Studies on Porcine Cytomegalovirus Relevant to Xenotransplantation

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by

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Abstract

Xenotransplantation of porcine organs into humans provides a potential solution to the current shortage of human donor organs, but raises considerable safety concerns including the potential transmission of porcine infectious agents, particularly viruses, to humans. Porcine cytomegalovirus (PCMV) is a betaherpesvirus, which causes generalised infection in newborn piglets. It is endemic in world pig populations including those of high health status, and is transmitted horizontally and *in utero*. PCMV represents a potential risk in xenotransplantation since its human counterpart, HCMV, is frequently transmitted from the organ donor and reactivates posttransplant, where it is associated with end-organ disease and graft rejection.

Qualitative and quantitative PCR assays were developed in order to investigate the quantity and organ distribution of PCMV in pigs being bred for xenograft organs, and to investigate when virus is acquired. PCMV was found to be a common infection in conventionally-reared CD55 transgenic Large White pigs, with virus detected in potential xenograft organs of adult pigs, and widely disseminated in tissues of 3-5 week-old piglets. Prospective monitoring of piglets from birth showed that virus was acquired post-natally, whilst no evidence of *in utero* infection was found in caesarean-derived, barrier-reared piglets. Although these results showed that PCMV could be eliminated from pigs being bred for xenograft organs, knowledge of antiviral susceptibility is essential as it would provide therapeutic options should clinical trials proceed. The effect of antiherpetics on the growth of PCMV in a pig fallopian tube cell line was determined by real-time PCR and indirect immunofluorescence (IIF). In this cell culture model, ganciclovir and cidofovir were the most effective against PCMV replication, however, some cytotoxicity was associated with the highest concentration of cidofovir tested. Finally, a recombinant protein-based serological assay for PCMV was developed, utilising PCMV glycoprotein B expression in a baculovirus system.

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Abbreviations

α1,2FT	α1,2-fucosyltransferase
α1,3GT	α1,3-galactosyltransferase
α2,3ST	α2,3-sialtransferase
αGal	Galα1-3Galβ1-4GlcNAc-R
AcMNPV	*Autographa californica* multiple nuclear polyhedrosis virus
AIDS	acquired immune deficiency syndrome
AlHV	alcelaphine herpesvirus
ALS	amyotrophic lateral sclerosis
AP	activator protein
AP	assembly protein
APS	ammonium persulphate
AVR	acute vascular rejection
AZT	zidovudine
BCMV	baboon cytomegalovirus
BLHV	baboon lymphotropic herpesvirus
BMT	bone marrow transplant
BNF	British National Formulary
bp	base pair
BSA	bovine serum albumin
C	cytosine
CC	consumptive coagulopathy
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
cm	centimetre
CMV	cytomegalovirus
CNS	central nervous system
CPE	cytopathic effect
CRP	complement regulatory protein
DAB	3,3'-diaminobenzidine
DAF	decay accelerating factor
ddGTP	dideoxyguanosine triphosphate
dH_2O	deionised water

DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpi	days post-infection
DR	direct repeat
E	early
EBV	Epstein-Barr virus
EC	endothelial cell
EC_{50}	50 % effective concentration
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EMCV	encephalomyocarditis virus
env	envelope
ER	endoplasmic reticulum
FCS	fetal calf serum
FDA	Food and Drug Administration
FeLV	feline leukaemia virus
FITC	fluorescein isothiocyanate isomer 1
g	glycoprotein
g	gram
G	guanosine
gag	group-specific antigen
gC	glycoprotein complex
GCV	ganciclovir
GnT-III	N-acetylglucosaminyltransferase III
GTP	guanosine triphosphate
H/h	human
HAR	hyperacute rejection
HCMV	human cytomegalovirus
HBV	hepatitis B virus
HEK	human embryonic kidney
HEPA	high-efficiency particulate
HERV	human endogenous retrovirus

HEV	hepatitis E virus
HHV	human herpesvirus
His	histidine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPMPC	cidofovir
hr	hour
HRP	horseradish peroxidase
HSV	herpes simplex virus
HTRC	human-tropic replication-competent
Hve	herpesvirus entry mediator
ICAM-1	intercellular adhesion molecule-1
IE	immediate early
IFN	interferon
Ig	immunoglobulin
IIF	indirect immunofluorescence
IL	interleukin
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase
kDa	kilodalton
kg	kilogram
L	late
LAT	latency-associated transcript
LB	Luria Broth
M	molar
mA	milliamp
MCMV	murine cytomegalovirus
MCP	major capsid protein
MCP	membrane cofactor protein
mC-BP	minor capsid binding protein
mCP	minor capsid protein
MEM	minimum essential medium
µg	microgram
mg	milligram

MHC	major histocompatibility complex
MIE	major immediate early
min	minute
µl	microlitre
ml	millilitre
µm	micrometre
µM	micromolar
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor κB
ng	nanogram
NK cell	natural killer cell
nm	nanometre
ORF	open reading frame
OvHV	ovine herpesvirus
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCMV	porcine cytomegalovirus
PCR	polymerase chain reaction
PCV	porcine circovirus
PERV	porcine endogenous retrovirus
PFA	foscarnet
PFT	pig fallopian tube
pfu	plaque forming units
PHS	Public Health Service
PLHV	porcine lymphotropic herpesvirus
pmoles	picomole
pol	polymerase
PRV	pseudorabies virus
PTLD	posttransplant lymphoproliferative disease
PVDF	polyvinylidene difluoride
QC	quantitative-competitive

R	correlation coefficient
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
s	second
SCID	severe combined immunodeficiency
SCP	smallest capsid protein
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SEW	segregated early weaning
SFM	serum-free medium
siRNA	short interfering ribonucleic acid
SLAM	signalling lymphocyte-activation molecule
SPF	specific-pathogen-free
T	thymidine
TAE	tris/acetate/EDTA
Taq	*Thermus aquaticus*
TBE	tris/borate/EDTA
TBS	tris buffered saline
TCS	tissue culture supernatant
TEMED	N,N,N',N' tetramethylethylenediamine
TFPI	tissue factor pathway inhibitor
TMB	3,3',5,5'-tetramethylbenzidine
TNE	tris/NaCl/EDTA
TPA	12-O-tetradecanoylphorbol 13-acetate
U	unit
UK	United Kingdom
UKXIRA	United Kingdom Xenotransplantation Regulatory Authority
UL	unique long
US	unique short
US	United States
UV	ultraviolet
V	volt

VGCV	valganciclovir
VZV	Varicella-zoster virus
v/v	volume to volume ratio
W	watt
w/v	weight to volume ratio
×g	relative centrifuge acceleration
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1: General introduction

1.1 Overview to xenotransplantation

1.1.1 Current organ shortages

Experimental transplantation for the treatment of organ failure was performed as early as the eighteenth century, although successful graft survival has only been achieved in the past 50 years. The first successful kidney and liver transplants were carried out in the United States in 1954 and 1967 respectively (Starzl, 2000; Starzl *et al*, 1963), while the first successful heart transplant was performed in South Africa in 1967 (Reitz, 2002). Following advances in tissue typing and immunosuppression, transplantation has become the favoured treatment for the failure of vital organs. In 2003, 25,457 whole-organ transplants were carried out in the US, while 2,222 were performed in the UK (The US Organ Procurement and Transplantation Network/Scientific Registry of Transplant Recipients (OPTN/SRTR) Annual Report, 2003; UK Transplant), with the most commonly transplanted organ being the kidney, although heart, liver, lung and pancreas transplants are also commonly performed (Figure 1.1). However, the increasing success of transplantation has lead to a chronic shortfall in human donor organs. This is illustrated in Figures 1.2 and 1.3, which show that the number of people awaiting solid-organ transplants in the US and UK has increased dramatically over the past 10 years, while the number of transplants being carried out has been limited by the number of available donor organs. Such chronic organ shortages mean that patients may wait a long time for a transplant, with many dying before a suitable donor organ is found. In the US in 2002, more than 6,000 patients were reported to have died while waiting for a transplant (The US OPTN/SRTR Annual Report, 2003). Since transplantation is becoming increasingly necessary in the treatment of many illnesses, this trend is likely to continue unless a solution can be found.

1.1.2 Potential solutions to the human organ shortage

Improvements in modern medicine mean that an increasing proportion of the human population is living into old age. With most people dying from cancer and heart disease, as well as a reduction in the number of deaths in road traffic accidents, fewer organs are becoming available for transplant. In addition, many potential donor organs are wasted because of a lack of public awareness of organ donation programmes, issues of consent,

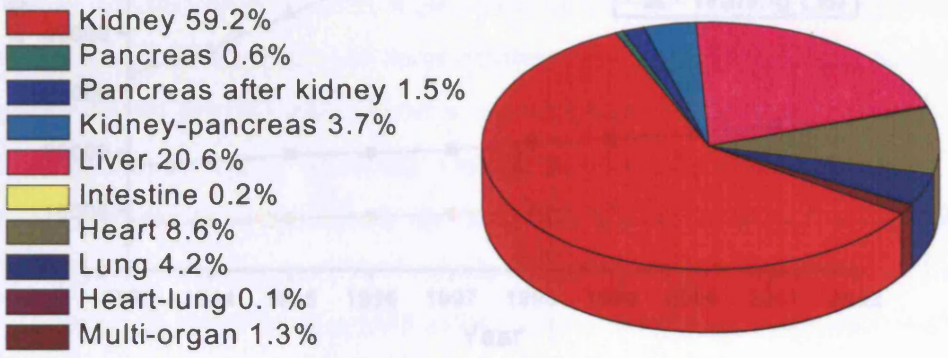


Figure 1.1. Transplants in the US in 2002 by organ type (N=24,544) (The US OPTN/SRTR Annual Report, 2003).

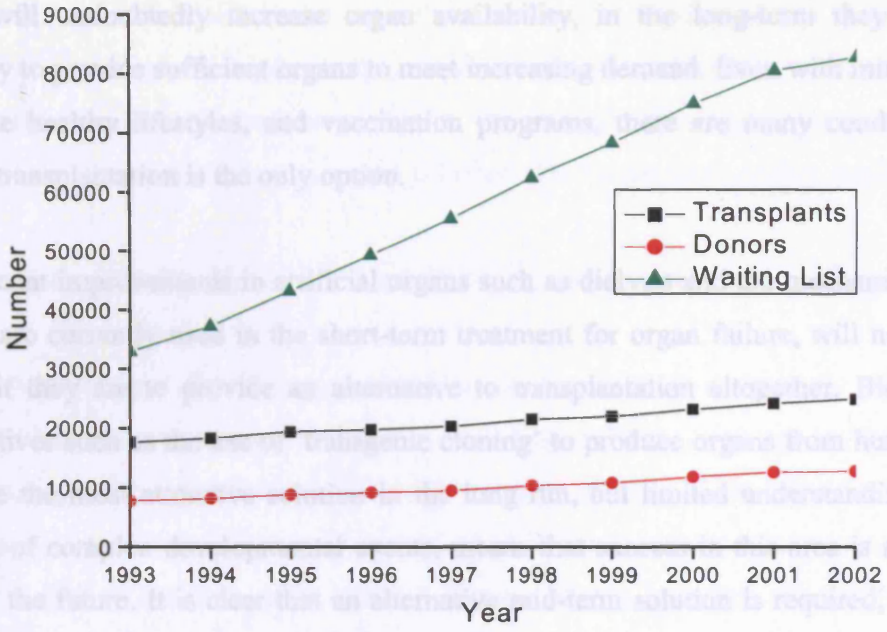


Figure 1.2. US waiting list and transplants 1993-2002 (The US OPTN/SRTR Annual Report, 2003).

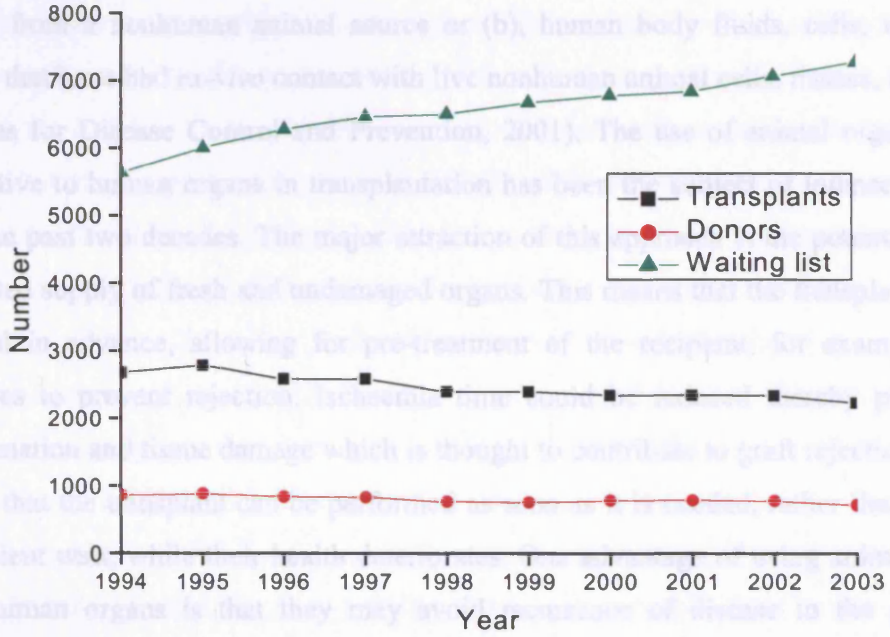


Figure 1.3. UK waiting list and transplants 1994-2003 (UK Transplant).

and problems associated with procurement of organs. While improvements in these areas will undoubtedly increase organ availability, in the long-term they are still unlikely to provide sufficient organs to meet increasing demand. Even with initiatives to promote healthy lifestyles, and vaccination programs, there are many conditions for which transplantation is the only option.

Significant improvements in artificial organs such as dialysis and the mechanical heart, which are currently used in the short-term treatment for organ failure, will need to be made if they are to provide an alternative to transplantation altogether. Bioartificial alternatives such as the use of 'transgenic cloning' to produce organs from human cells provide the most attractive solution in the long run, but limited understanding of the control of complex developmental events, means that success in this area is still some way in the future. It is clear that an alternative mid-term solution is required, with one possibility being xenotransplantation.

1.1.3 Xenotransplantation

Xenotransplantation is defined as any procedure that involves the transplantation, implantation, or infusion into a human recipient of either (a), live cells, tissues, or organs from a nonhuman animal source or (b), human body fluids, cells, tissues or organs that have had *ex-vivo* contact with live nonhuman animal cells, tissues, or organs (Centres for Disease Control and Prevention, 2001). The use of animal organs as an alternative to human organs in transplantation has been the subject of intense research over the past two decades. The major attraction of this approach is the potential for an unlimited supply of fresh and undamaged organs. This means that the transplant can be planned in advance, allowing for pre-treatment of the recipient, for example, with therapies to prevent rejection. Ischaemia time could be reduced thereby preventing inflammation and tissue damage which is thought to contribute to graft rejection. It also means that the transplant can be performed as soon as it is needed, rather than making the patient wait, while their health deteriorates. One advantage of using animal organs over human organs is that they may avoid recurrence of disease in the graft, for example, they may be resistant to viruses such as human immunodeficiency virus (HIV) or hepatitis B virus (HBV), already present in the recipient. In addition, animal donors can be genetically manipulated to improve organ acceptability and biochemical

functioning within the host, and through breeding techniques it may be possible to reduce the risk of transmission of animal pathogens. Although the definition of xenotransplantation encompasses a whole range of therapies including cellular xenografting and extracorporeal perfusion, the use of solid-organ xenografts is the 'Holy Grail' of xenotransplantation and will therefore be the focus of this thesis.

