of Burkitt's lymphoma tumors. [79] Primary infection occurs through oral contact with saliva of an infected individual.[80] Frequently, primary infection produces mild, if any clinical disease. In some instances, primary exposure may produce acute infectious mononucleosis.[81] It is hypothesized that the virus passively passes through the epithelium via transcytosis in order to encounter lymphocytes.[82] Upon interaction with the naïve B cell, EBV enters the cell via interaction of gp350/220 with the cell surface protein CD21 and also via interaction of three viral proteins gp42, gH, and gL with the MHC II molecule on the cell.[83–85] After entry, the virus enters a state of latency where the genome arranges itself as a closed circular episome.[78,86] Once infected, the latent virus induces cellular expression of proteins that signal for the cell's migration to the germinal center of the lymph node, in much the same way as if the naïve B cell

encountered its cognate antigen.[87] In the germinal center, the infected B cell undergoes rapid division and exits into the periphery as a memory cell.[88] Latently infected memory cells circulate and act as a reservoir of persistent infection within the host. As the circulating memory B cells transit near the oropharyngeal lymphoepithelium, they occasionally differentiate into a plasma cell. The exact stimulus of this differentiation is unknown: one possibility is that it encounters its cognate antigen and differentiates in order to produce antibody. Upon differentiation of a latently infected memory cell into a plasma cell, lytic viral gene expression is initiated, and the virus begins *de novo* replication of infectious virions.[89] It is from these plasma cell-produced virions that infection of the nearby epithelium occurs. Though more research is necessary to understand specific mechanisms permitting the infection of epithelial cells, it is likely the active lytic infection of these cells that produces the virus shed by the host through the saliva.[90]

Evidence of gamma herpesvirus involvement in IPF

Gamma herpesvirus infection, specifically infection with EBV, has long been suspected to be involved in IPF. Serological evidence has shown that individuals with IPF have higher serum IgA and IgG antibodies against the viral capsid antigen (VCA) of EBV when compared to subjects without signs of IPF. [91,92] Investigations of lung tissue reveal that EBV infection occurs in the lower airways of IPF patients at a significantly higher frequency than in other populations. Pulkkinen et al used PCR to detect EBV DNA in the lung tissues of 11/12 patients with IPF, while none of the 10 control lungs showed detectable levels of viral DNA. [93] Similarly, Stewart et al found EBV DNA in the lung tissues of 13/27 IPF patients, while only 4/28 controls had detectable levels. [21] Another study by Calabrese et al in 2013 found EBV DNA in the lung tissues of 22/55 IPF patients, while only 3/41 control lung tissues were positive. [94] It is evident

that despite the ubiquity of infection with EBV in the population, presence of EBV in the lower airway is not commonly detected in healthy lungs. The higher incidence of EBV DNA in IPF lungs makes a strong case that the virus may be involved with the etiology or exacerbation of IPF. However, though an association is likely, causality cannot be determined based on the above observations. It must also be noted that reports exist in which EBV was not detected within the lung tissue of IPF patients, and controversy still exists on whether infection of the lower airway with the virus is important in the pathogenesis of IPF.[95,96]

The virus associated with disease in IPF lungs are likely involved in active, lytic infection of the lung tissue, specifically the epithelial cells of the alveoli. Studies in the 1990s used immunohistochemistry to target the viral antigens gp 340/220 and VCA. These antigens are expressed during lytic replication of EBV. It was shown that the epithelial cells in lung biopsies taken from IPF patients were significantly more likely to stain positive for these antigens when compared to control lung biopsies. This not only supports the previous evidence that EBV may be associated with IPF disease, but also indicates that the virus is undergoing lytic replication. [21,97] Kelly, et al. found that of IPF lung tissue samples that were positive for EBV, 61% of these samples contained a rearranged genome fragment known as WZhet that is indicative of a lytic version of the virus. In this study, EBV DNA was found in the peripheral leukocytes of 75-85% of IPF patients and controls, but interestingly, the WZhet form was found in 16 of 27 IPF patients, but 0 out of 32 lung transplant recipients, and 1 out of 24 control blood donors. [98] Some studies involving animal models of viral associated lung fibrosis indicate that pulmonary cells latently infected with gHV up-regulate expression of pro-fibrotic factors and contribute to *in vivo* development of fibrosis.[99,100] Understanding the state of latency of the virus will give better

understanding as to its contributions to pathogenesis of disease. Regardless, detectable levels of gHV in healthy lungs is abnormal, and given the prevalence of evidence of EBV in the lungs of patients with IPF, an association between gHV infection of pulmonary cells and fibrotic disease is worthy of additional study in humans as well as other model systems.

Mechanisms by which EBV can contribute to IPF – evidence from murine models

Murine models have been a useful tool to help tease out the mechanisms involved in gHV related pulmonary fibrosis. Murine herpesvirus 68 (MHV68) is a gHV that naturally infects the bank vole (*Myodes glareolus*) and the wood mouse (*Apodemus sylvaticus*). [101,102] Because EBV is unable to establish infection in laboratory mice, MHV68 has been shown to establish infection within laboratory mice and used as models of human gHV infection. [103] However, the pathogenesis MHV68 disease is not completely similar in the laboratory mouse when compared to the natural host. [103,104]

The natural host of MHV68 does not develop significant pathology upon infection with the virus. [104] However, wild type laboratory mice will develop some lung inflammation, but no pulmonary fibrosis when infected with MHV68. [104] Bleomycin treated laboratory animals are a popular model for studying the mechanisms of pulmonary fibrosis *in vivo*, though this model only shares some histologic features of human pulmonary fibrosis.[105] Bleomycin is an antibiotic that is used clinically in humans as a chemotherapeutic cancer treatment. Bleomycin reacts with metal ions and oxygen to produce reactive oxygen species (ROS) which cleaves DNA leading to arrest of the cell cycle and death of the cancer cell.[106] However, in the lungs, the overproduction of ROS leads to unwanted cell damage and triggers an inflammatory response. This damage signals for wound healing mediators, such as TGF-β1, which initiates fibrosis.

Through this mechanism, the lung undergoes an acute phase injury due to neutrophilic and lymphocytic infiltration, and up-regulation of type 1 pro-inflammatory cytokines prior to fibroblast activation and excessive collagen deposition. In this first inflammatory phase, steroidal treatment can cease the progression of fibrosis and discontinuation of bleomycin exposure can halt disease progression.[107,108] In human IPF, a known injurious event is absent (or has long been resolved) upon clinical presentation. Anti-inflammatory treatments are ineffective, and the disease does not regress with time, but instead progresses despite the absence of a known inciting factor. Though the bleomycin model may elucidate some key mechanisms involved in the formation of fibrosis in general, the history and development of the resulting lesions differs from natural disease. In this way, information generated from this model may be limited.

MHV68 infection in the bleomycin model may provide clues to the development of gHV associated pulmonary fibrosis. Laboratory mice resistant to the effects of bleomcyin were infected with MHV68 8 days prior to a bleomcyin challenge. Mice that did not receive the virus did not develop pulmonary fibrosis in response to the bleomycin, but importantly those mice infected with MHV68 developed significant fibrotic pathology after belomycin injection. [109] These results indicate that MHV68 lytically infected animals will develop fibrosis when exposed to conditions that will not cause disease in an uninfected animal.

By 14 days post infection with MHV68, laboratory mice harbor the latent form of the virus within the lung [99,100] Latently infected laboratory mice have an enhanced fibrotic response when challenged with FITC or bleomycin as far as 70 days post infection with MHV68. [100] Latently infected alveolar macrophages, B-cells, and fibroblasts were harvested from mice 28

days post infection and analyzed for expression of pro-fibrotic factors. When compared to those from mock inoculated animals, the latently infected cells up-regulated cytokines and chemokines important for myfibroblast differentiation, leukocyte, and fibrocyte recruitment. [99] These results indicate that the presence of latent gHV may be enough to modulate the environment of the lung into a pro-fibrotic state.

MHV68 infection can also exacerbate established fibrosis. After established FITC induced fibrosis, mice infected with MHV68 had increased collagen deposition 7 days post infection when compared to uninfected mice. [110] In this study, it was observed that the lytic replicating form of the virus was responsible for the exacerbation of the fibrotic response. [110] Established fibrosis is not exacerbated by infection with other common viral or bacterial pathogens. Though infection with influenza or *Pseudomonas aeruginosa* increases the recruitment of inflammatory cells to the lungs, it does not increase collagen deposition in mice previously treated with bleomycin. MHV68 infection, however, does increase collagen deposition in mice previously treated with bleomycin when compared to uninfected animals, or those infected with influenza or *Pseudomonas aeruginosa*. Animals treated with bleomycin have an increase in lytically active MHV68 when compared to those infected with the virus, but not challenged with bleomycin. [111]

The above studies illustrate that MHV68 infection likely contributes to the development of fibrotic mechanisms in the lung. In these observations, the virus acts as a co-factor to an insult known to induce fibrosis. In contrast, in the cases of IPF observed in humans, fibrosis can develop in the absence of a known environmental stimulus. In those cases, the pulmonary environment is often characterized by a type 2 cytokine profile.[54,55] Knocking out the IFNγ receptor creates a mouse (IFNγR-/-) with a cytokine profile skewed towards a type 2 response.

Though both IFNγR-/- and wild type mice develop pulmonary inflammation following infection with MHV68, only the knockout mouse develops fibrosis without any other pro-fibrotic stimulus. Evidence of subpleural fibrosis in the lungs begins around 45 days post infection, with the disease advancing to interstial fibrosis by 150 days post infection. The alveolar wall continues to thicken at least until day 180 post infection. [112] The results of this study are substantial, as this model is not dependent on a single insult, such as a bleomycin or FITC challenge. Importantly, the disease observed in the type 2 biased mice was a progressive disease that continued to worsen with chronic herpesvirus infection. In this model, it seems that the progression of fibrosis can be halted by administering anti-virals to target MHV68, indicating that lytic replication of the virus is necessary for the disease progression. [113] Work with this model has helped draw conclusions that a type 2 environment in conjunction with gHV infection in the lung may play important roles in the pathogenesis of the human disease.

The prevalence of IPF increases with age, with the mean age of diagnosis being 66 years old. [6] In a senescence accelerated mouse model, it appears that the ageing lung is inherently more prone than a younger lung at developing fibrosis after exposure to bleomycin. [114] This is likely a result of the aged animal more likely expressing elevated levels of pro-fibrotic factors, such as TGF-β1, but interestingly aged mice also showed more circulating bone marrow derived fibrocyte recruitment. [114] Furthermore, evidence shows that aged mouse fibroblasts have more TGF-β1 receptors and therefore an increased collagen production response to TGF-β1 stimulation. [115] Aged mice are also more predisposed to gHV induced lung fibrosis. [115,116] The increase in gHV related fibrosis in these models corresponds with an up-regulation of pro-inflammatory as well as pro-fibrotic factors, notably TGF-β1 and the fibrocyte recruitment

chemokine, CCL12 [115,116] The epithelial cells in aged animals infected with MHV68 exhibit higher levels of ER stress, as well as higher levels of AEC II apoptosis when compared to infected younger animals. [116] These studies indicate that cellular factors associated with aging may contribute to the pro-fibrotic environment necessary to promote gHV associated fibrotic disease.

In summary, these laboratory animal experiments provide valuable evidence supporting the hypothesis that gHV can play a role as a cofactor in the development of pulmonary fibrosis. The studies indicate that gHV infected animals harboring the lytic or the latent form of the virus within the lower airways can develop fibrosis under conditions uninfected animals will not. gHV infection can also exacerbate established fibrosis, or initiate disease in lungs with a pro-fibrotic environment. Evidence from these models nicely complement the observations from human IPF patients and together provide a compelling argument that gHV contributes to fibrotic lung disease in humans. Future studies in laboratory mice will continue to reveal potential mechanisms by which gHV infection can influence the development of progression of pulmonary fibrosis. However, the disease created using MHV68 in laboratory mice differs in several key ways from human IPF. Laboratory mice are not the natural host for the virus, and in order to develop disease, an extreme fibrotic environment is necessary. Therefore, it will be difficult to tease out the co-factors that contribute to IPF development in the gHV infected lung using laboratory mouse models. In order to answer questions regarding pathogenesis of the natural disease, we can look towards the horse – as this species naturally develops a gHV associated fibrotic lung disease.

EQUINE HERPESVIRUS 5 (EHV-5)

Like EBV in humans, EHV-5 is a ubiquitous pathogen among the equine population. Viral DNA is routinely detected in nasal swab and peripheral blood cells in healthy and diseased animals.[117–123] EHV-5, similarly to gamma herpesviruses of humans, likely establishes latency in the lymphocytes. Though the specific target cell for latency has not been determined, sensitive assays can routinely detect viral DNA in the peripheral blood cells of horses.[118,119,121–123] Foals acquire EHV-5 early in life, likely by 6 months of age. The primary infection is not associated with any clinical signs of disease. [119] Because of its ubiquity and no noticeable clinical disease, until recently, EHV-5 infection was largely presumed to be insignificant for veterinary investigation.

Scientific and veterinary interest in this virus has grown in the past decade, prompting improved diagnostics as well as speculative investigations on the potential involvement in different pathologies. With the prevalence of EHV-5 nasal shedding throughout the equine population, it is tempting to hypothesize that infection with the virus can influence the pathogenicity of common upper respiratory diseases. EHV-5 DNA was reported in the nasal secretions of horses with infectious upper respiratory disease due to various respiratory pathogens. However, EHV-5 was also detected in the nasal swab samples from horses free from respiratory disease. The EHV-5 viral load was not significantly higher in diseased animals when compared to healthy ones, except in the case of those with EHV-4 respiratory disease. However, this cohort was much younger than any other group of animals tested, and it cannot be assumed that higher EHV-5 shedding was associated with EHV-4 disease, or other group

differences, such as age in this particular study. [117] However, it has been suggested that detection of EHV-5 may be influenced by age by other studies. One group found that it was significantly more likely to find EHV-5 DNA in the nasal swabs or PBMCs of horses 1-3 years old than in horses 4 years and older.[122] Rushton, et al. also found a higher rate of detection in nasal swabs of younger horses when compared to those of older horses. However, in this study though the horses were housed in different environments, with the younger animals being held in pens in larger groups. Therefore, it is unclear whether increased shedding is due to age or housing conditions.[121] Another group also found an effect on age with the detection of EHV-5 in the nasal swabs of horses. However in this group ages 2 or 4 were less likely to be positive, whereas it was more likely to detect the virus in horses aged 3, 5, or 8 years.[120] The results of this study must be carefully considered, as there little evidence that there are immunological distinctions between horses of these ages, and the variances in the viral load reported may largely be due to differences in environment, housing, and exposure between these groups, rather than age alone. It has been speculated that EHV-5 infection in young horses may predispose them to infection of other pathogens.[119] However, as evident by the results of the above studies, there is no significant evidence that EHV-5 infection induces or modulates the susceptibility to upper respiratory disease.

