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Primary Human Fibroblasts are Permissive for Porcine Cytomegalovirus *In Vitro*

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PRIMARY HUMAN FIBROBLASTS ARE PERMISSIVE FOR PORCINE CYTOMEGALOVIRUS *IN VITRO*

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In Fulfullment of the Requirements for the Degree of
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ABSTRACT

Xenotransplantation with pig organs is being considered to alleviate donor organ shortages; however, the risk of introducing porcine viruses to humans is exaggerated in this setting. Accordingly, the goal of this study was to determine the infectious potential of porcine cytomegalovirus (PCMV), a xenozoonotic virus of interest, in human fibroblasts *in vitro*. Confluent cells were incubated with either live PCMV or controls. Infection was investigated by light microscopy/neutral red staining, by RT-PCR and sequencing, and by Western blotting. Neutralization experiments were also performed. Cells incubated with PCMV demonstrated significant cytopathic effect by 7 days post-infection. Also, RT-PCR sequencing identified PCMV DNA polymerase in infected cells. In Western blots, monoclonal antibodies (mAbs) to human CMV glycoprotein B and pig serum presumed to contain anti-PCMV antibodies detected PCMV proteins by 19 days post-infection. Furthermore, one of these mAbs and the pig serum neutralized PCMV infection. Western blots and neutralization studies using a mAb to a human herpes virus 6 membrane glycoprotein provided no concrete evidence for a biological relationship between PCMV and human herpes virus 6. These results demonstrate that PCMV can infect human fibroblasts *in vitro*.

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LIST OF ABBREVIATIONS

α = alpha

β = beta

γ = gamma

α-Gal = galactose α1-3galactose

AHXR = acute humoral xenograft rejection

AIDS = acquired immunodeficiency syndrome

ATCC = American Type Culture Collection

BCA = bicinchonic acid

BLAST = basic local alignment search tool

BME = Basal Medium Eagle

bp = base pairs

BSA = bovine serum albumin

CMV = cytomegalovirus

CMVs = cytomegaloviruses

CO_2 = carbon dioxide

CPE = cytopathic effect

CXR = cellular xenograft rejection

DMEM = Dulbecco's Modified Eagle's Medium

DNTPs = deoxyribonucleotide triphosphates

DTT = dithiolthreitol

E = early

EDTA = ethylenediaminetetra acetic acid

FBS = fetal bovine serum

gB = glycoprotein B

gH = glycoprotein H

HAR = hyperacute rejection

hCRPs = human complement regulating proteins

HFF = human foreskin fibroblasts

HHV-6 = human herpes virus 6

HHV-7 = human herpes virus 7

HIV-1 = human immunodeficiency virus-1

HRP = horseradish peroxidase

IE = immediate early

IFA = immunofluorescent assay

IFAs = immunofluorescent assays

IgG = immunoglobulin G

kbp = kilobase pairs

kDa = kilodaltons

mAb = monoclonal antibody

mAbs = monoclonal antibodies

MHC-I = major histocompatability complex class I

NCBI = National Center for Biotechnology Information

NGS = normal goat serum

NKC = natural killer cell

NKCs = natural killer cells

PBS = phosphate buffered saline

PCMV = porcine cytomegalovirus

PERVs = porcine endogenous retroviruses

PPEC = primary porcine endothelial cells

PT-K75 = a porcine nasal fibroblast cell line

PVDF = polyvinylidene fluoride

SDS = sodium dodecyl sulfate

SIV = simian immunodeficiency virus

T75 = 75 cm^2 (60 mL max.) rectangular canted neck culture flasks with vented caps

TBS = tris buffered saline

U = unique

U_L = unique long

U_S = unique short

ZAP-70 = zeta-chain-associated protein kinase 70

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1.0 INTRODUCTION

One of the most significant concerns preventing the clinical application of xenotransplantation is the theoretical risk of infectious disease associated with the procedure. Porcine cytomegalovirus (PCMV) is a xenozoonotic virus in pigs and is of particular interest, as a number of *in vivo* studies by Mueller *et. al.*[49,128,130] have found PCMV DNA in numerous baboon tissues following pig-to-primate xenotransplantation. Since all cytomegaloviruses (CMVs) are normally considered species-specific, these findings may reflect a PCMV–associated cross-species infectivity potential in the specific context of pig to primate xenotransplantation. Given these accounts and the small evolutionary distance between humans and non-human primates, the studies described in this thesis were carried out in order to determine the infectious potential of PCMV in human fibroblasts.

1.1 XENOTRANSPLANTATION.

The clinical success of allotransplantation has created a crisis in donor organ and tissue shortages. Advancement of technology and medical techniques have made allotransplantation a routine and preferred disease treatment[1]. In Canada, the demand for organ transplantation has nearly doubled since 1995; whereas, the organ supply has remained fairly constant[2,3]. Xenotransplantation – defined as the transplantation of live cells, tissues, or organs across a species barrier – can potentially answer this shortfall in donor organ availability. If it is ever a viable solution, xenotransplantation can provide an unlimited supply of organs and tissues for permanent organ replacements, bridged organ transplants, cell-based therapy for the treatment of disease, or as part of a medical device[4]. Conversely, as discussed below, there are many reasons as to why this solution may never be realized.

1.1.1 A Brief History of Xenotransplantation.

Although the first attempts at

xenotransplantation can be traced back to the early 1500's, modern day, solid-organ xenotransplantation dates back to the 1960's and the realization of the importance of immunosuppressive drugs[5]. The first reported xenotransplantation attempt that included an immunosuppressive regime occurred in 1963 when Dr. Keith Reemtsma transplanted a rhesus monkey kidney into a 43-year-old man[5]. From then on, attempts at xenotransplantation and their relative successes parallel the discovery of improved immunosuppressive techniques[5]. Although, endeavors of successful xenotransplantation have continued over the past 40 years, progress has been hindered by the success of allotransplantation. Technology and research preferentially focused on allotransplantation since its was much more successful and promising than xenotransplantation. For example, no xenograft recipients, in early attempts and to date, have reached the 1-year patient/graft survival mark as seen with allograft recipients[4]. Being more compatible in all aspects, human organs were and still are preferred to treat end-stage organ failure. However, there is currently a renewed interest in xenotransplantation, as the supply of human organs continues to fall short of meeting an ever-increasing demand[4-7].

1.1.2 Why Xenotransplantation? Besides an unlimited supply of organs and tissues, xenotransplantation offers many potential advantages when compared to allotransplantation. These include: advanced planning of life-saving operations, elective surgery options for disease treatment, beginning graft accommodation treatment prior to transplantation, organ harvest only at the time required, potential pathogen-free source animals, and the pre-screening of potential organs for infectious substances prior to harvesting[4]. Xenotransplantation would also provide an alternative life-saving option in countries or under religious faiths where human organ transplantation is not accepted[8].

Furthermore, the xenograft has the potential to be immune to a viral infection or an autoimmune condition that may have caused the original organ failure[9]. Given the plethora of potential advantages, xenotransplantation seems like the perfect answer to the crises in organ shortages; nonetheless, there are disadvantages associated with xenotransplantation. Some of these include: the risk involved – both individually and to the society – of introducing new human pathogens from animals; the high level of immune suppression which may be needed in a xenograft recipient that would potentially increase susceptibility to any known or unknown infection; religious objections present in many different faiths; the potential physiological incompatibilities associated with a xenograft; and the many issues associated with animal welfare and genetic modification[6,8,9].

1.1.3 Species of Choice. As summarized by Levy[10], a species should possess a number of ideal traits to be considered for use in xenotransplantation. To begin with, the organs should be of comparable size and structure to human organs as well as having similar function. Additionally, the animal would ideally present no immunological barrier when transplanted into humans. Also, the potential for zoonotic infections from both xenograft to human and human to xenograft should not exist. Furthermore, the animal source should be able to provide the intended "unlimited supply" of organs at an inexpensive price and within a reasonable amount of time. Lastly, the general public should be accepting of the chosen species in order to curtail ethical objections. However, this may be hard considering that many individuals and religious beliefs are opposed to the use of any living mammal, including humans, for transplant therapy[11]. No animal can meet all these expectations; however, currently, the generally accepted donor species of choice is the domestic pig[10-15].

Historically, non-human primates were once the preferred animals due to the phylogenetic, structural, and functional similarities of their organs to human organs[4,10-15]. From an immunological viewpoint, xenografts from non-human primates would present less severe episodes of rejection than other mammals, and they may also possess resistance to certain human diseases[10]. Nonetheless, non-human primates present many disadvantages as xenograft donors, which far outweigh their benefit. As discussed in section 1.1.4-c, the most significant setback associated with xenotransplantation is the zoonotic risk that is associated with it, and this risk is particularly pronounced when using non-human primates as a donor source[10,11,14,15]. Although allotransplant teams attempt to minimize the transfer of infectious agents between human donor organs and recipients and vice versa, the phenomenon still occurs and is expected to occur in xenotransplantation. Because non-human primates and humans are closely related, the risk of an infectious agent transfer and subsequent human adaptation is more likely to occur in a xenotransplantation setting when using non-human primates as an organ source than when using a more distantly related mammal[10,11,14,15]. Since most non-human primates are currently either wild caught or housed under colony conditions for only a few generations, the potential for any pathogen to be present and transferred to the xenograft host is accordingly high[15]. Besides the prominent infectious disease risk, non-human primates do not make an ideal donor source for the following reasons: many species are considered endangered; many species would provide organs that are too small for use in humans; there are many ethical concerns due to intellectual and social human-like traits; there is little knowledge in the genetic modification of these animals; and the relative cost of large-scale breeding and husbandry is thought to be difficult, costly and untimely, as non-human primates reproduce slowly with long gestation periods, are slow to attain sexual

maturity, and normally have only single off-spring[4,10,11,14,15]. Conversely, the pig has many advantages as an organ source.

First, the infectious disease threat is lessened when using pigs due to a larger evolutionary distance from humans[4,10-15]. Second, domestic pigs are not endangered. Third, porcine organ size, structure, function, and lifespan are comparable to that of humans; although, some physiological differences do exist between them[4,10-15]. Fourth, ethical issues that surround the use of non-human primates are reduced or even eliminated when considering the pig since millions are bred for the sole purpose of consumption yearly; they have no outward human-like traits; and pigs have long been used to provide medical treatments for humans[4,10-15]. Fifth, much is known about the genetic manipulation of pigs allowing for the production of both transgenic and knockout versions of the animal to produce – from an immunological perspective – a more tolerated organ source[4,10,11,14-17]. Lastly, the relative cost of large scale breeding is considered much more feasible because pigs reproduce quickly with three and a half month gestation periods, reach sexual maturity within nine months, and have large litter sizes[4,10-15]. Nonetheless, although xenotransplantation with pig tissue has the potential to increase the quality of life for thousands, there are some obstacles surrounding the technology that need to be addressed before it can be safely applied.

1.1.4 Current Xenotransplantation Obstacles. If xenotransplantation is ever to become a viable alternative to allotransplantation, three main hurdles from a scientific/medical perspective must be overcome: (1) immunological hurdles, (2) physiological incompatibilities, and (3) infectious disease risks.

1.1.4-a Immunological Hurdles. Much of the research concerning xenotransplantation has focused on overcoming the major practical obstacle to successful xenotransplantation – immunological rejection[6]. Rejection in xenotransplantation is similar to that of allotransplantation and is divided into three core phases based on time and histopathology, as summarized in Table 1[18,19]. They are hyperacute rejection, acute humoral xenograft rejection, and cellular xenograft rejection[4,14,15,18-21].

Phase 1: Hyperacute Rejection (HAR). The first stage of rejection is designated HAR and occurs within hours of the initial xenograft transplant[6,15,18,22-24]. HAR is complement-mediated[6,15,18]. Due to a functional α1,3galactosyltransferase gene, the endothelium of pigs and "lower mammals" express a terminal carbohydrate residue – a galactose α1-3galactose (α-Gal) epitope – that is not present in humans, apes, and other Old World Primates[6,15,18]. Shortly after birth, humans develop antibodies specific for the α-Gal residue due to its expression on normal flora found in the gut[6,15,18,25]. Making up approximately 5% of human plasma, these "natural antibodies" instantly recognize and bind to the α-Gal epitope expressed on the xenograft vasculature[6,15,18,25,26]. This sets the complement cascade into motion via the classical route of activation, and subsequently results in the typical histopathology of HAR (Table 1)[6,15,18]. Much research has focused on overcoming HAR. Humanized knock-in pigs expressing human complement inhibitors such as CD55 and CD59 and knock-out pigs lacking the α-1,3galactosyltransferase gene have aided in overcoming the instantaneous effects of HAR[16,17,27-31].

Phase 2: Acute Humoral Xenograft Rejection (AHXR). The second stage of rejection is called AHXR and normally occurs within days of the initial xenograft introduction[4,15,18]. AHXR is similar to HAR in that it is also recognized as being an α-Gal

Table 1: The Phases of Rejection in Xenotransplantation and the Diagnostic Criteria used to Define them[18,24].

Diagnostic Criteria	Hyperacute Rejection	Acute Humoral Xenograft Rejection	Cellular Xenograft Rejection
Survival Time	< 24 hrs; immediately post-transplant	> 24 hrs post-transplant, usually within 1 week	> 1 week post-transplant
Graft Function	Graft has never been functional	Graft has been functional	Graft has been functional
Histopathology	Massive hemorrhage	Hemorrhage present	No hemorrhage
	Complement (MAC), Ig, and fibrin deposition on graft	Complement (MAC), Ig, and fibrin deposition	Low levels of complement possible; Ig and fibrin deposition rare
	PMN granulocytes present	PMN granulocytes present	T-cell & macrophage infiltration with graft destruction; Natural killer cell (NKC) activity
	Thrombosis possible	Thrombosis	No thrombosis
		Type II endothelial activation	Tissue injury/necrosis

antibody-mediated rejection; however, other antibodies are also involved[4,15,18]. These antibodies are a result of an immune response elicted towards many different xenogenic proteins that results in graft sensitization[4,15,18]. Complement plays a much smaller role in AHXR, and many other humoral immune mechanisms are believed to play a role in the type-II (pro-coagulant) endothelium activation seen in a xenograft that has gone through AHXR (Table 1) [4,15,18]. In 2002, Dai *et. al.*[29] and Lai *et. al.*[32] produced α-1,3galactosyltransferase heterozygous knock-out pigs, and in 2003, Phelps *et. al.*[31] developed double knock-out pigs totally lacking this gene. These developments provided important steps forward in potentially overcoming AHXR because when both Kuwaki *et. al*[17] and Yamada *et. al.*[30] transplanted porcine organs from pigs lacking the 1,3galactosyltransferase gene, the detrimental effects of AHXR were greatly reduced. These studies showed that hearts and

kidneys from these knock-out porcine donors will survive an average of approximately 3 months in baboon recipients while using clinically applicable immunosuppressive regimes.

Phase 3: Cellular Xenograft Rejection (CXR). Since it is becoming clear that both HAR and AHXR can be successfully managed, more research is beginning to focus on the third phase of rejection, CXR. Not much is known about CXR; however, it appears to be T-cell mediated, and natural killer cells (NKCs) appear to play an important role[15,18,20,24,33-36]. Specifically, molecular incompatibilities allow for a strong natural killer cell (NKC) response during CXR because the human inhibitory receptors on the NKC do not recognize porcine major histocompatability complex class I (MHC-I) molecules on the xenograft, and the innate NKC response is not terminated[24,33]. It is thought that current allograft immune suppression techniques will not be sufficient to prevent CXR[24]. This is because of the additional NKC response present during CXR that is not there during cellular allograft rejection and because of the belief that a xenograft will provide many more foreign epitopes that can induce a more diverse/strong cellular immune response than an allograft can[15,18,20,24,33]. Thus, the prolonged survival (greater than 1 year) of a xenograft will likely depend on new immune suppression techniques[14,18,20,24,30].

1.1.4-b Physiological Incompatibilities. With the immunological barriers associated with xenotransplantation becoming smaller, the issue of pig organ function in a human body is receiving more attention, and certain differences are becoming apparent. For example, pigs have a normal body temperature of approximately 39°C, whereas humans function best at a temperature of about 36.5°C[14,15]. It remains unclear if this discrepancy will affect the function of a porcine xenograft in a human environment[14,15]. Most researchers agree that physiologically simple functions, such as pumping blood, will likely remain intact

when a xenograft is placed within a human environment; however, metabolically complex organs, such as the liver, may lack functions or produce unusable proteins that are crucial to human survival[14,15,20,37]. As expressed by Platt[37], the physiological performance of the organ in its new environment may not be a question of perfect function, but rather a question of the function being sufficient enough to maintain human life. There is a major lack of knowledge concerning the physiological compatibility of porcine organs and protein within a human host[15,37]. If long-term xenotransplantation of porcine organs into non-human primates ever becomes a reality, then it will be possible for researchers to identify the physiological limitations of a xenograft and to study how/if these limitations can be overcome[15,37].

1.1.4-c Infectious Disease Risks. In allotransplantation, infectious agents are often transplanted into the recipient along with the donor organ. In fact, an organ recipient is routinely prescribed a prophylactic drug regime before any infection is evident. Zoonosis is defined as the transfer of infectious agents across a species barrier. Accordingly, xenozoonosis, or xenosis, is the terminology applied to this definition with respect to xenotransplantation[20]. It is believed that most new emerging human pathogens which possess the ability to cause disease and spread within the human population have resulted from the zoonotic transfer and subsequent human adaptation of an infectious agent that originated from an animal[38,39]. For example, the current acquired immunodeficiency syndrome (AIDS) pandemic is now known to be the result of the zoonotic transfer and subsequent human adaptation of simian immunodeficiency viruses (SIVs) found in African chimpanzees and monkeys[40,41]. These viruses are believed to have crossed over into the human population relatively few times[40,41]. In fact, the virus from which human immunodeficiency virus 1 (HIV-1) originated is believed to have made that jump only

once[40,41]. Also, the recent severe acute respiratory syndrome epidemic is believed to have originated through the zoonotic transfer of an animal corona virus[42]. Taking these examples of zoonosis into consideration, the theoretical infectious disease risk associated with xenotransplantation not only concerns the xenograft recipient, but also the general public as a whole if subsequent human-to-human pathogen adaptation should occur[4,6,20]. Although stringent animal husbandry protocols can significantly decrease the zoonotic risks associated with xenotransplantation, they, as described by Tackaberry and Ganz[6], will never be zero.

1.1.5 Xenozoonosis. Discussion and concern over the theoretical infectious disease risks associated with xenotransplantation have always existed. However, both Patience *et. al.*[43] and Le Tissier *et. al.*[44] brought the issue to the forefront in 1997 by providing evidence which supported the hypothetical zoonotic risk that was associated with xenotransplantation. The genomes of all species contain sequences that are known to be the proviral genomes of exogenous retroviruses[4]. These now endogenous retroviruses normally cause no harm to their native host; however, they have been associated with cross-species transmission and disease in animals[4]. For example, the virus which causes leukemia in Gibbon apes is believed to have originated from the zoonotic transfer of a mouse endogenous retrovirus[4]. The studies published by both Patience *et. al.*[43] and Le Tissier *et. al.*[44] showed that pig endogenous retroviruses (PERVs) could infect human cells *in vitro*. Furthermore, Patience *et. al.*[43] also reported that the PERVs could act as a helper virus to a Moloney retroviral vector in infected human cells, suggesting that PERVs could potentially aid in producing replication-competent human endogenous retroviruses following pig-to-human xenotransplantation. Since then, many reports have been published which confirm these initial reports, and research within the field of xenotransplantation now includes a zoonotic

focus as well as trying to solve the immunological and physiological hurdles (see sections 1.1.4-a and 1.14-b). For example, several studies evaluated different porcine viruses that were believed to be a xenozoonotic risk to humans[43,45-50,51-54]. In particular, Mueller *et. al.*[49] evaluated the infectivity potential of PCMV in a baboon model of xenotransplantation and concluded that PCMV will reactivate and infect non-native host tissues in a xenotransplantation setting. Similar to allotransplantation, the infectious disease risk will forever remain an important issue associated with xenotransplantation.

1.1.5-a An Increased Zoonotic Potential. The chance for a zoonosis to occur is greatly enhanced in the xenotransplantation setting and has been the subject of many reviews[4,39,55-58]. In particular, articles published by both Boneva *et. al.*[55] and Fishman *et. al.*[56] thoroughly discuss the issues surrounding xenozoonosis. First, the non-specific innate barriers of the immune system are bypassed allowing the potentially infectious agent favourable, direct entry into the human host without the need for a vector[4,39,55-60]. Second, the xenograft recipient will be in a severe and prolonged immune-suppressed state and will be unable to mount an effective immune response against any transplanted pathogen[4,39,55-60]. Third, the xenograft will be in contact with the human host for long periods of time[4,39,55-60]. This prolonged contact may allow the xenograft to serve as a "permissive niche" within the new environment permitting unnatural pathogen replication with subsequent persistence and adaptation to the xenograft host[4,39,55-60]. Fourth, no pre-existing immunity to the novel pathogen will likely exist in the xenograft recipient, making the host that much more susceptible to infection and greatly increasing the zoonotic risk[4,39,55-58]. Fifth, known host-pathogen interactions and clinical manifestations that occur as a result of infection in the native animal may be quite different in the unnatural human host and thus impossible to

identify: virulence may be increased or decreased, disease characteristics may be altered, or a microorganism that is non-pathogenic in the native host may become deadly in a new host[4,6,39,55-60]. For example, monkey B virus, which causes only mild lesions in Old World Macaca primates, exhibits greater virulence when inadvertently transferred to humans causing encephalitis, encephalomyelitis, and death[61,62]. Sixth, the porcine pathogen alone may not cause disease in the human host but may genetically recombine with a human pathogen to create a completely new pathogen[4,45,56]. Lastly, xenozoonotic concerns have been raised surrounding the use of transgenic pigs in xenotransplantation. Currently, transgenic pigs expressing human complement regulating proteins (hCRPs) such as CD55 and CD59, or transgenic pigs lacking the α-Gal epitope are being used in xenotransplantation to dampen the immune system and rejection associated with xenotransplantation. However, Takefman *et. al.*[63] has shown that PERVs which replicate within pig cells transgenic for human CD59 will acquire the protein and that the "humanized" pathogens are able to avoid complement-mediated lysis by human serum. Magre *et. al.*[64] has also shown that enveloped viruses budding from pig cells engineered to lack the α-Gal epitope or possess human CD55 are able to avoid complement-mediated lysis by human serum. These studies show that the acquisition of hCRPs or the lack of α-Gal epitopes may allow the pathogen to become less sensitive to natural human immunity[4,39,55,65]. Furthermore, some human pathogens use human complement regulating proteins as entry receptors to gain access to their host[4,39,55]. As discussed by Weiss[65], porcine organs that are not normally susceptible to certain human viruses may be rendered susceptible to infection by expressing the receptor used for entry. Pigs carry and are susceptible to many different pathogens, either intracellular or

extracellular. Although they all pose a risk to humans in terms of xenotransplantation, some pose a greater risk than others, and these are discussed below.