1.1.4 Experience with clinical solid-organ xenotransplantation

Several attempts at solid-organ xenotransplantation were made early in the 20th century using kidneys from various mammals including rabbits, pigs, goats, sheep and nonhuman primates (Table 1.1). Although in most of these cases there was no graft function, and grafts were lost to thrombosis within hours, patient survival was reported up to 16 days (Reemtsma *et al*, 1964b). Interest in this approach diminished after it became clear that both xenografts and allografts were subject to a powerful immune response, which resulted in graft failure.

In the 1960s, following demonstration of the effectiveness of immunosuppressive drugs, including azathioprine and corticosteroids to prevent rejection, there was renewed interest and success with human-to-human allotransplantation. However, problems associated with organ procurement and preservation lead to a shortage of human organs, and so the possibility of using animal organs was again explored. Hearts, livers and kidneys from animals were transplanted into human patients, although the greatest success in terms of graft survival was achieved with kidney xenografts. Of note, are the attempts by the groups of Reemtsma and Starzl. Reemtsma *et al* performed a series of transplants using kidneys derived from chimpanzees, with grafts surviving on average between 11 days to 2 months, with one patient surviving 9 months (Reemtsma *et al*, 1964a; Reemtsma *et al*, 1964b). Starzl *et al* transplanted kidneys from baboons with graft survival ranging from 19-60 days (Starzl *et al*, 1964). The majority of deaths in all cases were related to rejection or infection, often resulting from over-immunosuppression. The results of these studies suggested that chimpanzees, rather than baboons, provided the most suitable source of nonhuman primate donor organs, since the increased disparity between baboons and humans meant that their organs were rejected more aggressively than those from chimpanzees. Starzl also transplanted chimpanzee livers into three patients, but survival was limited to 9 days (Taniguchi &

Table 1.1. World experience in clinical solid-organ xenotransplantation (Taniguchi & Cooper, 1997).

Year	Surgeon	Donor	Number	Patient survival
RENAL				
1905	Princeteau	Rabbit (kidney slices)	1	16 days
1906	Jaboulay	Pig	1	3 days
		Goat	1	3 days
1910	Unger	Monkey	1	2 days
1913	Schonstadt	Monkey	1	?
1923	Neuhof	Sheep	1	9 days
1964	Reemtsma	Chimpanzee	12	<9 months
		Monkey	1	10 days
1964	Hitchcock	Baboon	1	5 days
1964	Starzl	Baboon	6	<60 days
1964	Hume	Chimpanzee	1	1 day
1964	Traeger	Chimpanzee	3	<49 days
1965	Goldsmith	Chimpanzee	2	<4 months
1966	Cortesini	Chimpanzee	1	31 days
HEART				
1964	Hardy	Chimpanzee	1	<1 day
1968	Cooley	Sheep	1	<1 day
1968	Ross	Pig	1	<1 day
1969	Marion	Chimpanzee	1	<1 day
1977	Barnard	Baboon	1	<1 day
		Chimpanzee	1	4 days
1984	Bailey	Baboon	1	20 days
1992	Religa	Pig	1	1 day
LIVER				
1966	Starzl	Chimpanzee	1	<1 day
1969	Starzl	Chimpanzee	2	<2-9 days
1969	Bertoye	Baboon	1	<1 day
1970	Lerger	Baboon	1	3 days
1970	Marion	Baboon	1	<1 day
1971	Poyet	Baboon	1	<1 day
1971	Motin	Baboon	1	3 days
1974	Starzl	Chimpanzee	1	14 days
1992	Starzl	Baboon	1	70 days
1993	Starzl	Baboon	1	26 days
1993	Makowka	Pig	1	<2 days

Cooper, 1997). In the 1970s, great apes including chimpanzees, were recognised as endangered species, limiting their use in xenotransplantation. In addition, at this time, alternative therapies such as dialysis and cadaveric transplantation became available, and since xenograft rejection could not be controlled by the available immunosuppression, the procedure was stopped.

In the past 20 years, since the advent of the immunosuppressive agent cyclosporin A, allotransplantation has become increasingly successful. In 1984, cyclosporine therapy was given when a baboon heart was transplanted into a 13-day-old patient with congenital heart disease (Bailey *et al*, 1985). The heart functioned for 20 days before the organ was rejected, probably, in part, due to ABO blood group incompatibility between the donor and recipient. This is currently the longest survival time for a clinical heart xenograft. This case was the start of renewed efforts in clinical xenotransplantation (Czaplicki *et al*, 1992; Starzl *et al*, 1993; Makowka *et al*, 1994). Having been refused a human organ, Starzl *et al* transplanted a baboon liver into a male patient with HBV and HIV infection (Starzl *et al*, 1993). Although there was little evidence of rejection, the patient died after 70 days from an infection resulting from over-immunosuppression. Since 1993, no more solid-organ xenotransplantations have been performed.

1.1.5 Experience with clinical cellular, tissue and extracorporeal xenografting

Although there has been little activity with solid-organ xenotransplantation in the past decade, throughout this time several hundred patients have participated in clinical trials using cellular, tissue or extracorporeal xenografts (Council of Europe, 2000; Groth *et al*, 1994; Buchser *et al*, 1996; Aebischer *et al*, 1996; Deacon *et al*, 1997; Schumacher *et al*, 2000; Levy *et al*, 2000; Pascher *et al*, 2002). The cellular xenografts used in these studies have been derived from a variety of animals (Table 1.2), and have been used in the treatment of diabetes, Parkinson's disease, Huntington's chorea, stroke, pain associated with cancer or neurologic pain, spinal injuries, epilepsy, amyotrophic lateral sclerosis (ALS), and acquired immune deficiency syndrome (AIDS), while tissue xenografts have been used in the treatment of burns. The results of these studies demonstrate long-term survival (months) of xenogeneic cells in humans without a significant immune response, however, they provide little conclusive data on the efficacy of the treatments (Groth *et al*, 1994; Buchser *et al*, 1996; Aebischer *et al*, 1996;

Table 1.2. World experience in clinical cellular xenografting during the 1990s (Council of Europe, 2000).

Graft	Indication	Number	Country
Neonatal bovine cromaffine cells	Pain	>100	Poland, Czech Republic, Switzerland & USA
Encapsulated transgenic hamster cells	ALS	6	Switzerland
Foetal porcine neurones	Parkinson's	21	USA
	Huntingdon's	12	USA
	Epilepsy	3	USA
	Stroke	3	USA
Foetal porcine islets	Diabetes	10	Sweden
Neonatal porcine islets	Diabetes	6	New Zealand
Foetal rabbit islets	Diabetes	Several 100	Russia
Baboon bone marrow	HIV	1	USA

Deacon *et al*, 1997; Schumacher *et al*, 2000). Extracorporeal perfusion of patient's blood or plasma through bioartificial organs, such as the HepAssist device containing porcine hepatocytes, or whole pig organs, has been used in the treatment of liver failure. In particular, since 1964, 270 patients with acute, subacute or chronic liver failure have been treated with extracorporeal liver perfusion, using livers from six different species, with the pig being the most frequently used source (Pascher *et al*, 2002). Early cases were far from successful with long-term survival rates of 20 %, 10 % and 37 % in the late 1960s, 1970s and early 1980s, respectively. Long-term survival rates improved in the past decade (to 52 %), when the treatment was used for bridging until a suitable human organ was available for liver transplantation. In the most recent cases, porcine livers, transgenic for human (h) CD59 and hCD55, were used in two patients as a bridge to successful liver transplantation (Levy *et al*, 2000).

1.1.6 Donor animals

As previously described, xenografts have been derived from a variety of animal sources including rabbits, goats, and sheep, with the majority being derived from either pigs or nonhuman primates. The greatest success was achieved using organs from nonhuman primates, and in particular chimpanzees, because they were subject to a less aggressive immune response. However, there are significant problems associated with the use of nonhuman primate organs rather than those from other species. The close phylogenetic proximity of humans and nonhuman primates increases the risk of cross-species transmission of infectious agents, particularly viruses, including retroviruses (e.g. simian foamy virus and simian T-cell lymphotropic virus) and herpesviruses (Allan, 1999; Michaels, 2003). In addition, nonhuman primates are expensive and time consuming to breed. With great apes including chimpanzees being protected species, they can no longer be used in xenotransplantation. Although baboons and several other small monkey species are available, it has been suggested that certain organs from these animals, such as the heart, would be too small for use in full-grown adult humans, which would restrict their use to children and small adults (Taniguchi & Cooper, 1997).

For a variety of practical and ethical reasons the pig has been identified as the most suitable potential donor animal for xenotransplantation. They have a high reproductive capacity, with a short gestation and large litters, providing a potentially unlimited

supply of organs. In addition, they are relatively inexpensive to breed and maintain, when compared with nonhuman primates, and could be raised to obtain pathogen-free herds. Recent studies, to be discussed later, have shown that pigs can be genetically modified, for either prevention of rejection or therapeutic benefit. Porcine organs are also comparable to the size of human organs, and could therefore be used in both adults and children. Finally, the use of porcine organs, compared with those from nonhuman primates, is generally considered to be more ethical, since pigs have been farmed by humans for thousands of years.

1.1.7 Obstacles to xenotransplantation

In order to provide an adequate alternative to allografts, the xenograft must undergo successful engraftment, avoid immune destruction, and be physiologically compatible with the recipient. It is also important that the graft does not transmit infectious agents to the recipient. Currently, the three main barriers to successful xenotransplantation are immune rejection, physiological incompatibilities, and the risk of transmission of porcine infectious agents. Each of these obstacles will be discussed further in the following sections.

1.2 Immune Rejection

Rejection of transplanted tissues occurs upon revascularisation of the graft, when immune components in the circulation of the recipient, recognise and respond to foreign antigens expressed on the graft, and is the major hurdle to allotransplantation.

1.2.1 Rejection of allografts

The events involved in transplanting an organ, including tissue excision, transport and implantation, result in ischaemia and inevitable tissue damage, which initiate early proinflammatory responses. These activities promote immune surveillance, attracting T cells, monocytes, neutrophils and macrophages into the graft site, which are subsequently involved in the process of rejection (Orosz, 2002). This process comprises a series of events, clinically classified according to time frame and histology into; hyperacute, acute and chronic rejection.

Hyperacute rejection is mediated by pre-formed, circulating, graft-specific antibodies which bind to the endothelial lining of blood vessels of the graft, and lead to the activation of complement, coagulopathy and graft destruction within minutes to hours of reperfusion. These alloantibodies target either ABO blood group proteins, or foreign alloantigens which have been encountered previously during pregnancy, blood transfusion or a previous transplantation (Goldsby *et al*, 2000). If hyperacute rejection can be avoided, for example, by matching of blood groups, or pre-transplant screening of the recipient for the presence of circulating donor-specific antibodies, then the graft is susceptible to acute rejection in the weeks to months following transplant, predominantly mediated by T lymphocytes. T cells are initially activated by graft proteins in the lymph nodes and undergo proliferation, before being released back into the circulation where they are restimulated by alloantigens in the graft to secrete proinflammatory cytokines and carry out other effector functions (Orosz, 2002). Proliferation of B cells to produce graft-specific alloantibodies also contributes to acute rejection.

To some extent, donor immunogenicity and thus the degree of acute rejection, can be prevented by tissue-matching of the major human leukocyte antigens (HLA) between the donor and recipient. However, since a perfect match is not always possible, acute rejection is generally controlled by immunosuppressive drugs which inhibit T cell responses. The most widely used immunosuppressive agents include azathioprine and corticosteroids, both developed in the early 1960s, and cyclosporin A developed in the late 1970s (Humar & Matas, 2001). However, these are associated with a number of side effects including an increased risk of infection, particularly with viruses which are normally controlled by T cells. Depending on the genetic disparity between the donor and recipient, and the strength of immunosuppression, rejection can be delayed for months to years before the graft is subject to chronic rejection. Chronic rejection is characterised by a gradual thickening and blocking of the graft blood vessels, and a steady decline in graft function. It is thought to be initiated early posttransplant following mechanical, ischaemic (Hauet *et al*, 2001), immunological and pharmacological injury, and develop further through prolonged and repeated vascular stress, such as high blood pressure. Since there is no therapeutic treatment for chronic rejection, a second transplant is usually required.

1.2.2 Rejection of xenografts

The initial immune response directed against the xenograft is to some extent determined by the type of xenograft used and how it derives it's blood supply. While whole-organ xenografts initially induce a strong immune response as soon as the recipient's blood passes through the donor blood vessels, cellular xenografts, which derive their blood supply primarily via host vessels, encounter a delayed response, and can additionally be protected by encapsulation, or by implantation into privileged sites such as the brain. Since the use of whole organs from pigs is the goal of xenotransplantation, immune rejection will be discussed in the following sections with reference to solid-organ porcine xenografts.

An overview of solid-organ xenograft rejection, as studied in pig-to-nonhuman primate models, is shown in Figure 1.4. Briefly, the xenograft is subject to hyperacute rejection (HAR) within minutes of reperfusion of the graft, mediated by the binding of pre-formed xenoreactive antibodies to the porcine vascular endothelium, and resulting in the activation of complement and graft destruction within hours (Lawson & Platt, 1996). If HAR can be prevented, the xenograft is then subject to acute vascular rejection (AVR), characterised by the binding of elicited anti-donor antibodies and endothelial cell (EC) activation. If AVR is prevented, for example, through the induction of accommodation, then the xenograft will be subject to either cellular rejection, chronic rejection or long-term xenograft survival.

1.2.3 Hyperacute rejection

In the 1960s, it was demonstrated that vascularised grafts, transplanted between widely disparate species, suffered immediate destruction by a humoral immune response, which was similar to that seen in hyperacute rejection of ABO-mismatched allografts. Hyperacute rejection of xenografts occurs within minutes of revascularisation of the graft, and is characterised by extensive intravascular thrombosis and extravascular haemorrhage, leading to graft destruction within hours (Auchincloss & Sachs, 1998).

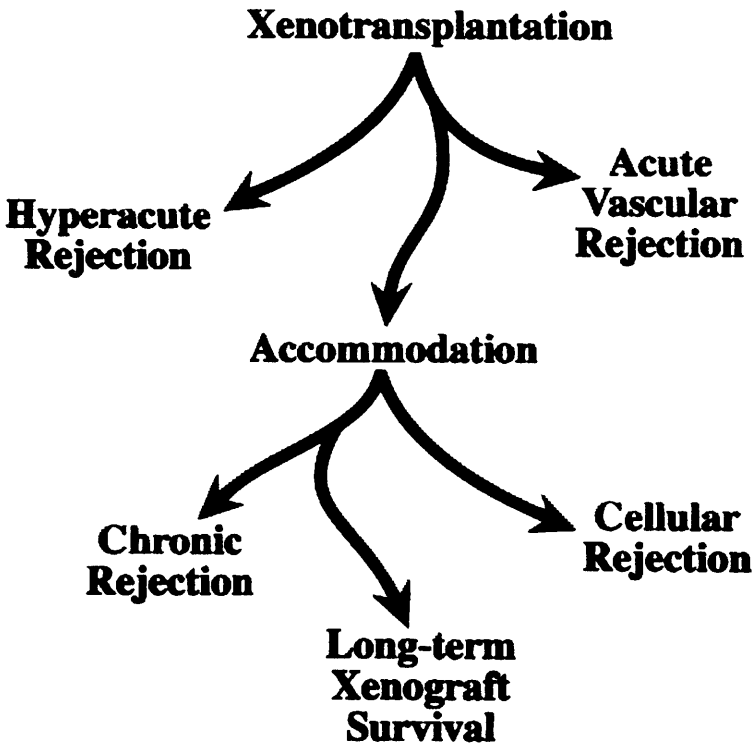


Figure 1.4. An overview of xenograft rejection, as studied in pig-to-nonhuman primate models (Lawson & Platt, 1996). Within minutes of reperfusion, the xenograft is subject to hyperacute rejection, mediated by preformed xenoreactive antibodies. If this can be prevented, for example, by the transient removal of such antibodies, then the xenograft is subject to acute vascular rejection when xenoreactive antibodies return. If acute vascular rejection can be prevented, for example, by the induction of accommodation, then the xenograft is subject to either cellular or chronic rejection, or long-term xenograft survival.