In one thorough study, Helena Back and her colleagues measured the EHV-5 viral load in the nasal swabs of 63 racehorses monthly, for over one year[120]. They found that 97% of these horses tested positive at least once for the virus, and that viral load did not have any association with respiratory disease or quality of performance. The findings from this investigation indicate that any observed variations in presence and viral load of nasal secretions

are most likely due to the natural temporal variation and epidemiology of this virus, than to a specific respiratory disease state.

Because of the ubiquity of EHV-5 in nasal swab samples of horses with respiratory disease, it is tempting to imagine that infection with EHV-5 is a co-factor in upper respiratory disease pathology. Whether EHV-5 infection makes the host susceptible to other pathogens, or it is possible that subsequent infections with other respiratory pathogens reactivates EHV-5 out of a latent state. While it is certainly possible that EHV-5 infection may influence the pathogenesis of other infectious disease, because EHV-5 can routinely be found in nasal swabs of healthy horses, it is difficult to make an association by quantifying level of shedding in healthy and sick animals, without careful attention to match each group in other factors such as age, breed, diet, sex, use, etc.

Equine multinodular pulmonary fibrosis

Though EHV-5 is prevalent in the nasal swabs and peripheral blood leukocytes of horses regardless of disease, it is rare to find the virus in the lower airways of healthy or diseased horses – with one notable exception.[124,125] Recently, EHV-5 infection of the lung has been linked to a newly recognized disease termed equine multinodular pulmonary fibrosis (EMPF) by Williams and colleagues in 2007.[1] In horses afflicted with this progressive fibrotic lung disease, EHV-5 infection of the lung has been confirmed with PCR of the lung tissue, however, tissue DNA is negative for the virus in horses with respiratory disease but not affected by EMPF.[1,124,126–133] Though the relationship of EHV-5 infection of the lung and the development of EMPF is still controversial, a study by Williams et al. found that experimental inoculation with EHV-5 into the lungs of healthy horses induced fibrosis in the lung. In this study, 6 horses were

inoculated with EHV-5 directly into the lung via endoscope, and 2 horses were mock inoculated. The horses were euthanized 97-108 days post inoculation and tissues were collected. 3 of the 6 horses had gross fibrotic lesions that resembled naturally occurring EMPF. Histologically, lung tissues contained significant more collagen deposition when compared to the control inoculated horses. Around the lesions of in the lungs of EHV-5 inoculated horses, immunohistochemistry identified numerous activated myofibroblasts, as well as EHV-5 antigen localized to the alveolar epithelial cells. These results provide compelling evidence that pulmonary infection with EHV-5 can induce the development of fibrotic disease.[2]

The clinical disease of EMPF – a review of case studies

Horses with EMPF typically present with a history of lethargy and weight loss. Examination usually reveals tachycardia, tachypnea, and pyrexia. Laboratory examination will often reveal leukocytosis, lymphopenia, and hyperfibrinogenemia. Hypoxia is also a common observation due to decreased gas-exchange area on the lung. Auscultation of the thorax is commonly abnormal in all lung fields, and includes crackles and wheezes. Thoracic radiographs will show a diffuse nodular interstitial pattern, and defined nodular lesions can usually be detected with ultrosonography. Transtracheal wash, bronchoalveolar lavage, or lung biopsy are collected if the case warrants in order to differentiate from other interstitial lung disease. Bacterial cultures of tracheal or lung aspirates are normally negative. Histopathology of lung biopsy will reveal expansion of the alveolar septum due to collagen deposition. The alveolar lumen will often contain an infiltrate of mixed inflammatory cells. Occasionally, macrophages with viral inclusion bodies can be detected. If the above conditions indicate fibrotic lung disease, and the lung tissue is positive for EHV-5 DNA using PCR analysis, a diagnosis of EMPF is

usually made. Treatment of the cases reviewed here varied, but most included corticosteroids, non-steroidal anti-inflammatory drugs, bronchodilators, or an anti-herpesviral drug. Once a diagnosis of EMPF is made, the prognosis is usually fair to poor – and less than 50% of the cases reviewed here recovered. Prognosis seems to improve if the diagnosis is made early before severe progression of the fibrosis. Post-mortem examination of the lung will reveal gross tan-white firm nodules throughout the lung with apparent healthy tissue surrounding the lesions. [1,124,126–133]

Though efficacy of acyclovir (a herpesvirus polymerase inhibitor) for treatment of EMPF is still largely anecdotal and theoretical, there seems to be some consensus that valacyclovir treatment (the prodrug form of acyclovir) for several weeks has the most potential to be effective. [124,134,135] Valacyclovir is preferred over acyclovir treatment, as evidence exists that to reach effective antiviral plasma concentrations in the horse acyclovir must be administered intravenously as a constant rate infusion. In contrast, valacyclovir has improved oral bioavailability, and therefore can be administered orally every 8 hours to achieve ideal plasma concentrations. [135–137] There is some controversy surrounding the use of corticosteroids for EMPF treatment. Corticosteroids have the potential to reduce the inflammatory state of the lung that is contributing to the development and progression of the fibrosis. [124,134] However, besides typical veterinary concerns surrounding long term administration of corticosteroids to horses, including the increased risks for conditions such as laminitis, some authors believe that the immunosuppressive properties will encourage an increase in EHV-5 replication. [124,129,135,138] As with human IPF cases, there seems to be mixed evidence that corticosteroid treatment is effective at improving the disease outcome of EMPF.

Based on the literature, it is not a stretch to conclude that nearly every horse has been infected with EHV-5, yet only a select few develop disease. This mimics observations in humans and EBV related lung disease. More investigations are necessary in order to elucidate the mechanisms by which EHV-5 infection contributes to the pathogenesis of fibrotic lung disease. Contrary to the lung fibrosis induced in murine models, EMPF is a naturally occurring spontaneous disease in the horse and is associated with a gHV that naturally infects the species. Understanding the pathogenesis of EHV-5 associated lung disease in the horse will provide insight that cannot be obtained by using laboratory mouse models. The ability to experimentally study the horse in a laboratory setting overcomes several obvious limitations to experiments using human disease. However, *in vivo* studies in equids have some practical, economical, and ethical limitations that murine models do not. *In vitro* cell culture models using equine cells and EHV-5 can answer important questions regarding viral pathogenesis and host interactions. Furthermore, information generated from these studies can lead to well supported hypotheses that can justifiably be tested *in vivo*. In this way, EHV-5 and the horse is an attractive way to investigate the role of gHV associated lung fibrosis by addressing obvious limitations to murine and human research.

PURPOSE AND HYPOTHESES

Though subclinical lifelong persistent infection of both EBV and EHV-5 appear to occur in the lymphocytes of the majority of their host species' populations, there is increasingly compelling evidence suggesting that infection of pulmonary epithelial cells with these viruses is associated with fibrotic lung disease. [1,2,21,97] In humans, EBV virus is persistently shed in significant quantities in the oral cavity of healthy individuals. This is likely due lytic replication in the oral epithelium, and thus the oral cavity can serve as a reservoir for the virus and subsequent transfer to another host.[90] *In vivo* evidence has identified lytic replication of EBV in normal epithelial cells, and that this infection occurs in the differentiated (suprabasal) layers of the epithelium.[139]

Until recently, specific mechanisms of EBV replication in the epithelium was poorly understood due to the inability to generate productive infection *in vitro* with human epithelial cells. However, recently lytic replication has been achieved by culturing human oropharangeal epithelial cells in an organotypic system, in which the bottom surface of the culture is exposed to liquid media through a porous membrane while the top surface is exposed to air. This model mimics the biological environment of the airway epithelium, and thus promotes a natural differentiation program of the cells. In this system, in agreement with *in vivo* observations, the differentiated cells are capable of maintaining lytic EBV infection.[140] While lytic viral gene expression is confined to the suprabasal layer of the epithelium, a low level of latent infection exists in the basal layer. Furthermore, transcription factors involved in normal epithelial differentiation can activate latent EBV into a lytic infection. These results indicate that latent

viral infection can be established in the basal epithelium, and as these cells differentiate lytic EBV replication can be activated. Consequently, high levels of virus can then be shed into the environment.[141]

In horses, detection of EHV-5 suggests that the virus has a similar cellular tropism, with the virus establishing low-level latent infection in the lymphocytes, and persistent active replication in nasal epithelium in order to shed progeny into the environment. Though these kinetics occur without clinical disease, it appears that infection of lower airway tissue may contribute to the development of pulmonary fibrosis. Observations of horses with EHV-5 associated fibrosis suggest that EHV-5 infection becomes established in the epithelial cells of the affected lungs, but not in the lungs of healthy animals.[2] Understanding mechanisms involved in EHV-5 infection of equine airway epithelium will provide valuable insight into the processes involved in the development of gHV associated fibrotic lung disease. Similar to the work done with EBV, an air-fluid-interface cell culture model of airway epithelium has been developed to study cellular mechanisms involved in infectious disease of the horse. This model develops a psuedostratified layer of fully differentiated epithelial cells that maintain key immunological properties of the natural airway.[142] Infection of this system with EHV-5 could provide useful information on viral kinetics in equine respiratory epithelial cells, and thus be used to generate knowledge with which to design future studies on the pathogenesis of EHV-5 associated lung disease in the horse.

It was our intention in this study to develop a reliable and sensitive method by which to quantify viral load of EHV-5 in cell culture samples, as well as clinical samples. Once developed, we sought to use this assay to investigate EHV-5 kinetics in epithelial cell cultures at different

stages of differentiation in order to better understand the mechanisms involved with lytic replication of the virus in the respiratory epithelium of the horse. Furthermore, we sought to use the assay to determine if the viral load of EHV-5 in the nasal secretions and peripheral blood lymphocytes of horses was affected by acute respiratory disease with equine herpesvirus 1 (EHV-1).

The specific aims of this work are to:

a) Develop a sensitive quantification method in order to detect and quantify viral load in a variety of equine clinical and cell culture samples. We hypothesize that the designed assay will reliably detect and quantify multiple isolates of EHV-5, while not detecting other equine herpesviruses.

b) Identify viral load in EHV-5 inoculated equine epithelial cell culture at the air fluid interface over time, and identify whether viral replication differs when fully differentiated and stratified epithelial cells are inoculated compared to a newly growing monolayer. We hypothesize that fully differentiated epithelial cells will support lytic infection of EHV-5, and that replication of EHV-5 will increase over time as newly seeded epithelial cells differentiate.

c) Identify viral load in lymphocytes and nasal secretions of a cohort of horses, and investigate whether acute EHV-1 infection influences EHV-5 viral quantity at these sites. We hypothesize that viral load of EHV-5 detected in lymphocytes and nasal secretions of horses will increase during acute infection of EHV-1.

CHAPTER 3.

Development and Application of a Quantitative PCR Assay to Study the Pathogenicity of Equine Herpesvirus 5

Lila M. Zarski[a], Emily A. High[a], Rahul K. Nelli[a], Steven R. Bolin[a], Kurt J. Williams[a], & Gisela Soboll Hussey[a]

[a]Department of Pathobiology and Diagnostic Investigation
College of Veterinary Medicine
Michigan State University
East Lansing, MI

Manuscript to be submitted for publication in: *The Journal of Virological Methods*

ABSTRACT

Equine herpesvirus 5 (EHV-5) infection is associated with pulmonary fibrosis in horses, but further studies on EHV-5 persistence in equine cells are needed to fully understand viral and host contributions to disease pathogenesis. We developed a quantitative PCR (qPCR) assay to measure EHV-5 viral copy number in equine cell culture, blood lymphocytes, and nasal swabs of horses. The PCR primers and a probe were designed to target gene E11 of the EHV-5 genome. Specificity was verified by testing multiple isolates of EHV-5, as well as DNA from other equine herpesviruses. Four-week old, fully differentiated (mature) and newly seeded (immature) primary equine respiratory epithelial cell (ERECs) cultures were inoculated with EHV-5 and the cells and supernatants collected daily for 12-14 days. Blood lymphocytes and nasal swabs were collected from horses experimentally infected with EHV-1. The qPCR assay detected EHV-5 at concentrations around 10^4 intracellular genomes per cell culture in experimentally inoculated mature ERECs, and these values remained stable throughout 12 days. Intracellular EHV-5 copies detected in the immature cultures increased over 14 days and reached levels greater than 10^6 genomes per culture. EHV-5 was detected in the lymphocytes of 97% of horses and in the nasal swabs of 88% of horses both pre and post EHV-1 infection. In conclusion, qPCR was a reliable technique to investigate viral load in *in vivo* and *in vitro* samples, and EHV-5 replication in equine epithelial cells may be influenced by cellular stages of differentiation.

INTRODUCTION

The gammaherpesvirinae form a subfamily of the herpesviridae and are known to establish lifelong latency in the lymphocytes of their host following primary infection. In addition, there is evidence that viruses in this subfamily may be associated with fibrotic lung disease in multiple species. In humans, idiopathic pulmonary fibrosis (IPF) is a chronic and progressive interstitial lung disease of unknown etiology. IPF is the most severe form of interstitial pneumonias with a median survival of 3-5 years after diagnosis, regardless of treatment. [6] DNA of the gamma herpesvirus Epstein-Barr Virus (EBV) has been detected in the lungs of IPF patients, and evidence has strongly suggested an association between viral infection of the lung and the fibrotic lung disease. [21,93,94] However, observations of EBV in human tissues does not substantiate causality. Because of this, animal models are necessary to evaluate the potential role of gamma herpesvirus infection in the pathogenesis of fibrotic lung disease.