1.1.5-b Pathogens of Interest. Pigs used for xenotransplantation will undoubtedly be screened against many pathogens. In fact, as stated by Weiss[39], "specific pathogen-free animals should be cleaner than 'free-range' human donors". However, he also comments that, "even with the most stringent regulations and the most rigorous barrier containment of source animals, we must recognize that accidents and errors will occur"[39]. Pigs raised and monitored in special environments can potentially be free of extracellular microorganisms, such as fungi, parasites, nematodes, and bacteria, thus providing microbiologically clean xenografts[55,56,66-68]. Moreover, any iatrogenic bacterial, fungal, or parasitic infection accidentally acquired as a result of xenotransplantation should be susceptible and thus treatable with antibiotic drugs or any similar disease-suppressing therapy[55,57,66]. Accordingly, bacteria, fungi, and parasites are not the main concern of xenosis. The main concern involves viruses[4,45,55-57,59]. Of the many viruses able to infect swine, only some are considered to be of xenozoonotic risk[4,56,57,66]. Viruses of concern include those that are (1) *unknown* in that they are non-pathogenic in the native host and are thus not yet identified; and (2) *known* pathogens which cause disease and are difficult to eliminate, which may result in a latent or intracellular persistent state, or impact on graft viablilty[4,56,57]. Endogenous retroviruses, circovirus types 1 and 2, reproductive and respiratory syndrome virus, encephalomyocarditis virus, swine influenza viruses, African swine fever virus, hepatitis E-like virus, pseudorabies virus, parvovirus, polyomaviruses, and herpesviruses are known porcine viruses of xenozoonotic interest[45,46,55-60,66-68]. This

discussion will focus on β-herpesviruses since they are the focus of the research presented in section 4.

1.2 β-HERPESVIRUSES.

The *Herpesviridae* viral family is found ubiquitously throughout nature[69]. The family is subdivided into three main subfamilies – alpha (α), beta (β), and gamma (γ) – based on distinctive biological properties[69]. β-herpesviruses are considered species specific, and genera of the *Betaherpesvirinae* subfamily include *Cytomegalovirus* and *Roseolovirus.* The prototype members for this subfamily are the human cytomegaloviruses[69].

1.2.1 Human CMV.

1.2.1-a Structural and Genomic Properties. Human CMV virions are made up of four main components: the core, the capsid, the tegument (or matrix), and the envelope. The structure and genomic properties of human CMV have been well studied and are described in detail by Mocarski and Courcelle in Fields Virology[70]. The core consists of a linear, double-stranded DNA molecule enclosed by an icosahedral-shaped capsid. The capsid is constructed from several different proteins with the major constituent being a 1,370 amino acid protein that is highly conserved among herpesviruses. Surrounding the capsid is a proteinaceous matrix called the tegument. The tegument can be of variable thickness and consists of up to 25 different phosphoproteins. Tegument proteins appear to be conserved among β-herpesviruses, but not across the *Herpesviridae* family. The core, capsid, and tegument are all contained within a lipid bilayer called the envelope. The envelope is composed of numerous different glycoproteins embedded in a lipid bilayer that the virus obtains from both nuclear and cytoplasmic intracellular membranes during its life cycle. There are several different major envelope glycoproteins, but gB is the most abundant.

Infectious, mature virions are large, ranging in size from 150-300 nm depending on the thickness of the tegument and the nature of the envelope.

Herpesviruses are known to be large, complex viruses, and human CMV is no exception. With an approximate 235 kilobase pair (kbp) genome that carries over 200 open reading frames, it is one of the largest animal virus genomes. The genome has directly repeated termini and is divided into two sections: unique long (U_L) and unique short (U_S) sequences are both flanked on each end by inverted repeats. The internal and terminal repeats vary in size depending on strain and passage history. For example, highly passaged strains tend to have deleted U_L DNA sequences and larger flanking inverted repeats. Many different strains of human CMV are believed to exist in nature. But all laboratory (highly passaged) human CMV strains studied to date are closely related with a 90-95% DNA sequence homology. Most data generated today are derived from experiments with laboratory-adapted strains of human CMV such as the AD169 strain or the Towne strain.

1.2.1-b Viral Life Cycle. The human CMV viral life cycle is also well described in Fields Virology[70,71] and in a review by Emery[72]. After attachment, the human CMV virion releases its capsid into the cytoplasm of a susceptible cell via fusion of the virion envelope with the cell surface. In permissive cells, the capsid makes its way into the nucleus where viral gene transcription, DNA replication, and new capsid assembly occur.

Human CMV gene expression is classified into three phases as a result of a temporally ordered cascade of protein expression. The first group of genes is called the immediate early (IE) genes, and expression of IE genes is not dependant on the expression of any other viral gene. Thus, IE gene expression begins immediately after viral penetration. IE gene products are not incorporated into progeny virions but function to control subsequent

gene expression. The second group of genes to be expressed is called the early (E) genes, and expression of E genes will only begin once functional IE gene products have been made. Like IE genes, E genes also play a role in regulating subsequent gene expression and hence do not become part of the final virion. DNA replication begins during this phase. The last phase of gene expression, called the late phase, is responsible for producing most of the virion structural proteins and is when virion assembly occurs. During an infection, viral glycoproteins are expressed in every cell-associated membrane, and assembled capsids obtain envelopes when they bud from the nucleus. Virions can also be re-enveloped in the cytoplasm by the Golgi apparatus. The human CMV viral life cycle is lytic, and *in vitro*, most new virions are released upon the bursting and simultaneous death of the infected cell.

In vivo, human CMV has the ability to productively infect several different human cell types during an active infection. These permissive cells include fibroblasts, epithelial cells, macrophages/monocytes, neuronal cells, and smooth muscle and endothelial cells; however, endothelial cells, neutrophils, and lymphocytes are predominantly infected. On the other hand, *in vitro*, the virus replicates best in primary differentiated human fibroblasts. In cultured fibroblasts, human CMV infection with highly passaged isolates at high multiplicity of infection is slow. DNA synthesis is first noted approximately 15 hours post-infection with levels gradually increasing thereafter. Mature virions are seen in the cytoplasm 72-96 hours post-infection, and progeny virus is found in tissue culture fluids 96-120 hours post-infection. The entire monolayer will show signs of CPE about one week after the initial infection. In fibroblasts, CPE includes substantial cell rounding and enlargement with nuclear and perinuclear inclusions. The *in vitro* infection of human fibroblasts with clinical isolates of

human CMV is even slower – it takes up to two weeks to see scattered foci of CPE, and the spread is believed to be via cell contact because little extracellular virus is seen.

It has become dogma that human CMV is restricted to replicating only in human cells[47,69,70,73]. In fact, all cytomegaloviruses (CMVs) are considered species-specific and are believed to replicate only in the species from which they originate[69]. Studies show that this species-specificity is a result of the ablation of gene expression in non-permissive cells and not a failure to enter the cells[74,75]. A recent study by Jurak *et. al.*[73] suggests that the induction of apoptosis following entry into a susceptible, but not permissive, cell across a species barrier may be the cause of this gene expression termination and that human CMV may not be as "species-specific" as previously thought. For example, human CMV has been shown to infect porcine endothelial cells *in vitro*[47].

1.2.1-c Epidemiology and Pathogenesis. Human CMV acquisition increases progressively in a population and is ubiquitous throughout the world. On average, 50–90% of the adult population will be infected by the age of 50[71,76-79]. Viral prevalence is related directly to socio-economic status: the virus is more prevalent in developing countries and in populations of low socio-economic status within developed countries[71,77-79].

After initial acquisition, infectious human CMV is found for months to years in blood, urine, semen, tears, saliva, breast milk, and cervical secretions of an infected individual, and human CMV virions are easily transmitted through direct contact with these bodily fluids[70,71,77-79]. Consequently, childcare centers are often a source of initial infection with human CMV[70,71,77-79]. In pregnant women, congenital human CMV transmission to the fetus is common during an active infection; however, newborn infants also acquire the virus through perinatal infection via breast milk and cervical fluids[71]. Infants who are infected

with human CMV in this manner will shed the virus for years and are an important source of viral spread to other children and adults[71]. Human CMV can also be readily acquired iatrogenically through blood transfusions and organ transplants[70,71,77-79].

Because human CMV is spread through bodily fluids, primary infection normally starts with viral replication in mucosal epithelium[70]. An acute systemic phase of infection follows and is associated with persistent viral shedding in bodily fluids as a result of secondary human CMV replication in the ductal epithelial cells of mucosal surfaces[70]. The virus is highly cell-associated, and dissemination of the virus to secondary sites of replication within the host occurs through the blood via monocyte-associated viremia[70]. Typical for all herpesvirus infections, primary human CMV infection will eventually result in a lifelong, latent infection[70,71,77-79]. Although the steps to achieving latency are not understood, human CMV is thought to maintain a latent reservoir in either myeloid hematopoietic stem cells or granulocyte-monocyte progenitor cells[71,80-83]. In circulation, $CD14^+$monocytes are believed to harbor the latent virus[71,80-83]. Furthermore, Söderberg-Nauclér *et. al.*[81] have shown that differentiation of these monocytes into macrophages via interferon gamma and allogenic stimulation *in vitro* leads to human CMV re-activation and replication.

In healthy individuals, human CMV infection is usually subclinical[70,71,77-79]. However, in rare cases, the virus will result in mononucleosis-like symptoms following primary infection[70,71,77-79]. Human CMV only becomes clinically important in situations where immune function is compromised. Several situations are of particular importance: (1) newborn infants who have acquired the virus congenitally; (2) patients with AIDS; (3) patients receiving immunosuppressive chemotherapy for treatment of cancer and/or other diseases; (4) patients with genetically acquired immune deficiencies; and (5) transplant

recipients who are under immune suppression[70-72,77-79]. Because the focus of the research presented in the following sections concerns xenozoonosis and PCMV, only human CMV infection in transplant recipients will be discussed.

1.2.1-d Human CMV Infection in Transplant Recipients. Symptomatic and subclinical human CMV infection in transplant recipients is common and can present in many different forms. Human CMV infections in allograft recipients can be due to reactivation, reinfection, or primary infection of the recipient[71,72,84,85]. The most severe forms of CMV disease are usually seen in recipients who were sero-negative for human CMV prior to transplantation and are thus undergoing a primary infection[71].

Disease-causing human CMV infection occurs in 30% of solid organ transplant recipients and in 25% of bone marrow transplants[71,86]. Symptomatic human CMV infection most commonly results in a mild "CMV syndrome" – persistent fever, malaise, leukopenia/thrombocytopenia, and human CMV dissemination. However, invasive, severe end-organ diseases, such as pneumonitis, gastrointestinal ulceration, hepatitis, and retinitis can also occur and may be fatal[71,72,77,78,84-88]. The allograft itself appears to be more susceptible to the aforementioned "direct effects" of human CMV reactivation[78,87]. For example, end-organ disease normally begins in the allograft itself (e.g. hepatitis in liver recipients) with subsequent systemic dissemination of virus (e.g. retinitis and/or pneumonitis in bone marrow recipients)[72,78,87].

The anatomical site and presentation of clinical human CMV disease will differ depending on the type of transplant preformed[71,72,84,88]. Recipients of bone marrow often experience human CMV-caused pneumonitis and gastrointestional tract disease; whereas recipients of solid organ allografts often experience human CMV-associated fever, hepatitis,

and gastrointestional tract disease[71,72,84,88]. Although the mechanisms by which human CMV causes disease are not completely known, the severity of the disease appears to reflect the amount of immune suppression present and the viral load in the blood: highly immune suppressed individuals and/or patients with high viremia titers are associated with an increased risk of severe, symptomatic human CMV disease[71,72,84,88].

Human CMV infection following allotransplantation has also been associated with a number of "indirect effects" including allograft injury and rejection[71,78,85,87]. Allograft-specific damage such as atherosclerosis in heart transplants or vanishing-bile-duct syndrome in liver transplants have been linked to inflammation of the allograft resulting from human CMV infection[71,78,85,87]. Complete rejection of the allograft has also been associated with human CMV reactivation. As discussed by Fishman *et. al.*[87], the association is controversial and can potentially be viewed as reciprocal. That is, the human CMV-associated immune response may trigger the rejection episode, or the rejection episode may trigger human CMV reactivation, which, in turn, intensifies the rejection episode[87].

1.2.2 PCMV.

1.2.2-a Genomic and Structural Properties. The morphological features of PCMV are typical for members of the β-herpesvirus subfamily (discussed in section 1.2.1-a). Mature virions range in size from 150-200 nm[89].

The full sequence of PCMV has not yet been determined and no information concerning genome size or structure is available[89]. Sequencing of the PCMV genome has been done, and three PCMV gene sequences are available: the DNA polymerase locus, the gB locus, and the major capsid protein locus[89-93]. Comparing amino acid sequences of two of these genes (Table 2) places PCMV phylogenetically closer to HHV-6 and human herpes

Table 2: Amino Acid Sequence Identities Between PCMV Proteins and other Human β-herpesviruses. The PCMV isolate 'B6' amino acid sequence for the indicated protein was entered into a National Center for Biotechnology Information (NCBI) Protein-Protein Basic Local Alignment Search Tool (BLAST: blastp) search. Some of the resulting matches for the human β-herpesviruses are listed in the table. The virus strain / isolate are included, and NCBI accession numbers are in parentheses. The PCMV B6 amino acid sequences were obtained form the NCBI database.

Virus	% Identity to PCMV					
	DNA Polymerase			Glycoprotein B		
PCMV	• **(AAF80107)**	**strain B6**	**100%**	• **(AAL47542)**	**strain B6**	**100%**
Human CMV	• (NP_039988)	strain AD169	43%	• (P06473)	strain AD169	35%
	• (AAP8837)	strain Towne	43%	• AAA45920	strain Towne	37%
	• (AAD30077)	isolate E763	44%	• (AAR31620)	isolate merlin	37%
HHV-6A	• (NP_042931)	isolate U1102	50%	• (NP_042932)	isolate U1102	43%
HHV-6B	• (AAD49652)	isolate Z29	50%	• (AAD49653)	isolate Z29	43%
	• (BAA78259)	isolate HST	50%	• (BAA78260)	isolate HST	43%
HHV-7	• (YP_073778)	isolate RK	50%	• (YP_073779)	isolate RK	41%
	• (AAC54700)	isolate JI	50%	• (AAC54701)	isolate JI	41%

virus 7 (HHV-7) of the β-herpesvirus genus *Roseolovirus* (discussed in section 1.2.3)[89-93]. Although different strains of PCMV have not yet been officially identified, comparing the protein sequences of gB and DNA polymerase of different isolates indicates a significant intra-species variation and suggests the presence of different strains[89-91,93].

1.2.2-b Viral Life Cycle. Like human CMV, PCMV replication occurs in the nucleus; however, detailed replication patterns are not known. The information that is available is well described in the current edition of Diseases of Swine[89]. The PCMV viral life cycle appears to be non-lytic *in vitro*. *In vivo*, PCMV has the ability to infect macrophages, monocytes, reticuloendothelium, and epithelial cells. *In vitro*, the virus has been propagated in porcine lung macrophages, primary testicle cells, turbinate nose cells, a fallopian tube cell line, and the PK-15 cell line, although yield is lower in cell lines than in primary systems. CPE includes cell swelling to about six times larger than uninfected cells,

nuclear inclusion bodies, and sometimes acidic cytoplasmic inclusions are present. In culture, infected cells show signs of CPE within 3 days post-infection. PCMV is believed to be species specific, and productive sites of PCMV replication have only been reported in pigs[48,89]. However, one study published by Mueller *et. al.*[49] showed that low-levels of PCMV DNA could be found in numerous baboon tissues following solid organ xenotransplantation. This finding challenges the species-specificity of PCMV and supports the hypothetical xenozoonotic risk associated with this virus.

1.2.2-c Epidemiology and Pathogenesis. PCMV is endemic in pig herds throughout the world with a prevalence of more than 90% in Europe, North America, and Japan[89,94]. The epidemiological and pathogenic features of PCMV are described in Diseases of Swine[89]. Like human CMV, PCMV is easily spread through bodily fluids and the virus is shed in nasal, ocular, and cervical fluids, as well as in urine. The length of virus shedding is short and lasts anywhere between 10 to 30 days. PCMV can also be spread congenitally; however, infection of piglets in commercial herds is normally achieved after birth. Most piglets will be infected within the first month of life and will subsequently shed the virus in nasal secretions during the second month of life.

Because PCMV is commonly spread via nasal droplets, primary infection normally begins with replication in the nasal mucosa and/or the mucosa of the lachrymal glands. Viral dissemination in the blood is cell-associated and follows approximately three weeks after initial acquisition and replication. The secondary sites of replication are associated with viral shedding, and they vary with age. In young pigs, the virus replicates mostly in epithelial cells of the nasal mucosal glands, lachrymal glands, kidney tubules and, in rare cases, the epididymis and mucous glands of the esophagus and duodenal epithelium. On the other

hand, in congenitally infected piglets, the virus replicates mostly in reticuloendothelial cells associated with the capillaries and sinusoids of the lymphoid tissue. Like all herpesviruses, PCMV establishes life-long, latent infections that have the ability to reactivate and result in virus replication and shedding. The virus maintains a latent reservoir in lung macrophages, blood monocytes, and $CD8^+$ T-cells, and reactivation can be stimulated via allogenic stimulation or immune suppression *in vivo*[89,95,96].

PCMV infection is normally subclinical in young pigs but is fatal if the virus is congenitally acquired. If clinical signs of an infection in either young pigs or congenitally infected pigs are noted, they normally present as respiratory signs such as shivering, sneezing, nasal discharge, coughing, neurological disease, poor weight gain or weight loss, and rhinitis. In fact, PCMV infection is commonly referred to as "inclusion body rhinitis" due to the phenotypic nuclear inclusions seen in the mucosal glands of turbinate cells following infection with PCMV. Congenitally infected fetuses are associated with a 100% death rate, and piglets infected in this manner are born shedding the virus, are normally symptomatic, and will continue shedding the virus until death.

1.2.3 HHV-6. First identified in 1986, HHV-6 is a member of the genus *Roseolovirus*[69,97]. The other human member of this genus is HHV-7[69]. It was first identified in 1990 and will not be discussed any further[97].

1.2.3-a Genomic and Structural Properties. The morphological features of HHV-6 are typical for members of the β-herpesvirus subfamily (see section 1.2.1-a)[98,99]. Mature virions range in size from 160-200nm[98,99].

The structure and genomic properties of HHV-6 have been well described in Fields Virology[97,98] and more recently by Bolle *et. al.*[99]. The HHV-6 genome, like most

herpesviruses, is a large, linear, double-stranded DNA molecule that is 160-170 kbp long and carries over 100 open reading frames. The genome has directly repeated termini and a central unique (U) region that is interrupted with three internal repeats. The U region gene organization is similar to the U_L genome organization of human CMV, and the direct terminal repeats are reminiscent of the telomeres of vertebrate chromosomes. HHV-6 exists in nature as two variants – HHV-6A and HHV-6B. The two are closely related with a 90% DNA sequence identity; however, they have very distinct restriction endonuclease profiles and vary in their ability to infect distinct T-cell lines *in vitro*, which allows them to be separately identified.

1.2.3-b Viral Life Cycle. HHV-6A and B strains are believed to use CD46, which is expressed on the surface of all nucleated cells, to gain entry into susceptible cells[98,99]. HHV-6 replication is similar to other β-herpesvirus with a temporally ordered cascade of gene expression and envelope acquisition as discussed in section 1.2.1-b[98,99]. Unlike human CMV, no viral glycoproteins are expressed on the surface of a cell during an infection[98,99,100]. The life cycle is lytic, and mature virions are released upon cell rupture[98,99].

In vivo, HHV-6 variants productively infect many cells such as $CD4^+$ T-cells, B-cells, NKCs, monocytes, macrophages, dendritic cells, tubular epithelial cells, endothelial cells, histocytes, neurons, and oligodendrycytes[97]. The use of CD46 as an entry receptor is believed to be responsible for the viruses' broad *in vivo* cell tropism[98,99]. *In vitro*, both virus strains replicate best in activated $CD4^+$ T-lymphocytes; however, human cell cultures of fibroblast cells, continuous liver cells, epithelial cells, endothelial cells, fetal astrocytes, oligodendrocytes, and microglia have all propagated the virus, albeit at low levels[99,101]. In cultured human T-cells, the complete HHV-6 life cycle takes approximately three days, and

the resulting viral titer is low[98,99]. Scattered foci of CPE are noted around 10 days post-infection and are characterized by a ballooning morphology and, in fetal astrocytes, by giant syncytia[97]. Like human CMV, HHV-6 strains are believed to be species specific, with humans being the only known natural reservoir[99].

1.2.3-c Epidemiology and Pathogenesis. Infection with one or both strains of HHV-6 is high, and over 95% of the adult population will have acquired the virus by the age of two[97,99,102]. Socio-economical standing plays no role in viral prevalence as it is equally ubiquitous in all corners of the globe[97]. Primary acquisition of the virus is highest between the ages of 6 to 15 months, and protective antibody obtained passively from the mother is believed to be the main reason why viral acquisition is delayed[97,99,102]. HHV-6B variant is more prevalent in the population than the A variant, and mostly B variants are isolated within the healthy adult population[99,103-105].

HHV-6 is believed to be mainly transmitted through saliva, and after initial infection, the majority of people will excrete infectious virion in this fluid for approximately one year[97,99,102]. Older children in the household to which an infant is born appear to be the source of transmission of HHV-6 and initial acquisition is normally the B variant[99,106]. Details on HHV-6A infection are unknown, but it is thought to occur after HHV-6B and to be asymptomatic[99]. Congenital transmission of HHV-6 to the fetus is also evident, although the frequency of occurrence is believed to be low[99,102,107,108]. HHV-6 DNA can be found in the cervix of some expectant mothers, but perinatal acquisition of the virus is unlikely[99,104]. Also, hereditary transmission of both HHV-6 variants is possible[109,110,111]. Tanaka-Taya *et. al.*[110] have found HHV-6 DNA integrated into the chromosomal DNA of peripheral blood

cells of healthy and sick individuals and their children, and Ward *et. al.*[111] have shown that the chromosomal integration is not restricted to blood cells.

Besides the saliva, HHV-6 DNA has also been found in blood, cervical fluids, fecal samples, brain tissue, liver tissue, tonsillar tissue, endothelium, and bone marrow[97-,99,104]. Because of this, HHV-6 is thought to disseminate throughout the body via the blood after primary infection; however, details on virus pathogenesis are missing[97,99]. Initial acquisition of the virus will result in a lifelong, latent infection as well as a low-level chronic infection[97,99]. Bone marrow progenitors are believed to harbor latent infections, and the salivary glands and brain tissues are believed to be the source of a persistent infection[97,99,105,109,112,113].

Primary infection of an infant with HHV-6B normally results in a benign exanthem subitum that lasts for about one week – fever, runny nose, and fussiness are frequent symptoms that are followed by a mild skin rash[7,99,102]. Serious complications are rare; nevertheless, central nervous system involvement has been reported as convulsions, meningoencephalitis, and encephalopathy[97,99,108]. Primary infections with HHV-6B are normally symptomatic, whereas primary infections with HHV-6A are almost always subclinical[97,99,105,106]. In healthy individuals, HHV-6B DNA can often be isolated from saliva; however, disease is normally not present[97,99,105]. HHV-6 has been associated with numerous different lymphoproliferative disorders and some central nervous system disorders such as multiple sclerosis; but, no direct link between HHV-6 and these diseases has been established[97,99,105,109,114]. As with other members of the β-herpesvirus family, HHV-6 is an opportunistic pathogen, and reactivation of HHV-6 becomes clinically important in situations

where immune function is compromised (listed in section 1.2.1-c)[97,99]. Because of the focus of this thesis, only HHV-6 infection in transplant recipients will be discussed.

1.2.3-d HHV-6 Infection in Transplant Recipients. Symptomatic HHV-6 infection in immunocompromised allotransplant recipients is believed to be mostly caused by the reactivation of HHV-6B within 20 days following transplantation, that is, earlier than human CMV reactivation[99,115-117]. Forty-eight percent of bone marrow transplant recipients and 32% of vasculated organ transplant recipients will experience some form of disease[99-118]. Direct effects associated with HHV-6 reactivation most commonly include fever, rash, and encephalitis, however, hepatitis, bone marrow suppression, and pneumonitis have also been observed[99,118-120]. Also, primary infection with HHV-6 in an organ recipient leads to increased morbidity and mortality rates[121-123]. The "direct" role HHV-6 plays following transplantation is far from being understood; however, it is thought that the indirect effects of the virus, as discussed below, may have a far greater impact[117-119].