Initiation of HAR

Hyperacute rejection is initiated by the binding of pre-formed natural antibodies, to xenoantigen on the surface of pig cells. These antibodies predominantly recognise a single carbohydrate epitope, this being Galα1-3Galβ1-4GlcNAc-R (αGal), expressed on the cell-surface of lower mammals as well as many bacteria, viruses and parasites (Cramer, 2000), with approximately 10^7 αGal epitopes present on the surface of pig cells (Galili *et al*, 1988). The αGal epitope is synthesised by the α1,3-galactosyltransferase (α1,3GT) enzyme (Galili, 2001), which transfers a galactose molecule to the terminal N-acetyllactosamine of glycoproteins and glycolipids. Although this gene is present in humans, apes and Old World monkeys (those of Asia and Africa), it is thought to have been inactivated approximately 20 million years ago when apes and monkeys diverged, and is therefore not transcribed (Galili, 2001). Antibodies specific for this epitope therefore arise during the first few years of life, upon stimulation by the normal bacterial flora of the gut, and comprise approximately 5 % of circulating IgM antibody (Cramer, 2000; McMorrow *et al*, 1997).

Pathogenesis of HAR

The binding of xenoreactive antibodies to the αGal epitope results in the activation of complement through the classical pathway. The human complement cascade is normally regulated by both plasma and membrane-bound complement regulatory proteins (CRPs), which prevent autologous damage (Lachmann, 1991). However, since these regulatory proteins are species-specific, activation of the complement cascade, leads to the rapid assembly of terminal complement complexes on the porcine endothelium, resulting in type I endothelial activation, intravascular thrombosis, extravascular hemorrhage, edema and loss of the graft (Auchincloss & Sachs, 1998).

1.2.4 Overcoming HAR

Since HAR results from the binding of pre-existing xenoreactive antibodies to the αGal epitope and subsequent complement activation, manipulations which interfere with these processes could potentially be used to overcome this immune response. Approaches to overcome HAR are summarised in Table 1.3. Although temporary

depletion of xenoreactive antibodies can be achieved by plasmapheresis or immunoadsorption (Taniguchi *et al*, 1996; Xu *et al*, 1998), and complement activation can be prevented to some extent by the administration of cobra venom factor and soluble complement receptor type 1 (Candinas *et al*, 1996), the two main approaches to overcome HAR involve genetic manipulation of the donor.

Table 1.3. Approaches to prevent hyperacute rejection (Cascalho & Platt, 2001b).

Approach	Method
Depletion of xenoreactive antibodies	Column absorption
Inhibition of complement	Cobra venom factor, sCR1
Genetic engineering for the expression of complement-regulatory proteins	DAF, CD59, MCP
Genetic engineering to decrease antigen expression	Knockout α-1,3-Galactosyltransferase gene and possibly other genes in pigs

DAF, decay accelerating factor; MCP, membrane cofactor protein; sCR1, soluble complement receptor type 1.

Regulation of complement activation

Uncontrolled complement activation can be prevented by the transgenic expression of human membrane-bound CRPs on the surface of the porcine vascular endothelium. A number of successful attempts have been made to produce pigs transgenic for such proteins; including the decay accelerating factor (DAF), CD55 (Cozzi *et al*, 2000; Bhatti *et al*, 1999), membrane cofactor protein (MCP), CD46 (Diamond *et al*, 2001; Adams *et al*, 2001), CD59 (Fodor *et al*, 1994; Diamond *et al*, 1996) or combinations of these (Chen *et al*, 1999). Organs from these transgenic pigs were not hyperacutely rejected when transplanted into nonhuman primates, and have been shown to survive for approximately 1 month without evidence of HAR (Diamond *et al*, 2001; Garcia *et al*, 2004). This can be extended to 2.5 months by the administration of soluble αGal glycoconjugates, which 'mop up' the returning natural antibodies that cause AVR (Teranishi *et al*, 2003; McGregor *et al*, 2003).

Removal of xenoantigen

The use of competitive glycosylation, to replace αGal epitopes with other carbohydrate moieties, to which humans are immunologically tolerant, is one approach to remove xenoantigen. Transgenic expression of α1,2-fucosyltransferase (α1,2FT), which synthesizes the H antigen, has been shown to reduce αGal expression by competing with α1,3GT for its common substrate lactosamine (Sharma *et al*, 1996). Other enzymes which could be used in this manner to reduce αGal expression include sialyltransferases (e.g. α2,3ST) and N-acetylglucosaminyltransferase III (GnT-III) (Galili, 2001).

Complete removal of the αGal epitope using gene knockout techniques to remove the α1,3GT gene is a more attractive option, and was first demonstrated in mice using embryonic stem cell technology (Tearle *et al*, 1996). Since porcine embryonic stem cell lines are not available, nuclear transfer techniques were used instead to clone pigs for this purpose (Polejaeva *et al*, 2000). Heterozygous knockout pigs, where one copy of the α1,3GT gene was removed, were produced early in 2002 by two groups (Lai *et al*, 2002; PPL Therapeutics, 2002), and later that year, homozygous knockout pigs were produced (Phelps *et al*, 2003). Although it is too early to tell if there will be any improvement in graft survival, the first *in vivo* results presented at the International Xenotransplantation Association conference in September 2003, showed the survival of an α1,3GT knockout pig vascularised thymic lobe and kidney xenograft in a baboon for 68 days without any evidence of HAR or AVR (Yamada *et al*, 2003). The baboon died during an operation to replace an infected catheter and its death was therefore unrelated to rejection. In addition, α1,3GT knockout pigs expressing human α1,2FT have been produced (Ramsoondar *et al*, 2003). Xenografts from these animals may be more effective than the null phenotype alone in overcoming humoral rejection.

1.2.5 Acute vascular rejection

If HAR is prevented, the xenograft is subject to rejection within days to weeks by a predominantly humoral response called acute vascular rejection (AVR), and sometimes referred to as delayed xenograft rejection, or acute humoral xenograft rejection. Since HAR is now largely preventable through one or a combination of therapies previously described, AVR is currently seen as the major immunological hurdle in pig-to-primate

xenotransplantation. Beginning within 24 hours of reperfusion of the graft, it is characterised by the activation and swelling of endothelial cells lining blood vessels in the graft, focal ischaemia and diffuse intravascular coagulation (Cascalho & Platt, 2001a).

Initiation and pathogenesis of AVR

Increasing evidence suggests that AVR is initiated by elicited anti-graft antibodies (primarily directed against αGal), since it is accompanied by an increase in the synthesis of xenoreactive antibodies, and can be delayed or prevented by the removal of anti-αGal antibodies (Lin *et al*, 2000). The binding of these xenoreactive antibodies to the vascular endothelium of the xenograft is thought to result in either complement-dependent or independent EC activation (Dorling, 2003). Complement-dependent EC activation is associated with an infiltrate of chronic inflammatory cells, including lymphocytes, macrophages and natural killer (NK) cells. EC activation is accompanied by interleukin 1 (IL-1) expression (Saadi *et al*, 2004), and an increased expression of pro-inflammatory molecules including intercellular adhesion molecule-1 (ICAM-1), and pro-coagulant molecules, such as tissue factor and other regulators of thrombosis, as well as the loss of thrombomodulin (Auchincloss & Sachs, 1998). These changes give rise to thrombosis and ischaemia, characteristic of AVR.

Molecular incompatibilities involved in AVR

As with HAR, there is evidence of the involvement of underlying molecular incompatibilities between species in AVR, which may contribute to disseminated intravascular coagulation. The loss of protective anti-coagulation factors normally present on the surface of human blood vessels, and the intrinsic failure of porcine endothelium to control the human coagulation cascade, is likely to contribute to the strength of AVR. The human coagulation cascade is partly regulated by the expression of tissue factor pathway inhibitor (TFPI), but the pig equivalent of this molecule fails to interact appropriately with the human factor Xa target molecule (Robson *et al*, 2000).

1.2.6 Overcoming AVR

A key feature of AVR is the induced xenoreactive antibody response to the αGal epitope, and the subsequent activation of the xenograft vascular endothelium. Approaches to overcome AVR aim to block these processes and are summarised in Table 1.4. Although methods employed to remove the αGal epitope in overcoming HAR, such as competitive glycosylation or gene knockout technology, could be used to prevent the production of xenoreactive antibody in AVR, it is possible that there may be other non-αGal xenoantigens, targeted by xenoreactive antibodies, which contribute to HAR and AVR (Macchiarini *et al*, 1998; Zhu, 2000). Therefore, the most attractive approaches to overcome AVR involve the induction of accommodation or immunological tolerance to xenoantigen.

Table 1.4. Approaches to prevent acute vascular rejection (Cascalho & Platt, 2001b).

Method	Result
Pre-transplant infusion with donor haematopoietic cells	Tolerance to αGal and other xenospecific antigens
Knockout α-1,3-Galactosyltransferase gene and possibly other genes in pigs	Decreased antigen expression
Suppression of pro-coagulant or pro-inflammatory genes	Inhibition of endothelial cell activation
Transient depletion of xenoreactive antibodies	Induction of accommodation

Accommodation, defined as an acquired resistance to humoral-mediated injury, despite the presence of anti-donor antibodies and complement, has been demonstrated in transgenic pig-to-nonhuman primate xenotransplantation (Lin *et al*, 2000). Since it is thought to be initiated by EC expression of anti-apoptotic genes, stimulated by low levels of anti-graft antibodies (Bach *et al*, 1997; Soares *et al*, 1999), it may be possible to induce a state of accommodation within the xenograft through over-expression of such genes (Auchincloss & Sachs, 1998).

Induction of tolerance could be used to prevent the production of anti-αGal antibodies and other potential xenoreactive antibodies by B cells. This could be achieved by transplanting porcine bone marrow expressing the αGal epitope into the xenograft recipient (Yang *et al*, 1998; Cooper *et al*, 2002), or by transducing recipient bone marrow with a retroviral vector containing the α1,3GT gene. The latter approach has been demonstrated in α1,3GT knockout mice, to prevent the production of xenoreactive antibodies towards αGal (Bracy & Iacomini, 2000).

1.2.7 Cell-mediated and chronic rejection

If the humoral responses in AVR can be overcome, then it is likely that the xenograft will be susceptible to cellular immune responses, predominantly from T cells, which characterise acute allograft rejection. As yet, the T cell responses involved in pig-to-primate xenotransplantation are poorly understood due to the strength and speed of the humoral response in HAR and AVR. However, studies have suggested that both CD8+ and CD4+ T cells are capable of recognising porcine antigens by direct and indirect pathways (Cunningham *et al*, 1994; Yamada *et al*, 1995; Olack *et al*, 2000). Cell-mediated rejection of allografts is generally controlled through immunosuppression, but this approach is unlikely to overcome cellular rejection of xenografts without resulting in increased toxicity and susceptibility to infections. Alternative approaches to overcome the cellular response include the induction of T cell tolerance to xenoantigens, or genetic manipulation of the donor to down-regulate the cellular response. In particular, it may be possible to inhibit the CD8+ T cell response by down-regulation of porcine major histocompatibility complex (MHC) class I molecules on the surface of pig cells by transgenic expression of viral genes such as herpes simplex virus (HSV) ICP47 (Crew & Phanavanh, 2003).

Again, due to the strength of HAR and AVR chronic xenograft rejection is poorly characterised, but is likely to occur months posttransplant, and be similar to chronic allograft rejection. In theory, the extent of xenograft injury could be reduced by careful pre-transplant planning to reduce ischaemia time, however, since xenotransplantation has the potential to provide an unlimited supply of organs, a chronically-rejected xenograft could be replaced.

1.3 Anatomical and physiological incompatibilities

Some of the molecular immunological incompatibilities between pigs and humans have already been discussed with respect to rejection. Similarly, anatomical and physiological incompatibilities may pose problems in the functioning of the graft. Indeed, long-term survival is dependent on the ability of the xenograft to function in the human body. Since allografts differ only in immunological factors, such as HLA class I and II antigens, and are otherwise anatomically, physiologically and biochemically identical, this aspect of xenotransplantation poses a novel problem to scientists. There is currently little information regarding the impact of anatomical and physiological differences following solid-organ xenotransplantation due to short survival times of porcine xenografts in humans and nonhuman primates. However, problems associated with physiological incompatibilities in baboon-to-human xenografted kidneys and livers have been reported (Reemtsma *et al*, 1964b; Starzl *et al*, 1993).

1.3.1 Anatomical differences

Anatomical aspects such as the unique upright posture of man, and differences in organ size and position of blood vessels, may affect functioning of the xenograft. Posture influences blood circulation and the functioning of organs. For example, the size of the heart valves varies depending on whether the organ is in an upright or horizontal position (Hammer, 1998). Similarly, posture can influence blood pressure in the lung, which, along with other species-specific factors such as blood viscosity, size of blood cells and haemoglobin, may ultimately affect oxygen supply. The size of the organ can also affect homogenous blood pressure, which is essential for parenchymal organs. Both over and undersized organs, especially the heart and liver, can be affected (Hammer, 1998). In addition, anatomical differences may make surgery more difficult.

1.3.2 Physiological differences

In addition to anatomical disparity, differences in physiological factors such as blood circulation, viscosity and pressure, as well as biochemical elements such as electrolytes, serum albumin, hormones and enzymes, may affect proper functioning of the xenograft. The activity of hormones and enzymes needs to be carefully controlled, and they may

only function in a species-specific manner. In this respect, it is likely that the extent of physiological incompatibilities may be dependent on which type of organ is transplanted. The heart depends on a fairly small number of biochemicals to function; electrolyte disturbances will have a major impact, whereas the influences of other biochemical elements will be much smaller. This is in contrast to the liver, which is the most important metabolic organ of the body, producing 2500 different kinds of enzymes (Schraa *et al*, 1999). As with immunological hurdles, it may be possible to overcome some of these physiological incompatibilities through genetic engineering of the donor. Although, the true impact of anatomical and physiological incompatibilities is likely to remain unclear until the current immunological hurdles have been overcome.