Laboratory mice have proven to be useful in generating evidence that infection with murine gamma herpesvirus 68 (MHV68) can exacerbate established lung fibrosis. [110,111] Furthermore, infection with MHV68 alone can induce pulmonary fibrosis in transgenic laboratory mice with an exaggerated type 2 cytokine response.[112] Despite the value of the evidence obtained from these studies, murine models have limitations when translating results to human disease. Wild type laboratory mice do not spontaneously develop lung fibrosis after MHV68 infection and infection in the virus's natural host produces no disease pathology.[104] In this way, these models incompletely mimic human IPF and as a result cannot answer important

questions regarding progressive fibrotic lung disease associated with viral infection in the natural host.

Equine herpesvirus 5 (EHV-5) is a gamma herpesvirus that naturally and ubiquitously infects the equine population. Like EBV in humans, infection with EHV-5 generally does not cause disease in the horse, with one notable exception. Equine multinodular pulmonary fibrosis (EMPF) is a progressive fibrotic lung disease of the horse that has been associated with the presence of EHV-5 DNA in the lungs of affected animals.[1] In contrast to murine models of gamma herpesvirus associated lung fibrosis, healthy horses develop lung fibrosis after experimental inoculation with EHV-5.[2] Thus, the horse has the potential to serve as a tool with which to study gamma herpesvirus associated lung fibrosis in the natural host. However, though not as limited as human IPF research, there are practical, ethical, and economical restrictions with *in vivo* equine EMPF research. Development of the disease can take several months, and housing horses in research facilities requires a large amount of labor and resources. In order to optimally design and justify further *in vivo* investigations, preliminary knowledge regarding EHV-5 pathogenesis should be generated *in vitro.*

Primary cell cultures provide a method in which to study specific mechanisms of virus – host interactions without the extensive resources of *in vivo* experiments. Epithelial cell injury has long been implicated in the dysfunctional wound healing response characteristic of fibrotic disease. [22,23] It is hypothesized that infection of epithelial cells in the lung with gamma herpesviruses dysregulates the epithelial signals involved with proper wound healing. EBV antigen can be found in the epithelial cells of the lungs of patients with IPF.[21,97] Similarly, in horses with both spontaneous as well as experimentally induced EMPF, EHV-5 antigen is

detected in the epithelial cells of the lungs.[2] Viral infection of these cells may play a role in the epithelial dysfunction and as a consequence, perpetuation of aberrant collagen deposition in these tissues. A primary cell culture system of equine respiratory epithelial cells (ERECs) has been established that retains key immunological features of the natural equine airway.[142] This model has been used to characterize host gene expression in response to infection with equine herpesvirus 1, an alpha herpesvirus.[143] A similar system in which to measure epithelial cell gene expression in response to infection with EHV-5 would be a valuable tool for understanding specific mechanisms involved in the pathogenesis of EMPF, and could be used to generate knowledge to use to design appropriate, economical and informative equine *in vivo* experiments.

In order to develop an *in vitro* equine cell model for EHV-5 infection, productive replication must be established and verified. Quantitative PCR (qPCR) can be used to measure EHV-5 genome copy in cell culture samples and thus evaluate viral replication in this system. In this study, we sought to develop a sensitive and specific qPCR test to be used to evaluate EHV-5 quantity in cell cultures and *in vivo* samples. In order to understand viral and host mechanisms involved in EHV-5 infection of equine cells, a cell culture system that promotes viral replication is necessary. Determining viral growth over time in equine cells could elucidate a system that supports lytic replication of EHV-5, and thus could serve as a model for future investigations into the pathogenesis of EHV-5 and associated disease.

MATERIALS AND METHODS

Virus isolation and propagation

EHV-1, EHV-2, and EHV-5 viruses were propagated in rabbit kidney 13 (RK-13) cells. For this, cell cultures were incubated at 37°C and 5% CO_2 with MEM-10 (Minimum Essential Medium Eagle (Sigma-Aldrich, St. Louis, MO USA) supplemented with 100 IU/ml penicillin, 100µg/ml streptomycin, 1% GlutaMAX® (GIBCO, Life Technologies, Carlsbad, CA USA), and 10% fetal bovine serum (FBS)). EHV-4 was propagated in equine dermal cells (NBL-6) (ATCC® CCL-57™, Manassas, VA USA), using MEM-10 supplemented with 1mM sodium pyruvate. After propagation, the cultures were frozen at -80°C and then thawed and centrifuged at 300*g for 10 minutes to remove cellular debris. The clarified supernatants were aliquoted and stored at -80°C as viral stock. The EHV-5 stock used to inoculate cell cultures in this study was isolated from lung tissue lesions from a horse diagnosed with EMPF.

EHV-5 stock titration

To determine infectious titer of EHV-5 viral inoculum for the cell culture experiments, $TCID_{50}$/ml was determined by inoculating monolayers of RK-13 cells in a 24 well plate. 200 µl of ten-fold serial dilutions of purified viral stock was added to the wells. After incubation at 37°C and 5% CO_2 for one hour, 800 µl media was added to each well. The cultures were incubated at 37°C and 5% CO_2 and the media replaced every 3-4 days. After 7 days, the cells were split 50% into a new 24 well plate. Cytopathic effect was recorded 17 days p.i. and $TCID_{50}$/ml was calculated via the method described by Reed and Muench.[144] The DNA titer of the inoculum was also determined by calculating EHV-5 genomes/ml using the qPCR assay described below.

DNA extraction

For assay development, viral DNA was extracted from 200µl of clarified supernatants from isolates of EHV-1, EHV-2, EHV-4, and EHV-5. For EREC culture samples, 200 µl of culture supernatants or thawed cell pellets from the protocol described below were re-suspended in 200 µl PBS and used for DNA extractions. For *in vivo* samples, peripheral blood mononuclear cell (PBMC) pellets re-suspended in 200 µl PBS or 200 µl nasal swab transport media was used. DNA extraction was performed on all samples using the QiAmp DNA Blood Mini Kit (Qiagen, Valencia, CA USA) according to the manufacturer's instructions.

Quantitative PCR (qPCR)

Primer and probe design

Primers and probes were designed using Primer Express software verson 3.0 by Applied Biosystems (Foster City, CA USA) and based off of the genome described by Wilkie, et al. (GenBank Accession no. KM924295).[145] The primer combinations were tested for possible cross-reactivity with unintended targets using the BLAST tool with the NCBI database. Candidate genes E6B and E11 were chosen as a target for EHV-5 detection as these genes are not present in the genome of EHV-2, a virus which shares a close sequence homology to EHV-5.[145] ORF8 (the gene that encodes the EHV-5 glycoprotein B protein) was chosen as it is well described by several independently derived sequences, and is highly conserved among strains. Additionally, the performances of the designed primers were compared against a previously published primer combination targeting ORF8.[123] Primers and probes were purchased from Sigma-Aldrich (St. Louis, MO USA) and are described in Table 1.

The primers and probe sequences for equine β actin reference gene were previously described and are as follows: equine β actin forward 5'-AGG GAA ATC GTG CGT GAC A-3'; equine β actin reverse 5'-GCC ATC TCC TGC TCG AAG TC-3'; equine β actin probe 5'[HEX]-CAA GGA GAA GCT CTG CTA TGT CGC CCT-[BHQ2]3'.[146]

Conventional and SYBR® Green PCR

Specificity of the primers for EHV-5 was tested using conventional PCR and SYBR® Green chemistry with viral DNA extracted from isolates of EHV-1, EHV-2, EHV-4, and EHV-5. SYBR® Green PCR was also performed using the designed primers on DNA obtained from 7 isolates of EHV-5 propagated from 6-10 passages of equine lung or blood samples in RK-13 cells. Conventional PCR was performed using 12.5 µl GoTaq® G2 Hot Start Green Master Mix (Promega, Madison, WI USA), 5 µl template DNA, and forward and reverse primers at a final concentration of 400 nM each with nuclease free water added for a total reaction volume of 25 µl. Thermocycling was performed with an Applied Biosystems Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA) using the following conditions: 94°C for 2 min; 40 cycles of 94°C for 15s, 61°C for 15s, and 72°C for 1 min. PCR products were separated by gel electrophoresis in 1.8% agarose gel and stained with GelRed nucleic acid stain (Phenix Research Products, Chandler, NC USA). The products were visualized with the ChemiDoc™ XRS+ system (Bio-Rad Laboratories, Hercules, CA USA).

SYBR® Green PCR used 10 µl SYBR® Select Master Mix (Applied Biosystems) and 5µl template DNA were used in each reaction. Forward and reverse primers were added at a concentration of 400 nM each, with nuclease free water added for a total reaction volume of 25 µl. Thermocycling was performed using the Applied Biosystems 7500 Fast Real-Time PCR

system with the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15s and 60°C for 1 min.

TaqMan® probe based qPCR

Probe based qPCR was performed using TaqMan® chemistry for all the samples collected for this study. Gene E11 was selected as the target gene for EHV-5 quantification based on conventional PCR and SYBR® Green analysis. The following concentrations and cycling conditions were used for both EHV-5 E11 and equine β actin assays: 10µl TaqMan® Gene Expression Master Mix (Applied Biosystems), 5 µl template DNA, forward and reverse primers to a final concentration of 500 nM, probe to a final concentration of 200 nM, and nuclease free water for a total reaction volume of 20 µl. Thermocycling was performed using the Applied Biosystems 7500 Fast Real-Time PCR system with the following conditions: 50°C for 2 min; 95°C for 10 min; 45 cycles of 95°C for 15s and 60°C for 1 min. All samples were run in triplicate and the quantification cycle (C_q) values were averaged; any outliers were excluded from the average. Plasmid DNA standards and negative template controls were included with each plate.

Plasmid DNA and standard curves

Gene segments of EHV-5 E11 and equine β actin were amplified from positive templates using the designed primers. The PCR products were cloned using the TOPO® TA Cloning Kit (Invitrogen, Life Technologies, Carlsbad, CA USA) according to manufacturer's instructions. Plasmid DNA concentration was determined by mass spectrophotometry using the NanoDrop 2000 (ThermoScientific, Waltham, MA USA), and copy number was calculated. Ten-fold serial dilutions were created from the stock plasmid DNA in order to create a standard curve and determine sensitivity for qPCR. The lowest ten-fold standard dilution that could be detected

and maintain a linear curve was considered the limit of detection. Intra-assay and inter-assay repeatability was determined from the CV of C_qs from 5 replicates in one plate or C_qs from 5 different runs respectively of ten-fold serial dilutions of plasmid copies from 100-10^8.

EHV-5 quantification

EHV-5 and β actin copy number per 5 µl template DNA was determined using the standard curve included with each assay, and copy number was determined by averaging the quantification cycles of triplicates of each sample. Triplicates that did not have at least 2 samples cross the threshold prior to cycle 45 were considered negative. Samples in which the C_q was later than the limit of detection of that plate's plasmid standard curve were considered positive, but below the quantifiable limit. For PBMC samples, EHV-5 copy number per 1 million copies of β actin was calculated. For cell culture samples, total EHV-5 genomes per culture well were determined by multiplying the total copies per qPCR reaction by 10 (to estimate the total copies per 50 µl total DNA elute). For cell culture supernatants, EHV-5 genome copies per culture well were determined by multiplying by 10 (as above) and the appropriate factor to consider original culture media volume. To control for differences in total material collected in the nasal swab transport media, DNA concentration was calculated using mass spectrophotometry for each sample, and EHV-5 copy number was determined per ng of DNA used in the reaction.

Quantification of EHV-5 following infection of equine dermal cell and equine respiratory epithelial cell (EREC) cultures

Equine dermal cell culture and inoculation

CCL-57™ equine dermal cells were purchased from ATCC® and were seeded into 24 well tissue culture plates at a density of 0.25 million cells per well in MEM-10 supplemented with 1mM sodium pyruvate and incubated at 37°C with 5% CO_2. The next day, the media was removed and 850 µl of EHV-5 stock ($10^{6.4}$ $TCID_{50}$/ml; 3.1 X 10^7 genomes/ml) or blank MEM was added. After 2 hours incubation at 37°C with 5% CO_2, the inoculum was removed and the cells were gently washed twice with media. 1.1 ml MEM-10 was added to each well, and 100 µl was removed from each well and pooled for a p.i. measurement. EHV-5 inoculated cells were harvested and stored as above 1-8, 10, 12, and 14 days p.i. Mock inoculated cells were collected days 1, 7, 10, and 14 p.i. Media was collected and replaced every 3 days.

Tissue collection for establishment of ERECs

A section of trachea was collected from 6 horses, aged 1-21 years old that were euthanized by intravenous overdose of pentobarbital for reasons unrelated to respiratory disease. Approximately 20 cm segments of trachea from just above the bifurcation of the bronchus were collected and transported immediately to the laboratory in chilled DMEM/F12 (GIBCO). All procedures were performed in compliance with the Institutional Animal Care and Use Committee of Michigan State University.

EREC isolation and preservation

EREC isolation was performed as previously described by Quintana, et al.[142] Briefly, the trachea mucosal tissue was washed in Dulbecco's Phosphate Buffered Saline (DPBS) (Sigma-

Aldrich) and then stored in enzymatic digestion media containing 1.4 mg/ml Pronase (Roche Applied Science, Indianapolis, IN USA) and 0.1 mg/ml deoxyribonuclease I (Sigma-Aldrich) for 3-5 days at 4°C. Following digestion, cells were rinsed away from the cartilage and incubated in a petri dish at 37°C with 5% CO_2 in order to reduce adherent fibroblasts. The non-adherent cells were counted, suspended in 10% DMSO, 30% FBS and 60% BEGM™ media (Lonza, Basel, Switzerland), and stored in liquid nitrogen until cultured.

EREC culture at the air-liquid interface

ERECs were thawed and suspended in DMEM/F12 (GIBCO) media supplemented with 5% FBS that was not heat inactivated, 1% MEM non-essential amino acid solution (GIBCO), 100 IU/ml penicillin, 100µg/ml streptomycin, and 1.25 µg/ml amphotericin B. 2-3 million cells in 500µl media were added to the top chamber of a type IV collagen coated Corning® Transwell® polyester membrane cell culture insert for 12 well plates (Corning, Inc., Corning, NY USA). 1 ml media was added to the bottom chamber below the insert. EREC cultures were incubated at 37°C with 5% CO_2. The following day, the media was removed from the top and bottom chambers. The media below was replaced with DMEM/F12 supplemented with 2% Ultroser™ G (Pall, Port Washington, NY USA), 100 IU/ml penicillin, 100µg/ml streptomycin, and 1.25 µg/ml amphotericin B.