Indirectly, HHV-6 reactivation appears to be important for the development of severe human CMV disease[118,119]. In fact, in a study of 540 liver transplant recipients, Härmä *et. al.*[120] showed that 84% of patients who developed symptomatic human CMV disease had an active HHV-6 infection that, in most cases, appeared before human CMV was noted. Although the interaction between the viruses is not known, high viral loads of human CMV and HHV-6 can be found in the blood of patients with human CMV disease, and research suggests that human CMV alone may not be responsible for the well-documented CMV syndrome[120,124-127]. As with human CMV, increased graft dysfunction and/or rejection is sometimes reported after HHV-6 associated disease is diagnosed[119,127]; however, whether the dysfunction and/or rejection episode is a result of infection in the allograft, or, as stated by

Dockrell and Paya[119], "is an epiphenomenon with viral reactivation being induced by the cellular activation associated with a rejection episode" is still unknown.

2.0 RATIONALE & STATEMENT OF OBJECTIVES

Viruses associated with a xenozoonotic risk include known pathogens in pigs which cause disease but are difficult to eliminate; known pathogens which result in a latent or intracellular persistent state; and known pathogens which may impact on graft viability[4,56]. From the information presented in section 1, PCMV meets these criteria, and, as discussed by both Yoo *et. al.*[57] and Mueller *et. al.*[58], it is clearly considered a xenozoonotic risk.

Given that PCMV is acquired early in life and normally results in inapparent infection, PCMV may be a difficult virus to eliminate from pig herds destined for xenotransplantation. Furthermore, PCMV resides latently for life in macrophages and monocytes of pigs after initial infection. These cells are found in every vascularized tissue and solid organ and hence will be easily transplanted into a naive xenograft recipient[46]. Studies by both Mueller *et. al.*[128] and Clark *et. al.*[129] suggest that PCMV-free pig herds can be generated through early weaning or through hysterotomy delivery with barrier-rearing, respectively. However, Tucker *et. al.*[50] has shown that PCMV DNA can be identified in hysterotomy delivered, specific-pathogen-free transgenic pig herds destined for xenotransplantation, even when serum from these same herds suggests there is no infection. Furthermore, no current human CMV antiviral therapy appears to be entirely successful at eliminating/controlling PCMV infection in both *in vivo*[130] and *in vitro*[131] studies.

The probability that latent PCMV would reactivate in an immunocompromised environment is high. In support of this probability, Mueller *et.al.*[96] have shown that PCMV will reactivate in healthy pigs put under an immunosuppressive regime similar to that used during pig-to-baboon xenotranplantation[96]. Given that xenograft recipients may be subject to immune suppression regimes that are more severe than the current treatments used following

allotransplantation and that PCMV can be easily transplanted with a vascularized organ, conditions for the xenozoonotic reactivation of PCMV in a human recipient are favourable. In fact, as first noted in section 1.2.2-b, a few studies by Mueller *et. al.*[49,128,130] have found PCMV DNA in baboon tissues following pig-to-primate xenotransplantation – the accepted human model for human xenotransplantation.

PCMV shares many biological properties with human CMV, and it is not known whether PCMV will behave similarly to human CMV if transferred into the human host along with a xenograft. Previous research supports a comparable behavior. Guedes *et. al.*[95] have shown that, like human CMV, PCMV will reactivate and replicate from peripheral blood mononuclear cells after allogenic stimulation *in vitro*, and Mueller *et. al.*[49,96,130] have reported a consistent increase in PCMV DNA copy number in the xenograft, as well as in recipient tissue, following pig-to-baboon xenotransplantation. Moreover, both Mueller *et. al.*[128] and Gollackner *et. al.*[132] have suggested that PCMV, like human CMV, plays an indirect role in the intravascular coagulation associated with xenograft rejection. Furthermore, Mueller *et. al.*[49] have shown that xenograft necrosis in the recipient is associated with PCMV reactivation, similar to the human CMV-linked allograft damage.

PCMV is an interesting virus. What little is known indicates that it shares biological properties with human CMV, yet its partially sequenced genome places it phylogenetically closer to HHV-6 and HHV-7, and a member of the *Betaherpesvirinae* genus *Roseolovirus*[57,70,71,89-93]. More research needs to be carried out to understand the full infectious potential of PCMV, in both pigs and humans[57]. Accordingly, this project was conceived in order to try to fill knowledge gaps concerning PCMV in the context of xenotransplantation. Its **overall goals** were to explore the interactions of PCMV with human

cells *in vitro*. The work presented in this thesis represents the initial research completed in realizing this goal.

Since laboratory-adapted human CMV preferentially replicates in cultured fibroblasts[70], it was hypothesized that PCMV also had the ability to infect these same cells *in vitro*. Accordingly, the **specific goals** of this Master's research were to investigate the interaction of PCMV with human fibroblasts *in vitro,* and then evaluate to what extent those cells were permissive for PCMV. The specific **project objectives** were defined as follows:

1. Determine if PCMV can infect human fibroblasts by visualizing any potential CPE generated after infection with PCMV.
2. Verify PCMV infection of human fibroblasts through identification of viral RNA in infected cells.
3. Confirm PCMV infection of human fibroblasts via detection of virus-specific proteins in infected cells.
4. Determine if antibodies specific for human CMV, HHV-6, and PCMV can neutralize PCMV infection of human fibroblasts.

3.0 MATERIALS AND METHODS

3.1 CELLS AND CELL CULTURE. The following three cell types were used to complete the objectives outlined in section 2: porcine turbinate cells, porcine fibroblasts, and human fibroblasts. All cells were cultured at 37°C in 5% carbon dioxide (CO_2) and 95% humidity. Subcultivation of all cell types was done with 0.5 % (v/v) trypsin – 0.2% (v/v) ethylenediaminetetraacetic acid (EDTA) [Invitrogen/Gibco #15400-054, Burlington, ON] with a 1:4 subcultivation ratio.

Primary porcine turbinate cells persistently infected with PCMV [American Type Culture Collection (ATCC) #VR-1499, Manassas, VA] were cultured in Dulbecco's Modified Eagle's Medium (DMEM) [ATCC #30-2002] supplemented with 10% (v/v) fetal bovine serum (FBS) [Invitrogen/Gibco #116000-044], 1% (v/v) of 200 mM L-glutamine [Invitrogen/Gibco #25030-081], and 0% antimicrobial agent. This is subsequently referred to as 10:1:0 DMEM. Cells were received at passage 17, stored in liquid nitrogen, and used between passage numbers 20 and 24 for all experiments. In 75 cm^2 (60 mL max.) rectangular canted neck culture flasks with vented caps (T75) [Fisher Scientific #10-126-37, Ottawa, ON], confluent monolayers represented approximately 5.0 x 10^6 cells per flask.

A **porcine nasal fibroblast cell line** (PT-K75) [ATCC #CRL-2528] was cultured in Basal Medium Eagle (BME) [Invitrogen/Gibco #21010-046] supplemented with 5% (v/v) FBS, 1% (v/v) of 200 mM L-glutamine, and 0% antimicrobial agent. This is subsequently referred to as 5:1:0 BME. Cells were received at passage number 19 but were re-designated passage number 1. They were stored in liquid nitrogen and used between passage numbers 3 and 6. In T75 flasks, confluent monolayers represented approximately 2.0 x 10^6 cells per flask.

Primary human foreskin fibroblasts (HFF) obtained from a frozen bank of low passage cells available in our laboratory[133] were cultured in 5:1:0 BME. The cells were stored in liquid nitrogen at low passage numbers and used between passage numbers 9 and 12 for all experiments. In T75 flasks, confluent monolayers represented approximately 2.0 x 10^6 cells per flask. In 24-well (3.4 mL max.) flat bottom tissue culture plates with condensation rings [Fisher #003526], confluent monolayers represented approximately 1.0 x 10^5 cells per well.

3.2 VIROLOGY.

3.2.1 PCMV Propagation and Generation of Heat-Killed Controls. PCMV stocks were generated by the co-cultivation of persistently infected porcine turbinate cells with PT-K75 cells. In T75 flasks, 1.5 x 10^6 cells of each cell type were seeded and co-cultured in 20mL of 10:1:0 DMEM. On days 4, 6, and 9 post co-cultivation, the supernatant containing PCMV was removed to ice and spun at 10,624 g for 10 minutes to remove cellular debris. This supernatant was subsequently aliquoted and stored at -85°C. Because PCMV generated no lytic CPE in porcine cells, a viral titer could not be determined. However, infectious PCMV was used exactly the same way for every experiment – by making a viral stock from equal volumes of supernatant from all three days post co-culture. Heat-killed virus was generated by incubating the viral stock in a dry oven at 95°C for 15 to 30 minutes.

3.2.2 PCMV Infection of PT-K75 Cells and HFF. Live PCMV-infected cells or heat-killed PCMV-infected control cells were generated in order to evaluate cell permissiveness for PCMV. A general infection protocol for both pig and human cells is described here, and specific details can be found in subsequent paragraphs. All live and heat-killed PCMV stock dilutions were done in 10:1:0 DMEM and kept on ice, as was the

medium intended for use in the mock infection. Cells to be infected were grown to a confluent monolayer in either T75 flasks or 24-well plates. On the day of infection, spent medium was removed and discarded, and the monolayer was washed with 10:1:0 DMEM. The cells were subsequently inoculated with the appropriate dilution of infectious PCMV, heat-killed PCMV, or 10:1:0 DMEM (medium alone, mock infection). The culture vessel was gently tilted a few times to ensure that the viral inoculum completely covered the monolayer. The monolayer was incubated for 90 minutes in normal culture conditions, and the culture vessel was rocked several times throughout the incubation period. After the incubation period, the inoculum was removed from the monolayer and discarded. The monolayer was then washed with 5:1:0 BME and treated as specified.

For PCMV infections in **T75 flasks**, HFF or PT-K75 cells were seeded at 4.0×10^5 cells/T75, grown to confluency, and then inoculated with either live PCMV, heat-killed PCMV, or medium alone (mock-infected control) by the method described above. All wash volumes were 10 mL, and 5.9 mL of the appropriate control or infectious PCMV dilution was used to inoculate the monolayers.

For PCMV infections in **24-well plates**, HFF were seeded at 1.0×10^4 cells/well, grown to confluency, and then inoculated with either live PCMV, heat-killed PCMV, or 10:1:0 DMEM alone (mock-infected control) by the method described above. All wash volumes were 1 mL, and 150 µL of infectious PCMV or the appropriate control was used to treat the monolayers.

3.3 DOCUMENTATION OF CPE. CPE generated in HFF by infection with PCMV was documented via light microscopy and neutral red staining. Confluent monolayers of HFF were infected in 24-well plates as described in section 3.2.2. After infection,

monolayers were immediately covered with 1 mL of 0.35% agar [Sigma-Aldrich #A9915, Oakville, ON] supplemented with 5:1:0 BME and then maintained in culture for up to 30 days. In order to make 0.35% agar in 5:1:0 BME, BME powder [Sigma-Aldrich #B-9638] was first made to a 2X concentration, sterile filtered [Millipore #SCGPU05RE, Mississauga, ON], prepared as 10:2:0 BME, and then mixed in equal volumes with sterile 0.7% agar. Once CPE was well-advanced, the agar-covered monolayer was stained with 0.5 mL of a sterile 0.01% (w/v) neutral red solution [Sigma-Aldrich #N-4638] overnight in normal culture conditions. CPE was subsequently visualized via light microscopy at different magnifications.

3.3.1 Time Course Studies. To visualize the progression of CPE over a one-month period, HFF monolayers infected with either live PCMV, heat-killed PCMV (negative CPE control), or medium alone (mock infected, negative CPE control) were stained with neutral red and photographed at a 10X magnification approximately every four days. An inverted microscope [Leica model #DMIRB, Richmond Hill, ON] equipped with phase contrast capabilities, a digital camera [Leica model #DCF320] attachment, and Leica IM software was used to acquire images of the stained monolayers. All assays were carried out in triplicate.

3.3.2 Antibody Neutralization Studies. To test for antibody neutralizing capacity, the CPE generated in an HFF monolayer that had been inoculated with PCMV, which had been pre-incubated with a test neutralizing antibody, was compared with the CPE generated by live PCMV alone (maximum CPE control) and the lack of CPE generated by 10:1:0 DMEM alone (no CPE control). Also, HFF monolayers inoculated with live PCMV pre-mixed with an irrelevant antibody was also included in each experiment as an added control.

Specifically, the following mouse monoclonal antibodies (mAbs) and pig serum were tested for any neutralizing capacity: (1) a mouse **monoclonal antibody** (mAb) specific for **human CMV gB [CMVB1**, IgG2a, in-house product][134]; (2) a mouse **mAb** specific for a **HHV-6 membrane glycoprotein** that is 60 kDa or 110 kDa in size [ViroStat #2002, clone #2002, IgG2, Portland, ME]; and (3) **pig serum** presumed to contain antibodies specific for PCMV.

The pig serum was obtained from the blood of two pigs at a local abattoir [Tom Henderson's Custom Meat Cutting, Chesterville, ON]. Specifically, blood was obtained via a neck cut using non-sterile conditions into Vacutainer SST tubes [BD Biosciences #366590, Mississauga, ON]. Blood was allowed to clot for one hour at room temperature, and subsequently spun for 10 minutes at 1200 g to pellet the erythrocyte clot. Separated serum was aliquoted and frozen at -20°C.

To make the neutralizing or irrelevant **mAb** preparations used during inoculation, mAb samples were diluted to various concentrations in DMEM with 10% (v/v) guinea pig serum as a source of complement [Sigma-Aldrich #S4639]. They were then mixed with an equal volume of live PCMV diluted 1:5 in 20:2:0 DMEM with 10% (v/v) guinea pig serum, and the final mAb preparations were a 1:10 dilution of live PCMV mixed with various amounts of the desired mAb in 10:1:0 DMEM with 10 % (v/v) guinea pig serum. Neutralizing or irrelevant **serum** solutions were prepared in a similar manner, except no additional complement in the form of guinea pig serum was added. The final serum preparations were a 1:10 dilution of live PCMV mixed with various amounts of the desired serum in 10:1:0 DMEM. The different mAb and serum concentrations tested for neutralizing capacity are listed in the following paragraph.

For **CMVB1 neutralizing experiments,** the antibody was tested for neutralizing

capacity at 1:10 (200 μg of protein), 1:1,000 (2 μg of protein), 1:10,000, and 1:100,000 dilutions. The irrelevant control was a mAb of the same isotype but specific for Zeta-chain-associated protein kinase 70 (ZAP-70) [Upstate Biologicals #05-253, clone #2F32, IgG2a, Lake Placid, NY]. It was used at a protein concentration of 200 μg and 2 μg. For **HHV-6 mAb neutralizing experiments,** the antibody was tested for neutralizing capacity at 1:10 (10 μg of protein) and 1:1,000 (0.1 μg of protein) dilutions. The irrelevant control was a mAb of the same isotype but specific for ZAP-70 [BD Biosciences #610239, clone #29, IgG2a]. It was used at a protein concentration of 10 μg and 0.1 μg. For **pig serum neutralizing experiments,** the serum was tested for neutralizing capacity at 1:10, 1:1,000, 1:10,000, and 1:100,000 dilutions. The irrelevant control serum was normal pooled rabbit serum [aliquoted and stored at -20°C, obtained from Animal Resources Division, Food Directorate, Health Canada] used at the same dilutions as the pig serum.

All final neutralizing preparations described above, as well as all controls, were then pre-incubated for one hour at 37°C in 0% CO_2 and 0% humidity on a shaking platform. The preparations were then used as infectious or control material to inoculate separate HFF monolayers according to the methods described in section 3.3. To visualize CPE, all monolayers were stained with neutral red and photographed at a 10X magnification on days 16 and 30 post-infection. All assays were carried out in triplicate.

3.4 RT-PCR AND SEQUENCING. PCMV-specific nucleic acid generated in HFF and in PT-K75 cells after infection was detected via nested RT-PCR. Confluent monolayers of either cell type were infected in T75 flasks as described in section 3.2.2. After the infection procedure, they were immediately covered with 15 mL of 2:1:0 BME and then maintained in culture for up to 30 days. Spent medium was removed and replaced every six days. The

monolayer of one T75 flask was collected via trypsinization and cell pelleting approximately every 3 days. The pellets were stored at -85°C. Total RNA was extracted from the frozen cell pellets [Qiagen #74104, Mississauga, ON], treated with DNase I [Qiagen #79254] to ensure no DNA remained in the samples of RNA, and subsequently aliquoted and stored at -85°C. RNA concentration was determined as an average of three individual absorbancies at 260nm.

All nested RT-PCR reactions were carried out in an automated thermocycler [Eppendorf Canada, Mastercycler, Mississauga, ON]. PCMV-specific RNA was reverse transcribed [Sigma-Aldrich #HSRT-20] into cDNA with an antisense primer, PCMV POL R1 (5' CCCTGAAAATCACCGTCTGAGAGA '3), specific for PCMV DNA polymerase[135]. In this reaction, 5 μg of RNA was used along with 0.4 μM of primer and 250 μM of all four deoxyribonucleotide triphosphates (dNTPs). The final reaction volume was 20 μL. Amplification of PCMV-specific cDNA was achieved with primers specific for PCMV DNA polymerase in a nested PCR reaction [Sigma-Aldrich #HSRT-20]. For both the first and nested PCR reactions, 0.4 μM of each primer, 200 μM each of all four dNTPs, and 5 μL of target DNA in PCR buffer containing 5 mM of magnesium chloride to a volume of 50 μL was used. All primers were generated by Invitrogen Canada. The first round primers, PCMV POL F1 (5'AAGCAGCAGCTTGCCCTCAAGGTG '3) and PCMV POL R1, amplified a product of 212 base pairs (bp)[135]. PCR cycle conditions were as follows: initial denaturation for 6 minutes at 95°C, followed by 40 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and extension for 30 seconds at 72°C. The final extension was for 10 minutes at 72°C. The nested primers, PCMV POL F2 (5' ACGTGCAATGCGTTTTACGGCTTC '3) and PCMV POL R2 (5'

ACTTCTCTGACACGTATTCTCTAG '3), amplified a product of 160 bp[135]. PCR cycle conditions were as follows: initial denaturation for 6 minutes at 95°C, followed by 40 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 58°C, and extension for 30 seconds at 72°C. The final extension was for 10 minutes at 72°C. Base pair markers [Invitrogen #15628-050] and the nested round PCR product (160 bp) were electrophoresed on a 3% agarose gel and stained with ethidium bromide. Reaction controls included nucleic acid extracted from PCMV-positive porcine turbinate cells as a positive control, and reactions containing no nucleic acid as internal experimental blanks. To ensure that the amplified products were PCMV DNA polymerase, they were sequenced by Michèle Lemieux in Dr. Bill Casley's laboratory at the Center for Biologics Research within Heath Canada. Before sequencing, the products were purified with either a Montage PCR Centrifugal Filter Device [Millipore #UFC7PCR50] or by agarose gel extraction [Invitrogen #K2100-12]. The reactions were carried out on an Applied Biosystems 3100 Genetic Analyzer instrument [Applied Biosystems, CA] equipped with ABI Prism 3100 Data Collection software version 1.1, at a concentration of 90 ng/μL. Sequences were edited with the Sequencher, Version 4.2 [Genes Codes Corporation, MI] software. The NCBI MegaBLAST program was used to identify the compiled sequences.

3.5 IMMUNOASSAYS.

3.5.1 IFAs.

Commercially available antibodies against PCMV proteins are not available. To determine whether available antibodies specific for human CMV proteins would cross-react with PCMV proteins, an immunofluorescent assay (IFA) was done. PCMV-positive porcine turbinate cells were grown to confluency on glass cover slips [Fisher Scientific #12-545-80] in 24-well plates. Once confluent, cells were washed 5 times for 3

minutes with 500 μL of room temperature phosphate buffered saline (PBS) [137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2] and then fixed and permeabilized with -20°C 80% (v/v) acetone for 8 minutes. Cells were subsequently washed in PBS again, and the cover slips were removed to Kim wipes and allowed to dry. Fixed and permeabilized cells were stored at -20°C for future immunoflurorescent staining.

The general immunofluorescent staining protocol is described here. Fixed and permeabilized cells were removed from storage and allowed to warm to room temperature in PBS. Non-specific epitope recognition was prevented by incubation with 200 μL of blocker (specified below) for 30 minutes at 37°C. Primary antibody diluted in blocker was then incubated with the cells for 1.5 hours at 37°C on a shaking platform. Next, the cells were detected with fluorescein-conjugated secondary antibodies that were allowed to incubate for 1 hour at 37°C on a shaking platform. All blots were washed 3 times for 5 minutes with rigorous shaking following primary and secondary antibody application with 0.5 mL of PBS for each wash. Cover slips were mounted cell-side down on glass plates with 90% (v/v) glycerol in PBS. Two different sources of primary antibody were tested: (1) a mouse **mAb** specific for **human CMV gB (CMVB1)** described in section 3.3.2, and (2) a rabbit **polyclonal antibody** specific for human CMV tegument, capsid, and membrane proteins [Chemicon #AB1131, Temecula, CA].

PCMV-positive porcine turbinate cells tested for cross-reactivity with **CMVB1** were blocked with 3% bovine serum albumin (BSA) [Sigma-Aldrich #A7030], and the primary antibody was used at a concentration of 1:25 diluted in 2% BSA. A fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody [Jackson ImmunoResearch Laboratories #115-095-164, West Grove, PA] diluted in 3% BSA with 0.008% Evan's Blue

(w/v) [Fisher Scientific #E515] as a counter stain. Mounted cover slips were examined by ultraviolet illumination with an excitation/emission filter that allowed visualization of the fluorescein (490 nm/560 nm) and Evan's Blue (550 nm/610 nm) signals simultaneously. Photographs were taken at different magnifications with Kodak Max 400 film. Exposure times ranged from 10 seconds to 30 seconds. PCMV-positive porcine turbinate cells tested for cross-reactivity with the **polyclonal antibody** were blocked with 10% normal goat serum (NGS) [Jackson ImmunoResearch Laboratories #005-000-121] and the antibody was used at a concentration of 1:400 diluted in 10% NGS. A fluorescein-conjugated goat anti-rabbit IgG secondary antibody [Jackson ImmunoResearch Laboratories #111-095-046] diluted in 11% NGS with 0.008% Evan's Blue (w/v) as a counter stain. Mounted cover slips were examined by ultraviolet illumination as described above. Photographs were taken at different magnifications with Kodak max 400 film. Exposure times ranged from 1 minute to 4 minutes.

3.5.2 Western Blots. PCMV-specific proteins expressed in HFF were detected via Western blot. Cells were grown to a confluent monolayer in T75 flasks and infected with either live PCMV or heat-killed PCMV by the method described in section 3.2.2. To identify viral specific proteins, monolayers were immediately covered with 15 mL of 2:1:0 BME after control or PCMV incubation and maintained in culture for up to 31 days. Spent medium was removed and replaced every six days. On various days post-infection, monolayers were collected via scraping into cold PBS with 2 mM EDTA (pH 8.0). Cells were subsequently pelleted and washed 3 times via re-suspension and pelleting with cold PBS containing 2 mM EDTA (pH 8.0) and stored at -85°C. Whole-cell lysates were generated from the frozen cell pellets via sonication in a buffer containing 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 1%

TritionX-100, 1% sodium dodecyl sulfate (SDS), 1 mM EDTA (pH 8.0), and 1X protease inhibitor cocktail mix [Roche #11-697-498-001, Mississauga, ON]. The lysate was centrifuged for 10 minutes at 10,624 g to remove cellular debris, and total protein concentration was determined via the bicinchonic acid (BCA) method [Pierce #23227, Rockford, IL]. Lysates were stored at -20°C.