1.4 The risk of transmission of porcine infectious agents to humans in xenotransplantation

1.4.1 The risk of zoonosis in xenotransplantation

Zoonosis is the transmission of infectious agents, such as bacteria, fungi, parasites, prions and viruses, between species, and can occur through many types of contact between humans and animals. In xenotransplantation, the potential for animal infectious agents to be transmitted to human xenograft recipients is sometimes referred to as xenosis. The clinical xenotransplantation protocol may facilitate this cross-species transmission, through intimate contact between species, via connection of the donor organ to the systemic circulation of the recipient, by local tissue ischaemia and the stimulation of inflammatory cytokines which may activate latent porcine viruses, and by the administration of potent immunosuppressive drugs.

Of all the potential infectious agents, which may be transmitted to humans in xenotransplantation, it is perhaps the viruses, which will present the greatest threat. Viral infections are a significant cause of long-term complications in allotransplantation and may be less amenable to therapy. In addition, viruses that are able to remain latent or which acquire new characteristics in the new host may be hard to detect. Such infectious agents may circulate within the population for some time before new clinical syndromes emerge, presenting a risk not only to the human xenograft recipient, but also to relatives, medical staff and the general population. Indeed, historic pandemics of

zoonotic human disease, including the 1918 influenza pandemic, and the current HIV/AIDS pandemic, highlight the potential zoonotic risk from xenotransplantation.

1.4.2 The effect of genetic manipulation of the donor on the risk of xenosis

Genetic manipulation of the donor organ to overcome immune rejection by the transgenic expression of CRPs and/or knocking-out α1,3GT, may increase the risk of viral infection. Evidence that some CRPs also act as receptors for human viruses, raises the possibility that xenografts from these transgenic animals may be susceptible to infection with human viruses. CD55 (DAF) has been shown to be the receptor mediating attachment and infection by several echoviruses, coxsackieviruses (B1, B3 and B5) and enteroviruses, all of which are human pathogens belonging to the picornavirus family (Bergelson *et al*, 1994; Ward *et al*, 1994; Shafren *et al*, 1995; Spiller *et al*, 2002). CD46 (MCP) has been shown to be the cellular receptor for both the Edmonston and vaccine strains of measles virus (wild-type strains use the signalling lymphocyte-activation molecule (SLAM)), and human herpesvirus (HHV) -6 (Dorig *et al*, 1993; Naniche *et al*, 1993; Santoro *et al*, 1999). More recently, CD46 has been shown to be a receptor for group B adenoviruses, which are associated with a variety of often fatal illnesses in immunocompromised individuals (Gaggar *et al*, 2003).

Since enveloped viruses such as retroviruses can be inactivated by complement, the transgenic expression of human CRPs on porcine cells, and incorporation of these proteins into the envelopes of viruses released from these cells, may protect them from attack by human complement. Initial studies showed that the transgenic expression of human CD59 on porcine cells resulted in incorporation of this protein into porcine endogenous retrovirus (PERV) particles produced from these cells, and inhibited complement-mediated virolysis (Takefman *et al*, 2002), but did not compromise the protective effects of human serum on the neutralisation of PERV.

Removing the αGal epitope from pig cells by gene-knockout technologies or reducing it's expression, by competitive glycosylation, may also allow enveloped viruses, such as gammaretroviruses, produced by these xenografts to evade neutralisation by human serum. PERV-B and feline leukaemia virus (FeLV) particles produced from porcine endothelial cells transduced with α1,2FT, α2,3ST or GnT-III, showed reduced

sensitivity to human serum (Kurihara *et al*, 2003). There is also a risk that human viruses may infect the xenograft, for example, human cytomegalovirus (HCMV) and HSV-1 and -2 have been shown to infect porcine endothelial cells *in vitro* (Degre *et al*, 2001; Leventhal *et al*, 2001).

1.4.3 Candidate porcine xenosis viruses

Porcine viruses have been categorised into groups according to the risk that they pose in xenotransplantation, these being; (i), those of known zoonotic potential, (ii), those having the ability to replicate in human cells or having some evidence for zoonotic potential, (iii), those with poor replication efficiency in humans but which have the potential to be oncogenic, (iv), those currently not defined as zoonotic but which have a wide host range, and finally (v), those which would be detrimental to the health of the herd or indicate a breakdown in biosecurity (Onions *et al*, 2000).

Porcine viruses categorised into the first group include; swine hepatitis E virus, swine influenza virus, paramyxoviruses such as Nipah virus, and encephalomyocarditis virus. Swine hepatitis E virus (HEV) (family *calicivirus*) is a recently identified virus, ubiquitous in ≥3 month-old pigs in the US (Meng *et al*, 1997). It was found to share 92 % nucleotide identity with a virus isolated from two US patients suffering from acute viral hepatitis, but only 74 % identity with other strains of human HEV, suggesting that swine HEV may have been transmitted to humans and be the cause of disease in these patients (Schlauder *et al*, 1998; Erker *et al*, 1999). Swine influenza A virus (family *orthomyxovirus*) is thought to be endemic in pigs, with infection commonly causing respiratory disease associated with fever, lethargy, coughing, and breathing difficulties (Olsen, 2002). Pigs are thought to act as 'mixing vessels' for human and avian influenza virus assortments that have been the cause of a number of influenza pandemics (Webster *et al*, 1992). However, viral sequences recently isolated from formalin-fixed, paraffin-embedded, lung tissue from a victim of the 1918 'Spanish' influenza pandemic, were found to be most closely related to early swine influenza strains (Taubenberger *et al*, 1997). More recently, in Malaysia, Nipah virus (family *paramyxovirus*) was the cause of fatal encephalitis among 28 people in close contact with pigs (Chua *et al*, 1999). Porcine encephalomyocarditis virus (EMCV) (family *picornavirus*) is endemic in many pig populations, causing acute myocarditis and sudden death in pre-weaned pigs,

and is associated with transplacental transmission resulting in foetal and neonatal deaths. EMCV infection, associated with fever, neck stiffness, lethargy, delirium, headaches and vomiting has been documented in humans, and the virus has been shown to infect human myocardial cells in culture (Brewer *et al*, 2001). In addition, porcine EMCV was transmitted to immunocompetent mice following transplantation of EMCV-infected pig islet cells to reverse diabetes, and resulted in acute EMCV disease 5 days following transplant (Brewer *et al*, 2004).

Other viruses, which have no known zoonotic potential but which are pathogenic in pigs, include porcine circoviruses (PCV) and coronaviruses. Although, PCV1 was first identified as a non-cytopathic contaminant of the pig kidney cell line PK15 and is non-pathogenic in pigs, PCV2 was identified as the etiologic agent of post-weaning multisystemic wasting syndrome in pigs (Nayar *et al*, 1997). PCV gene expression and replication has recently been shown to take place in human cells *in vitro*, however, infection was non-productive (Hattermann *et al*, 2004). Porcine coronaviruses are associated with diarrhoea, and respiratory and neurological syndromes in pigs, but do not appear to be zoonotic (Muir & Griffin, 2001).

1.4.4 Lessons from allotransplantation

Allotransplantation provides a useful model for the types of infection that are transmitted through organs and tissues, and therefore gives an indication of the types of organisms which may be transmitted in xenotransplantation. Micro-organisms such as bacteria, fungi, parasites, and viruses (including members of the herpesviruses, hepatitis B and C viruses, and HIV) have all been documented in recipients of allografts, and are a significant cause of posttransplant morbidity and mortality (Muir & Griffin, 2001). In the first month posttransplant, bacterial and fungal infections predominate. While later, following sustained immunosuppression, viruses such as HCMV, Epstein-Barr virus (EBV), hepatitis B and C viruses, and HSV, are a common cause of post-operative complications (Fishman & Rubin, 1998). Herpesviruses in particular, are a significant cause of end-organ disease and graft rejection in the posttransplant period, with HCMV long recognised as the most important pathogen affecting transplant recipients (Fishman & Rubin, 1998).

1.5 Xenotransplantation regulation and clinical protocols

1.5.1 Regulation of xenotransplantation

Considering the potential zoonotic risks involved, xenotransplantation requires close regulation and monitoring. This responsibility is carried out by a number of groups appointed in various countries, whose purpose is also to advise the government on issues relating to xenotransplantation. The United Kingdom Xenotransplantation Interim Regulatory Authority (UKXIRA) regulates xenotransplantation in the UK. Its roles are to provide a focal point for xenotransplantation activity and to consider applications for clinical trials to be undertaken in the UK. In addition, steering groups have been set up to monitor the production of xenografts and the long-term follow-up of xenograft recipients. At the time of the last UKXIRA annual report there were no clinical trials taking place in the UK (UKXIRA Fifth Annual Report, 2003). In the US, xenotransplantation is regulated by the Food and Drug Administration (FDA). In addition, guidelines have been drawn up by many health authorities in other countries including Canada, Australia, New Zealand, Japan and those of Europe, regarding health issues related to xenotransplantation (Sykes *et al*, 2003).

1.5.2 Guidelines for clinical trials

If xenotransplantation clinical trials are to proceed, protocols are needed regarding the generation of source animals, the clinical xenotransplantation site, pre- and post-xenotransplantation health screening and surveillance plans for source animals and xenograft recipients, and informed consent and patient education processes. To this end, the US Public Health Service (PHS) has set out guidelines on the infectious disease issues in xenotransplantation (Centres for Disease Control and Prevention, 2001). US PHS guidelines recommend that precautions to minimise the infection risk should be employed in all steps of the production of source animals for xenotransplantation, for example, during animal husbandry, procurement, and processing of xenotransplantation products. Source animals should be bred in closed herds, preferably using caesarian derivation, in facilities with adequate barriers to prevent the introduction and spread of infectious agents. These herds should then be actively monitored for infectious agents, including the individual source animal and the xenograft product itself. Sites of clinical

xenotransplantation should have accredited virology and microbiology laboratories which have the ability to isolate and identify unusual or newly recognised pathogens of human and animal origin.

The process of obtaining informed consent is also an important aspect of clinical trials. Relevant information regarding xenotransplantation should be provided to the patient, and made available to the family and contacts prior to, and at the time of consent. Patients should be informed of the potential for infection with both known and unknown zoonotic agents, and the potential for transmission of these agents to the recipient's family or close contacts. Patients additionally need to understand the importance of complying with long-term or life-long surveillance and the need for autopsy upon death.

Surveillance programs are important to monitor the introduction and propagation of xenogeneic infectious agents in the recipient, with the aim of detecting infection prior to dissemination into the general population. US PHS guidelines suggest that post-xenotransplantation surveillance include surveillance of the recipient(s), selected health care workers or other contacts, and the surviving source animal(s). Xenograft recipients should be monitored throughout their life-time, with regular evaluation immediately following xenotransplantation (i.e. every two weeks), and decreasing in frequency thereafter in the absence of any evidence of infection. A number of samples should be monitored including; serum, peripheral blood mononuclear cells (PBMCs), tissues and other body fluids. It is critical that adequate diagnostic assays and methods for surveillance of known infectious agents are available prior to initiation of the clinical trial. These assays should be specific, especially when xenogeneic viruses of concern have similar human counterparts. Surveillance methods should also be able to detect infectious agents known to establish persistent latent infections in the absence of clinical symptoms, such as herpesviruses, retroviruses, and papillomaviruses. Depending on the degree of immunosuppression, serological assays may or may not be of use in the surveillance procedure. All samples recovered during surveillance, or recovered from patients as a result of death or rejection of the xenograft, should be archived for 50 years beyond the date of xenotransplantation to allow retrospective analysis. In addition to surveillance, xenograft recipients and their contacts would be subject to strict behavioural procedures, including; refraining from donating body fluids and/or parts for use in humans, barrier contraception and refraining from having children.

1.6 Infection control: breeding pigs for xenograft organs

Experience from allotransplantation allows us to make reasonable predictions about the likely candidate viruses which may be transmitted to humans following xenotransplantation. As previously mentioned, and described further in section 1.10, the transmission of viruses, such as HCMV, within the allograft can have a considerable effect on the clinical outcome of the procedure. In allotransplantation, it is not always possible to exclude all serious pathogens due to the urgency of transplant, and the short supply of donor organs. However, in xenotransplantation, we are potentially able to have much greater control over the microbiological status of the donor, and this is especially important considering the risk of zoonosis. In light of the risks associated with zoonosis, all pigs destined for use as sources of organs for clinical xenotransplantation will need to be of an exceptionally high health status and free from a long list of porcine infectious agents (Onions *et al*, 2000). Indeed, the donor organ or 'xenograft product' will need to fulfil all of the safety specifications of a biological product. Comprehensive reviews of porcine infectious agents that should be excluded from donor pigs have been published (Onions *et al*, 2000; Muir & Griffin, 2001). Apart from the porcine equivalents of those viruses transmitted in human allotransplantation, other viruses that should be excluded from xenograft organs include; those which are known to be transmitted to and pathogenic in humans, those which are pathogenic in pigs, as well as those which have only been recently identified and therefore are poorly characterised (Onions *et al*, 2000). Pigs to be used in xenotransplantation should be bred from microbiologically characterised, inbred herds of domestic pigs, and some techniques used to control disease in commercial pig herds could be applied to the production of pigs for xenograft organs.

1.6.1 Disease control in commercial pig herds

In a commercial setting it is paramount to control infectious disease, since pig farming is often large-scale and infection can have a major economic impact on productivity. Infectious disease is most effectively controlled by altering breeding conditions. The production of high health status pigs often involves removing piglets from the sow and herd following early weaning, and is termed segregated early weaning (SEW) (Harris & Alexander, 1999). Piglets are protected from infection immediately after birth with strong humoral immunity passed to them from the sow's colostrum and milk. Removing

piglets from the sow and herd in the following days protects the piglets from infection when passive immunity fades. An even more effective, but costly method of producing high health status herds involves generating specific-pathogen-free (SPF) pigs, by caesarian delivery followed by gnotobiotic-rearing (Miniats & Jol, 1978). These SPF pigs are free from most infectious agents except those transmitted *in utero* (Harris & Alexander, 1999).

1.6.2 Breeding pigs for xenograft organs

A defined breeding method for the large-scale production of pigs for xenograft organs has been proposed by independent members of an advisory board (set up by Novartis Pharma AG), charged with advising on the microbiological safety criteria to be met before clinical trials of pig organ xenotransplantation (Onions *et al*, 2000), and is intended to maximise the health status and welfare of the herd while minimising cost. Similar recommendations for the exclusion of exogenous infectious agents have been set out by the US PHS (Centres for Disease Control and Prevention, 2001). Briefly, founder lines of transgenic SPF pigs such as those previously described, will be used to generate breeding herds. These breeding animals will be delivered by surgical techniques, such as hysterotomy or hysterectomy, and raised in gnotobiotic units for the first 2-3 weeks of life (Miniats & Jol, 1978), before being transferred to, and maintained for several months in high-welfare bioexclusion facilities, designed for the barrier-rearing of animals in closed herds. Surgical delivery of breeder animals, followed by gnotobiotic-rearing, is essential to exclude those potential infectious organisms transmitted peri- and post-natally, although those transmitted *in utero*, may still be passed on. The breeding herd may be maintained over a period of years in a barrier-rearing facility, and will be required for the production of subsequent source pigs, from which donor organs will be derived. Organ-source animals can either be delivered by hysterotomy of the breeding sow, followed by an initial period in isolators under gnotobiotic conditions, or can be naturally farrowed from breeding animals. This latter approach is preferable for the large-scale production of pigs for xenograft organs since it reduces costs and prevents the need to euthanise the sow. Following 5 days, naturally farrowed source animals can undergo SEW to minimise the transmission of infectious agents.