For the mature cultures, ERECs from 3 horses were maintained under these conditions with media replacement every 2-3 days. After 4 weeks of maturation, the cells were gently washed with DMEM/F12. Two days later the media in the bottom chamber was removed and 500 µl of EHV-5 viral stock ($10^{6.7}$ $TCID_{50}$/ml; 2.3 X 10^7 genomes/ml) or DMEM/F12 media was added to the top chamber for two hours at 37°C with 5% CO_2. After removal of the inoculum,

the top and bottom chambers were gently washed twice with DMEM/F12 and the insert was moved to a fresh plate containing 1.2 ml media. After the inserts were added to the fresh plate, 200 µl media was collected to quantify any residual viral inoculum in the bottom chamber supernatant. To harvest cells, media was removed and the cells were disassociated from the insert membrane by applying 300 µl Accumax™ (Innovative Cell Technologies, Inc., San Diego, CA USA) to the top chamber and incubating at 37°C with 5% CO_2. Once detached from the insert membrane, the cells were collected, pelleted, and frozen at -80°C until DNA extraction. EHV-5 and mock inoculated cells were collected daily in this manner for 12 days. Every 4 days, the media in the bottom chamber was replaced and the old media was collected and stored at -80°C until DNA extraction.

For immature EREC culture, cells from 3 horses were seeded as above. 24 hours following seeding, the media was removed from the top and bottom chambers, and 500 µl EHV-5($10^{6.2}$ - $10^{6.7}TCID_{50}$/ml; 2.3-4.7 X 10^7 genomes/ml) or DMEM/F12 media was added to the top chamber. After two hours of incubation at 37°C with 5% CO_2, inoculum was removed and the top and bottom chambers were gently washed twice, as above. EHV-5 inoculated cells were harvested daily for 14 days, as above, pelleted, and stored at -80°C. Mock inoculated cells were collected 1, 7, 10, and 14 days post inoculation (p.i.). The media in the bottom chamber of each well was collected and replaced each day through day 8, after which media was collected and replaced days 10, 12, and 14. Mock inoculated cell supernatants were analyzed by qPCR pre and immediately p.i., as well as 1, 7, 10, and 14 days p.i..

Detection of EHV-5 in PBMCs and nasal swabs collected from EHV-1 experimentally infected animals

25 yearling horses were experimentally infected with either a neuropathogenic strain of wild type EHV-1 (WT), an EHV-1 mutant with a mutation of the polymerase gene (N752), or an EHV-1 mutant with the glycoprotein D gene replaced with that of EHV-4 (gD4). 60 ml of blood was collected in heparinized syringes prior to EHV-1 infections, as well as 7 and 10 days post EHV-1 infection. PBMCs were isolated as previously described and frozen at -80°C in pellets of 20 million cells. [147] Nasal swabs were collected at the same time points and stored in virus transport media (phosphate buffered saline containing 5% glycerol, 800 U m/L Penicillin/Streptomycin, 200 U m/L Gentamycin, and 100 U m/L Nystatin) at -80°C until DNA extraction. To compare the temporal variability of EHV-5 viral load in horses not acutely infected with EHV-1, PBMCs and nasal swabs were collected at similar time intervals from 8 control horses.

RESULTS

qPCR assay validation

Conventional PCR verified that the designed primers for E6B and E11 specifically amplified their target genes in EHV-5, while DNA templates from EHV-1, 2, or 4 were not detected. Both the designed primer set and the previously published primer set targeting ORF8 amplified product when using DNA from both EHV-5 and EHV-2 (data not shown). SYBR® Green PCR confirmed these results (Figure 3A). The melting curve from the SYBR® Green assay confirmed the specificity of the primers and the absence of primer-dimers (data not shown). Further, primers targeting gene E6B and E11 effectively amplified DNA from 7 different isolates of EHV-5 (Figure 3B). Negative template controls were negative for all assays performed in this study.

Based on the preliminary results, a probe for use of a TaqMan® assay with gene E11 were ordered to test all further samples for this study. Using serial dilutions of the plasmid DNA, the assay was able to quantify E11 gene copy number per well with the limit of detection of 100 copies and within a linear range of 100-10^8 copies. The amplification efficiencies for each run were between 90%-105% and the curve was linear ($R^2 > 0.99$) (Figure 4). Intra-assay and inter-assay repeatability had CVs of 0.3-1.2% and 0.6-5.4% respectively.

EHV-5 replication in equine dermal cells

Equine dermal cells contained between 6 and 17 X 10^5 genomes per well throughout all days sampled (Figure 5A). The quantity of EHV-5 that accumulated in the culture supernatants

was slightly greater than in the cells, however no substantial trend was noticed at each 3-day media change interval (Figure 5B).

EHV-5 replication in mature ERECs

Mature ERECs were collected on days 1-12 p.i. with either media (mock) or EHV-5. Cytopathic effect was not observed in any culture (data not shown). The amount of EHV-5 peaked at around 40,000 genomes per well (Figure 6A). Viral DNA was not detected in the bottom chamber supernatants prior to inoculation or after washing away the inoculum. A small amount of DNA had accumulated in the bottom chamber supernatants in the first four days of culture in two of the three horses, but DNA was not detected days 5-8 or 9-12 p.i. (Table 2). Viral DNA was not detected in the ERECs that were mock inoculated or their supernatants (data not shown).

EHV-5 replication in immature ERECs

In the EREC cultures that were inoculated with EHV-5 24 hours post seeding, the copy number generally trended upward throughout the 14 days with peak values above 1 million genomes per culture well (Figure 6B). Cytopathic effect was not observed in any culture (data not shown). EHV-5 DNA was not observed in the bottom chamber supernatants prior to inoculation, and small amounts below the quantifiable limit was detected at nearly each time point following inoculation for all horses (Table 3). Viral DNA was not detected in the mock inoculated ERECs, or their supernatants (data not shown).

PBMCs and nasal swabs of horses infected with EHV-1

Low levels of EHV-5 DNA could be detected in the PBMCs of all but one horse on at least one time point (Table 4). Prior to and 7 days after EHV-1 infection, 24 of the 25 EHV-1 infected

horses had EHV-5 positive PBMC samples. However, 10 days post EHV-1 infection the PBMCs of 7 of these horses were negative for EHV-5.

EHV-5 could be detected in the nasal swab samples of all but four of the horses at least at one time point. The group of horses that were experimentally infected with EHV-1 gD4 mutant had higher pre-infection quantities when compared to the other groups. In this group, the quantity of EHV-5 detected in the nasal swabs increased 7 days post experimental infection with EHV-1 gD4, after which the quantity decreased by day 10 (Table 5).

DISCUSSION

EHV-5 can routinely be detected in the peripheral blood lymphocytes as well as the nasal mucosa of the majority of horses.[117–123] However, viral antigen detected in the alveolar epithelial cells of horses with EHV-5 associated fibrosis indicate that infection localized in the lung may be associated with the development of EMPF.[2] In our study, we have developed a sensitive and specific qPCR assay with which to reliably quantify viral load in equine cell culture and clinical samples. Horses are often co-infected with both EHV-5 and EHV-2, another gamma herpesvirus, which shares a close sequence homology to that of EHV-5.[117–121,123] The developed primers for genes E11 and E6B did not detect DNA from EHV-2, in contrast to primers targeting the ORF8 region of the genome that detected EHV-2 and EHV-5 DNA. However, it is important to note that the previously published primer combination was designed to be used with a flourogenic probe, which enhances the specificity of the assay. Our designed primers targeting E11 and E6B can be used with or without a probe to specifically identify EHV-5 with conventional PCR or SYBR green real-time chemistry, in addition to a probe based reaction. Further, next generation sequencing has identified four unique genotypes of EHV-5, therefore it was necessary to develop a robust assay that is capable of detecting multiple strains of the virus.[148] The designed primers targeting E11 and E6B were able to detect DNA from different EHV-5 isolates obtained from the blood or tissues of 7 different horses.

Moreover, we identified EHV-5 copy number over time in multiple equine cell culture systems with the intent to develop a model that supported lytic infection. Viral copy number remained fairly stable in equine dermal cells – between 6 and 17 X 10^5 genomes per well. A

pattern of growth was not observed within 14 days of culture. Quantifiable levels of EHV-5 could be detected in the supernatants of the cells at each 3 day interval, however, a clear trend was not observed to allow for conclusions regarding viral replication and release from this cell line. In this system, the equine dermal cells were grown in a monolayer submerged in media. Therefore, the supernatants collected likely contained not only free virus, but also intracellular virus from cells that had sloughed off the monolayer.

The site of lytic replication of EHV-5 within horses is not known. However, the prevalence of nasal shedding, as well as identification of EHV-5 viral protein antigen localized within the epithelial cells of lungs from horses with pulmonary fibrosis suggests that epithelial cells can support lytic replication of EHV-5 and release of progeny virions. Human gamma herpesviruses undergo lytic replication in the suprabasal differentiating cells of the epithelium, while the undifferentiated cells of the basal layer harbor latent, non-replicating virus. [140,141,149] As the cells undergo differentiation, the virus begins to replicate and is subsequently shed into the environment. This "switch" from the latent to lytic form is closely linked to the up-regulation of host transcription factors involved in cellular differentiation. [141] In this study, *in vitro* inoculation of the apical surface of fully differentiated ERECs with free EHV-5 virus results in a low level of intracellular infection that peaks at around 40,000 genomes per culture. A substantial increase in viral copy number is not noted after 12 days of culture. When inoculating an immature monolayer of epithelial cells, EHV-5 viral load tends towards an increase as the cells begin to divide and mature. Peak viral load in this system surpasses 1 million genomes per culture. Though the state of latency could not be determined in our systems, cytopathic effect was not observed in any equine cell.

In ERECs, negligible amounts of virus is detected over time in bottom chamber supernatants of both immature and mature cultures, indicating that little to no virus is being released from the basolateral layer of epithelium in this system. Recent work has suggested that systemic infection with EBV occurs as a result of transcytosis through the epithelium, rather than apical entry and basolateral release of newly synthesized virus.[82] With EBV, infection of basal epithelial cells likely occurs as a result of proximity to infected lymphocytes.[90] The basal cells may remain latently infected until the cells begin their differentiation program.[141] It is possible that EHV-5 employs a similar mechanism, as it was possible to achieve increasing viral copy numbers when infected immature cells underwent maturation.

Measurements from *in vivo* samples support previous evidence that EHV-5 establishes low level infection within the peripheral lymphocytes in most horses, and is shed in higher quantities through nasal secretions. In this study, 32 of 33 yearling horses had low levels of EHV-5 in the peripheral blood at least at one time point, indicating latent chronic infection due to previous exposure to the virus. Viral load did not increase or decrease in horses following experimental infection with EHV-1, indicating acute respiratory infection with EHV-1 does not influence the level of EHV-5 circulating in the lymphocytes. Based on these observations, it is unlikely that EHV-1 infection or reactivation functions as a trigger to initiate EHV-5 replication in the circulating lymphocytes.

In the same cohort, EHV-5 was detected in nasal secretions of all but 4 horses at least at one time point. These observations are consistent with previous studies on the prevalence of horses shedding the virus in other field studies throughout the world.[117–123] In this study, the horses that were experimentally infected with the gD mutant of EHV-1 trended toward an

increase in EHV-5 shedding 7 days p.i. with EHV-1. It is possible that the other groups had a similar trend below the limit of quantification for the assay, and this effect was merely amplified in the gD mutant group as this group had higher levels of EHV-5 shedding at baseline when compared to the other groups. In humans, oral shedding of EBV remains relatively stable over short periods of time but can vary several logs over several months. Viral shedding is believed to be the result of sporadic infection of the epithelium by lymphocytes. The variability in mucosal EBV output is a result of number of epithelial plaques, and the frequency of epithelial infection can be modulated by the local immune system.[90] It has been previously demonstrated that horses exhibit marked immunosuppression after infection with EHV-1, an effect that peaks 7 days p.i.[147] In our study, it is possible that immunosuppression of the host contributed to the higher viral load of EHV-5 in the nasal mucosa of horses. Our observations as well as others' in horses support the model that the peripheral blood lymphocytes maintain a low level of infection throughout the equine population, and perhaps these cells are responsible for recurrent infection of the nasal epithelium. This could result in the variable degrees of nasal shedding that has been observed between horses, and within individuals over time. [120]

With this assay, we demonstrate that EHV-5 does not readily establish lytic infection in differentiated epithelial cells when free virus is inoculated onto the apical surface. Additionally we found that inoculation of immature epithelial cells as a monolayer may lead to viral replication as the cells divide and mature. In order to more completely understand the mechanisms involved in infection of epithelial cells with EHV-5, an epithelial system that can be readily infected and allow for lytic viral replication would be valuable. Future work will focus on

modulating our current mature and immature EREC system with factors to promote viral entry and lytic replication. Co-culturing the EREC system with infected equine lymphocytes that contact the basal layer of cells may more closely mimics the putative *in vivo* mechanism of equine epithelial cell infection. Furthermore, though the EREC model has previously been shown to be fully differentiated after 4 weeks of culture, the temporal process of differentiation including when and which specific differentiation markers become up-regulated is unknown.[142] To ensure temporal uniformity of differentiation in these cultures, we will use techniques to promote differentiation such as wounding the tissue or adding factors, such as 12-O-tetradecanoylphorbal-13-acetate.[140,150] Viral gene expression involved in lytic and latent infection of EHV-5 has not been studied. Future assay development is necessary in order to identify and detect viral transcripts and proteins involved in different states of latency.

CHAPTER 4.

Concluding Discussion

CONCLUDING DISCUSSION

Based on current knowledge, EHV-5 in horses shares many similarities with EBV in humans. Infection with each is ubiquitous in their respective species. Both viruses establish latency in the lymphocytes of the host, and are shed in higher and variable quantities through the oral or nasal epithelium. Importantly, it appears that infection of the alveolar epithelium of each species is associated with the development of fibrotic lung disease. Existing murine models for IPF incompletely mimic the natural disease in humans, and of course data collection on humans is limited to clinical observations and basic sample collections. Because of this, experimental designs utilizing the horse as a natural host model could be a valuable tool with which to study the pathogenesis of gamma herpesvirus associated pulmonary fibrosis. In order to design economical and efficient *in vivo* studies, more information regarding viral and host interactions is needed.