After cellular lysates were generated, proteins were separated via SDS-polyacrylamide gel electrophoresis and detected in a Western blot. The general protocol is described here. Samples were mixed 2:1 in a sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 0.1 M dithiothreitol (DTT), 2% (w/v) SDS, 0.025% (w/v) bromophenol blue, and 20% (v/v) glycerol, boiled for 5 minutes at 100°C, and then electrophoreded in a SDS-polyacrylamide gel (10%) [BioRad #161-0158, Mississauga, ON] at 200V for approximately 45 minutes with the Mini-PROTEAN III system [BioRad]. The separated proteins were subsequently transferred to polyvinylidene fluoride (PVDF) membrane [BioRad #162-0177] at 100V for 90 minutes at 4°C with the Mini Trans-Blot Cell [BioRad]. The PVDF blotting membrane was blocked to reduce non-specific binding for 1 hour at room temperature and then incubated overnight in primary antibody diluted in blocker (specified below). The blots were subsequently stained with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in blocker for 1 hour at room temperature and then treated with a chemiluminescent substrate. Bands were detected via exposure to film for various amounts of time. All blots were washed 3 times for 10 minutes each with rigorous shaking following primary and secondary antibody application. The wash buffer was 36 mL of tris buffered saline (TBS) [10 mM Tris, 150 mM NaCl, pH 8.0] with 0.05% (v/v) Tween-20.

Three different sources of primary antibody were used to detect PCMV-specific proteins in the cellular lysates. They included: (1) a mouse **mAb pool** of many antibodies specific for **human CMV gB** as follows: Virusys Corporation #P1201 and #P1206 [Sykesville, MD], Applied Biosystems #13-127-1000 [Foster City, CA], Austral Biologicals #CMM-1401-5 [San Ramon, CA], ViroGen Corporation #024-A [Watertown, MA], Accurate Chemical and Scientific Corporation #YVS0811 and #YVS0826 [Westbury, NY], and CMVB1 (in-house mAb, described in section 3.3.2). Ten mL of a 1:100 stock mAb pool diluted in TBS with 0.04% sodium azide as a preservative was made, and the mAbs were used at a ratio of 1:1:1:1:1:1:1:3, respectively; (2) an **anti-HHV-6 membrane glycoprotein mouse mAb**; and (3) **pig serum** presumed to be sero-positive for PCMV antibodies. These latter two reagents have been previously described in section 3.3.2.

Membranes probed with the **human CMV gB specific mAb pool** were blocked with 10X SuperBlock [Pierce #37536], and the 1:100 stock antibody pool was further diluted and used at a final concentration of 1:500 in 10X SuperBlock. An HRP-conjugated goat anti-mouse IgG secondary antibody [Pierce #1858413] was used at a concentration of 1:2,000 diluted in 10X SuperBlock, and SuperSignal West Pico [Pierce #34078] was used as the chemiluminescent substrate. Membranes were exposed to film [Kodak #178-8207, Toronto, ON] for 10 – 20 minutes. Recombinant human CMV gB (140 kDa) derived from CHO cells [Austral Biologicals, discontinued] was included as an extra control in these experiments in order to ensure that the mAb pool would detect human CMV gB. Membranes probed with the **HHV-6 specific mAb** were blocked with 1X Western Blocking Reagent [Roche #11-921-673-001], and the antibody was used at a concentration of 1:100 diluted in 1X Western Blocking Reagent. An HRP-conjugated goat anti-mouse IgG secondary antibody was used at

a concentration of 1:10,000 diluted in 0.5X Western Blocking Reagent, and SuperSignal West Femto [Pierce #34096] was used as the chemiluminescent substrate. Membranes were exposed to film for 0.5 – 6 minutes. Cellular lysate from human CMV-infected normal human dermal fibroblasts [EastCoast Bio #CV001, North Berwick, ME] was included as an extra control in these experiments in order to ensure that the mAb was truly specific for HHV-6. Membranes probed with the **pig serum** were blocked with 1X Western Blocking Reagent, and the antibody was used at a concentration of 1:7500 diluted in 1X Western Blocking Reagent. An HRP-conjugated goat anti-swine IgG secondary antibody [Jackson ImmunoResearch Laboratories #114-035-003] was used at a concentration of 1:20,000 diluted in 0.5X Western Blocking Reagent, and SuperSignal West Pico was used as the chemiluminescent substrate. Membranes were exposed to film for 2 – 10 minutes. For a loading control in some Western blots, the PVDF membrane was stripped of all antibodies and blockers in TBS containing 100 mM of β-mercaptoethanol and 2% (w/v) SDS. The membrane was re-blocked with the appropriate blocker (described above, depended on the primary antibody) and then re-probed with an HRP-conjugated mAb specific for β-actin [Sigma-Aldrich #A3854] at a concentration of 1:50,000 diluted in blocker. SuperSignal West Pico was used as the chemiluminescent substrate, and bands were detected following exposure to film for 0.5 – 5 minutes.

4.0 RESULTS

The major goal of this research was to determine whether PCMV had the ability to infect human fibroblasts *in vitro* and subsequently to evaluate how permissive these human cells were for PCMV. The first step taken was to visually assess if PCMV was able to infect HFF.

4.1 CPE TIME COURSE STUDIES. PCMV generates no lytic CPE in porcine cells. In order to determine if PCMV was able to infect human cells and whether the infection generated CPE, studies to visualize any PCMV-generated CPE in human cells were initiated. Briefly, HFF were seeded in 24-well plates, grown to confluent monolayers, and then inoculated with either live PCMV, heat-killed PCMV, or medium alone (mock infection control). Directly following infection, the cells were covered with agar and then maintained in culture for 30 days. On specific days post-infection, cells were stained with neutral red overnight, and CPE was visualized via light microscopy at different magnifications. As shown in Figure 1, row E, CPE was first noted on day 5 post-infection when the PCMV viral stock was used at a 1:10 dilution. When compared with experimental controls (Figure 1, rows A, B, and C), CPE was characterized by a less confluent monolayer and a rounding/enlargement of some cells. By day 7 post-infection, CPE was well-progressed and included large areas of cell death throughout the monolayer. Typical of an *in vitro* PCMV infection of porcine cells, a few giant cells (Figure 1, row F, day 7, arrow) were seen, as were nuclear inclusions in some cells[89] (Figure 1, row F, day 12, arrows). Total destruction of the monolayer was noted approximately one month post-infection (data not shown).

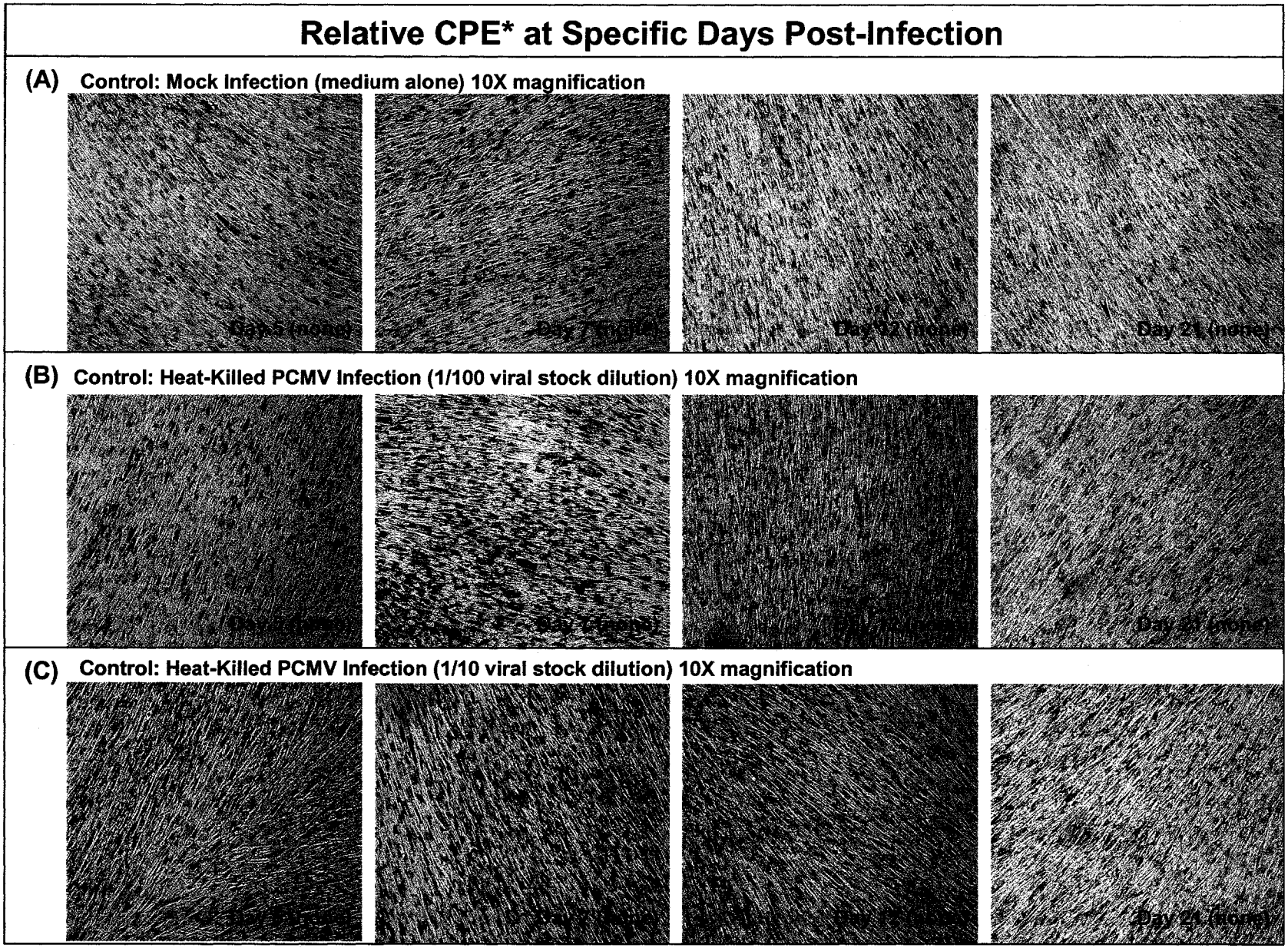
Figure 1 also shows that PCMV-generated CPE was directly related to viral concentration. When compared with the CPE generated by a 1:10 dilution of live PCMV

Figure 1: Progression of PCMV-Generated CPE in HFF. These results are representative of two individual experiments, each done in triplicate. Briefly, HFF were seeded in 24-well plates, grown to a confluent monolayer, and subsequently infected with either **(A)** medium alone (mock infection control), **(B)** heat-killed PCMV at 1/100 dilution of stock, **(C)** heat-killed PCMV at 1/10 dilution of stock, **(D)** live PCMV at 1/100 dilution of stock, or **(E)** / **(F)** live PCMV at 1/10 dilution of stock. Directly following infection, the cells were covered with agar and then maintained in culture for 30 days. On specific days post-infection, cells were stained overnight with neutral red, and CPE was subsequently visualized via light microscopy at different magnifications, as indicated. Arrows are indicating either giant cells (Row F, day 7), or cells with nuclear inclusions (Row F, day 12).

*CPE noted is relative to experimental controls.

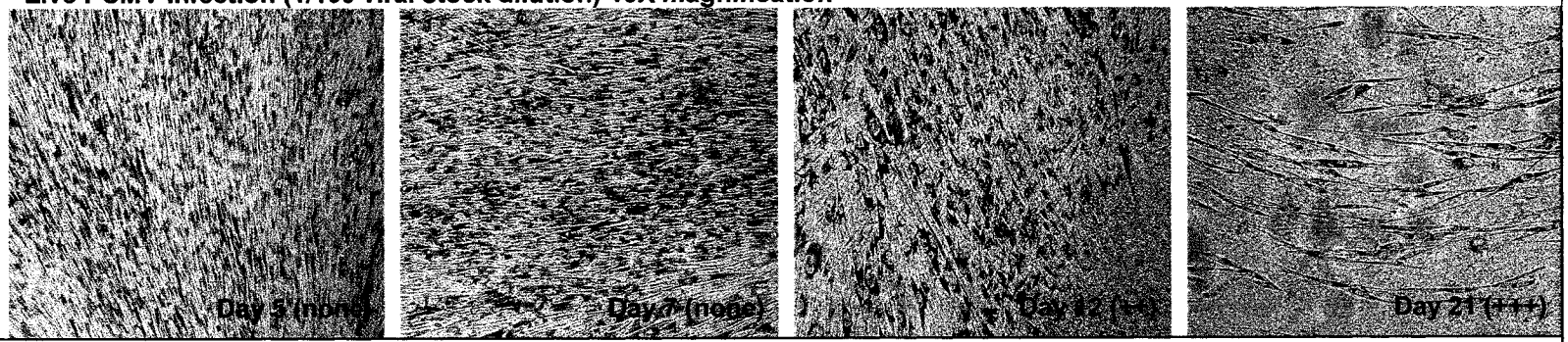
Figure legend:

+	cells becoming larger and more rounded, monolayer slightly less confluent
++	plaque-like areas of cell death in monolayer, remaining cells much less confluent
+++	more than 50% of monolayer dead, very few remaining cells
++++	entire monolayer dead
none	no CPE noted

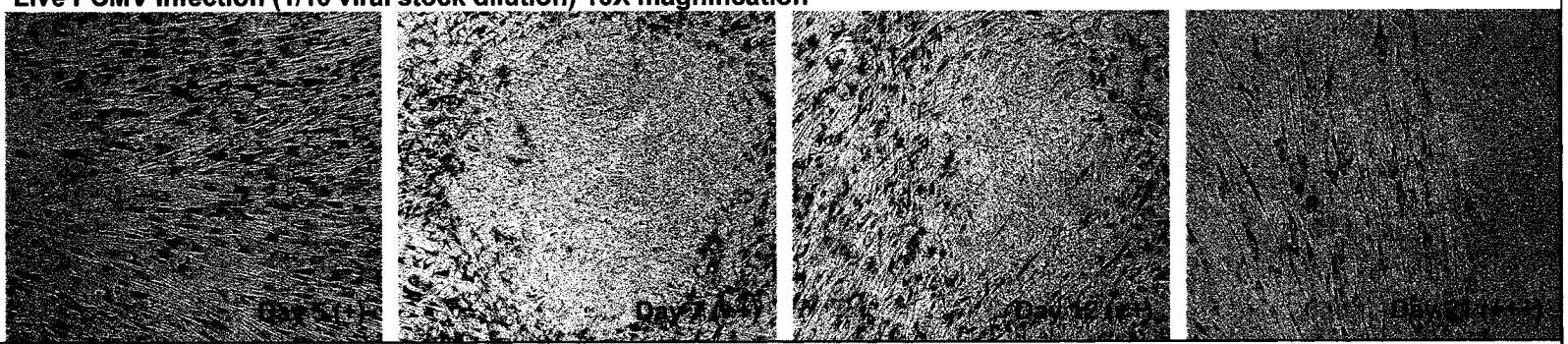

Relative CPE* at Specific Days Post-Infection
(A) Control: Mock Infection (medium alone) 10X magnification
Day 5 (none)
Day 7 (none)
Day 12 (none)
Day 21 (none)
(B) Control: Heat-Killed PCMV Infection (1/100 viral stock dilution) 10X magnification
Day 21 (none)
(C) Control: Heat-Killed PCMV Infection (1/10 viral stock dilution) 10X magnification
Day 21 (none)

Relative CPE* at Specific Days Post-Infection

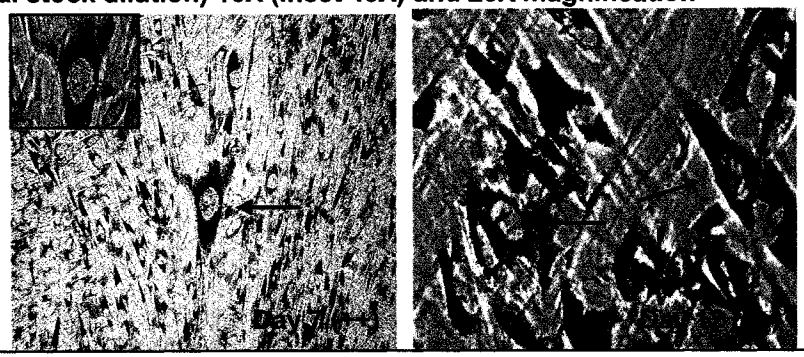
(D) Live PCMV Infection (1/100 viral stock dilution) 10X magnification



(E) Live PCMV Infection (1/10 viral stock dilution) 10X magnification



(F) Live PCMV Infection (1/10 viral stock dilution) 10X (inset 40X) and 20X magnification



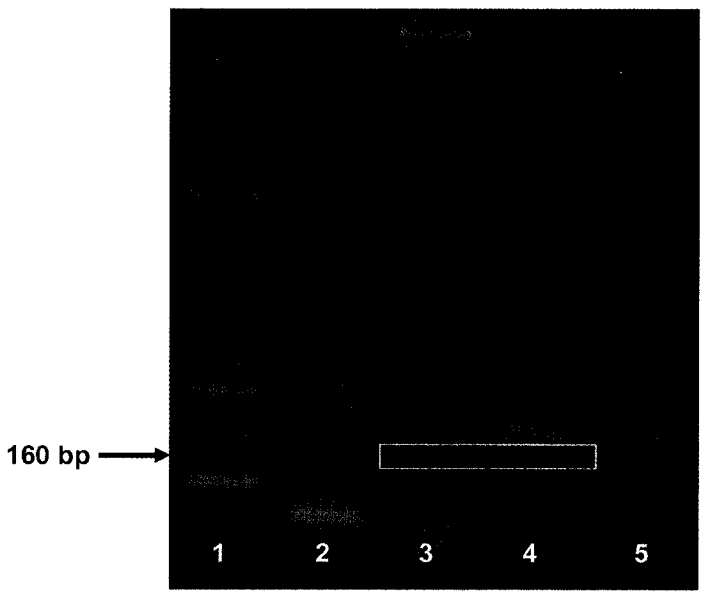
(Figure 1, row E), the development of CPE was delayed when the viral stock was used at a 1:100 dilution (Figure 1, row D). The shading differences seen in background colour (Figure 1) are the result of a learning curve in using the microscope. Improper white balancing with a new photography system were the cause of the blue tones; however, this background colour is unrelated to the CPE. Overall, these studies suggested that PCMV infection of human cells *in vitro* resulted in significant CPE, which was directly related to viral concentration.

4.2 RT-PCR. The studies described above indicated that HFF were susceptible for PCMV. In other words, they indicated that PCMV had, at minimum, entered the cells. Furthermore, the large plaque-like formation suggested that PCMV had replicated and spread within the monolayer thus indicating that HFF were not only susceptible, but also permissive, for PCMV. In order to assess whether HFF were permissive for PCMV, experiments to identify viral transcripts were initiated. First, RT-PCR experiments were performed using RNA extracted from a pig nasal fibroblasts cell line (known as PT-K75 cells) experimentally infected with PCMV. PT-K75 cells are identified to be permissive for PCMV, and the experiments were completed in order to ensure that the infection process was valid. Briefly, PT-K75 cells were seeded in T75 flasks, grown to confluent monolayers, inoculated with either live PCMV or medium alone (mock infection), covered with 2:1:0 BME, and maintained in culture. Total RNA was extracted from one T75 flask, and nested RT-PCR reactions were performed with primers specific for PCMV DNA polymerase. The resulting product was electrophoresed on an agarose gel and stained with ethidium bromide. A 212 bp product was expected after one round of PCR, and a 160 bp product was expected after the nested round of PCR[135]. As shown in Figure 2, lane 3, the studies illustrated that PCMV-specific RNA was detected in PT-K75 cells after the inoculation process and that the

Figure 2: Detection of PCMV RNA in Infected PT-K75 Cells. These results are representative of numerous infections and many RT-PCR experiments. Briefly, PT-K75 cells were seeded in T75 flasks, grown to confluent monolayers, infected with either live PCMV or medium alone (mock infection), covered with 2:1:0 BME, and maintained in culture. Total RNA was extracted from one T75 flask, and nested RT-PCR reactions were performed with primers specific for PCMV DNA polymerase. The resulting product was electrophoresed in a 3% agarose gel and stained with ethidium bromide. The **red** arrow indicates the nested round product of the PCR reaction expected at 160 bp[135], and the yellow box indicates products sequenced to be PCMV DNA polymerase. Here, RNA was extracted on day 12 post-infection.

Figure Legend:

Lane 1 base-pair ladder
Lane 2 **(-) control:** RT-PCR product amplified with RNA extracted from mock-infected (placebo) PT-K75 cells
Lane 3 RT-PCR product amplified with RNA extracted from PT-K75 cells infected with live PCMV
Lane 4 **(+) control:** RT-PCR products amplified with RNA extracted from PCMV-positive porcine turbinate cells
Lane 5 **(-) control**: internal experimental blank (no RNA)


160 bp
1
2
3
4
5

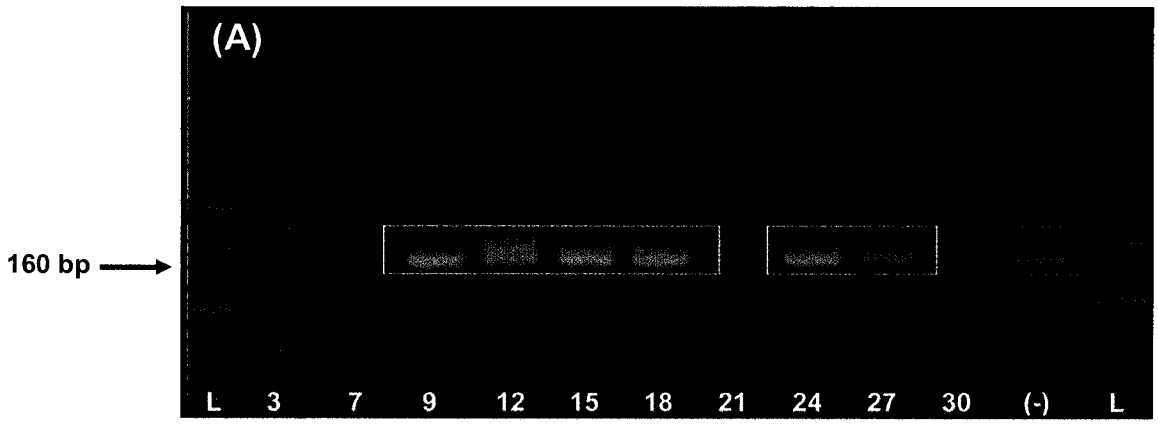
inoculation process was successful and valid. Moreover, the identical nested PCR product was also identified in PCMV-positive porcine turbinate cells (positive control) (Figure 2, lane 4) and was not present in mock-infected (negative) control cells (Figure 2, lane 2). Two bands are noted in the PCMV-positive porcine turbinate control nested round PCR product (Figure 2, lane 4). One was the 160 bp nested round PCR product, and the other was assumed to be the 212 bp first round product. These two bands were present in most positive control reactions. The nested round PCR products amplified with RNA extracted from PT-K75 cells infected with PCMV, as well as the product amplified with RNA extracted from PCMV-positive porcine cells (Figure 2, yellow box), were confirmed to be PCMV DNA polymerase by sequencing and NCBI MegaBLAST searching (Appendix I, Figure 11).