Whichever method is chosen for delivery and initial care, source pigs will be barrier-reared for several months in cohorts (litters) of approximately 10-20 animals, in strict isolation from the breeding herd, before being moved to a separate quarantine room approximately 1 month before excision of the organs. In the bioexclusion, barrier-rearing facility, breeding or organ-source animals will have no direct contact with pigs outside their cohort, but occasional contact with humans. Positive air pressure and high-efficiency particulate (HEPA) filtered air should exclude most airborne infectious agents, while water and feed will be sterilised, and contain no animal proteins, except milk-derived lactose for pre-weaned piglets. Antibiotics should be excluded to reduce the risk of selection for antibiotic-resistant bacteria, and vaccination of pigs should be avoided as it may be unreliable, may mask the clinical signs of infection and impair the ability to diagnose infection by monitoring the antibody response (Onions *et al*, 2000). A standard procedure to prevent microbial contamination during surgical harvest of porcine xenografts, equivalent to that used in an operating room for human graft retrieval, has also been evaluated (Tucker *et al*, 2004).

1.6.3 Monitoring the SPF status of source pigs

Although ideal, it would not be feasible to assess the SPF status of organ-source pigs by extensive microbiological evaluation of each animal on the day of harvesting organs, because of the time taken to perform all of the necessary tests. Therefore, the proposed microbial screening of cohorts and individual source animals involves routine monitoring, by physical examination and serologic testing, of all pigs in each cohort, soon after SEW, on transfer to the barrier-rearing facility, at 2 months of age, and on transfer to the final quarantine barrier (Onions *et al*, 2000). This would involve bacteriologic screening of nasal and faecal swabs, as well as virological testing for infectious virus, viral antigen, nucleic acid and/or antibodies. In addition, at two months of age and prior to the approval of the cohort for human clinical use of the organs, sentinel animals, representative of the microbiologic status of the entire cohort, would undergo necropsy including extensive bacterial and parasite testing, histopathological examination and virological testing of appropriate organs. At the time of organ excision, necropsy and sample archiving would allow retrospective confirmation of the SPF status of individual source animals.

The applicability of this system in terms of microbiological safety has been examined (Tucker *et al*, 2002a). The aim of this study was to evaluate the feasibility of producing pigs to recommended SPF specifications, and to evaluate the validity of the proposed cohort health monitoring programme. In this study, 9 cohorts were delivered by caesarian section, and reared for 14 days in isolator tanks under gnotobiotic conditions at the Ontario Veterinary College. Gnotobiotic pigs were then transferred to a barrier-rearing facility at the Toronto General Hospital and reared in closed herds for 4-5 months. These breeding techniques were shown to eliminate a wide range of bacterial, parasitic, fungal, and viral agents from transgenic pigs being bred for potential use in clinical xenotransplantation (Tucker *et al*, 2002a). In addition, the baseline clinical pathology and sizes of potential xenograft organs from these SPF pigs was determined (Tucker *et al*, 2002b). The high health status of these pigs was confirmed by low leukocyte counts and serum globulin concentrations, while the growth rates of these SPF pigs were shown to be above the targets currently set out for the commercial production of conventionally-reared pigs. The results obtained in this study will enable estimation and selection of the appropriate organ size prior to transplant (Tucker *et al*, 2002b).

1.6.4 Problematic viruses

Experience from allotransplantation has highlighted the porcine equivalents of retroviruses, herpesviruses and hepatitis viruses as potential candidate viruses which may be transmitted to humans following xenotransplantation (Fishman & Rubin, 1998). Retroviruses and herpesviruses in particular, cause latent, persistent infection, and will be difficult to eradicate from donor animals using the proposed breeding method described above, since they are transmitted *in utero* or in the germ-line (in the case of endogenous retroviruses). Therefore, the following sections will focus on porcine viruses of particular concern in xenotransplantation; these being porcine endogenous retroviruses and the porcine herpesviruses.

1.7 Porcine endogenous retroviruses

Retroviruses comprise a family of enveloped viruses (Table 1.5), with a unique life cycle. Upon entry into the host cell, the single-stranded RNA genome is transcribed into

double-stranded DNA, and integrated into the host cell chromosomes in the form of a provirus, which then serves as a template for viral RNA and protein production (Goff, 2001). Endogenous retroviruses result from integration of the provirus into the genome of germ-line cells, and as a result, are transmitted vertically through generations. The majority of endogenous retroviruses are inactive fossil remnants of ancient germ-line infections, and have undergone many mutations which render them defective, however some, particularly more recent endogenous proviruses can still give rise to infectious retroviruses (Patience *et al*, 1997b). The genomes of all the replication-competent retroviruses contain at least three large genes termed; group-specific antigen (*gag*), polymerase (*pol*) and envelope (*env*).

Table 1.5. Retrovirus genera (adapted from Goff, 2001).

Name	Example
Alpharetrovirus	Rous sarcoma virus
Betaretrovirus	Mouse mammary tumour virus
Gammaretrovirus	Murine leukaemia viruses
	Feline leukaemia virus
	Gibbon ape leukaemia virus
Deltaretrovirus	Human T-lymphotropic virus
	Bovine leukaemia virus
Epsilonretrovirus	Walleye dermal sarcoma virus
Lentivirus	HIV-1, HIV-2
Spumavirus	Human foamy virus

Porcine endogenous retroviruses (PERV) are gammaretroviruses that were first described in the 1970s as C-type particles released from porcine cell lines. Subsequently, two PERV subtypes A and B, capable of infecting human cells, were shown to be produced by the porcine cell line PK15 (Patience *et al*, 1997a). PERV-A and -B were shown to be highly homologous (92 % amino acid identity), and closely related to gibbon ape, feline, and murine leukaemia viruses (63-66 % amino acid identity) (Le Tissier *et al*, 1997). Sequences from a third subtype, PERV-C (also known as PERV-MSL), were then amplified from the lymphocytes of miniature swine

(Akiyoshi *et al*, 1998). These three viruses differ in the sequence of their *env* gene, host range and receptor interference (Takeuchi *et al*, 1998), while the *gag* and *pol* genes remain highly conserved. PERV-A and -B are found in the genome of all pigs tested so far, while PERV-C is only found in certain animals (Akiyoshi *et al*, 1998). In addition, PERV expression has been identified in a variety of pig strains and breeds, including SPF pigs and CD55 transgenic pigs, in tissues such as the kidney, lung, liver, heart, skin and pancreas (Martin *et al*, 1998; Clemenceau *et al*, 1999; Bosch *et al*, 2000; Langford *et al*, 2001), with PERV-A and -B more prevalent than PERV-C.

1.7.1 PERV tropism

With the revival of xenotransplantation in the 1990s the susceptibility of humans cells to PERV infection was examined. PERV-A and -B infection of the 293 human embryonic kidney (HEK293) cell line, following incubation with cell-free virus, was demonstrated by measurement of reverse transcriptase (RT) activity in supernatant and RT-PCR of PERV-specific RNA, while co-cultivation with PK15 cells resulted in a broader range of human cell infection (Patience *et al*, 1997a). In addition, a PERV-A/C recombinant (PERV-NIH), produced by mitotic stimulation of PBMCs of miniature swine, was found to infect HEK293 and HeLa cell lines (Wilson *et al*, 1998). Importantly, co-cultivation of HEK293 cells with pig primary aortic endothelial cells, which will form an interface between the xenograft and recipient, were shown, without mitotic stimulation, to result in productive infection of these human cells (Martin *et al*, 1998). Meanwhile, PERV infection of primary human endothelial cells, vascular fibroblasts and mesangial cells, provides further evidence of the risk posed by PERVs in xenotransplantation (Martin *et al*, 2000). Infection of human primary foreskin fibroblasts as well as nonhuman primate primary cells and cell lines has also be demonstrated (Blusch *et al*, 2000). Recently, two human PERV-A receptors (HuPAR-1 and -2) were identified and shown to be expressed on a wide range of human tissues including PBMCs (Ericsson *et al*, 2003).

In vivo experiments of PERV transmission have been limited to date. Cross-species transmission of PERV was demonstrated *in vivo*, following transplantation of porcine pancreatic islets cells into severe combined immunodeficiency (SCID) mice (van der Laan *et al*, 2000). The pig islets were shown to release infectious PERV that

productively infects human epithelial cells *in vitro*, and following transplantation of the islets into SCID mice, expression of PERV p30 Gag and spliced PERV *env* mRNA was detected. However, to date, no PERV mRNA or PERV-specific immune responses have been detected in nonhuman primate recipients of porcine xenografts (Switzer *et al*, 2001). In this study, 23 nonhuman primates were analysed for PERV infection following transplantation of either porcine heart or skin grafts, or encapsulated islet cells. All plasma samples tested negative for PERV RNA, and PERV antibodies. Similarly, PERV sequences were not detected in PBMC, spleen or lymph node samples from these animals.

1.7.2 The potential for PERV infection in xenotransplantation

Although pigs have been estimated to contain approximately 50 copies of PERV-A and -B, integrated into the genome (Patience *et al*, 1997a; Le Tissier *et al*, 1997), many of these are likely to be defective, owing to mutations introduced over many years. However, it is possible that mutations, genetic recombination or complementation between defective PERV copies, or other related endogenous viruses in xenograft recipients could give rise to infectious virus. Such chimeric PERV sequences have been detected in pig genomes (Lee *et al*, 2002; Oldmixon *et al*, 2002; Klymuik *et al*, 2003). One recombinant, PERV-NIH, containing receptor binding domains of PERV-A, and cell fusion domains of PERV-C, was generated by passaging of human-tropic PERV in HEK293 cells (Wilson *et al*, 2000). In addition to the three known envelope subgroups of PERV, other novel endogenous beta- and gammaretroviruses have been identified (Ericsson *et al*, 2001; Patience *et al*, 2001). In the xenotransplantation setting, activation of porcine endothelial cells by human cytokines could stimulate the release of PERVs, although *in vitro*, this stimulation was not mirrored by an increase in infectivity towards human cells (Cunningham *et al*, 2004). PERV adaptation or recombination with human endogenous retrovirus (HERV) sequences could potentially enhance human-to-human transmission, although a recent study suggested that the potential for recombination of PERV and HERV sequences was low (Suling *et al*, 2003).

In addition to the potential for recombination in xenograft recipients, genetic modifications of donor pigs and immunosuppression of the xenograft recipient may increase the risk of PERV infection. As described in section 1.4.2, the use of pigs

transgenic for human CRPs, or those which do not express the α-Gal epitope, may influence recognition and immune responses to enveloped viruses such as PERVs. In contrast to the work by Takefman *et al*, PERV produced from hDAF transgenic pig aortic endothelial cells were protected from inactivation by human serum (Magre *et al*, 2004), while PERV produced from α1,3GT knockout pigs was found to be resistant to inactivation by neutralising antibodies and human serum (Quinn *et al*, 2004). If transmission to humans did occur, PERV infection could result in a range of clinical syndromes, such as neoplasia, immunosuppression or autoimmunity, which have been associated with other types of endogenous retroviruses (Muir & Griffin, 2001).

1.7.3 Evidence of PERV transmission to human xenograft recipients

Retrospective analysis of human recipients of porcine foetal pancreatic islet and mesencephalic cells, skin grafts, and extracorporeal connection to porcine spleens, livers and kidneys, as well as bioartificial livers containing porcine hepatocytes, has revealed no evidence of transmission of PERV to humans, despite long-term microchimerism of up to 8.5 years in some patients (Heneine *et al*, 1998; Patience *et al*, 1998; Paradis *et al*, 1999; Pitkin & Mullon, 1999; Schumacher *et al*, 2000; Dinsmore *et al*, 2000; Levy *et al*, 2000). In the most comprehensive study, PBMC samples were collected from 160 patients, up to 12 years following treatment with various porcine xenografts (Table 1.6) (Paradis *et al*, 1999).

Exposure of patients' to porcine cells varied from minutes in the case of extracorporeal perfusion, to over a year in recipients of pancreatic islets. All samples tested negative for PERV DNA, RNA and PERV-specific antibodies by PCR, RT-PCR and protein immunoblot antibody assays, although samples from 23 patients receiving extracorporeal splenic perfusion indicated the presence of microchimerism. However, despite the positive results of these findings it is far from conclusive that PERVs will not pose a risk in xenotransplantation. The majority of subjects in these studies did not receive pharmacological immunosuppression, and those who did, received low doses. In most cases, the time that the recipient was exposed to the xenograft was very limited, reducing the potential for PERV transmission. In addition, human PBMCs were often analysed for evidence of PERV infection, and at present, studies of the susceptibility of these cells to PERV infection have yielded conflicting results (Wilson *et al*, 2000;

Table 1.6. Results of the Paradis *et al* study (Paradis *et al*, 1999), of PERV infection in patients treated with living pig tissue (adapted from Cunningham *et al*, 2001).

Patient numbers	Treatment	Exposure	PERV infection	Microchimerism present?	PERV RNA	PERV antibody response (anti-Gag)
1	Extracorporeal liver perfusion	4.25 hrs	Negative	NT	Negative	Negative
2	Extracorporeal kidney perfusion	15 and 65 min	Negative	NT	Negative	Negative
28	Bioartificial liver	11.7 hrs average	Negative	NT	Negative	1 positive[c] (preprocedural)
14	Pancreatic islets	1-460 days	Negative	NT	Negative	1 positive (preprocedural)
15	Skin grafts	10 days average	Negative	NT	Negative	Negative
100	Extracorporeal splenic perfusion	50-60 min	96 negative, 1 untested[a] 3 uninterpretable[b]	23 positive, 1 untested 76 negative	Negative	2 weak positive[d] (but negative for PERV DNA)

Microchimerism was present in samples from 23 patients. PCR results from three patients were uninterpretable. All samples were RT-PCR negative. Anti-Gag antibodies were found in four cases. These antibodies were considered to be cross-reacting, as all four samples tested negative for Env antibodies and in two cases were present prior to exposure to porcine cells (preprocedural). Thus, no evidence of infection with PERV was found in these patients.

[a] RT-PCR and antibody negative.

[b] Uninterpretable: the quantity of sample available was insufficient for retesting but patient was RT-PCR and antibody negative.

[c] Serum taken within 3 days of treatment, before antibodies could develop, also tested positive.

[d] Serum was positive at one (of two) testing centres only and PERV DNA or RNA was not detected in samples from these patients.

Specke *et al*, 2001). Furthermore, the majority of xenografts used were not derived from pigs transgenic for human CRPs, therefore it is not clear whether the expression of these proteins on pig cells will reduce the susceptibility of PERVs to inactivation by human complement. The only study to use xenografts from transgenic pigs involved extracorporeal liver perfusion in two patients for 6.5 and 10 hours, using pig livers transgenic for human CD55 (hDAF) and CD59, prior to successful allotransplantation (Levy *et al*, 2000). In this study, serial monthly PBMC samples taken up to 4 and 10 months following perfusion, tested negative for PERV DNA by PCR.

1.7.4 Overcoming the risk of PERV transmission

In order to prevent the transmission of PERVs to xenograft recipients, it would be ideal to create pigs which lack all human-tropic PERVs, i.e. PERV-A and PERV-B. Although PERVs are present in multiple copies in pig genomes, many of these are defective (Bosch *et al*, 2000; Herring *et al*, 2001), and as such it may be possible just to remove the critical human-tropic replication-competent (HTRC) PERV loci through selective breeding and gene knockout technologies. Indeed, pigs that fail to transmit PERV to human cells *in vitro* have already been reported (Oldmixon *et al*, 2002). In this study, those PERVs that were able to replicate in human cells were recombinants of PERV-A and -C, and these HTRC PERV-A/C recombinants were later found to be exogenous viruses that were absent from the genome of miniature swine (Wood *et al*, 2004; Scobie *et al*, 2004).