We were able to develop a specific and reliable method of quantifying EHV-5 in a variety of equine samples. While a previously designed quantitative assay targeted a section of the glycoprotein B gene, a highly conserved region of the EHV-5 genome, we found that these primers also detected DNA from a genetically similar virus when used with conventional or SYBR Green PCR.[123] This previously published assay was intended to be used with a flourogenic probe to ensure specificity. However, it was our intention to design a robust assay that could be adapted to different PCR applications, with or without the use of a probe. Our designed primers targeting gene E11, a gene unique to EHV-5, was specific for EHV-5 using conventional and SYBR Green, as well as the probe based TaqMan PCR chemistries.

Using this assay, we identified EHV-5 in the lymphocytes and nasal swabs of a cohort of horses that were experimentally infected with the alpha herpesvirus, EHV-1, which causes acute respiratory disease. Horses maintained very low levels of detectable virus within the lymphocytes at all time points before and after EHV-1 infection. Levels of EHV-5 nasal shedding did not vary greatly in response to infection with EHV-1, however, there appeared to be a trend towards an increase in a few of the horses. It is possible this was due to changes in the immune system as a result of the acute infection. However, the trend was minor and other natural causes unrelated to EHV-1 infection cannot be excluded. The results from these samples are congruent with previous work and support the current knowledge that EHV-5 is a highly prevalent pathogen among equines. [117–123]

In order to better understand potential viral mechanisms that contribute to the establishment or perpetuation of fibrotic disease, it is useful to establish EHV-5 replication in equine epithelial cell culture. EHV-5 antigen is detected within the lung epithelial cells of horses with pulmonary fibrosis, but not healthy horses, indicating that infection of these cells can occur *in vivo* and is associated with the disease.[2] Recent cell culture work with EBV has revealed that lytic replication can be established in primary epithelial cell cultures. This replication occurs in the suprabasal differentiated cells of this culture, while latent infection can be established in the undifferentiated basal layer.[140,141] A similar system has been previously established using equine respiratory epithelial cells (ERECs), and was used to study the pathogenicity of other viral pathogens.[142,143] In our work, we were unable to develop high levels of viral replication within 12 days following apical inoculation with free virus of mature and differentiated ERECs.

In contrast, we were able to reach peaks greater than 1 million genomes in cultures that were inoculated as a newly formed monolayer and allowed to grow for up to 14 days. This indicates that like EBV, EHV-5 may establish a lytic infection in conjunction with upregulation of cellular transcription factors associated with differentiation.[141] However, these levels were variable between individual cell cultures, indicating that a more robust system needs to be established.

Due to the prevalence of nasal shedding in addition to the previous identification of EHV-5 protein antigen in the alveolar epithelial cells of horses, it is likely that the equine epithelium can support productive replication of EHV-5. Future work is needed to understand the mechanisms that promote this lytic infection.

With current knowledge, specific cellular and viral surface proteins involved in viral entry are unknown. In human epithelial cell culture, EBV infection is more successful when infected lymphocytes are cultured with the epithelial cells compared to cell free culture. Furthermore, infection was achieved by wounding the culture indicating that the virus must be exposed to different layers of the epithelium for proper entry.[140] Our future work with EHV-5 and ERECs will consider this. We plan on inoculating equine lymphocytes and co-culturing these cells with ERECs. In this system, the lymphocytes will be exposed to the basal layer of the epithelium, a more representative model of the putative method of epithelial cell infection *in vivo*.

Additionally, though the EREC model has been shown to be fully differentiated after 4 weeks of culture, the temporal process of differentiation including when and which specific differentiation markers become upregulated is unknown in these cells.[142] Variability in the differentiation of these cells could account for the variability we observed in the viral copy

number in the immature ERECs. To ensure uniformity of the upregulation of differentiation associated cellular transcription factors, we plan to use the chemical, 12-O-tetradecanoylphorbal-13-acetate to induce cellular differentiation.[150] We expect this will promote lytic replication of EHV-5 in the EREC system.

In summary, we have developed a method by which to detect and quantify EHV-5 in *in vivo* and *in vitro* samples. With this assay, we attempted to validate a cell culture system that represents the natural airway and supports lytic replication of EHV-5. Though we were unable to reliably produce lytic replication in this culture, we plan on using other techniques to increase the reliability of lytic replication in this system. Once developed this cell culture model will be a valuable tool with which to study the viral and cellular mechanisms involved in EHV-5 infection of the epithelium.

APPENDICES

APPENDIX A: TABLES

Table 1. EHV-5 primers and probe

Target		Sequence (5'-3')	Amplicon length (bp)
ORF8 Published Sequence	Forward	TCG CTG CCA TTG ACA AAC TTG A	63
	Reverse	TGG ACC AAG AGA GCG TGT TTG	
ORF8	Forward	GGC CTC TCT GAT GTG GTA CGA	80
	Reverse	CAG ACA CGG GTC TCC CAT AGA	
E6B	Forward	TCC GGC GGG TCA AAC TC	75
	Reverse	ATG GGC TCT GAC CTT ACA CTG TGT	
E11	Forward	AAG TCT TTG TGG TGG AAC CTG TCT	60
	Reverse	CGC CCC TGC TAA AAT CCA TA	
	Probe	[6FAM]-CCT GCC GCT GGC T-[BHQ1]	

Table 2. EHV-5 copy number in bottom chamber supernatants of mature ERECs post inoculation with EHV-5

Days post inoculation	EHV-5 E11 copy number		
	Horse 1	Horse 2	Horse 3
Pre-inoculation	0	0	0
Post-inoculation	0	0	0
1-4	+	+	0
5-8	0	0	0
9-12	0	0	0

[+] indicates that the sample was positive for EHV-5 but copy number of E11 was below the quantification limit of the assay

Table 3. EHV-5 copy number in bottom chamber supernatants of immature ERECs post inoculation with EHV-5

Days post inoculation	EHV-5 E11 copy number		
	Horse 4	Horse 5	Horse 6
Pre-inoculation	0	0	0
Post-inoculation	+	+	+
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+
8	+	+	+
9-10	+	+	+
11-12	+	+	+
13-14	0	+	+

[+] indicates that the sample was positive for EHV-5 but copy number of E11 was below the quantification limit of the assay

Table 4. EHV-5 quantity in PBMCs of horses experimentally infected with EHV-1 (E11 copy number per 1E6 β actin)

Horse ID	Pre EHV-1 infection	7 days post EHV-1 infection	10 days post EHV-1 infection
1	+	+	+
2	+	+	0
3	+	+	+
4	+	+	+
5	+	+	0
6	+	+	+
7	+	+	no data
8	+	+	no data
9	+	+	0
10	+	+	+
11	0	0	0
12	+	+	0
13	+	+	+
14	+	+	+
15	+	+	+
16	+	+	0
17	+	+	+
18	+	+	+
19	145	+	+
20	33	+	+
21	+	+	0
22	+	+	22
23	+	65	+
24	+	+	+
25	+	+	+

Horse ID	Baseline	3 months post baseline
26	+	+
27	+	+
28	+	+
29	+	+
30	+	+
31	+	no data
32	+	+
33	+	no data

[+] indicates that the sample was positive for EHV-5 but copy number of e11 was below the quantification limit of the assay prior to normalization with β actin.

Table 4 (cont'd)
Horses 1-8 were experimentally infected with wild type EHV-1; 9-17 with N752 mutant EHV-1; 18-25 with gD4 mutant EHV-1.Horses 26-33 were uninfected controls.

Table 5. EHV-5 quantity in nasal swabs of horses experimentally infected with EHV-1 (E11 copy number per ng of DNA)

Horse ID	Pre EHV-1 infection	7 days post EHV-1 infection	10 days post EHV-1 infection
1	21	+	+
2	37	85	1132
3	0	0	0
4	274	93	333
5	+	+	+
6	+	10	+
7	109	4	no data
8	+	+	no data
9	0	0	0
10	33	26	+
11	0	0	0
12	0	0	0
13	+	+	+
14	39	+	+
15	8	+	+
16	+	+	0
17	+	180	50
18	297	1499	75
19	1567	157	+
20	504	1616	331
21	+	136	19
22	910	6756	18
23	1879	2460	992
24	+	41	+
25	686	1343	6
Horse ID	Baseline	21 days post baseline	3 months post baseline
26	64	10	18
27	327	271	570
28	182	359	99
29	+	+	+
30	139	52	252
31	209	149	271
32	271	55	362
33	+	+	+

[+] indicates that the sample was positive for EHV-5 but copy number of E11 was below the quantification limit of the assay prior to normalization with ng of DNA

Table 5 (cont'd)
Horses 1-8 were experimentally infected with wild type EHV-1; 9-17 with N752 mutant EHV-1; 18-25 with gD4 mutant EHV-1. Horses 26-33 were uninfected controls.

APPENDIX B: FIGURES

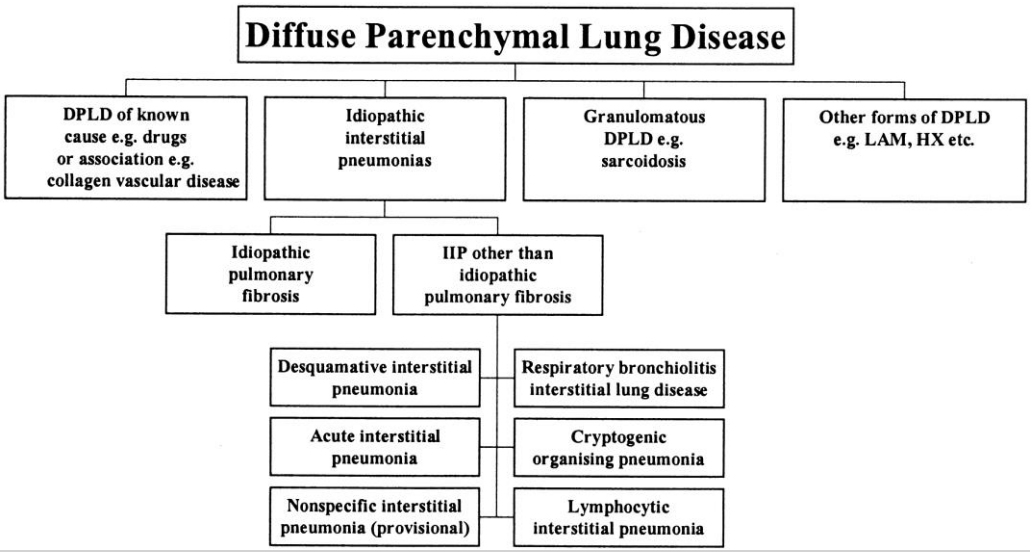

Figure 1. Classification of parenchymal lung disease. Interstitial lung diseases are classified as idiopathic interstitial pneumonia (IIP) if the cause is unknown. Distinction between idiopathic pulmonary fibrosis (IPF) and other IIPs is based the presence of usual interstitial pneumonia (UIP) upon radiographic and histologic examination. (Adapted from reference [4]).

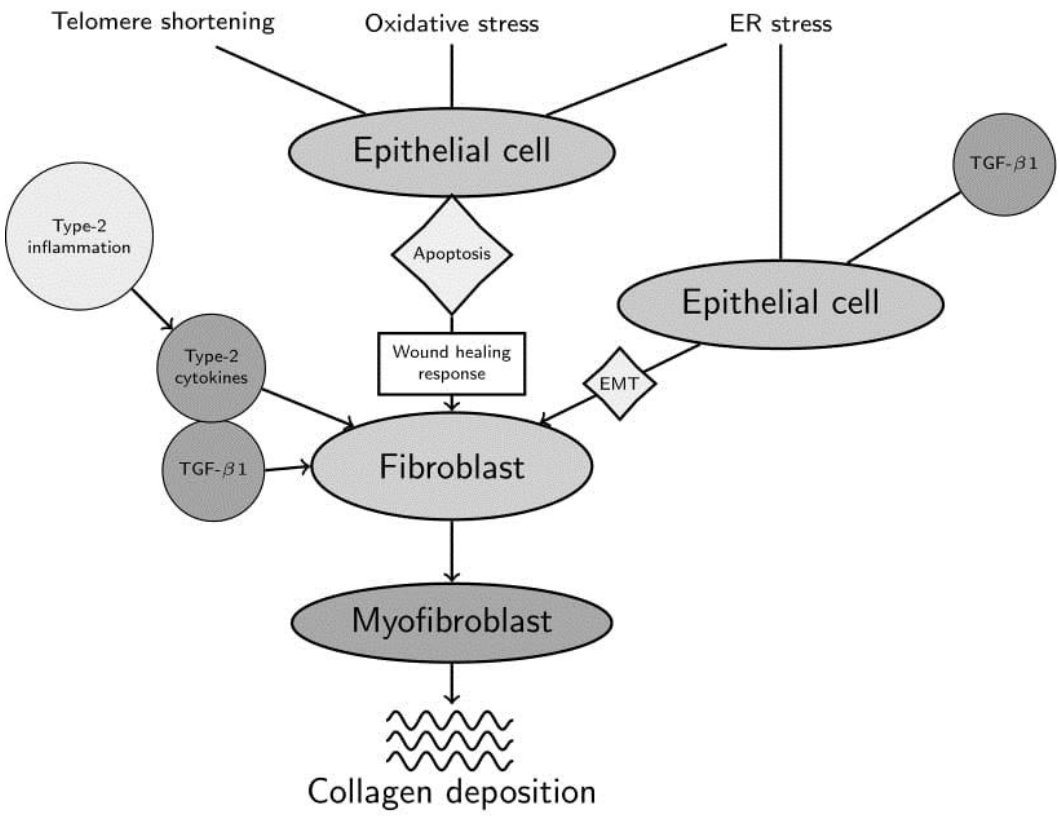

Figure 2. Mechanisms of fibrosis. Aberrant collagen deposition by myofibroblasts occurs as a result of activation of fibroblasts. This is a consequence of many environmental and cellular factors. ER=endoplasmic reticulum; TGF=transforming growth factor; EMT=epithelial mesenchymal transition.