Once the infection process was shown to be valid in permissive cells, identical RT-PCR reactions were performed with RNA extracted from HFF infected with live PCMV. Again, HFF were seeded in T75 flasks, grown to a confluent monolayer, inoculated with either live PCMV or heat-killed PCMV and maintained in culture exactly as the PT-K75 cells were. Total RNA was extracted approximately every 3 days over a one-month period, and nested RT-PCR reactions were performed with primers specific for PCMV DNA polymerase[135]. The resulting products were electrophoresed in an agarose gel and stained with ethidium bromide. Figure 3 illustrates the results of these experiments. PCMV-specific RNA was identified in HFF by day 9 post-infection (Figure 3A-9). This transcript remained expressed through to day 30 post-infection, although a decrease in the nested round PCR product was noted on day 21 and day 30 post-infection (Figure 3A-21, 3A-30). No similar PCR product was present in cells infected with heat-killed PCMV (negative control) over 31 days (Figure 3B-6 to 31), although a very faint nested round PCR product was observed at

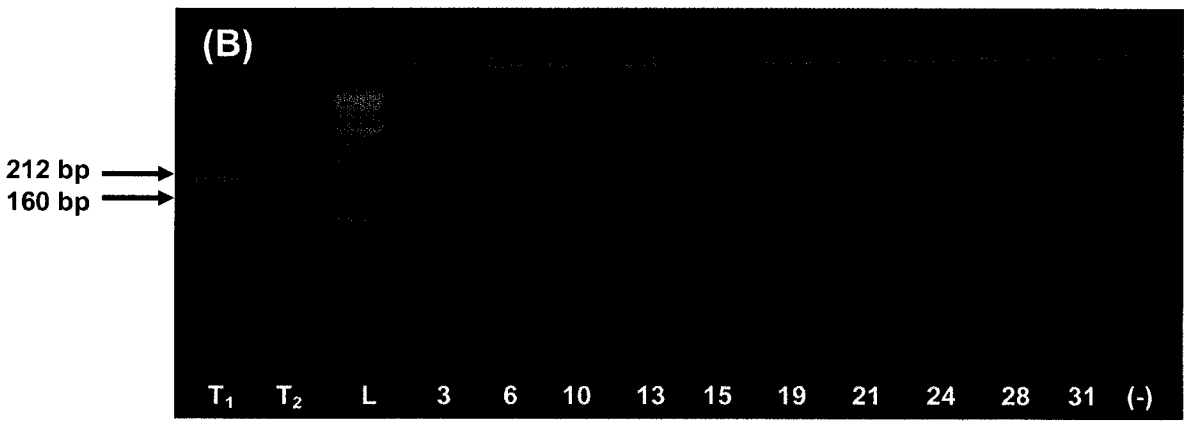
Figure 3: The Appearance and Monitoring of PCMV RNA in Infected HFF. These results are representative of three individual experiments. Briefly, HFF were seeded in T75 flasks, grown to confluent monolayers, infected with either live PCMV or heat-killed PCMV, covered with 2:1:0 BME, and maintained in culture. Total RNA was extracted from one T75 flask approximately every 3 days. To identify PCMV specific nucleic acid, nested RT-PCR reactions were performed with primers specific for PCMV DNA polymerase and either **(A)** RNA extracted from HFF infected with LIVE PCMV or **(B)** RNA extracted from HFF infected with HEAT-KILLED PCMV. The resulting products were electrophoresed on 3% agarose gels and stained with ethidium bromide. The **blue** arrow indicates the first round product of the PCR reaction expected at 212 bp[135], and the **red** arrow indicates the nested round product of the PCR reaction expected at 160 bp[135]. **(C)** RNA samples extracted from HFF infected with HEAT-KILLED PCMV were also electrophoresed on a 1% agarose gel and stained with ethidium bromide to show that the RNA used for the nested RT-PCR experiments in (B) were present and of high quality. The **pink** arrow indicates the 28S rRNA subunit, and the **green** arrow indicates the 18S rRNA subunit.

Figure Legend:

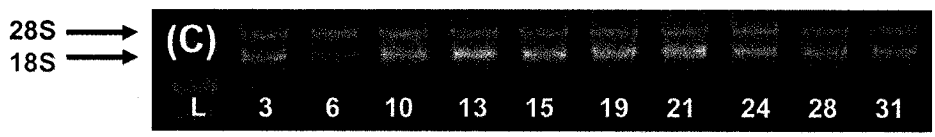
L	base-pair ladder
T_1	**(+) control:** PCMV-positive porcine turbinate cells, first round product (212 bp)
T_2	**(+) control:** PCMV-positive porcine turbinate cells, nested round product (160 bp)
(-)	**(-) control**: internal experimental blank (no RNA in reaction)
Numbers 3 – 31	specific day post-infection
Yellow box	PCR product confirmed as PCMV DNA polymerase by sequencing (Appendix I, Figure 12)
Purple box	reaction artifact; no known match to any sequence found in NCBI database (Appendix I, Figure 13)



Days Post-Infection with Live PCMV



Days Post-Infection with Heat-Killed PCMV



Days Post-Infection, Heat-Killed RNA only

day 3 post-infection (Figure 3B-3). Figure 3C represents an internal experimental control, where RNA extracted from HFF infected with heat-killed PCMV was electrophoresed on a non-denaturizing agarose gel (1%). This gel showed that the RNA used for the heat-killed PCMV nested RT-PCR experiments (Figure 3B) was present and of high quality.

PCR products amplified with RNA extracted from HFF infected with live PCMV (Figure 3A, yellow boxes) were confirmed to be PCMV DNA polymerase by sequencing and NCBI MegaBLAST searching (Appendix I, Figure 12). The band found in an internal experimental blank for these reactions (Figure 3A, purple box) was also sequenced and resulted in a 138 bp sequence that did not match to any known sequence found in the NCBI database (Appendix I, Figure 13). Thus, conclusions were drawn that it was an artifact. Overall, these studies verified that HFF were permissive for PCMV *in vitro*.

4.3 IFAs. Once it was determined that HFF were permissive for PCMV, additional studies were initiated to identify PCMV proteins in infected HFF. Because commercially available antibodies towards PCMV proteins are not available, PCMV-positive porcine turbinate cells were stained in an IFA to determine whether antibodies specific for human CMV proteins would cross-react with PCMV proteins and thus have the potential to be useful in different immunoassays. Briefly, PCMV-positive porcine turbinate cells were grown to confluency on glass cover slips, fixed, permeabilized, and probed with antibodies specific for human CMV epitopes. Cells were then stained with fluorescein-conjugated secondary antibodies, and Evan's Blue was used as a counter stain. Cells were visualized via fluorescent microscopy at different magnifications. As shown in Figure 4, these experiments determined that a mAb specific for human CMV gB (CMVB1)[134] (Figure 4A) and a polyclonal antibody specific for matrix, capsid, and membrane proteins of human CMV

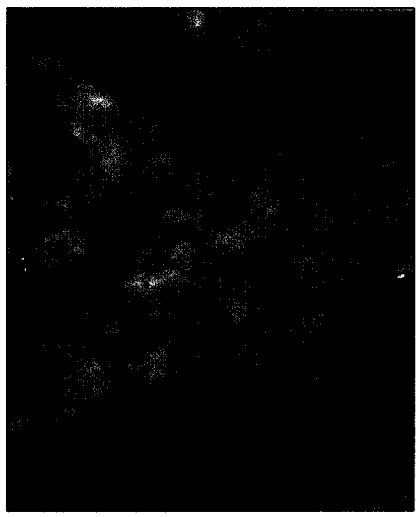
Figure 4: PCMV-Positive Porcine Turbinate Cells Fixed and Stained with Antibodies Specific for Human CMV Proteins in an IFA. These experiments are representative of one individual experiment for each antibody, done in duplicate. Briefly, PCMV-positive porcine turbinate cells were fixed, permeablized, and probed with antibodies specific for human CMV proteins as specified. Cells were then stained with fluorescein-conjugated secondary antibodies, and Evan's Blue was used as a counterstain. Cells were subsequently visualized via fluorescent microscopy at different magnifications.

(A) The primary antibody was a 1:25 dilution of a mouse mAb specific for gB of human CMV (CMVB1)[134], which was followed by a fluorescein-conjugated goat anti-mouse IgG secondary antibody. 20X magnification.

(B) The primary antibody was a 1:400 dilution of a rabbit polyclonal antibody specific for matrix, capsid, and membrane proteins of human CMV, which was followed by a fluorescein-conjugated goat anti-rabbit IgG secondary antibody. 40X magnification.

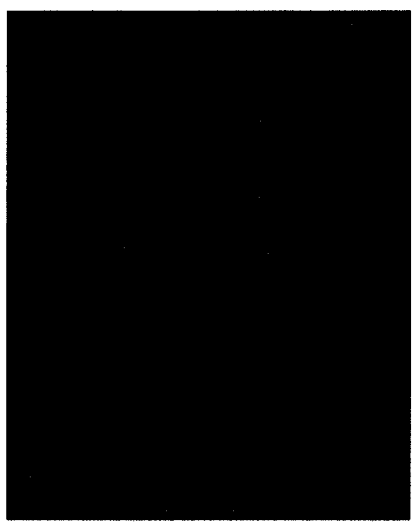
(C) (-) control: PBS was substituted for the primary antibody, which was followed by either a fluorescein-conjugated goat anti-mouse IgG secondary antibody (shown here) or a fluorescein-conjugated goat anti-rabbit IgG secondary antibody. 20X magnification.

(A)



(B)

(C)



(Figure 4B) cross-reacted with what were assumed to be similar epitopes found on PCMV proteins. Because high background problems occurred when these antibodies were used to detect PCMV proteins in infected HFF (data not shown), no further IFAs were done. However, the IFA that was completed with PCMV-positive porcine turbinate cells (Figure 4) suggested that these antibodies may be useful for detecting PCMV in a Western blot.

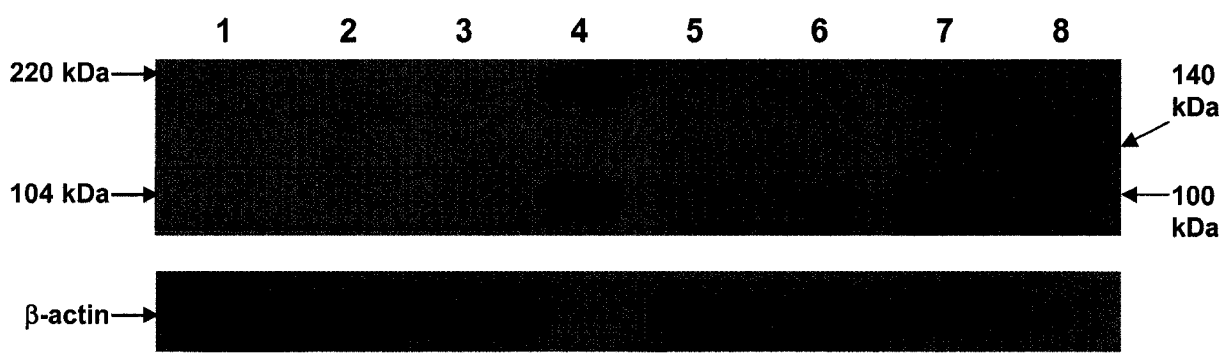
4.4 WESTERN BLOTS. After it was determined that antibodies specific for human CMV would cross-react with similar epitopes on PCMV proteins, Western blot studies were initiated to extend previous findings that identified PCMV in infected HFF. Briefly, HFF were seeded in T75 flasks, grown to confluent monolayers, inoculated with either live PCMV or heat-killed PCMV, and maintained in culture. Total cellular lysates were subsequently generated from several T75 flasks on various days post-infection. To identify PCMV specific proteins generated from inoculated monolayers, proteins in lysates were separated in a SDS-polyacrylamide gel, transferred to PVDF, and probed with one of the three antibodies described below. The membrane was then stained with an HRP-conjugated secondary antibody, treated with a cheminluminescent substrate, and exposed to film.

4.4.1 Probed with Anti-Human CMV gB. In a Western blot, a mAb pool specific for different epitopes of human CMV gB was able to detect a protein of 100 kilodaltons (kDa) in HFF infected with live PCMV, on day 19 and day 31 post-infection (Figure 5, lanes 5 and 6). The band seen in lysates from day 31 post-infection was more pronounced than the band on day 19 post-infection; however, this may reflect the slightly greater amount of lysate loaded into the gel for day 31 post-infection when compared with day 19 post-infection (Figure 5, β-actin, lanes 5 and 6). This mAb pool also detected the same 100 kDa protein in PCMV-positive porcine turbinate cells (positive control) (Figure 5, lane 7) as well as

Figure 5: Western Blot Showing Detection of PCMV Proteins in HFF Cell Lysate Using mAbs Specific for Human CMV gB. This blot is representative of four individual experiments performed with the lysates generated from two individual infections. Briefly, HFF were seeded in T75 flasks, grown to confluent monolayers, infected with either live PCMV or heat-killed PCMV, and maintained in culture. Total cellular lysates were generated from several T75 flasks on various days post-infection as specified. To identify PCMV specific proteins, 150 µg per lane of lysate was electrophoresed on a 10% SDS-polyacrylamide gel, transferred to PVDF, and probed with a mAb pool specific for different epitopes of human CMV gB at a 1:500 dilution in blocker. The membrane was then stained with an HRP-conjugated secondary antibody, treated with a chemiluminescent substrate, and exposed to film for 20 minutes. For a loading control, the blot was stripped, re-blocked, and then re-probed with an HRP-conjugated mAb specific for β-actin at a 1:50,000 dilution in blocker. The blot was treated with a chemiluminescent substrate and exposed to film for 1 second.

Blot Legend:

Lane 1 **(-) Control:** 150 µg of HFF lysate, non-infected
Lane 2 **(-) Control:** 150 µg of HFF lysate, infected with heat-killed PCMV, 19 days post-infection
Lane 3 **(-) Control**: 150 µg of HFF lysate, infected with heat-killed PCMV, 31 days post-infection
Lane 4 MW Marker
Lane 5 150 µg of HFF lysate, infected with live PCMV, 19 days post-infection
Lane 6 150 µg of HFF lysate, infected with live PCMV, 31 days post-infection
Lane 7 **(+) Control:** 150 µg of pig turbinate cell lysate, PCMV-positive
Lane 8 **(+) Control:** 5 ng of recombinant human CMV gB, 140 kDa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220 kDa
104 kDa
140 kDa
100 kDa
β-actin

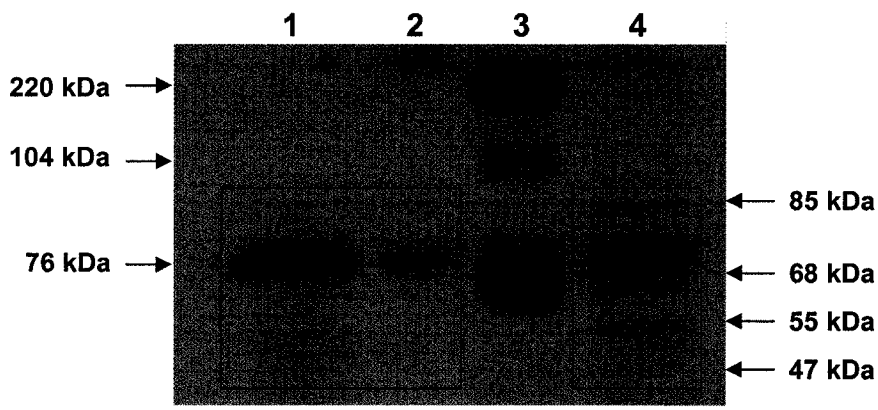
recombinant human CMV gB included as an extra positive control (Figure 5, lane 8). However, similar proteins were not seen in non-infected HFF (Figure 5, lane 1) or in HFF infected with heat-killed PCMV (negative controls) (Figure 5, lane 2 and 3). Overall, these experiments identified proteins that exist in both PCMV-infected HFF and PCMV-positive porcine turbinate cell and suggested that HFF were permissive for PCMV *in vitro*.

4.4.2 Probed with Anti-HHV-6 Membrane Glycoprotein. Because PCMV is phylogenetically closer to HHV-6 than to human CMV (section 1.2.2-a), a preliminary Western blot was done to determine if a mAb specific for a 60 kDa and a 110 kDa membrane glycoprotein of HHV-6 would be useful in identifying PCMV proteins in a Western blot. Blotting experiments using only PCMV-positive and PCMV-negative control lysates were first completed in order to establish optimal experimental conditions for using this mAb. Unfortunately, neither a HHV-6 infected cell lysate nor a HHV-6 recombinant protein was available to include as a genuine positive control. The procedure here is slightly different than the one described in section 4.4 and will thus be reviewed here. Briefly, HFF or PCMV-positive porcine turbinate cells were seeded and grown to confluent monolayers in separate T75 flasks. No infection took place. Total cellular lysates were generated from several T75 flasks once confluency was reached. After the lysate was prepared, proteins were separated in a SDS-polyacrylamide gel, transferred to PVDF, and probed with a mAb specific for an HHV-6 membrane glycoprotein of 60 kDa and 110 kDa. The membrane was then stained with an HRP-conjugated secondary antibody, treated with a chemiluminescent substrate, and exposed to film. Results of these experiments are shown in Figure 6. The HHV-6 mAb recognized a common epitope on a protein of 76 kDa in all cells tested, which included: porcine turbinate cells (PCMV positive control) (Figure 6, lane 1); human CMV-positive

Figure 6: Western Blot with mAb Specific for a 60 kDa and 110 kDa Membrane Glycoprotein of HHV-6. This blot is representative of two individual experiments performed with total cellular lysates that were generated once. Briefly, HFF and PCMV-positive porcine turbinate cells were seeded and grown to confluent monolayers in T75 flasks. Total cellular lysates were then generated from several T75 flasks. In order to establish PCMV-positive and PCMV-negative experimental controls for using this mAb, 150 μg of lysate generated from PCMV-positive porcine turbinate cells (positive control), 10 μg of lysate from human CMV-positive normal human dermal fibroblasts (recommended loading amount by manufacturer), and 150 μg of lysate generated from non-infected HFF were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to PVDF, and probed with the HHV-6 mAb diluted 1:100 in blocker. The membrane was then stained with an HRP-conjugated secondary antibody, treated with a cheminluminescent substrate, and exposed to film for 1 minute.

Blot Legend:

Lane 1 **(+) Control:** 150 μg of pig turbinate cell lysate, PCMV-positive
Lane 2 **(-) Control:** 10 μg of normal human dermal fibroblast cell line lysate, human CMV-positive and enriched for viral proteins, confirmed negative for HIV and hepatitis B
Lane 3 MW Marker
Lane 4 **(-) Control:** 150 μg of lysate of HFF, non-infected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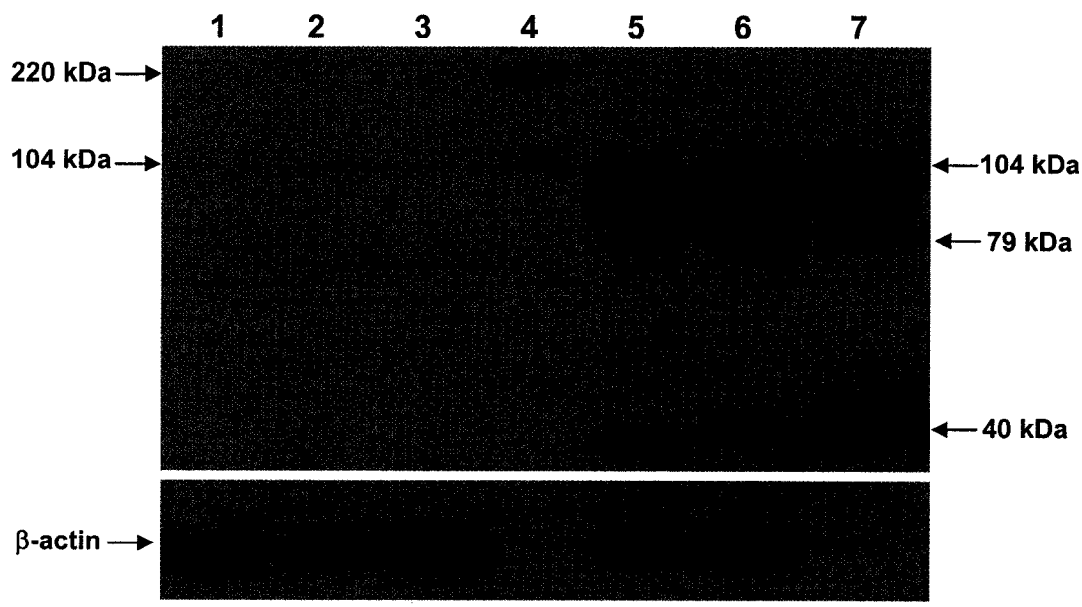
85 kDa
68 kDa
55 kDa
47 kDa

epithelial cells (Figure 6, lane 2); and non-infected HFF (PCMV negative control) (Figure 6, lane 4). The mAb also identified four faint, but distinct, bands of 85 kDa, 68 kDa, 55 kDa, and 47 kDa in the non-infected HFF lysate (Figure 6, lane 4). Because both PCMV-positive and PCMV-negative controls gave similar results and because PCMV could thus not be positively distinguished in porcine turbinate cells (PCMV-positive controls), PCMV-infected HFF cell lysate was not tested with this mAb. Overall, these results suggested that a mAb specific for a 60 kDa and a 110 kDa membrane glycoprotein of HHV-6 would not be useful in a Western blot to specifically identify PCMV proteins. They also showed that the HFF primary culture used for these experiments contained epitopes that were recognized by the HHV-6 specific mAb.

4.4.3 Probed with Pig Serum. PCMV is endemic in pig herds, and most herds will test sero-positive for PCMV. In an attempt to locate PCMV-specific antibodies, porcine blood was obtained from a local abattoir with the assumption that it would contain antibodies against PCMV. A pool of serum was then prepared and used to detect PCMV proteins in a Western blot. Results of these experiments showed that the pig serum was able to detect a series of protein bands ranging in size from 104 kDa to 79 kDa, and a protein of 40 kDa in HFF infected with live PCMV on day 19 and 31 post-infection (Figure 7, lanes 5 and 6). This serum also detected the identical protein bands in PCMV-positive porcine turbinate cells (positive control) (Figure 7, lane 7). Moreover, the serum did not detect any proteins in non-infected HFF or in HFF infected with heat-killed PCMV (negative controls) (Figure 7, lanes 1, 2, and 3). The series of protein bands ranging from 104 kDa to 79 kDa (Figure 7, lanes 5, 6, and 7) could not be distinguished because of the different intensities of the bands, and the similar migration patterns. The β-actin loading control suggested that very little PCMV-

Figure 7: Western Blot Showing Detection of PCMV Proteins in HFF Cell Lysate Using Pig Serum. This blot is representative of four individual experiments performed with the lysates generated from two individual infections. Briefly, human fibroblasts were seeded in T75 flasks, grown to confluent monolayers, infected with either live PCMV or heat-killed PCMV, and maintained in culture. Total cellular lysates were generated from several T75 flasks on various days post-infection. To identify PCMV specific proteins, 100 μg of lysate was electrophoresed on a 10% SDS-polyacrylamide gel, transferred to PVDF, and probed with pig serum diluted 1:7,500 in blocker. The membrane was then stained with an HRP-conjugated secondary antibody, treated with a cheminluminescent substrate, and exposed to film for 5 minutes. For a loading control, the blot was stripped, re-blocked, and re-probed with an HRP-conjugated mAb specific for β-actin at a 1:50,000 dilution in blocker. The blot was then treated with a chemiluminescent substrate and exposed to film for 1.5 minutes.