In addition to removing or inactivating PERV genomes, it may be possible to prevent PERV expression through the use of short interfering (si) RNA technology. This technology involves expression of siRNA sequences, which activate an innate cellular process that results, not only in the degradation of the invading siRNA molecule, but other identical single-stranded RNA sequences (Shuey *et al*, 2002). Synthetic siRNAs for the PERV *pol* region reduced expression of PERV p27 Gag protein in PERV-B-infected HEK293 cells by up to 90 % (Karlas *et al*, 2004). A further study demonstrating that porcine endothelial cells expressing siRNA from the *gag/pol* region of PERV, were less infective to HEK293 cells, suggests that siRNA technology could be important in reducing the risks posed by PERVs in xenotransplantation (Miyagawa *et al*, 2003).

If PERV infection did occur, either by direct infection or by recombination, it may be possible to inhibit virus with antiretroviral agents. The effect of antiretroviral drugs currently licensed for use in the treatment of HIV-1 on PERV has been evaluated (Qari *et al*, 2001; Wilhelm *et al*, 2002). Although PERV was sensitive to zidovudine (AZT) and dideoxyguanosine triphosphate (ddGTP), no activity was detected with the other RT and protease inhibitors tested.

1.8 Herpesviruses

1.8.1 Herpesvirus properties and classification

Herpesviridae are a family of large enveloped viruses comprising over 100 members, which are well adapted to their hosts, and highly disseminated in nature, infecting a wide range of vertebrates and some invertebrates. Although herpesviruses vary greatly in pathology and biology, they share four significant biological features. Firstly, they encode unique proteins involved in nucleic acid metabolism, DNA synthesis and the processing of proteins. Secondly, viral DNA synthesis and assembly of herpesviral capsids occurs in the nucleus. Thirdly, the production of progeny virus is invariably accompanied by cell death, and finally, they have the ability to establish life-long latent infection in specific host cells following primary infection (Roizman & Pellett, 2001).

Herpesviruses were divided into three subfamilies; *Alpha-*, *Beta-* and *Gammaherpesvirinae* according to biological properties, and more recent genome sequence analysis confirms similarities amongst viruses of the same subfamily. Alphaherpesviruses have a variable host range, short growth cycle spreading rapidly in culture, and are neurotropic, establishing latency primarily but not exclusively in sensory ganglia. Betaherpesviruses in contrast have a restricted host range, long reproductive cycle spreading slowly in culture with large infected cells (cytomegaly), and establish latency in secretory glands, lymphoreticular cells, kidneys and other tissues. Gammaherpesviruses infect lymphoblastoid cells *in vitro*, being specific for T or B lymphocytes, are associated with lymphoproliferative diseases, and establish latency in lymphoid tissue (Roizman & Pellett, 2001). All three subfamilies are believed

to have evolved from a common ancestor more than 200 million years ago (Figure 1.5) (Davison & Clements, 1998).

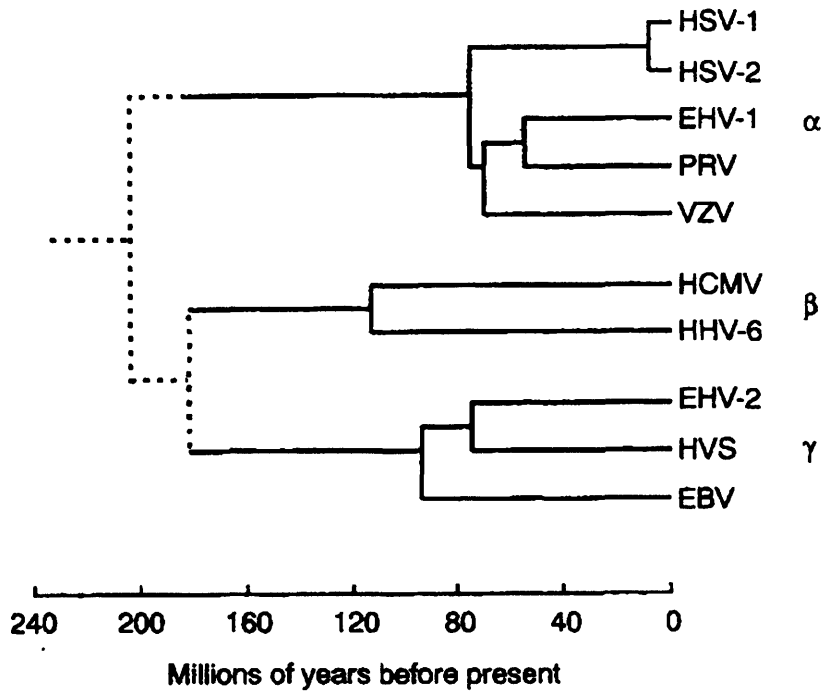


Figure 1.5. A phylogenetic tree for selected herpesviruses, derived from sequence comparisons (Davison & Clements, 1998). Broken lines indicate regions of lower confidence.

1.8.2 Porcine herpesviruses

So far, five herpesviruses have been identified in domestic pigs, these being; pseudorabies virus, porcine cytomegalovirus, and three recently identified lymphotropic herpesviruses -1, -2, and -3. Recently, an extensive search for unrecognised herpesviruses in commercial, experimental, and wild pigs from Germany, Spain, Sweden, France and the US, by pan-herpes consensus PCR, failed to identify further viruses (Chmielewicz *et al*, 2003a). Herpesviral DNA polymerase sequences were amplified using degenerate, deoxyinosine-containing primers, from blood and a range of tissue samples from 294 animals. Sequence analysis attributed all PCR products to the known porcine herpesviruses. However, five novel herpesviruses were recently

identified in suid species (other than the domestic pig, *S. scrofa*) from Africa and South-East Asia (Ehlers & Lowden, 2004). Three of these viruses were closely related to known porcine beta- and gammaherpesviruses, while two species were more closely related to bovine herpesvirus 4.

1.8.3 Pseudorabies virus

Pseudorabies virus (PRV), sometimes termed suid herpesvirus 1, is an alphaherpesvirus, classified alongside Varicella-zoster virus (VZV) in the genus *Varicellovirus*. It is an important veterinary pathogen, causing systemic and often fatal central nervous system (CNS) and respiratory disorders (Aujeszky's disease), in swine (Borie *et al*, 1998). PRV has a world-wide distribution, but has been eradicated from some areas, including the UK and Canada (Yoo & Giulivi, 2000; Muir & Griffin, 2001). In adult pigs, infection, although usually asymptomatic, can result in fever, growth arrest, as well as CNS, respiratory and gastrointestinal disorders. Meanwhile, infection in piglets is associated with fever, vomiting, diarrhoea, CNS involvement, and has a high mortality rate with death occurring 6-8 days following infection (Sawitzky, 1997). Virus is transmitted via oral or nasal routes, replicating in the oropharynx and spreading through the cranial nerves in the CNS. *In utero* transmission is also possible since infection in pregnant sows is associated with foetal deaths (Sawitzky, 1997). PRV is neurotropic, establishing latency in sensory ganglia, tonsils and lymphoid tissue, with occasional intermittent shedding of virus. Like HSV, PRV has a wide host range, infecting a variety of human cells *in vitro* (Sawitzky, 1997). It is also occasionally transmitted from pigs to cattle, sheep, goats, horses, dogs, cats, and wild animals (Muir & Griffin, 2001). PRV is thought to enter cells using the same cellular receptors as HSV, namely members of the nectin family including herpesvirus entry mediator (Hve) -B and -C (Campadelli-Fiume *et al*, 2000). Transmission to humans, resulting from close contact with domestic animals, has been reported to be associated with a variety of neurological symptoms, but is likely to be rare (Mravak *et al*, 1987). Since PRV is not common in high health status pigs and has been eradicated from pigs in the UK and Canada, it should be possible to exclude this virus from pigs being bred for xenotransplantation.

1.8.4 Porcine lymphotropic herpesviruses

Porcine lymphotropic herpesviruses (PLHV-1 and -2) are gammaherpesviruses classified in the genus *Rhadinovirus*, and were first detected in 1999 in porcine spleen and PBMC samples from German and Spanish pigs (Ehlers *et al*, 1999). The viral DNA polymerase sequences amplified from these samples showed highest amino acid identity with bovine gammaherpesviruses including alcelaphine herpesvirus 1 (AlHV-1), ovine herpesvirus type 2 (OvHV-2), and bovine lymphotropic herpesvirus (BLHV), and were prevalent in 87 % of PBMC, and 95 % of spleen samples from German pigs tested. Subsequently, the entire DNA polymerase gene of PLHV-1 and -2 was sequenced, and found to share 95 % amino acid identity with each other, and 60 % amino acid identity with AlHV-1 (Urich *et al*, 1999). These gammaherpesviruses are not pathogenic in the natural host, but cause serious lymphoproliferative disease in other species. In particular, AlHV-1 and OvHV-2 are non-pathogenic in their respective hosts, but cause malignant catarrhal fever, an often fatal lymphoproliferative and inflammatory disease, if transmitted to cattle and other animal species including pigs (Ensser *et al*, 1997; Albini *et al*, 2003). Both PLHV-1 and particularly PLHV-2 were prevalent in domestic and feral swine, suggesting the possibility of viral transfer between feral and domestic pig populations (Ulrich *et al*, 1999). More recently, a third lymphotropic herpesvirus PLHV-3 has been identified in blood and lymphoid organ samples from domestic and feral pigs from different geographic locations (Chmielewicz *et al*, 2003b), and is more distantly related to PLHV-1 and -2 than they are to each other.

Although PLHV appears not to be pathogenic in pig populations, it has been associated with a high incidence of posttransplant lymphoproliferative disease (PTLD) in miniature swine undergoing allogeneic haematopoietic cell transplantation (Huang *et al*, 2001), with a high mortality rate in these pigs. This PTLD in swine was morphologically and histologically similar to EBV-associated PTLD observed in human allotransplantation. PLHV sequences found in swine with PTLD, were later shown to be identical to those of PLHV-1 (Goltz *et al*, 2002), suggesting that PLHV-1 is active during PTLD and may be involved in the etiology of the disease. However, activation of PLHV-1 was not detected in baboon recipients of porcine thymokidney, kidney, thymus and heart xenografts (Mueller *et al*, 2004).

Although there is no evidence that PLHVs can infect humans, the fact that other gammaherpesviruses can cross the species barrier, and that PLHVs can cause lymphoproliferative diseases in the transplant setting, demonstrates that PLHVs will have to be eliminated from pigs being bred for xenograft organs.

1.9 Porcine cytomegalovirus

Porcine (P) CMV, sometimes termed suid herpesvirus 2, was first described by Done in 1955, as large basophilic intranuclear inclusions in cytomegalic cells, and was later classified as a betaherpesvirus. Considering that HCMV is frequently transmitted in the allograft, where it reactivates and is associated with end-organ disease and graft rejection (Fishman & Rubin, 1998), it is believed that the porcine equivalent, PCMV may pose a risk to human recipients of porcine xenografts. This risk was highlighted by the recent reported transmission of baboon (B) CMV to a human recipient of a baboon liver (Michaels *et al*, 2001). Replication-competent BCMV was isolated from the peripheral blood of the patient 4 weeks posttransplantation, and 10 days after discontinuation of ganciclovir (GCV) prophylaxis. The presence of BCMV was confirmed by BCMV-specific PCR analysis of a CMV-like virus isolated from human foreskin fibroblast and MRC5 human foetal lung fibroblast cultures, that had been inoculated with the patient's peripheral blood leukocytes. Replication-competence of this BCMV isolate was demonstrated by serial passage for up to five times in human foreskin fibroblast and MRC5 cells, and by plaque purification. The absence of BCMV in further blood samples from the patient following reinitiation of GCV therapy suggested that BCMV was susceptible to GCV. The patient died 70 days following xenotransplantation from an acute haemorrhage of the brain, secondary to disseminated aspergillus infection. No pathologic evidence of active CMV disease was seen at autopsy, and immunity to BCMV was not determined. BCMV is currently the only known infectious virus to have been transmitted to humans following xenotransplantation.

1.9.1 PCMV DNA sequence analysis

Although PCMV was initially characterised as a cytomegalovirus based on the identification of cytomegalic inclusions which are characteristic of CMV infections

(Mattes *et al*, 2000), recent sequence analysis of the DNA polymerase, glycoprotein B and major capsid protein genes of PCMV show that it is more genetically related to HHV-6 and -7, than to HCMV and murine (M) CMV. This was first suggested following phylogenetic analysis of partial sequences of the viral DNA polymerase gene of PCMV and comparison with other herpesviruses (Widen *et al*, 1999; Rupasinghe *et al*, 1999). Later, characterisation of the entire DNA polymerase gene of PCMV showed 48.2 % and 48.5 % amino acid sequence identity with HHV-6 and HHV-7 respectively, compared with 37 % identity with HCMV (Goltz *et al*, 2000). In addition, analysis of a German isolate and British and Japanese strains showed differences of 0.4-1 % on the nucleotide and amino acid level. Characterisation of the major capsid protein gene of PCMV showed 53.2 % and 51.9 % amino acid sequence identity with HHV-6 and -7 respectively, compared with 44.3 % and 46.8 % identity with HCMV and MCMV (Rupasinghe *et al*, 2001). More recently, the sequence of the glycoprotein B gene was determined and comparisons were made between different strains of PCMV and between other herpesviruses (Widen *et al*, 2001). Again, the highest amino acid sequence identities were found with HHV-6 and -7 (43.4 % and 42.6 % respectively). Strain differences of 0.6-1.7 % on a nucleotide level and 3.4 % at the protein level were found between five different PCMV strains and isolates from the UK, Germany, Spain, Japan and Sweden, indicating intra-species variation.

1.9.2 Epidemiology and transmission

PCMV is endemic in world-wide pig populations including those of high health status, with several strains and isolates of PCMV being identified. In the UK, serological evidence indicates that over 90 % of herds have been exposed to the virus (Edington, 1999). Whilst studies carried out in Japan and the Netherlands showed 99.4 % and 93 % of pigs were seropositive, respectively (Tajima *et al*, 1993; Rondhuis *et al*, 1980). PCR analysis of lung tissue and nasal scrapings of Canadian pigs showed that 59 % were positive for PCMV (Hamel *et al*, 1999).

Virus can be recovered from nasal and ocular secretions, urine, cervical fluids from primary infections in pregnant sows, and from the testis and epididymis (Edington, 1999). Virus is thought to be transmitted horizontally and *in utero*, with transmission

most commonly occurring via the nasal route (Plowright *et al*, 1976), but also by saliva and contaminated urine.

It has been reported that PCMV appears to be host-specific *in vivo* and *in vitro* and that the virus has failed to replicate in rabbits, mice, hamsters, chick embryos, and cattle (Edington, 1999).