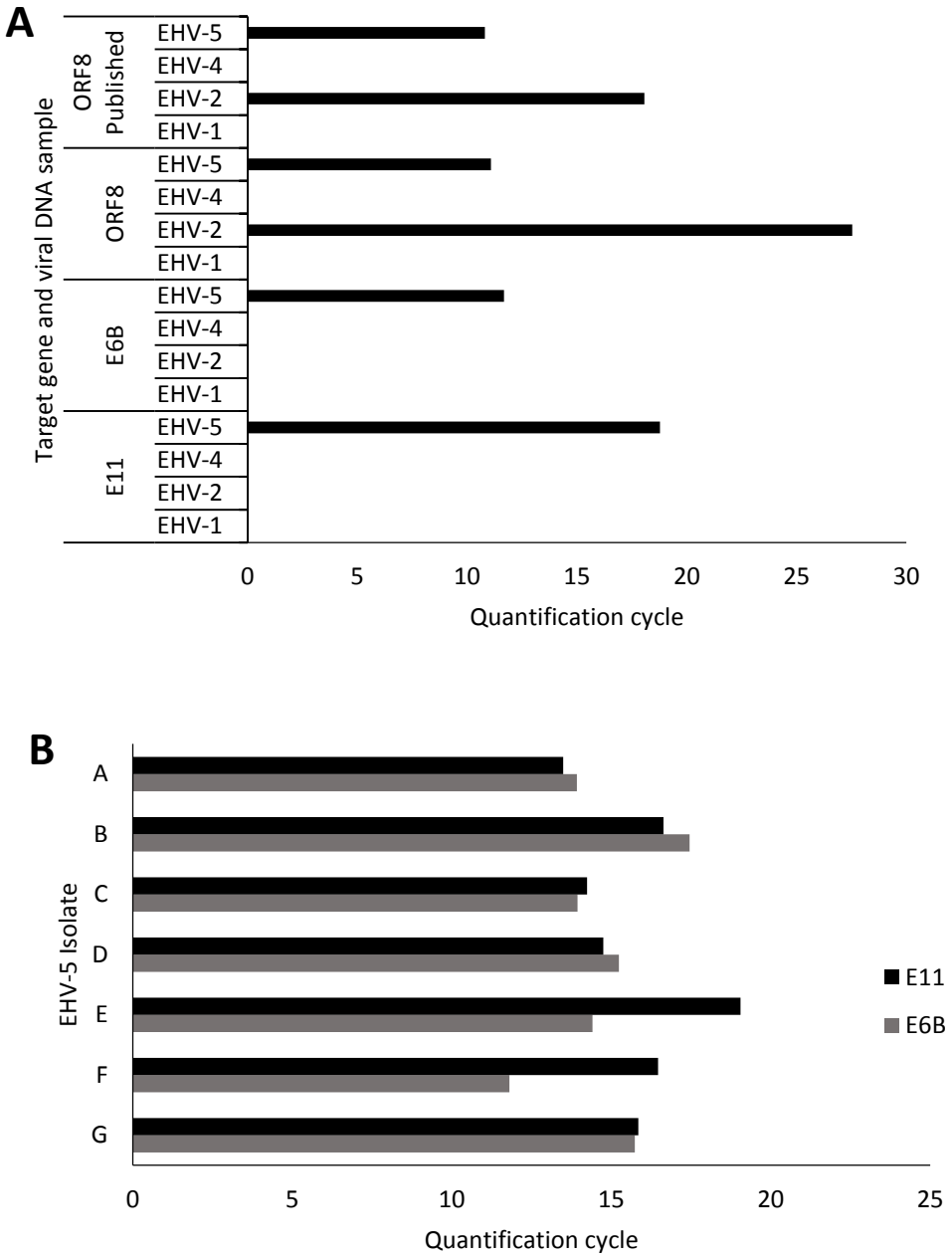

Figure 3. (A) Quantification cycles of SYBR green PCR of equine herpesvirus DNA using 3 designed primer sets and 1 previously published primer set. Primers targeting EHV-5 genes E6B and E11 specifically amplified DNA from the EHV-5 isolate. (B) Quantification cycles of SYBR green PCR of DNA from multiple EHV-5 isolates using designed primers targeting genes E11 and E6B. Primers targeting both genes E11 and E6B were able to detect the DNA from EHV-5 isolated from several cases.

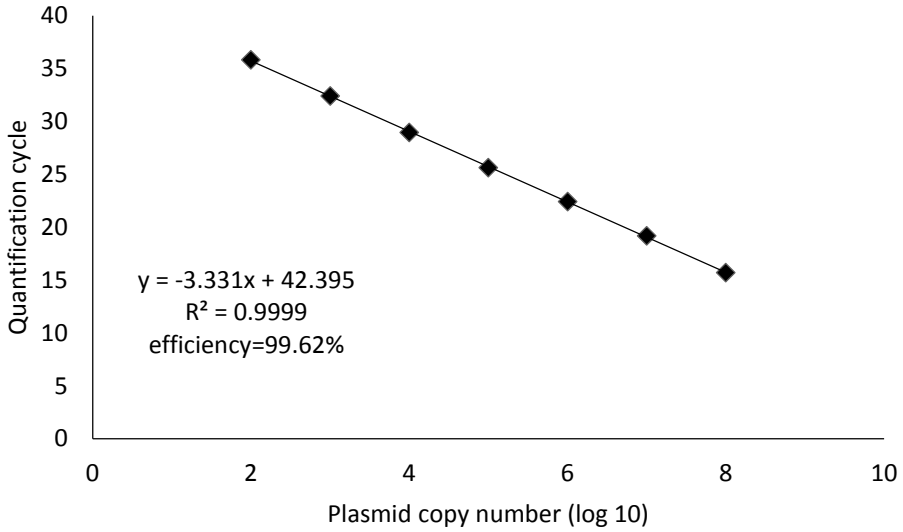

Figure 4. Plasmid standard curve using TaqMan primers and probe targeting gene E11.

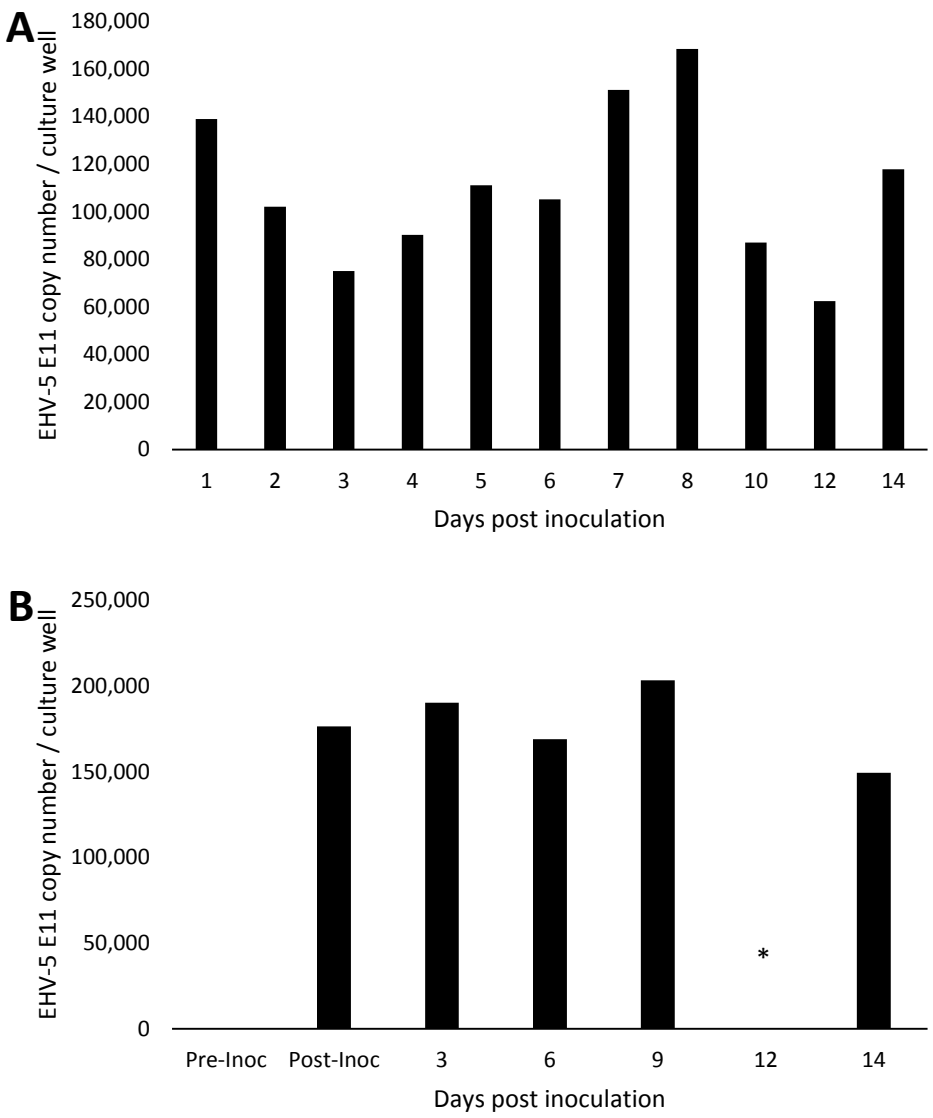

Figure 5. EHV-5 copy number per culture well in equine dermal cells (A) and supernatants (B) following inoculation with EHV-5. Mock-inoculated cells and supernatants were negative for E11 on all days collected (data not shown). *equine dermal cell supernatants collected on day 12 p.i. were positive for E11 below the quantifiable limit of detection.

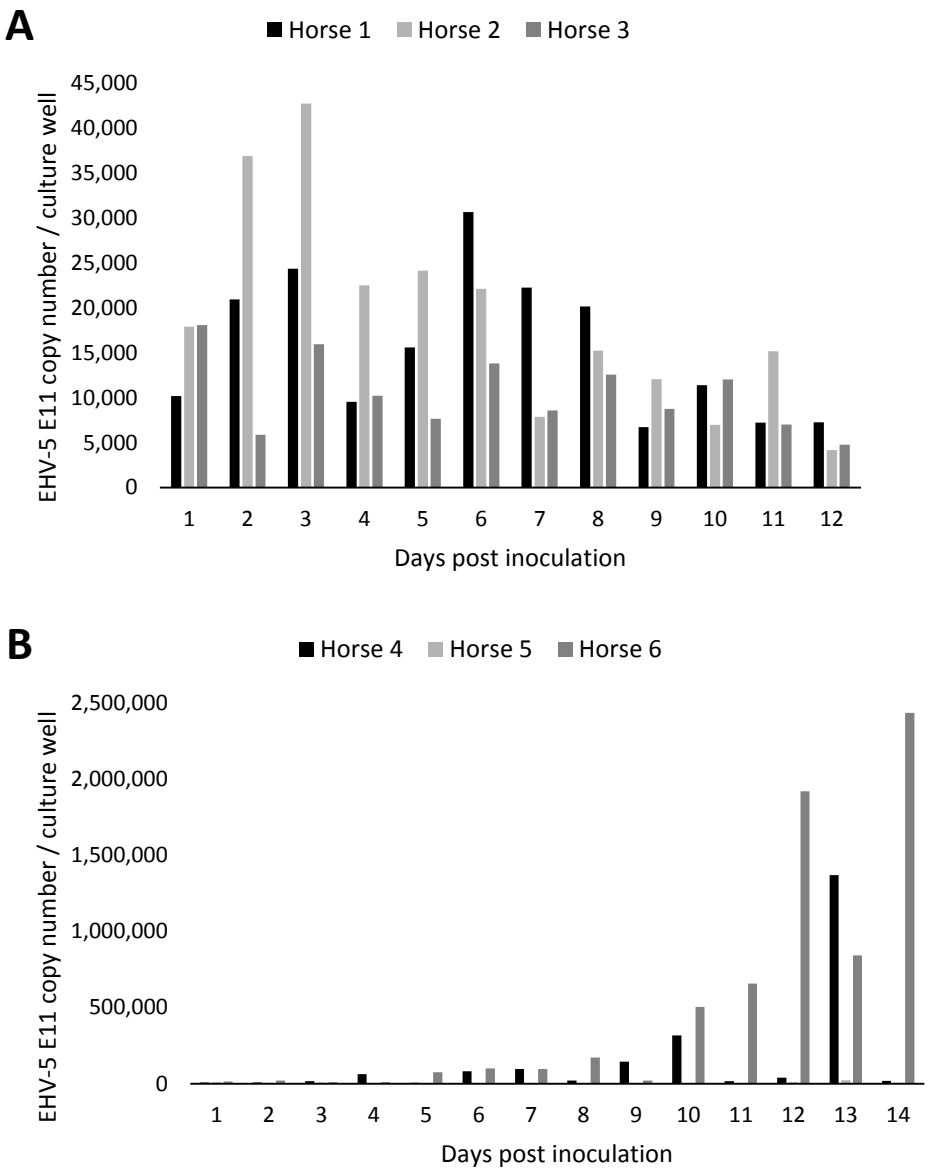

Figure 6. EHV-5 copy number in mature (A) and immature (B) ERECs following inoculation with EHV-5. EHV-5 copy number in mature ERECs peaked at approximately 40,000 E11 copies per EREC culture well. EHV-5 copy number in immature cells trended towards an increase throughout 14 days, with peak values nearing 2.5 million per EREC culture well. Mock-inoculated cells were negative for E11 all days collected (data not shown).

REFERENCES

REFERENCES

1. Williams KJ, Maes R, Del Piero F, et al. Equine multinodular pulmonary fibrosis: a newly recognized herpesvirus-associated fibrotic lung disease. Vet Pathol. 2007;44(6):849-862.

2. Williams KJ, Robinson NE, Lim A, et al. Experimental induction of pulmonary fibrosis in horses with the gammaherpesvirus equine herpesvirus 5. *PLoS One*. 2013;8(10):e77754.

3. Travis WD, Costabel U, Hansell DM, et al. An Official American Thoracic Society/European Respiratory Society Statement: Update of the International Multidisciplinary Classification of the Idiopathic Interstitial Pneumonias. *Am J Respir Crit Care Med*. 2013;188(6):733-748.

4. American Thoracic Society, European Respiratory Society. American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. *Am J Respir Crit Care Med*. 2002;165(2):277-304.

5. Raghu G, Collard HR, Egan JJ, et al. An Official ATS/ERS/JRS/ALAT Statement: Idiopathic pulmonary fibrosis: Evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med*. 2011;183(6):788-824.

6. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. *Am J Respir Crit Care Med*. 2000;161(2):646-664.

7. Crystal RG, Fulmer JD, Roberts WC, Moss ML, Line BR, Reynolds HY. Idiopathic pulmonary fibrosis. Clinical, histologic, radiographic, physiologic, scintigraphic, cytologic, and biochemical aspects. *Ann Intern Med*. 1976;85(6):769-788.

8. Crystal RG, Fulmer JD, Baum BJ, et al. Cells, collagen and idiopathic pulmonary fibrosis. *Lung*. 1978;155(1):199-224.

9. Hunninghake G, Gadek J, Weinberger S, et al. Comparison of the alveolitis of sarcoidosis and idiopathic pulmonary fibrosis. *Chest*. 1979;75(2):266-267.

10. Hunninghake GW, Gadek JE, Lawley TJ, Crystal RG. Mechanisms of neutrophil accumulation in the lungs of patients with idiopathic pulmonary fibrosis. *J Clin Invest*. 1981;68(1):259-269.

11. Weinberger SE, Kelman JA, Elson NA, et al. Bronchoalveolar lavage in interstitial lung disease. *Ann Intern Med*. 1978;89(4):459-466.

12. Richeldi L, Davies HR, Ferrara G, Franco F. Corticosteroids for idiopathic pulmonary fibrosis. *Cochrane database Syst Rev*. 2003;(3):CD002880.

13. Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER. Cell number and cell characteristics of the normal human lung. *Am Rev Respir Dis*. 1982;126(2):332-337.

14. Adamson IY, Bowden DH. Derivation of type 1 epithelium from type 2 cells in the developing rat lung. *Lab Invest*. 1975;32(6):736-745.

15. Adamson IY, Bowden DH. Origin of ciliated alveolar epithelial cells in bleomycin-induced lung injury. *Am J Pathol*. 1977;87(3):569-580.

16. Laurent GJ, McAnulty RJ. Protein metabolism during bleomycin-induced pulmonary fibrosis in rabbits. *Am Rev Respir Dis*. 1983;128(1):82-88.

17. Raghu G, Freudenberger TD, Yang S, et al. High prevalence of abnormal acid gastro-oesophageal reflux in idiopathic pulmonary fibrosis. *Eur Respir J*. 2006;27(1):136-142.

18. Steele MP, Speer MC, Loyd JE, et al. Clinical and pathologic features of familial interstitial pneumonia. *Am J Respir Crit Care Med*. 2005;172(9):1146-1152.

19. Gribbin J, Hubbard R, Smith C. Role of diabetes mellitus and gastro-oesophageal reflux in the aetiology of idiopathic pulmonary fibrosis. *Respir Med*. 2009;103(6):927-931.

20. Miyake Y, Sasaki S, Yokoyama T, et al. Occupational and environmental factors and idiopathic pulmonary fibrosis in Japan. *Ann Occup Hyg*. 2005;49(3):259-265.

21. Stewart JP, Egan JJ, Ross AJ, et al. The detection of Epstein-Barr virus DNA in lung tissue from patients with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 1999;159(4 Pt 1):1336-1341.

22. Wang R, Ibarra-Sunga O, Verlinski L, Pick R, Uhal BD. Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. *Am J Physiol Lung Cell Mol Physiol*. 2000;279(1):L143-51.

23. Kuwano K, Kunitake R, Maeyama T, et al. Attenuation of bleomycin-induced pneumopathy in mice by a caspase inhibitor. *Am J Physiol Lung Cell Mol Physiol*. 2001;280(2):L316-25.

24. Sisson TH, Mendez M, Choi K, et al. Targeted injury of type II alveolar epithelial cells induces pulmonary fibrosis. *Am J Respir Crit Care Med*. 2010;181(3):254-263.

25. Uhal BD, Joshi I, Hughes WF, Ramos C, Pardo A, Selman M. Alveolar epithelial cell death adjacent to underlying myofibroblasts in advanced fibrotic human lung. *Am J Physiol*. 1998;275(6 Pt 1):L1192-9.

26. Kuwano K, Nakashima N, Inoshima I, et al. Oxidative stress in lung epithelial cells from patients with idiopathic interstitial pneumonias. *Eur Respir J*. 2003;21(2):232-240.

27. Cantin AM, North SL, Fells GA, Hubbard RC, Crystal RG. Oxidant-mediated epithelial cell injury in idiopathic pulmonary fibrosis. *J Clin Invest*. 1987;79(6):1665-1673.

28. Waghray M, Cui Z, Horowitz JC, et al. Hydrogen peroxide is a diffusible paracrine signal for the induction of epithelial cell death by activated myofibroblasts. *FASEB J*. 2005;19(7):854-856.

29. Nogee LM, Dunbar AE, Wert SE, Askin F, Hamvas A, Whitsett JA. A mutation in the surfactant protein C gene associated with familial interstitial lung disease. *N Engl J Med*. 2001;344:573-579.

30. Thomas AQ, Lane K, Phillips J, et al. Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. *Am J Respir Crit Care Med*. 2002;165(9):1322-1328.

31. Mulugeta S, Nguyen V, Russo SJ, Muniswamy M, Beers MF. A surfactant protein C precursor protein BRICHOS domain mutation causes endoplasmic reticulum stress, proteasome dysfunction, and caspase 3 activation. *Am J Respir Cell Mol Biol*. 2005;32(6):521-530.

32. Lawson WE, Grant SW, Ambrosini V, et al. Genetic mutations in surfactant protein C are a rare cause of sporadic cases of IPF. *Thorax*. 2004;59(11):977-980.

33. Lawson WE, Crossno PF, Polosukhin V V, et al. Endoplasmic reticulum stress in alveolar epithelial cells is prominent in IPF: association with altered surfactant protein processing and herpesvirus infection. *Am J Physiol Lung Cell Mol Physiol*. 2008;294(6):L1119-26.

34. Korfei M, Ruppert C, Mahavadi P, et al. Epithelial endoplasmic reticulum stress and apoptosis in sporadic idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med*. 2008;178(8):838-846.

35. Tsuji T, Aoshiba K, Nagai A. Alveolar cell senescence in patients with pulmonary emphysema. *Am J Respir Crit Care Med*. 2006;174(8):886-893.

36. Armanios MY, Chen JJ-L, Cogan JD, et al. Telomerase mutations in families with idiopathic pulmonary fibrosis. *N Engl J Med*. 2007;365:1317-1326.

37. Tsakiri KD, Cronkhite JT, Kuan PJ, et al. Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc Natl Acad Sci*. 2007;104(18):7552-7557.

38. Zhang X, Mar V, Zhou W, Harrington L, Robinson MO. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev*. 1999;13(18):2388-2399.

39. Willis BC, Liebler JM, Luby-Phelps K, et al. Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in

idiopathic pulmonary fibrosis. *Am J Pathol*. 2005;166(5):1321-1332.

40. Kim KK, Kugler MC, Wolters PJ, et al. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proc Natl Acad Sci*. 2006;103(35):13180-13185.

41. Kasai H, Allen JT, Mason RM, et al. TGF-β1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respir Res*. 2005;6(1):56.

42. Tanjore H, Cheng D-S, Degryse AL, et al. Alveolar epithelial cells undergo epithelial-to-mesenchymal transition in response to endoplasmic reticulum stress. *J Biol Chem*. 2011;286(35):30972-30980.

43. Zhong Q, Zhou B, Ann DK, et al. Role of endoplasmic reticulum stress in epithelial-mesenchymal transition of alveolar epithelial cells: effects of misfolded surfactant protein. *Am J Respir Cell Mol Biol*. 2011;45(3):498-509.

44. Tanjore H, Xu XC, Polosukhin V V., et al. Contribution of epithelial-derived fibroblasts to bleomycin-induced lung fibrosis. *Am J Respir Crit Care Med*. 2009;180(7):657-665.

45. Kim KK, Wei Y, Szekeres C, et al. Epithelial cell α3β1 integrin links β-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. *J Clin Invest*. 2009;119(1):136-151.

46. Kim BS, Siracusa MC, Saenz SA, et al. TSLP elicits IL-33–independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med*. 2013;5(170).

47. Kim HY, Chang Y-J, Subramanian S, et al. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J Allergy Clin Immunol*. 2012;129(1):216-27-6.

48. Hams E, Armstrong ME, Barlow JL, et al. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc Natl Acad Sci U S A*. 2014;111(1):367-372.

49. Neill DR, Wong SH, Bellosi A, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. 2010;464(7293):1367-1370.

50. Hoffmann KF, Cheever AW, Wynn TA. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol*. 2000;164(12):6406-6416.

51. Cheever AW, Williams ME, Wynn TA, et al. Anti-IL-4 treatment of Schistosoma mansoni-infected mice inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while decreasing egg-induced hepatic fibrosis. *J Immunol*. 1994;153(2):753-759.

52. Zhu Z, Homer RJ, Wang Z, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest*. 1999;103(6):779-788.

53. Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest*. 1999;104(6):777-785.

54. Park S-W, Ahn M-H, Jang HK, et al. Interleukin-13 and its receptors in idiopathic interstitial pneumonia: clinical implications for lung function. *J Korean Med Sci*. 2009;24(4):614-620.

55. Wallace WA, Ramage EA, Lamb D, Howie SE. A type 2 (Th2-like) pattern of immune response predominates in the pulmonary interstitium of patients with cryptogenic fibrosing alveolitis (CFA). *Clin Exp Immunol*. 1995;101(3):436-441.

56. Saito A, Okazaki H, Sugawara I, Yamamoto K, Takizawa H. Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. *Int Arch Allergy Immunol*. 2003;132(2):168-176.

57. Munder M, Eichmann K, Morán JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol*. 1999;163(7):3771-3777.

58. Pechkovsky D V., Prasse A, Kollert F, et al. Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. *Clin Immunol*. 2010;137:89-101.

59. Broekelmann TJ, Limper AH, Colby T V, McDonald JA. Transforming growth factor beta 1 is present at sites of extracellular matrix gene expression in human pulmonary fibrosis. *Proc Natl Acad Sci U S A*. 1991;88(15):6642-6646.

60. Sempowski GD, Derdak S, Phipps RP. Interleukin-4 and interferon-gamma discordantly regulate collagen biosynthesis by functionally distinct lung fibroblast subsets. *J Cell Physiol*. 1996;167(2):290-296.

61. Taipale J, Miyazono K, Heldin CH, Keski-Oja J. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. *J Cell Biol*. 1994;124(1-2):171-181.

62. Fontana L, Chen Y, Prijatelj P, et al. Fibronectin is required for integrin v 6-mediated activation of latent TGF- complexes containing LTBP-1. *FASEB J*. 2005;19(13):1798-1808.

63. Munger JS, Huang X, Kawakatsu H, et al. A Mechanism for Regulating Pulmonary Inflammation and Fibrosis: The Integrin αvβ6 Binds and Activates Latent TGF β1. *Cell*. 1999;96(3):319-328.

64. Ignotz RA, Massagué J. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem*. 1986;261(9):4337-4345.

65. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol*. 1993;122(1):103-111.

66. Khalil N, O'Connor RN, Unruh HW, et al. Increased production and immunohistochemical localization of transforming growth factor-β in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol*. 1991;5(2):155-162.

67. Khalil N, O'Connor RN, Flanders KC, Unruh H. TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. *Am J Respir Cell Mol Biol*. 1996;14(2):131-138.

68. Yang L, Chan T, Demare J, et al. Healing of burn wounds in transgenic mice overexpressing transforming growth factor-β1 in the epidermis. *Am J Pathol*. 2001;159(6):2147-2157.

69. Koch RM, Roche NS, Parks WT, Ashcroft GS, Letterio JJ, Roberts AB. Incisional wound healing in transforming growth factor-beta1 null mice. *Wound Repair Regen*. 2000;8(3):179-191.

70. Chakir J, Shannon J, Molet S, et al. Airway remodeling-associated mediators in moderate to severe asthma: Effect of steroids on TGF-β, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol*. 2003;111(6):1293-1298.

71. Oursler MJ, Riggs BL, Spelsberg TC. Glucocorticoid-induced activation of latent transforming growth factor-beta by normal human osteoblast-like cells. *Endocrinology*. 1993;133(5):2187-2196.

72. Darby I, Skalli O, Gabbiani G. Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest*. 1990;63(1):21-29.

73. Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G. A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol*. 1986;103(6 Pt 2):2787-2796.

74. Kuhn C, Boldt J, King TE, Crouch E, Vartio T, McDonald JA. An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *Am Rev Respir Dis*. 1989;140(6):1693-1703.

75. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol*. 2001;166(12):7556-7562.

76. McGeoch DJ. Molecular evolution of the -Herpesvirinae. *Philos Trans R Soc B Biol Sci*. 2001;356(1408):421-435.

77. International Committee on Taxonomy of Viruses. Family – Herpesviridae. In: King AMQ, Lefkowitz E, Adams MJ, Carstens EB, eds. *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*. Elsevier; 2012:111-122.

78. Adams A, Lindahl T. Epstein-Barr virus genomes with properties of circular DNA molecules in carrier cells. *Proc Natl Acad Sci U S A*. 1975;72(4):1477-1481.

79. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. *Lancet*. 1964;1(7335):702-703.

80. Gerber P, Lucas S, Nonoyama M, Perlin E, Goldstein L. Oral excretion of Epstein-Barr virus by healthy subjects and patients with infectious mononucleosis. *Lancet*. 1972;300(7785):988-989.

81. Henle G, Henle W, Diehl V. Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. *Proc Natl Acad Sci U S A*. 1968;59(1):94-101.

82. Tugizov SM, Herrera R, Palefsky JM. Epstein-Barr virus transcytosis through polarized oral epithelial cells. *J Virol*. 2013;87(14):8179-8194.

83. Nemerow GR, Wolfert R, McNaughton ME, Cooper NR. Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2). *J Virol*. 1985;55(2):347-351.

84. Fingeroth JD, Weis JJ, Tedder TF, Strominger JL, Biro PA, Fearon DT. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. *Proc Natl Acad Sci U S A*. 1984;81(14):4510-4514.

85. Li Q, Spriggs MK, Kovats S, et al. Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes. *J Virol*. 1997;71(6):4657-4662.