Blot Legend:
Lane 1 **(-) Control:** 100 μg of HFF lysate, non-infected
Lane 2 **(-) Control:** 100 μg of HFF lysate, infected with heat-killed PCMV, 19 days post-infection
Lane 3 **(-) Control**: 100 μg of HFF lysate, infected with heat-killed PCMV, 31 days post-infection
Lane 4 MW Marker
Lane 5 100 μg of HFF lysate, infected with live PCMV, 19 days post-infection
Lane 6 100 μg of HFF lysate, infected with live PCMV, 31 days post-infection
Lane 7 **(+) Control:** 100μg of pig turbinate cell lysate, PCMV-positive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104 kDa
104 kDa
79 kDa
40 kDa
β-actin

positive porcine turbinate cell lysate was applied to this lane (Figure 7, β-actin, Lane 7); however, the blot displayed a strong reaction with the serum and indicated plenty of protein (Figure 7, Lane 7). Overall, these experiments identified proteins that exist equally in PCMV-infected HFF and PCMV-positive porcine turbinate cell and suggested that HFF were permissive for PCMV *in vitro*.

4.5 ANTIBODY NEUTRALIZATION STUDIES. Following the Western blot studies, experiments to test the neutralizing capacity of these same antibodies were initiated in order to confirm that infectious PCMV virions were present in the viral stock and that they were causing the observed CPE. Briefly, HFF were seeded in 24-well plates, grown to confluent monolayers, and subsequently infected with one of the following: (1) a 1:10 dilution of live PCMV mixed with complement and various concentrations of a neutralizing antibody (described below); (2) a 1:10 dilution of live PCMV mixed with complement and various concentrations of an irrelevant control antibody (negative control) (described below); (3) live PCMV alone (maximum CPE control); or (4) medium alone (no CPE control). Directly following infection, the cells were covered with agar and then maintained in culture for 30 days. On specific days post-infection, cells were stained with neutral red overnight, and CPE was then visualized via light microscopy.

4.5.1 Using Anti-Human CMV gB. Neutralization assays were first completed with a mAb specific for human CMV gB (CMVB1)[134]. As shown in Figure 8, CMV-neutralizing mAb rows C and D, column 16, this mAb efficiently neutralized PCMV infection of HFF on day 16 post-infection, as manifested by a reduction in CPE when compared with experimental controls (Figure 8, CMV-neutralizing mAb rows A and B, column 16). This occurred in a dose-responsive manner related to mAb concentration

Figure 8: Neutralization of PCMV Infection in HFF Using a mAb Specific for Human CMV gB. These results are representative of two individual experiments, each done in triplicate. Briefly, HFF were seeded in 24-well plates, grown to confluent monolayers, and subsequently infected with one of the controls or PCMV-neutralizing / irrelevant mAb preparations listed below. For all PCMV preparations, a 1:10 dilution of the viral stock was mixed with complement and the specified amount of mAb protein. Directly following infection, the cells were covered with agar and then maintained in culture for 30 days. On specific days post-infection, cells were stained overnight with neutral red, and CPE was subsequently visualized via light microscopy.

Row A: **No CPE control**: medium alone (mock infection)

Row B: **Maximum CPE control**: live PCMV alone

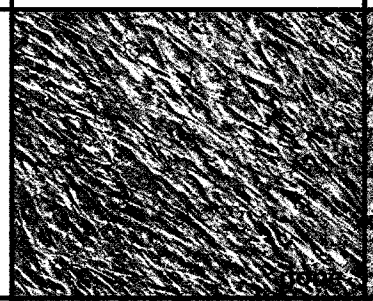
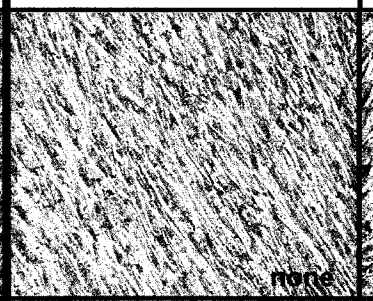
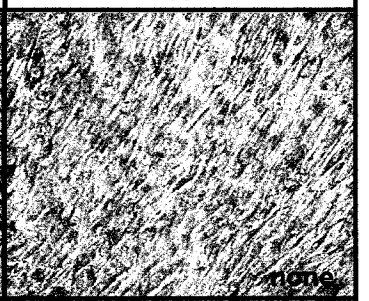
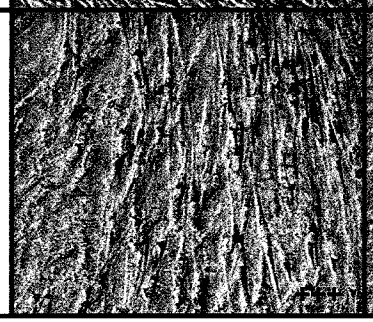
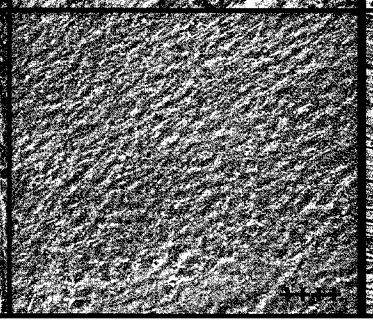
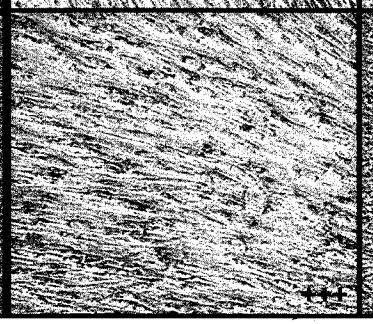
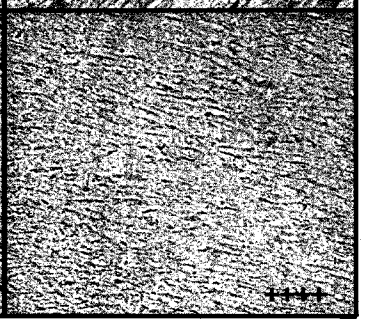
Row C: mAbs used at 2 μg (neutralizing or irrelevant)

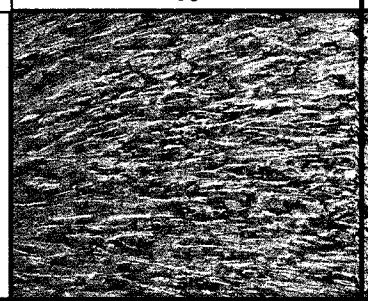
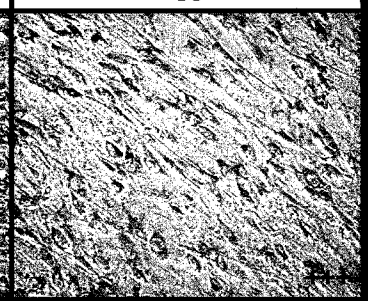
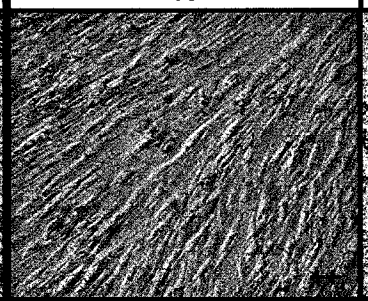
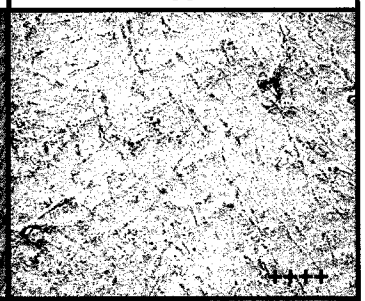
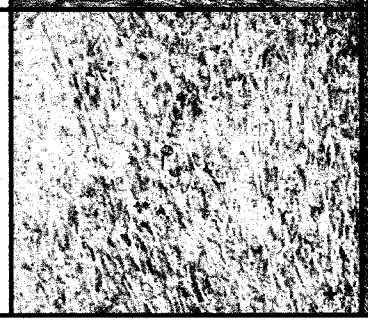
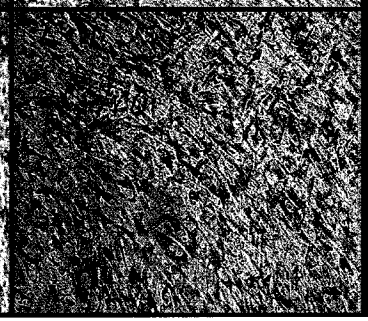
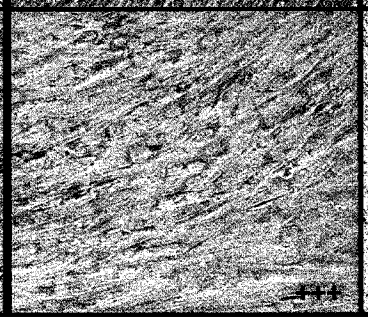
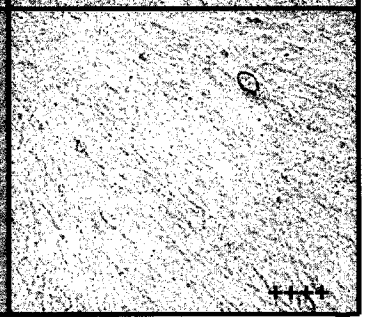
Row D: mAbs used at 200 μg (neutralizing or irrelevant)

*CPE noted is relative to experimental controls.

Figure legend:

+	cells becoming larger and more rounded, monolayer slightly less confluent
++	plaque-like areas of cell death in monolayer, remaining cells much less confluent
+++	more than 50% of monolayer dead, very few remaining cells
++++	entire monolayer dead
none	no CPE

Monolayer Treatment	Relative CPE*			
	CMV-Neutralizing mAb (anti-human CMV gB)		Irrelevant mAb (anti-ZAP-70)	
	Days Post-Infection		Days Post-Infection	
	16	30	16	30
(A) <u>No CPE Control</u>: Mock infection (medium alone) 10X magnification				
(B) <u>Max. CPE Control</u>: PCMV + medium alone 10X magnification				

Monolayer Treatment	Relative CPE*			
	CMV-Neutralizing mAb (anti-human CMV gB)		Irrelevant mAb (anti-ZAP-70)	
	Days Post-Infection		Days Post-Infection	
	16	30	16	30
(C) Live PCMV + 1/1,000 mAb dilution (2 µg of mAb protein) 10X magnification				
(D) Live PCMV + 1/10 mAb dilution (200 µg of mAb protein) 10X magnification				

(Figure 8, CMV-neutralizing mAb rows C and D, column 30), when compared with experimental controls (Figure 8, CMV-neutralizing mAb rows A and B, column 30). As seen in Figure 8, CMV-neutralizing mAb row D, column 30, 200 μg of anti-human CMV gB mAb was required to completely neutralize PCMV over a one-month period. Moreover, as shown in Figure 8, irrelevant mAb rows C and D, columns 16 and 30, an irrelevant mAb control of the same isotype and protein concentration did not exhibit any significant neutralizing activity when compared with experimental controls (Figure 8, irrelevant mAb rows A and B, columns 16 and 30). These results showed that a mAb specific for human CMV gB was useful in neutralizing PCMV infection of HFF, when used at high concentrations.

4.5.2 Using Anti-HHV-6 Membrane Glycoprotein. Neutralization assays were then performed with the mAb specific for an HHV-6 membrane glycoprotein of 60 kDa and 110 kDa in size. As shown in Figure 9, neutralizing mAb rows C and D, column 16, this mAb exhibited some neutralizing activity relative to controls at day 16 post-infection, especially when 10 μg of mAb protein was used (Figure 9, neutralizing mAb row D, column 16). This neutralization was observed as a reduction in CPE when compared with experimental controls (Figure 9, neutralizing mAb rows A and B, column 16). However, Figure 9, neutralizing mAb rows C and D, column 30, suggest that no PCMV neutralization had occurred, as clearly marked by the total CPE observed on day 30 post-infection when compared with controls (Figure 9, neutralizing mAb rows A and B, column 30). Moreover, as shown in Figure 9, irrelevant mAb rows C and D, columns 16 and 30, an irrelevant mAb control of the same isotype and protein concentration did not exhibit any significant neutralization when compared with experimental controls (Figure 9, irrelevant mAb rows A

Figure 9: Initial Neutralization of PCMV Infection in HFF Using a mAb Specific for a HHV-6 Membrane Glycoprotein of 60 kDa and 110 kDa. These results are representative of two individual experiments, each done in triplicate. Briefly, HFF were seeded in 24-well plates, grown to confluent monolayers, and subsequently infected with one of the controls or PCMV-neutralizing / irrelevant mAb preparations listed below. For all PCMV preparations, a 1:10 dilution of the viral stock was mixed with complement and the specified amount of mAb protein. Directly following infection, the cells were covered with agar and then maintained in culture for 30 days. On specific days post-infection, cells were stained overnight with neutral red, and CPE was subsequently visualized via light microscopy.

Row A: **No CPE control**: medium alone (mock infection)

Row B: **Maximum CPE control**: live PCMV alone

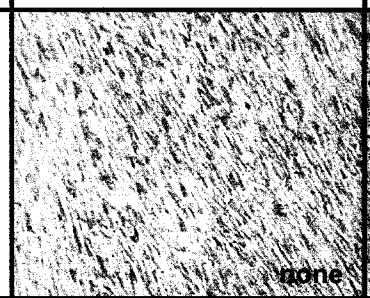
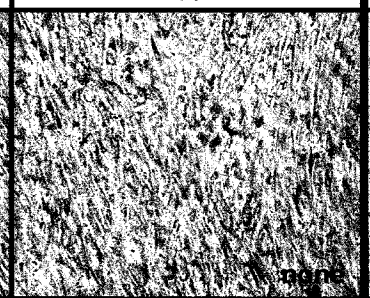
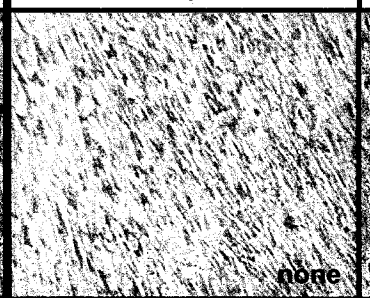
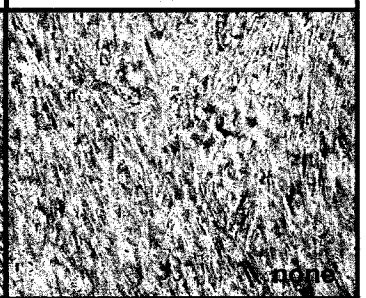
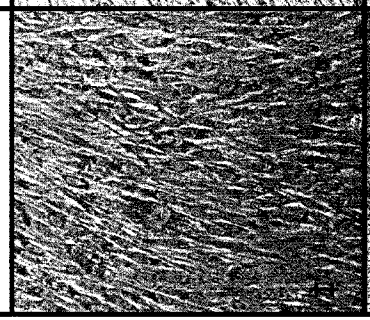
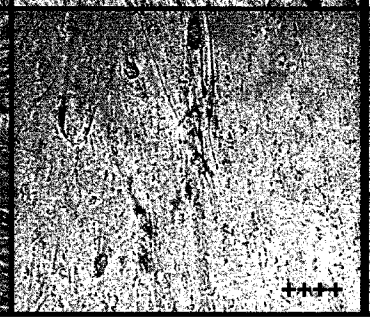
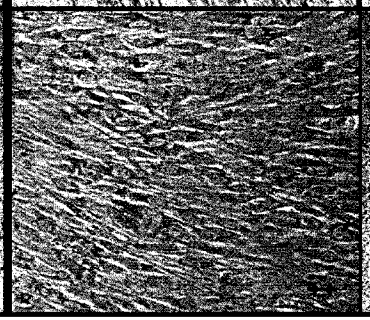
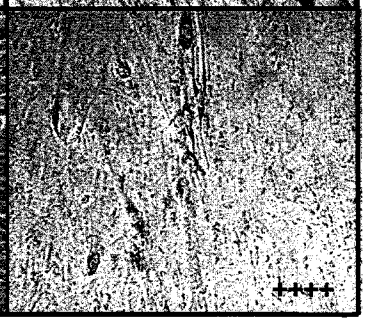
Row C: mAbs used at 0.1 μg (neutralizing or irrelevant)

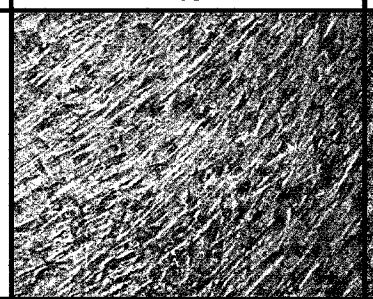
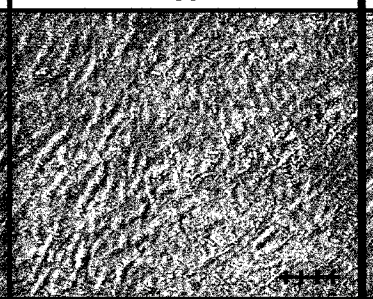
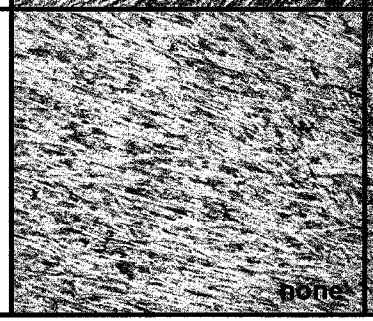
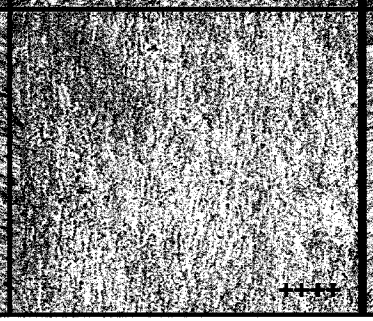
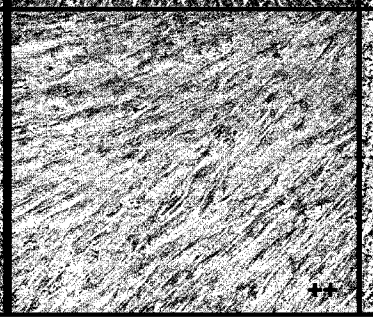
Row D: mAbs used at 10 μg (neutralizing or irrelevant)

*CPE noted is relative to experimental controls.

Figure legend:

+	cells becoming larger and more rounded, monolayer slightly less confluent
++	plaque-like areas of cell death in monolayer, remaining cells much less confluent
+++	more than 50% of monolayer dead, very few remaining cells
++++	entire monolayer dead
none	no CPE

Monolayer Treatment	Relative CPE*			
	Neutralizing mAb (anti-HHV-6 glycoprotein)		Irrelevant mAb (anti-ZAP-70)	
	Days Post-Infection		Days Post-Infection	
	16	30	16	30
(A) <u>No CPE Control</u>: Mock infection (medium alone) 10X magnification				
(B) <u>Max. CPE Control</u>: PCMV + medium alone 10X magnification				

Monolayer Treatment	Relative CPE*			
	Neutralizing mAb (anti-HHV-6 glycoprotein)		Irrelevant mAb (anti-ZAP-70)	
	Days Post-Infection		Days Post-Infection	
	16	30	16	30
(C) Live PCMV + 1/1,000 mAb dilution (0.1 µg of mAb protein) 10X magnification		 ++++		 ++++
(D) Live PCMV + 1/10 mAb dilution (10 µg of mAb protein) 10X magnification	 none	 ++++	 ++	 ++++

and B, columns 16 and 30). Overall, these results showed that a mAb specific for a HHV-6 membrane glycoprotein of 60 kDa and 110 kDa could neutralize PCMV infection of HFF at two weeks post-infection; however, infection was clearly evident and thus not neutralized at one month post-infection.

4.5.3 Using Pig Serum. Lastly, neutralization assays were carried out with the pig serum. As shown in Figure 10, neutralizing serum row E, column 16, this serum neutralized PCMV infection of HFF on day 16 post-infection when used at high concentration. This neutralization was clearly displayed as a reduction in CPE when compared with experimental controls (Figure 10, neutralizing serum rows A and B, column16). As shown in Figure 10, neutralizing serum rows C, D, and E, columns 16 and 30, this neutralization occurred in a dose-responsive manner related to serum dilution, as manifested by a increased reduction in CPE with higher concentration of pig serum when compared with experimental controls (Figure 10, neutralizing serum rows A and B, columns 16 and 30). However, Figure 10, neutralizing serum, rows D and E, column 30, suggested that neutralization of all infectious PCMV virions had not occurred. This observation was clearly visible as an increase in CPE on day 30 post-infection from the CPE noted on day 16 post-infection for these preparations (Figure 10, neutralizing serum, rows D and E, column 16), even though the CPE at day 30 post-infection still showed that efficient neutralization of PCMV had occurred when compared with controls (Figure 10, neutralizing serum, row B, column 30). As shown in Figure 10, irrelevant serum rows C, D, and E, columns 16 and 30, an irrelevant serum control used at the same dilution exhibited no neutralizing activity when compared with experimental controls (Figure 10, irrelevant serum rows A and B, columns 16 and 30). Overall, these results showed that pig serum used at high concentrations neutralized PCMV infection of HFF.

Figure 10: Neutralization of PCMV Infection of HFF with Pig Serum. These results are representative of one individual experiment, done in triplicate. Briefly, HFF were seeded in 24-well plates, grown to confluent monolayers, and subsequently infected with one of the controls or PCMV-neutralizing / irrelevant serum preparations listed below. For all PCMV preparations, a 1:10 dilution of the viral stock was mixed the specified dilution of serum. Directly following infection, the cells were covered with agar and then maintained in culture for 30 days. On specific days post-infection, cells were stained overnight with neutral red, and CPE was subsequently visualized via light microscopy.