1.9.3 Pathogenesis and clinical manifestations

In commercial pig herds infection occurs between 5 and 8 weeks of age, coinciding with the mixing of litters 3 weeks after birth (Plowright *et al*, 1976). Virus can be recovered from nasal secretions from 5 weeks of age, with antibody production detected between 8 and 11 weeks. Clinical features of PCMV infection vary depending on the age of the pig. In adults, infection is predominantly silent, although infected pregnant sows often appear anorexic and lethargic (Edington *et al*, 1977). Infection in foetuses and neonates leads to fatal generalised disease, with most piglets either being born dead or dying soon after birth (Edington *et al*, 1976a; Edington *et al*, 1976b; Edington *et al*, 1977). In older piglets, infection is characterised by rhinitis, respiratory distress and poor weight gain (Plowright *et al*, 1976).

From studies of experimental infections in piglets of varying ages, piglets infected with PCMV at less than 2 weeks of age showed clinical symptoms of disease. In one study where piglets were infected intranasally at 1 day of age (Edington *et al*, 1976b), viraemia was detected at 5-19 days post-infection (dpi). Three out of 6 piglets died within 5 weeks as a result of generalised PCMV infection, while in the remaining piglets infection was accompanied by poor growth. Findings were similar in a second study where piglets were infected intranasally with PCMV at 1 and 8 days of age (Edington *et al*, 1976a). Symptoms of PCMV infection were detected at 10-18 dpi, and most piglets infected at this age died or showed signs of dying as a result of PCMV infection. At necropsy there were widespread haemorrhages, particularly in the lungs and kidneys. PCMV was detected in many tissues, particularly reticulo-endothelial cells; lung macrophages, liver, spleen, lymph nodes, adrenal gland and capillary endothelium.

Where piglets were infected at greater than 2 weeks of age, piglets showed no clinical symptoms of disease. In piglets infected at three weeks of age, viraemia was detected at 14-16 dpi but all piglets remained healthy (Edington *et al*, 1976b). In addition, no clinical abnormalities were observed when piglets were infected at 14 and 16 days of age (Edington *et al*, 1976a). Intranuclear inclusions and cytomegaly were mainly found in epithelial cells, for example, in the mucous glands of the nasal mucosa and kidney tubule cells. Infection was also observed in the epithelial cells of salivary, lacrimal, and Harderian glands, mucous glands of the oesophagus, epithelial lining of the duodenum and hepatic cells.

Following these observations, the clinical manifestations of PCMV infection in neonates was termed fatal generalised disease, while manifestations in older piglets were termed asymptomatic generalised infection (Edington *et al*, 1976a).

1.9.4 Evidence for *in utero* infection

PCMV congenital infection was first reported by Raq in 1961, when characteristic intranuclear inclusion bodies and cytomegaly were observed in the nasal mucous glands of a day-old pig (Edington *et al*, 1977). Congenital infection has since been demonstrated following experimental PCMV infection of pregnant sows, with or without the presence of circulating antibody.

Six seronegative sows were intranasally infected with PCMV between 31 and 85 days of gestation (Edington *et al*, 1977). Eighteen out of 60 foetuses died during the gestation period, and evidence of PCMV infection was found in stillborn foetuses and other piglets, which died within a week of birth. There was no correlation between the stage of gestation in which sows were infected, and the number of foetal deaths. In a second study, two serologically positive sows were intranasally infected with PCMV at day 69 of gestation (Edington *et al*, 1988a). Four out of 24 foetuses died during gestation and virus was detected in various foetal tissues either by virus isolation or indirect immunofluorescence (IIF). Virus was detected in a further 4 apparently normal piglets. This suggests that PCMV can cross the placental barrier in spite of circulating antibodies. In a third study, congenital infection resulting from experimental infection of the sow, was demonstrated in the early stages of gestation. Nine sows from a SPF

herd were intranasally infected at day 2 of gestation (Edington *et al*, 1988b). Sows were sacrificed 28-33 dpi and embryos examined for evidence of PCMV infection. Seven out of 85 embryos were positive for PCMV by virus isolation and IIF. *In utero* infection seemed to correlate with the time of maternal shedding of the virus. From these results it is evident that infection *in utero* can occur at any stage of gestation, when sows are experimentally infected with PCMV. However, it is still unclear how common transplacental transmission is in a conventional setting.

1.9.5 Growth in culture

In vitro cultivation of PCMV has proved difficult. The virus has been grown in lung macrophages, but because these cells do not replicate, primary cultures must be obtained. More recently, PCMV has been shown to replicate in fibroblast- and epithelial-like pig fallopian tube (PFT) cell lines, designated 19-PFT-F and 19-PFT-E, with 19-PFT-F cells being more susceptible to PCMV (Kawamura *et al*, 1992). Virus is slow growing in these cells, with characteristic cytomegalic cytopathic effect (CPE) seen 14 dpi. However, the addition of the mitogen 12-O-tetradecanoylphorbol 13-acetate (TPA) to the culture medium was reported to increase the rate of virus replication in these cells, with CPE and cytomegaly observed 2 days after inoculation (Kawamura & Matsuzaki, 1996).

1.9.6 Detection and prevention

In the 1970s, when the majority of studies into the epidemiology and pathogenesis of PCMV infection were performed, PCMV was detected by virus isolation and culture of blood and swab samples in porcine lung macrophages, as well as by IIF of serum, and histopathology on tissue samples to determine the presence of cytomegalic inclusions (Plowright *et al*, 1976; Edington *et al*, 1976a; Edington *et al*, 1976b; Edington *et al*, 1977). In commercial pig herds, the presence of PCMV is most often confirmed by detecting antibody by IIF (Rondhuis *et al*, 1980), or enzyme-linked immunosorbent assay (ELISA) (Tajima *et al*, 1993). Recently, PCR assays have been developed to detect viral DNA. Primers in these assays have been used to amplify regions of the viral DNA polymerase (Widen *et al*, 1999; Hamel *et al*, 1999) and major capsid protein genes (Rupasinghe *et al*, 2001) of PCMV. Meanwhile, no specific treatment for PCMV

infection has been developed or used. This is perhaps because endemic PCMV infection does not present major economic problems in well-managed pig herds.

1.9.7 The potential for the transmission of PCMV in xenotransplantation

To assess the likelihood of the transmission of PCMV to humans in xenotransplantation, attempts have been made to determine whether PCMV can infect human cells *in vitro*. However, following co-cultivation of human B cells (Raji cells) and HEK293 cells with porcine alveolar macrophages infected with PCMV, no PCMV DNA was detected in the human cells by PCR (Tucker *et al*, 1999). To date, these are the only cells in which this type of study has been carried out. There are also no data available on human exposures to PCMV, since much of the recent research on the transmission of porcine viruses to human xenograft recipients has focused on PERVs.

Reactivation of latent PCMV has been demonstrated following administration of immunosuppressive agents in pigs. Administration of corticosteroids 56-62 dpi resulted in excretion of PCMV from gnotobiotic pigs 70 days following infection with PCMV and 38 days since the last detection of PCMV in nasal secretions (Edington *et al*, 1976b). In addition, administration of prednisolone in pigs inoculated with PRV resulted in reactivation of PCMV, with basophilic inclusion bodies seen in endothelial cells (Narita *et al*, 1985).

1.10 Human betaherpesviruses

At the time of initiating this thesis, PCMV was thought to be most closely related to HCMV, however, as previously described (section 1.9.1), recent sequence analysis shows the virus to be most closely related to HHV-6 and -7. HCMV (genus *Cytomegalovirus*), together with HHV-6 and -7 (both genus *Roseolovirus*), make up the human betaherpesvirus subfamily. Given the lack of characterisation of the biology of PCMV, the following section will describe certain aspects of human betaherpesvirus biology, with particular emphasis on HCMV, since this is the prototype member, and the most extensively studied of the betaherpesvirus subfamily. HCMV is one of the most common viral causes of congenital infection, and a significant cause of disease in the immunocompromised, being the most important pathogen affecting transplant

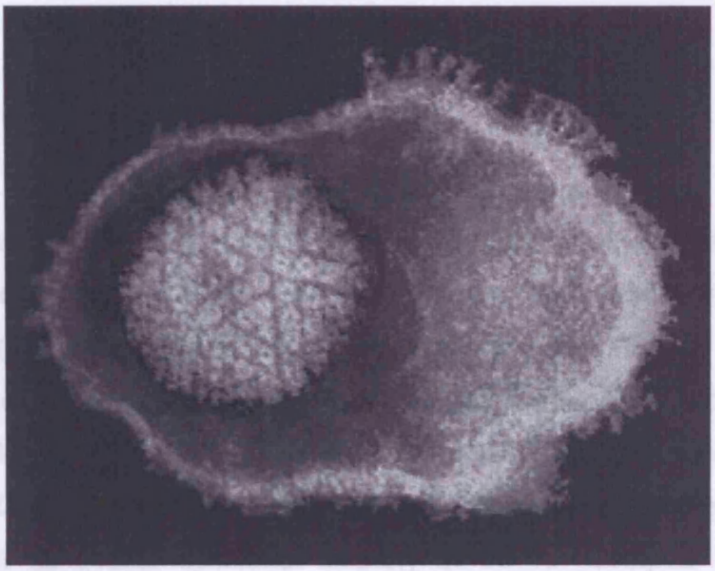
recipients (Fishman & Rubin, 1998). HHV-6 and -7 are more recently identified lymphotropic viruses, and are therefore less well characterised than HCMV. HHV-6 was first isolated during routine culture in 1986, from the peripheral blood of patients with lymphoproliferative disorders and HIV (Salahuddin *et al*, 1986), while HHV-7 was first isolated from CD4+ T cells of a healthy adult in 1990 (Frenkel *et al*, 1990). As with HCMV, both viruses may be reactivated from latency following primary infection, and can result in disease in the immunocompromised.

1.10.1 Betaherpesvirus biology

Virion morphology

Betaherpesvirus morphology is typical of all herpesviruses (Figure 1.6). The HCMV virion is 150-200 nm in size, and consists of a linear double-stranded DNA genome, encased in an icosahedral capsid, surrounded by an amorphous protein layer called the tegument, and a lipid bilayer envelope consisting of cellular polyamines and lipids, and viral glycoproteins. The virion capsid is made up of 162 capsomers composed of four proteins, all homologous to ones identified in HSV including; the major and minor capsid proteins (MCP and mCP), the minor capsid binding protein (mC-BP), and the smallest capsid protein (SCP) (Mocarski & Courcelle, 2001). The HCMV virion envelope carries three prominent glycoprotein complexes; gCI, gCII and gCIII, conserved among herpesviruses. One of these, gCI, is made up of covalently-linked, proteolytically-processed, homodimers of glycoprotein (g) B, while gCIII is composed of gH, gL and gO. Both are essential for virus entry into cells (Navarro *et al*, 1993), and are important targets for neutralising antibodies. Glycoprotein B is the major heparin sulphate proteoglycan-binding protein, and binds to cellular receptors including the recently identified epidermal growth factor receptor (EGFR) (Wang *et al*, 2003). A third glycoprotein complex gCII is comprised of gM and gN, and also binds neutralising antibodies. At least 25 proteins are located in the tegument layer of the virion; some of these, such as pp150 and pp65 are highly immunogenic, while others function as transactivators (Mocarski & Courcelle, 2001). In addition to infectious virions, HCMV-infected cells produce dense bodies, consisting of enveloped tegument proteins without a nucleocapsid, and noninfectious enveloped particles, which contain capsids but lack a

A



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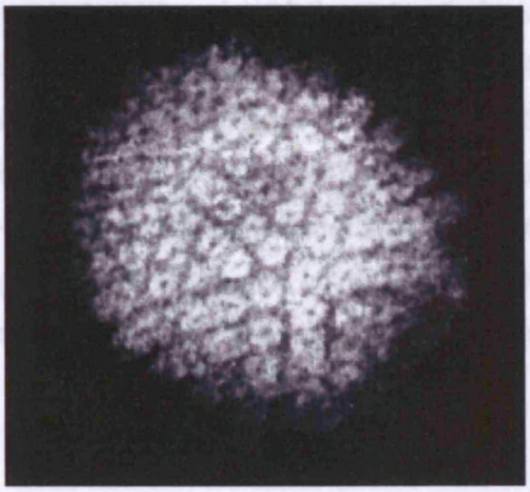


Figure 1.6. A negatively stained enveloped herpesvirus particle (A) and icosahedral capsid (B) (Stannard, 1995). The herpesvirus virion consists of a core containing a linear double-stranded DNA genome, surrounded by an icosadeltahedral capsid of approximately 100-110 nm in diameter and made up of 162 capsomers. The capsid itself is surrounded by an asymmetrical, amorphous protein layer called the tegument, and a lipid bilayer envelope consisting of cellular polyamines and lipids, and viral glycoproteins.

DNA core. Neither of these two particles are capable of infection and replication in cells.

Viral genome

At 235 kb in size, the HCMV genome is the largest of all the herpesviruses, and is comprised of unique long (UL) and short (US) regions, each flanked by direct and inverted repeats (TRL/IRL and IRS/TRS) (Dolan *et al*, 2004). Within these repeat sequences are the herpesvirus-conserved elements *pac*1 and *pac*2, which mediate cleavage and packaging of the viral genome. HHV-6 comprises variants A (prototype strain U1102) and B (prototype strains HST and Z29), which are approximately 160 kb in size and share an overall nucleotide identity of 90 % (Gompels *et al*, 1995; Dominguez *et al*, 1999; Isegawa *et al*, 1999). These strains are predicted to contain 119 (strains U1102 and Z29) and 115 (HST strain) ORFs, 67 % of which, are homologous to those of HCMV (Gompels *et al*, 1995). Meanwhile, isolates JI (145 kb) and RK (153 kb) of HHV-7 have been completely sequenced (Nicholas, 1996; Megaw *et al*, 1998). HHV-7 strain J1 proteins were found to share 41-75 % nucleotide sequence identity with HHV-6 proteins (Nicholas, 1996). The majority of HHV-6 and -7 genes are arranged in the unique region (U) of the genome, which is flanked by direct repeat (DR) sequences. At present, the structure of the PCMV genome remains undefined.

Virus tropism

Classification of HCMV as a betaherpesvirus was originally based on its slow growth *in vitro* and restricted species specificity. HCMV is poorly adapted to growth in culture; with a slow appearance of CPE, primary differentiated human skin or lung fibroblasts are the most permissive cell type for replication, and therefore commonly used in routine culture (Mocarski & Courcelle, 2001). In contrast, *in vivo*, HCMV replicates dynamically with a doubling time of approximately 1 day during active infection (Emery *et al*, 1999), in most of the major cell types including; fibroblasts, macrophages, endothelial cells, epithelial cells, smooth muscle cells, stromal cells, neuronal cells, neutrophils and hepatocytes (Sinzger *et al*, 1995; Sinzger *et al*, 1999; Jarvis & Nelson, 2002). The presence of HCMV within histopathological samples is routinely determined by the visualisation of 'owl's eye' intranuclear inclusions, and are a characteristic

unique to HCMV infections (Mattes *et al*, 2000). As described previously, PCMV infection is also characterised by the presence of cytomegalic inclusions (Plowright *et al*, 1976; Edington *et al*, 1976a; Edington *et al*, 1976b; Edington *et al*, 1977; Edington *et al*, 1988b).

HHV-6 and -7 are predominantly lymphotropic herpesviruses, replicating in cells of T cell lineage, with HHV-7 predominately targeting CD4+ lymphocytes (Takahashi *et al*, 1989). *In vitro*, both HHV-6 and -7 are routinely cultured in continuous T cell lines such as Jurkat and SupT-1 cells, inducing CPE characterised by large ballooning cells (Gompels, 2004). *In vivo*, HHV-6 has a wider tropism, including monocytes, and epithelial, endothelial and neural cells (Yamanishi, 2001). Interestingly, inclusion bodies are not associated with HHV-6 and -7 infection (Mattes *et al*, 2000).