86. Decker LL, Klaman LD, Thorley-Lawson DA. Detection of the latent form of Epstein-Barr virus DNA in the peripheral blood of healthy individuals. *J Virol*. 1996;70(5):3286-3289.

87. Birkenbach M, Josefsen K, Yalamanchili R, Lenoir G, Kieff E. Epstein-Barr virus-induced genes: first lymphocyte-specific G protein-coupled peptide receptors. *J Virol*. 1993;67(4):2209-2220.

88. Babcock GJ, Decker LL, Volk M, et al. EBV persistence in memory B cells in vivo. *Immunity*. 1998;9(3):395-404.

89. Laichalk LL, Thorley-Lawson DA. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J Virol*. 2005;79(2):1296-1307.

90. Hadinoto V, Shapiro M, Sun CC, Thorley-Lawson DA. The dynamics of EBV shedding implicate a central role for epithelial cells in amplifying viral output. Speck SH, ed. *PLoS Pathog*. 2009;5(7):e1000496.

91. Vergnon JM, de Thé G, Weynants P, Vincent M, Mornex JF, Brune J. Cryptogenic fibrosing alveolitis and Epstein-Barr virus: an association? *Lancet*. 1984;324(8406):768-771.

92. Manika K, Alexiou-Daniel S, Papakosta D, et al. Epstein-Barr virus DNA in bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis. *Sarcoidosis Vasc Diffuse Lung Dis*. 2007;24(2):134-140.

93. Pulkkinen V, Salmenkivi K, Kinnula VL, et al. A novel screening method detects herpesviral DNA in the idiopathic pulmonary fibrosis lung. *Ann Med*. 2012;44(2):178-186.

94. Calabrese F, Kipar A, Lunardi F, et al. Herpes virus infection is associated with vascular remodeling and pulmonary hypertension in idiopathic pulmonary fibrosis. Kolb MR, ed. *PLoS One*. 2013;8(2):e55715.

95. Wangoo A, Shaw RJ, Diss TC, Farrell PJ, du Bois RM, Nicholson AG. Cryptogenic fibrosing alveolitis: lack of association with Epstein-Barr virus infection. *Thorax*. 1997;52(10):888-891.

96. Zamò A, Poletti V, Reghellin D, et al. HHV-8 and EBV are not commonly found in idiopathic pulmonary fibrosis. *Sarcoidosis Vasc Diffuse Lung Dis*. 2005;22(2):123-128.

97. Egan JJ, Stewart JP, Hasleton PS, Arrand JR, Carroll KB, Woodcock AA. Epstein-Barr virus replication within pulmonary epithelial cells in cryptogenic fibrosing alveolitis. *Thorax*. 1995;50(12):1234-1239.

98. Kelly BG, Lok SS, Hasleton PS, Egan JJ, Stewart JP. A rearranged form of Epstein–Barr virus DNA is associated with idiopathic pulmonary fibrosis. *Respir Crit care Med*. 2012;166(4):510-513.

99. Stoolman JS, Vannella KM, Coomes SM, et al. Latent infection by γherpesvirus stimulates profibrotic mediator release from multiple cell types. *Am J Physiol Lung Cell Mol Physiol*. 2011;300(2):L274-85.

100. Vannella KM, Luckhardt TR, Wilke CA, Dyk LF van, Toews GB, Moore BB. Latent herpesvirus infection augments experimental pulmonary fibrosis. *Am J Respir Crit Care Med*. 2012;181(5):465-477.

101. Blaskovic D, Stanceková M, Svobodová J, Mistríková J. Isolation of five strains of herpesviruses from two species of free living small rodents. *Acta Virol*. 1980;24(6):468.

102. Blasdell K, McCracken C, Morris A, et al. The wood mouse is a natural host for Murid herpesvirus 4. *J Gen Virol*. 2003;84(Pt 1):111-113.

103. François S, Vidick S, Sarlet M, et al. Comparative study of murid gammaherpesvirus 4 infection in mice and in a natural host, bank voles. *J Gen Virol*. 2010;91(Pt 10):2553-2563.

104. Hughes DJ, Kipar A, Sample JT, Stewart JP. Pathogenesis of a model gammaherpesvirus in a natural host. *J Virol*. 2010;84(8):3949-3961.

105. Usuki J, Fukuda Y. Evolution of three patterns of intra-alveolar fibrosis produced by bleomycin in rats. *Pathol Int*. 1995;45(8):552-564.

106. Claussen CA, Long EC. Nucleic acid recognition by metal complexes of bleomycin. *Chem Rev*. 1999;99(9):2797-2816.

107. Chaudhary NI, Schnapp A, Park JE. Pharmacologic differentiation of inflammation and fibrosis in the rat bleomycin model. *Am J Respir Crit Care Med*. 2006;173(7):769-776.

108. Izbicki G, Segel MJ, Christensen TG, Conner MW, Breuer R. Time course of bleomycin-induced lung fibrosis. *Int J Exp Pathol*. 2002;83(3):111-119.

109. Lok SS, Haider Y, Howell D, Stewart JP, Hasleton PS, Egan JJ. Murine gammaherpes virus as a cofactor in the development of pulmonary fibrosis in bleomycin resistant mice. *Eur Respir J*. 2002;20(5):1228-1232.

110. McMillan TR, Moore BB, Weinberg JB, et al. Exacerbation of established pulmonary fibrosis in a murine model by gammaherpesvirus. *Am J Respir Crit Care Med*. 2008;177(7):771-780.

111. Ashley SL, Jegal Y, Moore TA, van Dyk LF, Laouar Y, Moore BB. γ-Herpes virus-68, but not Pseudomonas aeruginosa or influenza A (H1N1), exacerbates established murine lung fibrosis. *Am J Physiol Lung Cell Mol Physiol*. 2014;307(3):L219-30.

112. Mora AL, Woods CR, Garcia A, et al. Lung infection with gamma-herpesvirus induces progressive pulmonary fibrosis in Th2-biased mice. *Am J Physiol Lung Cell Mol Physiol*. 2005;289(5):L711-21.

113. Mora AL, Torres-González E, Rojas M, et al. Control of virus reactivation arrests pulmonary herpesvirus-induced fibrosis in IFN-γ receptor–deficient mice. *Am J Respir Crit Care Med*. 2007;175(11):1139-1150.

114. Xu J, Gonzalez ET, Iyer SS, et al. Use of senescence-accelerated mouse model in bleomycin-induced lung injury suggests that bone marrow-derived cells can alter the outcome of lung injury in aged mice. *J Gerontol A Biol Sci Med Sci*. 2009;64(7):731-739.

115. Naik PN, Horowitz JC, Moore TA, Wilke CA, Toews GB, Moore BB. Pulmonary fibrosis induced by γ-herpesvirus in aged mice is associated with increased fibroblast responsiveness to transforming growth factor-β. *J Gerontol A Biol Sci Med Sci*. 2012;67(7):714-725.

116. Torres-González E, Bueno M, Tanaka A, et al. Role of endoplasmic reticulum stress in age-related susceptibility to lung fibrosis. *Am J Respir Cell Mol Biol*. 2012;46(6):748-756.

117. Pusterla N, Mapes S, Wademan C, White A, Hodzic E. Investigation of the role of lesser characterised respiratory viruses associated with upper respiratory tract infections in horses. *Vet Rec*. 2013;172(12):315.1-315.

118. Dunowska M, Wilks C, Studdert M, Meers J. Equine respiratory viruses in foals in New Zealand. *N Z Vet J*. 2002;50(4):140-147.

119. Bell SA, Balasuriya UBR, Gardner IA, et al. Temporal detection of equine herpesvirus infections of a cohort of mares and their foals. *Vet Microbiol*. 2006;116(4):249-257.

120. Back H, Ullman K, Treiberg Berndtsson L, et al. Viral load of equine herpesviruses 2 and 5 in nasal swabs of actively racing Standardbred trotters: Temporal relationship of shedding to clinical findings and poor performance. *Vet Microbiol*. 2015;179(3):142-148.

121. Rushton JO, Kolodziejek J, Tichy A, Nell B, Nowotny N. Detection of equid herpesviruses 2 and 5 in a herd of 266 Lipizzaners in association with ocular findings. *Vet Microbiol*. 2013;164(1):139-144.

122. Marenzoni ML, Coppola G, Maranesi M, et al. Age-dependent prevalence of equid herpesvirus 5 infection. *Vet Res Commun*. 2010;34(8):703-708.

123. Akkutay AZ, Osterrieder N, Damiani A, Tischer BK, Borchers K, Alkan F. Prevalence of equine gammaherpesviruses on breeding farms in Turkey and development of a TaqMan MGB real-time PCR to detect equine herpesvirus 5 (EHV-5). *Arch Virol*. 2014;159(11):2989-2995.

124. Wong DM, Belgrave RL, Williams KJ, et al. Multinodular pulmonary fibrosis in five horses. *J Am Vet Med Assoc*. 2008;232(6):898-905.

125. Fortier G, van Erck E, Fortier C, et al. Herpesviruses in respiratory liquids of horses: Putative implication in airway inflammation and association with cytological features. *Vet Microbiol*. 2009;139(1):34-41.

126. Hart KA, Barton MH, Williams KJ, Flaminio MJBF, Howerth EW. Multinodular pulmonary fibrosis, pancytopenia and equine herpesvirus-5 infection in a Thoroughbred gelding. *Equine Vet Educ*. 2008;20(9):469-476.

127. Spelta C, Axon J, Begg A, et al. Equine multinodular pulmonary fibrosis in three horses in Australia. *Aust Vet J*. 2013;91(7):274-280.

128. Dunowska M, Hardcastle M, Tonkin F. Identification of the first New Zealand case of equine multinodular pulmonary fibrosis. *N Z Vet J*. 2014;62(2):226-231.

129. Niedermaier G, Poth T, Gehlen H. Clinical aspects of multinodular pulmonary fibrosis in two warmblood horses. *Vet Rec*. 2010;166(14):426-430.

130. Poth T, Niedermaier G, Mermanns W. Equine Multinodular Pulmonary Fibrosis in association with an EHV-5 infection in 5 horses. *Wien Tierarztl Monatsschr*. 2009;96(7-8):203-208.

131. Soare T, Leeming G, Morgan R, et al. Equine multinodular pulmonary fibrosis in horses in the UK. *Vet Rec*. 2011;169(12):313.

132. Verryken K, Saey V, Maes S, et al. First report of multinodular pulmonary fibrosis associated with equine herpesvirus 5 in Belgium. *Vlaams Diergeneeskd Tijdschr*. 2010;79(4):297-301.

133. Back H, Kendall A, Grandón R, et al. Equine Multinodular Pulmonary Fibrosis in association with asinine herpesvirus type 5 and equine herpesvirus type 5: a case report. *Acta Vet Scand*. 2012;54(1):57.

134. Wilkins PA. Equine multinodular pulmonary fibrosis: Diagnosis and treatment. *Equine Vet Educ*. 2013;25(8):393-397.

135. Schwarz B, Schwendenwein I, van den Hoven R. Successful outcome in a case of equine multinodular pulmonary fibrosis (EMPF) treated with valacyclovir. *Equine Vet Educ*. 2013;25(8):389-392.

136. Garré B, Baert K, Nauwynck H, Deprez P, de Backer P, Croubels S. Multiple oral dosing of valacyclovir in horses and ponies. *J Vet Pharmacol Ther*. 2009;32(3):207-212.

137. Garré B, van der Meulen K, Nugent J, et al. In vitro susceptibility of six isolates of equine herpesvirus 1 to acyclovir, ganciclovir, cidofovir, adefovir, PMEDAP and foscarnet. *Vet Microbiol*. 2007;122(1):43-51.

138. Marenzoni ML, Passamonti F, Lepri E, et al. Quantification of Equid herpesvirus 5 DNA in clinical and necropsy specimens collected from a horse with equine multinodular pulmonary fibrosis. *J Vet Diagn Invest*. 2011;23(4):802-806.

139. Walling DM, Flaitz CM, Nichols CM, Hudnall SD, Adler-Storthz K. Persistent productive Epstein-Barr virus replication in normal epithelial cells in vivo. *J Infect Dis*. 2001;184(12):1499-1507.

140. Temple RM, Zhu J, Budgeon L, Christensen ND, Meyers C, Sample CE. Efficient replication of Epstein-Barr virus in stratified epithelium in vitro. *Proc Natl Acad Sci U S A*. 2014;111(46):16544-16549.

141. Nawandar DM, Wang A, Makielski K, et al. Differentiation-dependent KLF4 expression promotes lytic Epstein-Barr virus infection in epithelial cells. *PLoS Pathog*.

2015;11(10):e1005195.

142. Quintana AM, Landolt GA, Annis KM, Hussey GS. Immunological characterization of the equine airway epithelium and of a primary equine airway epithelial cell culture model. *Vet Immunol Immunopathol*. 2011;140(3-4):226-236.

143. Soboll Hussey G, Ashton L V, Quintana AM, et al. Innate immune responses of airway epithelial cells to infection with equine herpesvirus-1. *Vet Microbiol*. 2014;170(1-2):28-38.

144. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints,. *Am J Epidemiol*. 1938;27(3):493-497.

145. Wilkie GS, Kerr K, Stewart JP, Studdert MJ, Davison AJ. Genome sequences of equid herpesviruses 2 and 5. *Genome Announc*. 2015;3(2).

146. Breathnach CC, Rudersdorf R, Lunn DP. Use of recombinant modified vaccinia Ankara viral vectors for equine influenza vaccination. *Vet Immunol Immunopathol*. 2004;98(3):127-136.

147. Soboll Hussey G, Hussey SB, Wagner B, et al. Evaluation of immune responses following infection of ponies with an EHV-1 ORF1/2 deletion mutant. *Vet Res*. 2011;42(1):23.

148. Back H, Ullman K, Leijon M, et al. Genetic variation and dynamics of infections of equid herpesvirus 5 in individual horses. *J Gen Virol*. 2016;97(1):169-178.

149. Johnson AS, Maronian N, Vieira J. Activation of Kaposi's sarcoma-associated herpesvirus lytic gene expression during epithelial differentiation. *J Virol*. 2005;79(21):13769-13777.

150. Li QX, Young LS, Niedobitek G, et al. Epstein-Barr virus infection and replication in a human epithelial cell system. *Nature*. 1992;356(6367):347-350.