Row A: **No CPE control**: medium alone (mock infection)

Row B: **Maximum CPE control**: live PCMV alone

Row C: serum used at 1:100,000 dilution (neutralizing or irrelevant)

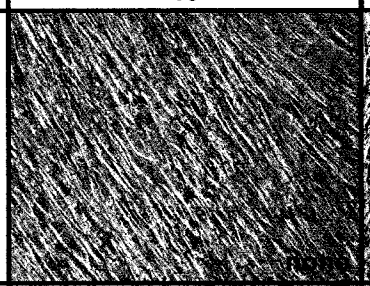
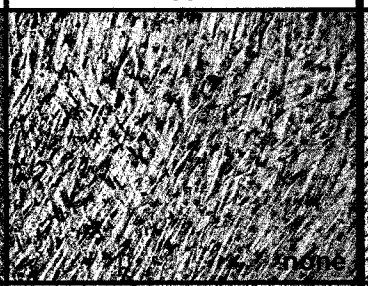
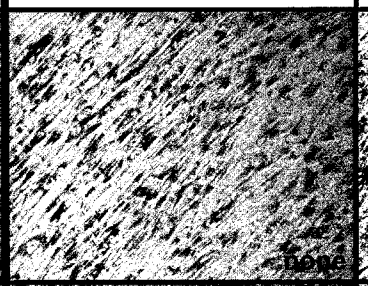
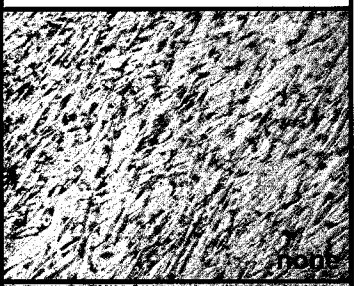
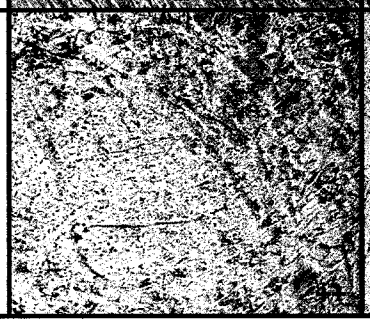
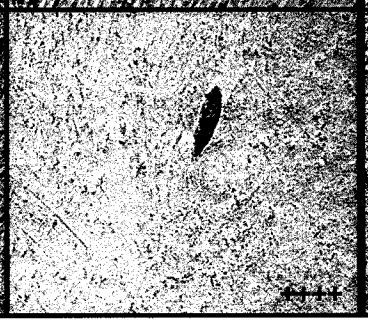
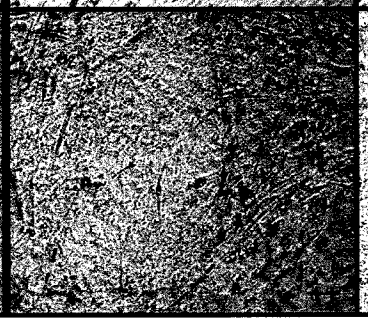
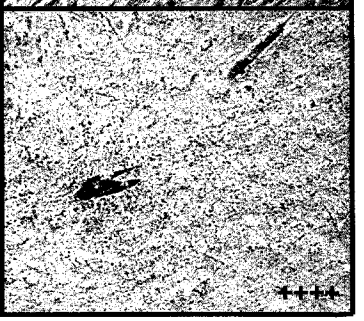
Row D: serum used at 1:1000 dilution (neutralizing or irrelevant)

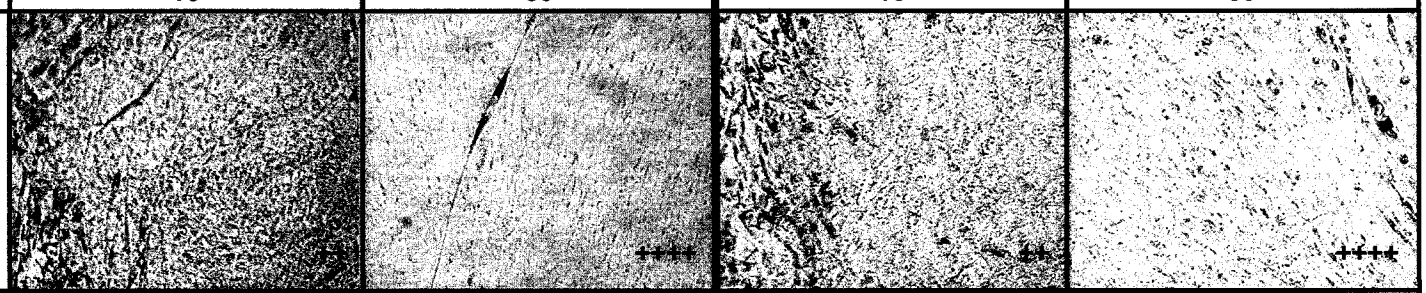
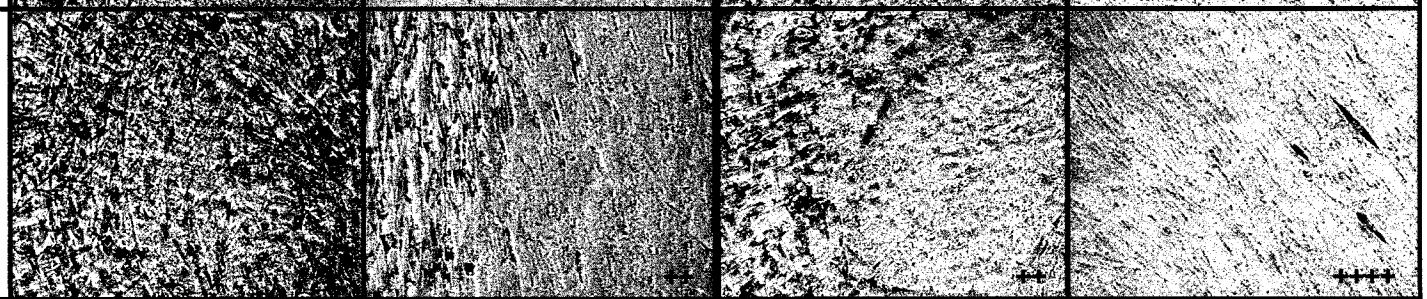
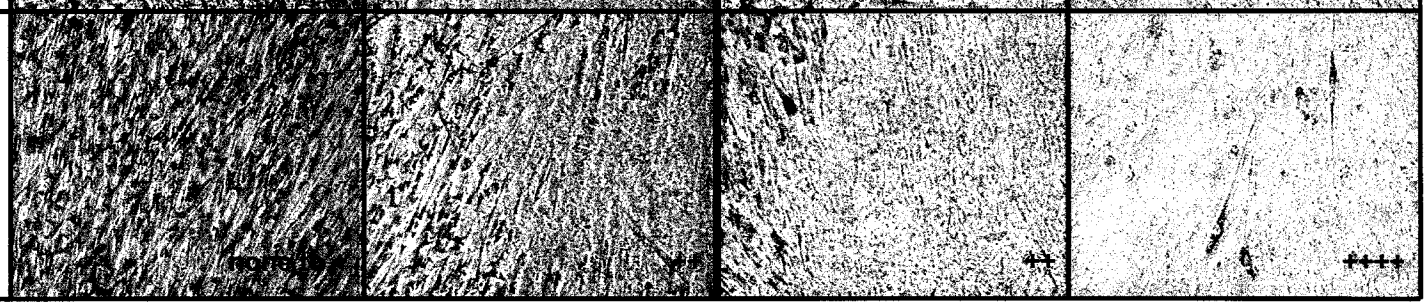
Row E: serum used at 1:10 dilution (neutralizing or irrelevant)

*CPE noted is relative to experimental controls.

Figure legend:

+	cells becoming larger and more rounded, monolayer slightly less confluent
++	plaque-like areas of cell death in monolayer, remaining cells much less confluent
+++	more than 50% of monolayer dead, very few remaining cells
++++	entire monolayer dead
none	no CPE

Monolayer Treatment	Relative CPE*			
	Neutralizing Serum (Pig)		Irrelevant Serum (Rabbit)	
	Days Post-Infection		Days Post-Infection	
	16	30	16	30
(A) <u>No CPE Control</u>: Mock infection (medium alone) 10X magnification				
(B) <u>Max. CPE Control</u>: PCMV + medium alone 10X magnification				

Monolayer Treatment	Relative CPE*			
	Neutralizing Serum (Pig)		Irrelevant Serum (Rabbit)	
	Days Post Infection		Days Post Infection	
	16	30	16	30
(C) Live PCMV + 1/100,000 serum dilution 10X magnification				
(D) Live PCMV + 1/1,000 serum dilution 10X magnification				
(E) Live PCMV + 1/10 serum dilution 10X magnification				

5.0 DISCUSSION

The experimental research described in this thesis was completed in order to determine the infectivity potential of PCMV in human fibroblasts. HFF were chosen to assess the xenozoonotic risk of PCMV *in vitro* because it was hypothesized that PCMV would replicate in these cells for two main reasons. First, PCMV is known to infect porcine fibroblasts, and the virus accordingly had the potential to replicate in HFF. Second, the PCMV stock generated for these experiments had been highly passaged (passage 12 in porcine lung cells, passage 15 in porcine testicle cells, and passage 25 in porcine turbinate cells). Highly passaged strains of human CMV replicate best in primary differentiated fibroblasts, and the highly passaged stock of PCMV had the potential to behave similarily[70,89]. In addition, HFF were readily available, and they were easy to work with.

The first objective of this research was to determine whether PCMV could infect HFF by visualizing any CPE generated after infection. The literature describes PCMV-induced CPE in porcine cells as cell swelling to about six times larger than uninfected cells, nuclear inclusion bodies, and occasional acidic cytoplasmic inclusions within 3 days of infection[89]. This description was confirmed in the current study by the co-cultivation of PCMV-positive porcine turbinate cells with porcine PT-K75 cells, which resulted in cell rounding and substantial nuclear and cytoplasmic inclusions by day 3 post co-cultivation (personal observation). Studies that visually assessed infection of HFF by PCMV revealed that the virus generated CPE and that the cells appeared to be supporting viral replication and spread. The PCMV-generated CPE in HFF was characterized by cell rounding and swelling, and nuclear inclusion bodies (Figure 1, rows E and F). This was followed by large plaque formation and eventual monolayer destruction. The PCMV-generated CPE in HFF was first

identified by day 5 post-infection, and 7 days were needed for it to be well established. Although the cell swelling and nuclear inclusions observed in infected HFF were similar to the reported / observed PCMV-generated CPE in porcine cells, two distinct differences existed. First, when PCMV was inoculated with HFF, a slightly delayed progression of CPE was observed when compared with the progression of PCMV-generated CPE in the porcine PT-K75 cells. Second, the PCMV life cycle in porcine cells is described as non-lytic *in vitro*[89], and cell death is not normally noted; however, substantial cell death was observed when PCMV infected HFF. Given these two differences, further comparisons were then made between the PCMV-generated CPE in HFF and the CPE generated by both HHV-6 and human CMV.

Considering HHV-6, scattered foci of CPE in culture T-lymphocytes have been reported in the literature around day 10 post-infection, and this CPE is characterized by a ballooning morphology[97]. Of particular interest is literature that reports no nuclear inclusions[97] and no CPE when HHV-6 infects human fibroblasts[101]. In the experiments carried out in this thesis, it was concluded that the pattern and CPE of a PCMV infection in HFF did not parallel that of a HHV-6 infection in cultured human cells, given the timing and morphological appearance of the PCMV-generated CPE in HFF. On the other hand, when considering the human CMV viral life cycle in HFF, the literature reports that the life cycle is lytic *in vitro* and that most new virions are released upon the bursting and simultaneous death of an infected cell[70]. In fact, previous infection of these same HFF with a high multiplicity of infectious human CMV resulted in a destruction of the monolayer that was similar to the PCMV-induced destruction (E. Tackaberry, personal communication). Furthermore, the cell rounding and nuclear inclusions seen in HFF following inoculation with PCMV appears

similar to the literature-described human CMV-generated CPE in HFF, which includes substantial cell rounding and enlargement with nuclear and perinuclear inclusions[71]. From these comparisons, it was concluded that the pattern of CPE in HFF following inoculation with PCMV was somewhat similar to the pattern of human CMV-generated CPE in these same human cells following high multiplicity inoculums.

Given that the PCMV life cycle in porcine cells is described as non-lytic *in vitro*[89], the cause of the PCMV-induced cell death observed in HFF was not clear. However, all cells die via one of two methods – either necrosis or apoptosis. A report published in 2006 by Jurak *et al.*[73] suggested that the PCMV-induced death in HFF may be a result of necrosis due to a lytic viral life cycle in these cells. Jurak *et. al.*[73] successfully infected human cells that were incapable of inducing apoptosis with rat CMV; furthermore, the cell death noted by the authors on day 7 post-infection was very similar to the large areas of cell death observed in our experiments on the same day post-infection (Figure 1, row E). Of particular interest, Jurak *et. al.*[73] showed that CMVs could not stop apoptosis when infecting cells of a different species, and the authors subsequently suggested that this may be reason for the species-specific dogma that has been associated with all CMVs. Considering this, it is reasonable to assume that the HFF cell death observed following PCMV infection was the result of necrosis. PCMV may have prevented apoptosis in HFF, which subsequently allowed for viral replication, lytic spread, and necrotic cell death. On the other hand, destruction of the HFF monolayer following PCMV infection could have been the result of apoptosis and not necrosis. Given the phylogenetic similarity between PCMV and HHV-6 and the lack of knowledge surrounding the full infectivity potential of PCMV, it is of value to consider that apoptosis has been linked with the HHV-6 viral life cycle. In the literature, HHV-6A and B

have been associated with the *in vivo* apoptosis of infected $CD4^+$ lymphocytes and macrophages and the *in vitro* apoptosis of cultured blood mononuclear cells[99,136,137]. Considering this, the cell death observed in HFF following inoculation with PCMV could potentially be due to a HHV-6-like apoptosis. More research is needed to investigate the mechanism surrounding the substantial monolayer destruction following infection of HFF with PCMV.

It is also important to mention that the total monolayer destruction associated with infection of HFF by PCMV was only observed during CPE studies, when HFF were covered with agar. When HFF were infected with the same concentration of PCMV and maintained in liquid BME, substantial destruction of the monolayer was not observed (data not presented). Evidence of some CPE did occur, and it was characterized by significant cell rounding with nuclear inclusion bodies. Foci of extremely rounded and granular cells could be seen throughout the monolayer, which appeared increasingly less confluent as the infection progressed. Furthermore, the liquid culture supernatant from these studies was filled with non-adherent cellular debris after only a few days of culture. Also, the appearance of CPE was much slower, taking a minimum of 10 days to be noted. The reason for this difference in pathogenesis is not clear. The agar was tested for toxicity during experimental set-up, and thus it can be ruled out as the cause of the cell death (also observed as no CPE in non-infected and heat-killed PCMV-infected experimental controls of Figure 1). Two explanations have been considered. First, the large plaque formation and gradual monolayer destruction observed after PCMV infection of HFF suggested lytic viral replication and spread. Lytic life cycles normally terminate with the necrotic death of an infected cell. Necrosis can trigger death in surrounding cells, and perhaps the death noted in agar-covered

HFF after infection with PCMV was enhanced by sustained contact with necrotic cellular debris that was slow to diffuse away from the agar-covered monolayer, as well as by efficient progeny virion spread. Second, Inoue *et. al*[136] have shown that UV irradiated / ultracentrifuged supernatant from an HHV-6 positive culture can induce apoptosis in the presence of TNF-α. Perhaps the supernatant derived from cells infected with PCMV was able to cause an HHV-6-like apoptosis in HFF, and this apoptosis was enhanced in PCMV-infected HFF covered with agar, again, because of slow diffusion. As before, the actual cause of the observed cell death cannot be determined from these experiments, and a definite explanation would require further research.

The second objective of this research was to verify that HFF were permissive for PCMV by identifying viral nucleic acid in infected cells. CPE studies had suggested cell permissiveness for PCMV, and it was decided that RT-PCR would be used to realize this objective. This method was chosen because CMVs are double-stranded DNA viruses[69], and if viral RNA was identified in infected cells, it would reflect the fact that PCMV had entered HFF and had began the process of producing progeny virions. RT-PCR studies confirmed the conclusions suggested from numerous CPE studies: that HFF are permissive for PCMV infection *in vitro*. PCMV RNA was first identified in infected HFF 9 days post-infection. This detection correlated with the observed CPE, which took a minimum of 10 - 14 days to appear when cells were maintained in medium. The weak PCR product seen on day 21 post-infection (Figure 3A-21) is thought to reflect a poor extraction / handling of total RNA and not a drastic decrease of viral transcription on that particular day post-infection. On the other hand, the weak PCR product seen on day 30 post-infection (Figure 3A-30) is thought to reflect a decrease in the number of cells remaining in the monolayer. The band in the

internal experimental blank (Figure 3A, lane (-)) was clearly an artifact since sequencing showed it to be irrelevant. This artifact was believed to be a result of poor technique during sample handling. Turning to the nucleic acid data generated with RNA extracted from HFF infected with heat-killed PCMV, it is important to mention the very faint band that was seen on day 3 post-infection (Figure 3B-3). The origin of this band is unknown, and no clear explanation was evident.

The RT-PCR experiments were completed three times, starting with the infection process and ending with the RT-PCR reactions. The characteristic PCR product of approximately 160 bp seen in the infected HFF was present after all three inoculations; however, the product was only weakly visible or was not present in random samples following some of the inoculations. This observation is similar to findings by Degré *et. al.*[47] who investigated the infectivity potential of human CMV in porcine endothelial cells. One explanation for the weakly visible/ missing data may reflect the quality of viral stock. The inoculations that produced weakly visible / inconsistent data were performed with viral stock that had been stored for several (more than 6) months at -85°C. Perhaps the PCMV stock had diminished in the number of infectious virions after spending many months in the freezer. This speculation was supported by the same trend in CPE studies, where the noted pattern and severity of PCMV-generated CPE in HFF would decrease after the virus had been stored for more than 6 months at -85°C. Also of interest is the study completed by Degré *et. al.*[47]. The authors found that only a small portion of porcine endothelial cells were infected with human CMV following inoculation and that the resulting titer was low. Perhaps this is the same for PCMV in HFF. Accordingly, the PCMV DNA polymerase transcripts would be

present in these cultures in low amounts or possibly not at all, especially if the infectious titer of the PCMV stock had diminished due to extended viral storage at -85°C.

The third objective of this research was to provide further evidence that HFF were permissive for PCMV by detecting virus-specific proteins in infected cells, and the last objective was to determine if PCMV infection of human fibroblasts could be neutralized with the same antibodies used to complete objective three. Once RT-PCR experiments had verified that PCMV infected HFF *in vitro*, studies were initiated to achieve these last two objectives. Western blot analysis provided further support that HFF were permissive for PCMV *in vitro*, and the neutralization assays confirmed that the CPE generated in HFF following incubation with live PCMV was indeed caused by infectious virions. Specifically, a mAb pool recognizing human CMV gB, and pig serum, were both able to positively identify PCMV proteins in total cell lysates from HFF that had been infected with PCMV. These antibodies were subsequently shown to be able to neutralize PCMV infection of HFF as well.

Human CMV gB is a type-I integral membrane protein and the most highly conserved glycoprotein across the entire *Herpesviridae* family[70]. Compared with PCMV gB, there is an approximate 36% amino acid sequence identity between the two (Table 2). Human CMV gB is produced as a single, 906 / 907 amino acid stable precursor that is about 150 kDa in size[70]. The precursor is proteolytically cleaved between amino acids 460 and 461 by a Golgi-associated host cell protease and is inserted into every membrane of an infected cell and progeny virions as two distinct subunits linked by a disulfide-bond[70,138]. The two cleavage products of 93 and 55 kDa, which represent the amino and carboxy termi respectively, are highly glycosylated with both N- and O-linked sugars; however, the amino terminus is more

heavily glycosylated[70,138]. Therefore, in the membranes of a human CMV- infected cell and in the viral envelope, gB is seen as two glycosylated subunits of approximately 110 kDa and 60 kDa respectively[70,138]. Comparatively speaking, very few studies have been done to characterize the gB of PCMV. One study by Widen *et. al.*[91] sequenced and analyzed the putative gB gene. They found that the gene and its product align best with the gB open reading frames of HHV-6 and HHV-7 (Table 2). The PCMV open reading frame produces a protein of approximately 860 amino acids[91], and from this amino acid sequence, the product is estimated to be approximately 100 kDa in size. The protein contains 15 potential N-linked sugar residues, an N-terminus signal sequence (amino acids 7-19), two putative transmembrane regions (amino acids 706-726 and amino acids 730-747), and a cleavage site (amino acids 439-442)[91].

The Western blot analysis of PCMV-infected HFF lysate showed that the mAb pool specific for human CMV gB was able to cross-react with a protein of approximately 100 kDa (Figure 5, lanes 5 and 6). As discussed above, unprocessed PCMV gB is estimated to be 100 kDa in size, and given the size of the protein detected in this blot, the experiment suggested that the mAb pool was cross-reacting with unprocessed PCMV gB. The literature reports that the PCMV viral titer normally takes 10 to 14 days to reach a maximum of approximately 10^6 $TCID_{50}$ in porcine cells[89]. As previously mentioned, Degré *et. al.*[47]. found that only a small portion of porcine endothelial cells were infected with human CMV following inoculation and that the resulting titer was low. The later detection of PCMV gB seen in our blots may also reflect a slow viral life cycle in HFF and / or a low viral titer. Perhaps many days were needed in order for enough viral protein to have accumulated within infected HFF for identification in a Western blot, although it is noted that PCMV-infected HFF lysate

extracted before two weeks post-infection was not tested after optimal blotting conditions were established for this mAb pool.

Neutralization studies completed with a single mAb specific for human CMV gB (CMVB1) provided supporting evidence that infectious PCMV virions present in the viral stock were causing the documented CPE, by significantly neutralizing PCMV infection of HFF (Figure 8, CMV-neutralizing mAb, row D, columns 16 and 30). However, 200 μg of CMVB1 mAb protein was needed in order for PCMV to be neutralized 30 days post-infection (Figure 8, CMV-neutralizing mAb, row D, column 30). As reported by Tackaberry *et. al.*[139], CMVB1 is a highly neutralizing antibody for human CMV. Accordingly, the large quantity of antibody that was needed to neutralize PCMV infection of HFF was unexpected. This observation can potentially be explained by one of two different reasons. First, CMVB1, considering it is specific for human CMV gB, was either weakly recognizing PCMV, and therefore 200 μg of antibody was needed in order to neutralize PCMV. Or, second, the titre of the PCMV stock used to complete the objectives of this thesis was very high, and a large amount of antibody was needed in order to neutralize PCMV.

Overall, the experimental results obtained with the mAb CMVB1 indicated that the antibody was recognizing an epitope on PCMV gB and that this protein was a useful target for neutralizing PCMV infection of HFF. Considering human CMV, initial attachment to the surface of susceptible cells is believed to be through an interaction of viral gB and cellular heparan sulfate; furthermore, the well-documented neutralizing humoral immune response generated by human CMV is normally directed towards gB – the glycoprotein is highly immunogenic and the main protein constituent of the human CMV envelope[71,138,140,141]. Also, the region between amino acid residues 589-645 of human CMV gB falls within an

antigenic domain (amino acids 476 to 645) of the human CMV gB protein that induces important neutralizing antibodies[138]. This region was found to have an overall higher sequence identity (46%) to a region between residues 521 and 623 of PCMV gB[91]. Taking all of this into consideration, it is reasonable to consider that PCMV gB may play a role in viral attachment and entry in HFF, and that neutralizing antibodies specific for either human CMV gB or PCMV gB may be useful as a prophylactic treatment in preventing PCMV spread within a xenograft recipient. However, that being said, it is important to note that the role of the neutralizing humoral immune response to human CMV infection is unclear[71,78,142]. Schoppel *et. al.*[140] have shown that high titers of gB-specific and glycoprotein H (gH)-specific neutralizing antibodies may be beneficial in controlling blood-borne human CMV viremia, and Alberola *et. al.*[141] have shown that high titers of neutralizing gB-specific antibodies may be beneficial in limiting the severity of human CMV-associated disease. On the other hand, Ludwig *et. al.*[142] and Eggers *et. al.*[143] have shown that high titers of gB and gH-specific neutralizing antibodies are associated with an increase in human CMV disease, and Muñoz *et. al.*[144] found no correlation between neutralizing antibodies and the appearance or lack of human CMV disease. Investigations into the role, if any, that PCMV gB plays in its life cycle would provid further insight into whether an antibody specific for this protein (or one that cross-reacts with it) would have any potential therapeutic application in dampening PCMV replication and spread post-xenotransplantation.

Pig serum generated from the blood of two meat hogs was obtained and used in Western blot and neutralization experiments identical to the ones discussed above. As mentioned in section 1, PCMV is endemic in pig herds throughout the world with a prevalence of more than 90% in Europe, North America, and Japan[89,94]. Furthermore, within

an infected herd, almost all pigs will have acquired the virus[93]. Not much information is available concerning the porcine immune response to PCMV; however, it is known that the response in pigs does involve antibody production[89]. Antibody can be detected 2 to 3 weeks post-infection with maximum antibody titers peaking around 6 weeks post-infection[89]. Antibody levels will remain present for approximately three months post-infection, and development of detectable serum antibody is associated with a disappearance of PCMV in the blood, although the virus will still be shed in bodily fluids for the next couple of weeks[89]. The exact age of the pigs who provided the serum for this research is not known; however, commercial meat hogs are normally between 4 to 6 months of age when they are slaughtered[145]. Given that most piglets will acquire PCMV during the first month of life[89], pigs between the ages of 4 and 6 months would likely be sero-positive for PCMV. It was accordingly assumed that the serum used in immunoblot and neutralization assays was sero-positive for PCMV.

A Western blot analysis with the same PCMV-infected HFF lysate used in the CMVB1 blot showed that the pig serum was able to detect a series of protein bands ranging from 104 kDa to 79 kDa, as well as a protein of 40 kDa (Figure 7, lanes 5 and 6). Unprocessed, PCMV gB was estimated to be approximately 100 kDa in size and this, along with the immunoblot completed with CMVB1, suggested that protein bands located near the top of the range (95kDa to 104 kDa) may include unprocessed PCMV gB. As previously discussed, the 150 kDa human CMV gB precursor is proteolytically cleaved into two subunits of 93 kDa and 55 kDa[70,138]. Furthermore, Widen *et. al.*[91] identified a potential cleavage site in PCMV gB, and the protein bands located near the bottom of the range (85 kDa to 79 kDa), as well as the 40 kDa protein could potentially be PCMV gB subunits.

Widen *et. al.*[91] also suggested that PCMV gB protein contains 15 potential N-linked sugar residues. The range of protein bands between 104 kDa and 79 kDa detected by the pig serum may also represent different glycoforms of one, or both, of the PCMV gB subunits. The β-actin loading control for this blot shows that very little positive control protein was separated in the gel (Figure 7, lane 7). Because the blot initially displayed a strong reaction with the pig serum, this weak reaction is believed to be the result of a number of different procedural errors. For example, the bottom corner of the blot could have adhered to the side of the container away from the anti-β-actin mAb solution during incubation. Or, perhaps the bottom corner of the blot was not allowed to incubate with the chemiluminescent substrate. If either on of these situations had occurred, no signal would be present.

Neutralization studies completed with the pig serum confirmed that infectious PCMV virions were present in the viral stock and that they were causing the documented CPE by displaying neutralizing activity towards PCMV infection of HFF (Figure 10, neutralizing serum rows D and E, columns 16 and 30). The assay also provided supporting evidence that the serum was recognizing PCMV-specific proteins in the Western blot; however, because of the many bands seen in the Western blot and the nature of polyclonal anti-serum, a single target for the neutralization activity could not be identified. In fact, during a human CMV infection, both gB-specific and glycoprotein H (gH)-specific antibodies appear to have some neutralizing capacity towards the virus[140,141]. During the neutralization assays, the pig serum could be diluted no more than 1:10 for PCMV to be neutralized approximately 2 weeks post-infection (Figure 10, neutralizing serum, row E, column16), and no more than 1:1,000 for PCMV to be neutralized approximately one month post-infection (Figure 10, neutralizing serum, row E, column 30). Furthermore, given the increase in CPE noted from day 16 post-

infection to day 30 post-infection at these two serum dilutions (Figure 10, neutralizing serum rows D and E, columns 16 and 30), as well as the high concentration of serum that had to be used for neutralization to occur, it was concluded that the pig serum was not as efficient as CMVB1 in neutralizing PCMV infection of HFF. However, considering that neutralizing antibodies develop slowly and are only present at low levels during the porcine immune response to PCMV[89], the fact that a high concentration of serum had to be present in order for significant neutralization of infection in HFF to occur was not unexpected.

Because PCMV is phylogenetically closer to HHV-6, a Western blot was performed with an antibody specific for a HHV-6 protein. A mAb specific for a membrane glycoprotein of 60 kDa and 110 kDa that would recognize both HHV-6A and HHV-6B was chosen as a probe for the blot, and although the actual specificity of this mAb is not known, it was chosen for these experiments with the possibility that it was specific for gB of both HHV-6 variants. Very few studies have been done to characterize gB of HHV-6. HHV-6A and B variants encode a protein of approximately 830 amino acids, and the protein is translated as 112 kDa and 102 kDa proteins, respectively[98]. Both are proteolytically cleaved and inserted into the membranes as disulfide-linked subunits of 64 kDa and 58 kDa, and 59 kDa and 50 kDa, respectively[981,146]. There is a 96% amino acid sequence similarity between the two variants, and given the sizes of the precursor and cleaved products of both variants, there exists a possibility that the mAb recognizing a membrane glycoprotein of 60 kDa and 110 kDa of HHV-6A and B used in these experiments was specific for HHV-6 gB.

In establishing optimal blotting conditions for using this mAb, it was shown that this particular mAb would not be useful in a Western blot to specifically identify PCMV proteins (Figure 6); however, the mAb did identify four faint, but distinct, bands of approximately 85

kDa, 68 kDa, 55 kDa, and 47 kDa in the non-infected HFF lysate (Figure 6, lane 4). Two possibilities were considered as an explanation for these bands. First, the mAb could have been cross-reacting non-specifically with similar epitopes found on proteins normally present in HFF. Second, the mAb could have been either recognizing different glycoforms of the HHV-6 specific membrane glycoprotein or cross-reacting with other HHV-6 viral proteins. HHV-6 has a broad host cell tropism both *in vivo* and *in vitro*, and acquisition of the virus results in a lifelong, latent infection as well as a low-level chronic infection[97-99]. Furthermore, congenital transmission of HHV-6 to the fetus has been reported, along with hereditary transmission[99,102,107-111]. Considering these points, it is possible that the infant who provided the foreskin which was used to generate the HFF culture was already infected with HHV-6. It is possible that this acquisition was subsequently reflected as a latent HHV-6 infection in the primary HFF culture generated. Accordingly, the HHV-6 specific mAb could potentially be recognizing HHV-6 proteins in the total cellular lysate of the HFF culture, which were expressed in order to maintain the latent infection. Three distinct sets of glycoproteins have been identified in the literature as antigens in HHV-6 infected cells by immunoprecipitation[97]. These sets are (1) glycoprotein (gp) 105 and gp82; (2) gp116, gp64, and gp54; and (3) a single gp of 102 kDa[97]. The four faint, but distinct, bands of approximately 85 kDa, 68 kDa, 55 kDa, and 47 kDa in the non-infected HFF lysate (Figure 6, lane 4) are very similar in size to the sets of antigens listed above and further support the explanation that the mAb was recognizing HHV-6 proteins.

Even though the anti-HHV-6 mAb was shown to be unusable in a Western blot, a neutralization assay was performed with this antibody in case it was specific for a neutralization-associated and conformational epitope on PCMV, in addition to the Western

blot bands. Neutralization studies completed with the mAb specific for a 60 kDa and 110 kDa membrane glycoprotein of HHV-6 showed that the mAb displayed slight neutralizing ability towards PCMV at two weeks post-infection but not at one month post-infection (Figure 9, neutralizing mAb, rows C and D, columns 16 and 30). A few explanations were considered in order to describe the initial neutralizing activity.

First, the HHV-6 specific mAb could be recognizing non-specific epitopes from proteins expressed on the surface of HFF. This recognition could have potentially blocked the entry of PCMV into some cells, if the antibody was cross-reacting with the entry receptor PCMV uses to infect these cells. This would have lowered the number of cells initially infected with PCMV. Accordingly, perhaps very little PCMV was present at two weeks post-infection, and one month was needed for PCMV to accumulate in sufficient quantities in order to generate notable CPE. It is noted here that the antibody is not believed to be recognizing HHV-6 specific proteins on the surface of the monolayer, even though the Western blot suggested that the monolayer might be latently infected with the virus. This is because, unlike human CMV, no HHV-6 viral glycoproteins are expressed on the surface of a cell during an infection[98-100]. Second, as previously discussed, the antibody used for these experiments was potentially specific for HHV-6 gB. The gB of HHV-6A and B variants share a 43% amino acid sequence homology with PCMV gB (Table 2). Perhaps the mAb was recognizing a conformational epitope on PCMV gB, but this recognition was either weak or not present in high enough quantities (a maximum of 10 μg of mAb protein was used for these experiments). Thus, the mAb would have been unable to neutralize all virions and would have served only to lower the number of infectious viral particles thus resulting in a delayed pattern of CPE. Last, the delay in CPE may not be a consequence of neutralization,

but a reflection of the quality of viral stock. It has been previously discussed that the infectivity potential of the PCMV viral stock would diminish after being stored for stored for several months at -85°C, and the HHV-6 neutralization assays were performed with "old" PCMV stock. Accordingly, the delay in CPE may reflect a low number of infectious PCMV virions / progeny present in the culture at two weeks post-infection.

Collectively, the research completed for this thesis shows that HFF are permissive for PCMV *in vitro*, and, in conjunction with PCMV reactivation / replication studies[49,95,96,128,130], it suggests that PCMV has the potential to reactivate and replicate within human fibroblasts post-xenotransplantation. However, one report published by Tucker *et. al.*[48] draws a different conclusion. In this report, the authors also evaluated PCMV as a potential xenozoonotic virus *in vitro* and concluded that PCMV "was unlikely to be a significant zoonotic agent in clinical xenotransplantation of pig organs to humans". Specifically, Tucker *et. al.*[48] showed that PCMV from persistently infected porcine alveolar macrophages could not infect either a human B cell line (RAJI) or a human epithelial cell line (293). A number of different explanations have been considered for the opposing experimental outcomes. First, perhaps the difference in cell permissiveness lies in the type of culture used: primary cells in this thesis and cell lines by Tucker *et. al.*[48] . PCMV may only replicate in primary human cells. In fact, the literature has reported that in cultured pig cells, PCMV displays a restricted cell tropism, and its yield is lower in cell lines than in primary culture systems (see 1.2.2-b)[57,89]. Second, the difference in cell permissiveness could also be explained by the type of cell cultured. Similar to human CMV replication *in vitro* (see 1.2.1-b), maybe PCMV will not replicate in either human B cells or human epithelial cells – only human fibroblasts may be permissive for PCMV *in vitro*. Last, the source of PCMV used by Tucker *et. al.*[48]

was different than the source used in this thesis, and this may have played a role in the infectivity potential of the virus.

Although our conclusion is different from that drawn by Tucker *et. al.*[48], our results are supported by Degré *et. al.*[47]. In this report, the authors concluded that "the possibility of cross-species infectivity of human CMV to porcine cells" exists. Specifically, Degré *et. al.*[47] showed that human CMV productively infected primary porcine endothelial cells (PPEC) *in vitro*. Similar to our methodology, the PPEC in Degré's experiments were infected with human CMV and then maintained in liquid medium for two weeks. This infection resulted in "distinct morphological alterations", which included substantial cell swelling and nuclear inclusions that were visible after 10 to 14 days post-infection[47]. This observation was strikingly similar to the PCMV-generated CPE noted in HFF when they were maintained in culture with liquid medium (discussed previously). Furthermore, Degré *et. al.*[47] found that the number of PPEC infected with human CMV was directly related to concentration of the virus inoculate, which is similar to our conclusions that PCMV-generated CPE in HFF occurred in a dose-responsive manner related to viral concentration. Our conclusion regarding the *in vitro* cross-species infectivity of CMVs is also supported with a report published by Jurak *et. al.*[73]. The authors showed that rat CMV was able to infected a human cell line unable to induce apoptosis. In particular, the report shows a large plaque-like formation following inoculation with rat CMV on day 7 post-infection that is strikingly similar to the large areas of cell death observed in our experiments on day 7 post-infection (Figure 1, row E).

6.0 CONCLUSIONS

The research described in this thesis is the first account of PCMV productively infecting human cells *in vitro*. The overall goal of this research project was to explore the interactions of PCMV with human cells *in vitro*, and the work presented in this thesis represents the initial research completed in realizing this goal. The major hypothesis of this thesis was that PCMV had the ability to productively infect primary human fibroblasts *in vitro*. Experiments using primary HFF revealed that they were permissive for PCMV replication *in vitro*.

Initial CPE studies indicated that the cells were permissive for PCMV, as manifested by CPE that suggested viral replication and spread. This occurred in a dose-responsive manner, related to viral concentration. In PCMV-infected HFF, nested RT-PCR resulted in products that were identified over a one-month period at the expected size for PCMV DNA polymerase. Through DNA sequencing, the products were identified definitively as PCMV DNA polymerase, and this data verified that HFF were permissive for PCMV. The conclusion that PCMV productively infects HFF was further supported with Western blot data which showed that monoclonal antibodies specific for human CMV gB and pig serum presumed to contain anti-PCMV antibodies were able to detect PCMV proteins in infected HFF. Finally, assays completed with a mAb specific for human CMV gB and pig serum provided further evidence that HFF were permissive for PCMV by reducing the CPE and neutralizing infectious PCMV virions in the inoculum.

Because PCMV is phylogenetically closer to HHV-6, preliminary Western blot and neutralization studies were completed with a mAb specific for a HHV-6 membrane glycoprotein. Overall, no concrete conclusions could be drawn from these experiments.

Further research into the full infectivity potential of PCMV in its native host may provide some insight into its genetic relationship with HHV-6.

As discussed by Webby *et. al.*[147], "for a virus to emerge successfully in a human population, it must achieve two feats. The first feat is replication in human cells and the second is human-to-human transmission". The results of this research indicate that, in an *in vitro* setting, PCMV has the ability to replicate and spread in human fibroblasts. This finding re-enforces concerns that PCMV may have the ability to infect human cells following direct introduction into this species and has significant implications for human xenotransplantation with porcine-derived tissues and / or organs.

As argued by Lien and Platt[7], the advancement of medical technology combined with the resulting extension of life, and the growing demand for a life free of disability, will continue to increase the need for organ transplantation. Accordingly, this will widen the interest in a life-saving alternative to allotransplantation. With continued, regulated research, xenotransplantation has the potential to help alleviate the current and anticipated demand for replacement organs. The research presented in this thesis has helped answer questions concerning the safety of xenotransplantation in terms of zoonosis and will help to provide solid evidence for regulatory bodies to make educated decisions concerning the clinical use of xenotransplantation.

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8.0 APPENDIX I

SEQUENCES AND MEGABLAST MATCH HITS OF RT-PCR PRODUCTS.

Nested round RT-PCR products amplified with RNA extracted from PT-K75 cells infected with live PCMV, with RNA extracted from PCMV-positive porcine turbinate cells (positive control), and with RNA extracted from HFF infected with live PCMV were sequenced in order to confirm that the resulting product(s), expected to be 160 bp[135], were PCMV DNA polymerase.

The nested product of the RT-PCR reactions that used RNA extracted from PT-K75 cells infected with live PCMV and RNA extracted from PCMV-positive porcine turbinate cells (Figure 2, yellow box) were sequenced to result in a 173 bp product (Figure 11A). By NCBI MegaBLAST searching, the sequence matched 98% with the PCMV DNA polymerase gene from PCMV isolate B6 (Figure 11B).

The nested product of the RT-PCR reactions that used RNA extracted from HFF infected with live PCMV on day 9, 12, 15, 18, 24, and 27 post-infection (Figure 3A, yellow boxes) were sequenced to result in a 166 bp product (Figure 12A). Nested round PCR product on day 21 and day 30 post-infection could not be sequenced due to too little product available after purification. By NCBI MegaBLAST searching, the sequence matched about 94% with the PCMV DNA polymerase gene from many isolates (Figure 12B).

The RT-PCR artifact found in an internal experimental blank (Figure 3A, purple box) for reactions that were used to detect PCMV RNA in HFF infected with live PCMV was also sequenced. The product was determined to be 138 bp (Figure 13A), and by NCBI MegaBLAST searching, the sequence did not match any known sequence found in the NCBI database (Figure 13B).

Figure 11: Sequence and MegaBLAST Match of Nested RT-PCR Products Amplified with RNA Extracted from PT-K75 Cells Infected with Live PCMV and with RNA Extracted from PCMV-Positive Porcine Turbinate Cells. [see Figure 2, yellow box]. Briefly, PT-K75 cells were seeded in T-75 flasks, grown to confluent monolayers, infected with either live PCMV or an appropriate control, and maintained in culture. PCMV-positive porcine turbinate cells were seeded in T-75 flasks and grown to a confluent monolayer. Total RNA was extracted from one T75 flask on various days post-infection or when the flask was confluent, respectively. To identify PCMV specific nucleic acid, nested RT-PCR reactions were performed with primers specific for PCMV DNA polymerase. The resulting nested PCR products were purified, sequenced, and NCBI MegaBLAST searched for any matching sequences.

(A) 173 bp sequence, representative of the two nested round RT-PCR products sequenced. One product that was sequenced was amplified with RNA extracted from PT-K75 cells infected with live PCMV on day 12 post-infection and the other was amplified with RNA extracted from PCMV-positive porcine turbinate cells.

(B) Top two NCBI MegaBLAST matches to the sequence.

(A) GGAACGSGTTGTTGATAAAGTCACTCGTCTGCCTAAGCATGTCCCGCCCT
ATGCGGTCMACTCGCCGCNGGGGRAGAARGAGAGACGG:TAGCAGACCGT
GTTTC:CCTCGCCCGTGAAGCCGTTTTTTCTTCCCCCNNNGGGGGGGGGGG
GGGGGAAAAAAAAAAAARAAAAA

(B) NCBI MegaBLAST search of sequence gives the following hits:

```
# BLASTN 2.2.14
# Database: nr
# Fields: subject ids: % identity, alignment length, mismatches,
          evalue, bit score
# 2 hits found:
  I)  2706629|emb|AJ222640.1|PCAJ640:  98.00, 50, 0, 3e-14, 86.1
  II) 17865441|gb|AF268039.2|AF268039: 98.00, 50, 0, 3e-14, 86.1
```

Identity of Hits:

I) AJ222640 = Porcine Cytomegalovirus gene encoding DNA polymerase (partial)
gi|2706629|emb|AJ222640.1|PCAJ640[2706629]

II) AF268039 = Porcine cytomegalovirus strain B6 ORF 40-like protein gene, partial cds; and glycoprotein B (gB) and DNA polymerase (pol) genes, complete cds
gi|17865441|gb|AF268039.2|AF268039[17865441]

Figure 12: Sequence and MegaBLAST Match of Nested RT-PCR Products Amplified with RNA Extracted from HFF Infected with Live PCMV. [see Figure 3A, yellow box]. Briefly, human fibroblasts were seeded in T-75 flasks, grown to confluent monolayers, infected with either live PCMV or an appropriate control, and maintained in culture. Total RNA was extracted from one T75 flask approximately every 3 days. To identify PCMV specific nucleic acid, nested RT-PCR reactions were performed with primers specific for PCMV DNA polymerase. The resulting nested PCR products were purified, sequenced, and NCBI BLAST searched for any matching sequences.

(A) 166 bp sequence, representative of the sequenced nested-round RT-PCR products from day 9, day 12, day 15, day 18, day 24, and day 27 post-infection. All six products were amplified with RNA extracted from HFF infected with live PCMV.

(B) Top 7 NCBI MegaBLAST matches to the sequence.

(C) Sequence alignment of nested-round 166 bp product [query] and PCMV DNA polymerase sequence, B6 "strain" (AF268039) [subject]

(A) TACTTCTCTGACACGTATTCTCTAGACGAGAGGACATWGTTGATAAAGTC
ACTCGTCTGCCTAAGCATGTCCCGCCCTATGTCTCTGGKGATGGACGCC
GCTAGAGGGAGACWCGGCAGCAGACCGTNNNACGGCTCCCGTGAAGC
CGTAAAACGCATTGCACGTA

(B) NCBI MegaBLAST search of sequence gives the following hits:

BLASTN 2.2.14
Database: nr
Fields: subject ids: % identity, alignment length, mismatches, e value, bit score
7 hits found:

I)	5545334|dbj|AB029823.1:	93.90, 164, 6, 2e-66, 259
II)	2706629|emb|AJ222640.1|PCAJ640:	93.90, 164, 6, 2e-66, 259
III)	17865437|gb|AF268041.2|AF268041:	93.90, 164, 6, 2e-66, 259
IV)	17865441|gb|AF268039.2|AF268039:	93.90, 164, 6, 2e-66, 259
V)	17865439|gb|AF268040.2|AF268040:	93.90, 164, 6, 2e-66, 259
VI)	8810237|gb|AF268042.1|AF268042:	93.90, 164, 6, 2e-66, 259
VII)	6552722|gb|AF191044.1|AF191044:	92.50, 120, 5, 1e-42, 180

Identity of Hits:

I) AB029823 = **Porcine cytomegalovirus OF-1** gene for DNA polymerase

II) AJ222640 = **Porcine Cytomegalovirus** gene encoding DNA polymerase

III) AF268041 = **Porcine cytomegalovirus** strain **OF-1** glycoprotein B and DNA polymerase (DPOL) genes

IV) AF268039 = **Porcine cytomegalovirus** strain **B6** ORF 40-like protein gene; and glycoprotein B (gB) and DNA polymerase (pol) genes

V) AF268040 = **Porcine cytomegalovirus** isolate **55b** glycoprotein B (gB) and DNA polymerase (DPOL) genes

VI) AF268042 = **Porcine cytomegalovirus** glycoprotein B (gB) gene; DNA polymerase (DPOL) and ORF 37-like protein genes; and ORF 36-like protein gene

VII) AF191044 = **Porcine cytomegalovirus** DNA-dependent DNA polymerase gene

(C)

```
Query  2     ACTTCTCTGACACGTATTCTCTAGACGAGAGGACATWGTTGATAAAGTCACTCGTCTGCC  61
             ||||||||||||||||||||||||||||| ||||||| ||||||||||||||||||||||
Sbjct  5277  ACTTCTCTGACACGTATTCTCTAGACGAAAGGACATTGTTGATAAAGTCACTCGTCTGCC  5218

Query  62    TAAGCATGTCCCGCCCTATGTCTCTGGKGATGGACGCCGCTAGAGGGAGACWCGGCAGCA  121
             |||||||||||||||||||   |||| ||||||||||||||||||||| ||||||||
Sbjct  5217  TAAGCATGTCCCGCCCTATG---CTGGTGATGGACGCCGCTAGAGGGAGACACGGCAGCA  5161

Query  122   GACCGTNNNACGGCTCCCGTGAAGCCGTAAAACGCATTGCACGT  165
             ||||||   ||||||||||||||||||||||||||||||||||||
Sbjct  5160  GACCGT-GCACGGCTCCCGTGAAGCCGTAAAACGCATTGCACGT  5118
```

Figure 13: Sequence and MegaBLAST Match of Artifact Identified in Internal Experimental Blank for RT-PCR Reactions that were used to Detect PCMV mRNA in HFF Infected with Live PCMV. [see Figure 3A, purple box]. Briefly, human fibroblasts were seeded in T-75 flasks, grown to confluent monolayers, infected with either live PCMV or an appropriate control, and maintained in culture. Total RNA was extracted from one T75 flask approximately every 3 days. To identify PCMV specific nucleic acid, nested RT-PCR reactions were performed with primers specific for PCMV DNA polymerase. A reaction that contained no RNA was included as an internal experimental negative control. The resulting nested PCR products, including the internal blank was run in an agarose gel and detected with ethidium bromide. The resulting artifact identified in the internal experimental blank was purified, sequenced, and NCBI MegaBLAST searched for any matching sequences.

(A) 138 bp sequence of artifact identified in internal experimental blank.

(B) NCBI MegaBLAST match of sequence resulting from internal experimental blank.

(A) GGCAGACGAGTGACTTTATCTTTNTTCTCCTCTCGTCGNGAGAATACGTG
TCAGAGAAGTAATCTCCCGNNGGGCGGGGGGAGCACGGAGGCAGACCG
TTCTTTCGCTCCCGGGGAGAAAAAAAACGCATTGCACGTA

(B) NCBI MegaBLAST search of sequence gives the following hit:

```
BLASTN 2.2.14 [May-07-2006]
RID: 1154972406-18525-75849928018.BLASTQ4

Database: All GenBank+EMBL+DDBJ+PDB sequences (4,300,551
sequences; 17,917,232,271 total letters)
```

No significant similarity found.

```
Number of Sequences: 4300551
Number of Hits to DB: 221268
Number of extensions: 0
Number of successful extensions: 0
Number of sequences better than 10: 0
Number of HSP's better than 10 without gapping: 0
Number of HSP's gapped: 0
Number of HSP's successfully gapped: 0
Length of query: 138
```