Virus attachment and penetration

Initial interaction between HCMV and the cell involves the low-affinity binding of gCI (gB) and gCII (gM/gN) to heparin sulphate proteoglycans (Compton *et al*, 1993), which are ubiquitously distributed on the cell surface. Following initial attachment, more stable interaction is mediated by gB and gH binding to non-heparin cellular receptors. One such receptor has been identified as EGFR (Wang et al, 2003). Penetration follows fusion of the virion envelope and the cell surface, involving gCIII (gH/gL/gO) and gB (Compton, 2004). Nucleocapsids and tegument proteins are released into the cytoplasm, and rapidly transported to the nucleus (Landolfo *et al*, 2003). Viral attachment and subsequent activation via cell surface receptors alters host cell gene expression, while tegument proteins and regulatory proteins expressed after penetration, regulate cellular signalling pathways and gene expression, as infection proceeds (Simmen *et al*, 2001; Song & Stinski, 2002).

The cellular receptor for both variants of HHV-6 is CD46 (Santoro *et al*, 1999), which contributes to its apparent broad tropism *in vitro*, whereas CD4 is the cellular receptor for HHV-7 (Lusso *et al*, 1994). As with HCMV, HHV-6 and -7 encode homologues of glycoproteins gB, gH, gM and gL, which mediate virus attachment and penetration (Gompels *et al*, 1995; Nicholas, 1996). At present, the cellular receptor for PCMV is not characterised.

Gene expression and regulation

As with all herpesviruses, gene expression and replication of the betaherpesviruses follows a temporal pattern of expression of different classes of genes termed; immediate early (IE), early (E) or late (L) (Figure 1.7), which are transcribed in the nucleus by the host cell RNA polymerase II. The expression of many HCMV genes is regulated by complex transcriptional elements including enhancers and modulators, which are situated upstream of the start site of each gene, and contain dense collections of binding sites for various host transcription factors and viral transactivators (Mocarski & Courcelle, 2001). These enhancer and modulator elements confer cell tropism and also, along with mRNA splicing, allow more than one protein to be produced from the same open reading frame (ORF). In permissive human fibroblast cells, transcription of HCMV IE genes begins within 1 hour (hr) of infection, with the majority of expression arising from the major (M) IE locus, and encoding proteins involved in the regulation of E, L and host genes (Mocarski & Courcelle, 2001). E genes are transcribed 4-24 hrs postinfection, and encode proteins involved in DNA replication as well as immunomodulation, while L genes are transcribed following DNA replication (later than 24 hrs postinfection), and encode proteins involved in virion morphogenesis and maturation. It is highly likely that PCMV gene expression will follow a similar temporal cascade.

DNA replication

HCMV DNA replication takes place within distinct sub-nuclear regions, similar to sites of cellular DNA synthesis. The HCMV genome circularises in the nucleus of infected human fibroblasts within 4 hrs postinfection, while DNA replication begins at 16 hrs, with several thousand copies produced in each infected cell (Mocarski & Courcelle, 2001). Eleven HCMV gene loci are required for replication (Pari & Anders, 1993). Six HSV-1-homologous proteins make up the replication fork; including the DNA polymerase, polymerase-associated processivity factor, single-stranded DNA-binding protein, and three other proteins which form the helicase-primase complex. The polymerase-associated processivity factor prevents dissociation of the polymerase from the template DNA, while the single-stranded DNA-binding protein prevents

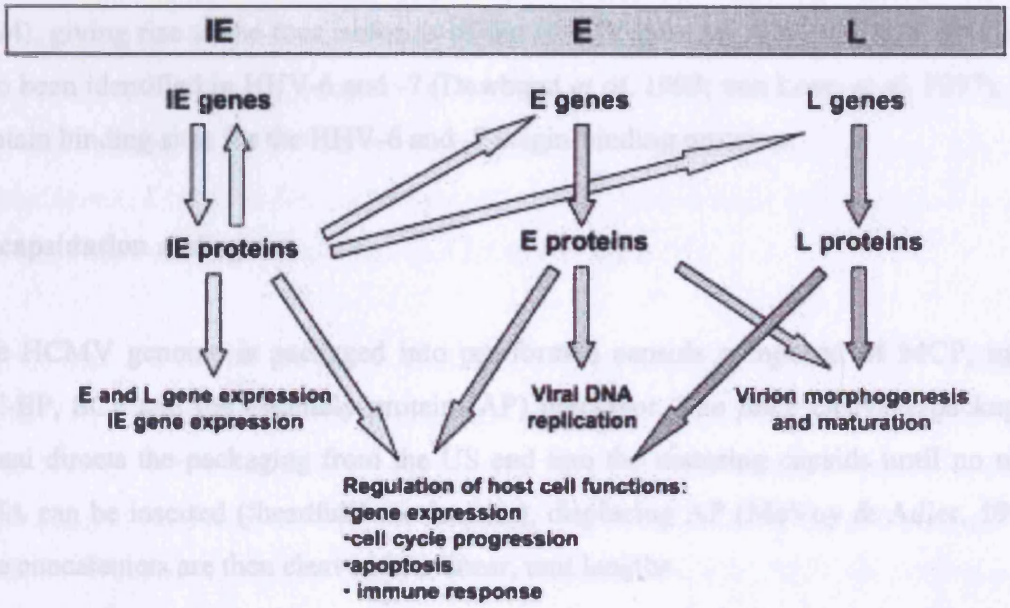


Figure 1.7. HCMV gene expression and viral gene product functions during productive infection (Landolfo *et al*, 2003).

re-annealing of the DNA strands following unwinding by the helicase-primase complex. In addition, three viral transactivators, as well as two further proteins, are required for optimal replication. Unlike HCMV, HHV-6 and -7 encode an origin-binding protein homologue, U73, which is found in alphaherpesviruses (Krug *et al*, 2001). HCMV replication starts from a single origin site (*ori*Lyt), located within the UL region (Masse *et al*, 1992), and proceeds bi-directionally by a rolling-circle mechanism producing concatemeric molecules. Following replication, concatemeric viral DNA molecules mature by inversion of both L and S components in the nucleus (McVoy & Adler, 1994), giving rise to the four isoforms of the HCMV genome. Single *ori*Lyt sites have also been identified in HHV-6 and -7 (Dewhurst *et al*, 1993; van Loon *et al*, 1997), and contain binding sites for the HHV-6 and -7 origin-binding proteins.

Encapsidation and egress

The HCMV genome is packaged into pre-formed capsids composed of MCP, mCP, mC-BP, SCP and the assembly protein (AP) precursor. The *pac*2 cleavage/packaging signal directs the packaging from the US end into the maturing capsids until no more DNA can be inserted ('headfull' mechanism), displacing AP (McVoy & Adler, 1994). The concatemers are then cleaved into linear, unit lengths.

Accumulation of nucleocapsids within the nucleus form characteristic nuclear owl's eye inclusions that are readily observed by light microscopy. From the nucleus, progeny nucleocapsids bud into the perinuclear cisternae, acquiring an initial envelope modified with viral glycoproteins. Nucleocapsids then undergo a series of de-envelopment and re-envelopment processes as they are transported through the cytoplasm, where the tegument is added (Mettenleiter, 2002). The virion is then transported in vesicles via the Golgi, where envelope glycoproteins are glycosylated, to the cell surface prior to fusion with cellular membranes and release beginning at 72 hrs postinfection (Landolfo *et al*, 2003).

Latency

The HCMV genome is maintained as a covalently closed circle in the nucleus of lineage-committed myeloid cells including progenitors of macrophages (monocytes),

granulocytes, dendritic cells and endothelial cells (Jarvis & Nelson, 2002). In some latently infected cells, HCMV latency-associated transcripts (LATs) derived from the MIE region and encoding several proteins, have been identified (Kondo & Mocarski, 1995), but are thought to play a minimal role in the regulation of HCMV latency and reactivation (Jarvis & Nelson, 2002). In an alternative theory, latency is thought to result from the repression of MIE gene expression (Sissons *et al*, 2002). Subsequent reactivation of HCMV and productive infection is thought to result from immune activation, in response to pathogenic or allogeneic stimulation, which induces proinflammatory cytokine (IL-2 and interferon (IFN) -γ) production by T cells, and differentiation of latently infected progenitor cells into a permissive cell type which supports viral replication (Soderberg-Naucler *et al*, 1997; Jarvis & Nelson, 2002). In immunocompromised individuals reduced immune surveillance leads to uncontrolled virus replication, systemic infection and HCMV disease.

HHV-6 and -7 establish latency in cells of the lymphoreticular system. HHV-6 sites of latency are thought to include monocytes and early bone marrow progenitor cells (Kondo *et al*, 1991; Luppi *et al*, 1999), with viral LATs detected in latently infected macrophages (Kondo *et al*, 2002). Meanwhile, monocytes also provide a possible site for HHV-7 latency (Zhang *et al*, 2001). An alternative form of HHV-6B persistence is characterised by high viral loads in blood and integration of the genome into host cell chromosomes (Luppi *et al*, 1993), in particular at the telomeric extremity of the short arm of chromosome 17 (Torelli *et al*, 1995).

Immunomodulation

All betaherpesviruses encode immunomodulatory functions which enable them to evade immune responses and persist within the host (Mocarski, 2002). In particular, they encode proteins involved in the down-regulation of MHC class I (Jones *et al*, 1996; Gewurz *et al*, 2001; Shamu *et al*, 2001; Hewitt *et al*, 2001; Hudson *et al*, 2001), and class II molecules (Tomazin *et al*, 1999; Miller *et al*, 2001), and encode viral chemokines (Penfold *et al*, 1999; Zou *et al*, 1999), and chemokine receptors (Chee *et al*, 1990; Isegawa *et al*, 1998; Milne *et al*, 2000; Nakano *et al*, 2003).

1.10.2 Virus transmission

There are significant similarities in the transmission routes, and time of acquisition, between human betaherpesvirus and PCMV infections. Human betaherpesvirus infections are ubiquitous in the human population. Primary infection can occur by horizontal and vertical transmission, while recurrent infection may result from either reactivation or re-infection with a second virus strain. Horizontal transmission of HCMV is thought to occur by close contact between individuals including the transfer of saliva, and other body fluids during sexual intercourse. In addition, HCMV is frequently transmitted by organ transplantation, with a seropositive organ transmitting virus to a seronegative donor in 60-80 % of cases (Griffiths & Emery, 2002).

HCMV can be transmitted vertically from a mother undergoing primary or recurrent HCMV infection to her baby either; *in utero* via the placenta, perinatally through ingestion of infected cervical secretions, or post-natally through ingestion of infected breast milk. Early publications suggested a relationship between the presence of maternal antibodies and congenital infection, with intrauterine infection occurring in approximately 30-40 % of pregnancies following primary infection (Stagno *et al*, 1986), and in 1 % of women following recurrent infection (Stagno *et al*, 1982). However, in a recent survey of studies performed between 1977 and 1997, a high seroprevalence was associated with a high incidence of congenital infection (Gaytant *et al*, 2002), suggesting that pre-existing maternal immunity is unable to completely prevent transmission of virus to the foetus.

Perinatal transmission occurs through exposure to infected maternal genital secretions during birth. Approximately 10 % of women are reported to shed HCMV from the vagina or cervix, and of these, 50 % are reported to transmit HCMV to the newborn (Pass, 2001). The most common route of vertical transmission is through infected breast milk, such that in countries where seroprevalence and breastfeeding are common, more than 50 % of infants acquire HCMV by the age of 1 year (Pass, 2001).

1.10.3 Pathogenesis and clinical manifestations

HCMV infection is generally asymptomatic in healthy individuals, and causes disease only in the immunologically naïve, such the foetus, and in immunocompromised individuals, such as AIDS and transplant patients. During the systemic phase of primary infection, HCMV disseminates throughout the body by leukocyte-associated viraemia, leading to the seeding of ductal epithelia. Following initial infection, HCMV is shed in saliva, urine, tears, semen, cervicovaginal fluid and breast milk (Pass, 2001).

Infection in the immunocompromised

HCMV is one of the most common opportunistic infections in the immunocompromised, causing a variety of end-organ diseases, 'HCMV diseases', as well as indirect effects (Table 1.7) (Griffiths, 2002). The most severe infections are seen in recipients of bone marrow transplants (BMT) and AIDS patients with low CD4+ T cell counts, however, disease is also common following solid-organ transplantation.

The onset of HCMV infection in recipients of solid organ transplants is generally 1-4 months posttransplant, when the patient is most profoundly immunosuppressed (Fishman & Rubin, 1998). HCMV can be reactivated from latency in the recipient or donor organ, by inflammatory cytokines, while immunosuppressive agents promote persistence and spread of the virus by suppressing host antiviral immune responses, leading to uncontrolled viral replication. In general, those without prior immunity are at a higher risk of uncontrolled viral replication and disease (Patel & Paya, 1997). Indeed, primary infection, viraemia and viral load, have all been identified as risk factors for HCMV disease in transplant recipients (Cope *et al*, 1997b), with a high viral load being the primary determinant (Cope *et al*, 1997a; Hassan-Walker *et al*, 1999), and primary infection and viraemia being secondary to this. Direct end-organ effects are usually seen in the transplanted organ, for example, nephritis in kidney recipients, hepatitis in liver recipients, myocarditis in heart recipients, and pneumonitis in lung recipients, while indirect effects can contribute to acute and chronic graft rejection (Fishman & Rubin, 1998).

Table 1.7. HCMV diseases in the immunocompromised (Griffiths, 2002).

Symptom	Solid organ Transplantation	BMT	AIDS
Fever/hepatitis	++	+	+
Gastrointestinal	+	+	+
Retinitis	+	+	++
Pneumonitis	+	++	
Myelosuppression		++	
Encephalopathy			+
Polyradiculopathy			+
Immunosuppression	+		
Rejection/GVHD	+	?	
Atherosclerosis	+		
Death		+	+

+ clinical problem, ++ major clinical problem, ? controversial

GVHD, graft vs. host disease

HHV-6 and -7 infections posttransplantation are common, and are thought to result primarily from reactivation because of the high seroprevalence of these viruses. Direct clinical effects of HHV-6 infection in BMT and solid-organ transplant recipients include pneumonitis (Cone *et al*, 1993; Singh *et al*, 1997), encephalitis (Drobyski *et al*, 1994; Singh *et al*, 2000a), hepatitis (Ljungman *et al*, 2000; Ward *et al*, 1989) and bone marrow suppression (Drobyski *et al*, 1993; Carrigan & Knox, 1995). While HHV-7 has been associated with encephalitis in BMT patients (Chan *et al*, 1997) and hepatitis in liver transplant recipients (Griffiths *et al*, 1999).

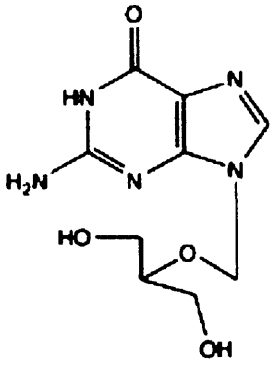
1.10.4 Prevention and treatment of HCMV

In some cases, the use of seronegative or leukocyte-depleted blood products could be used to prevent transmission of HCMV to the immunocompromised or pregnant women (Ljungman, 2004). However, the use of seronegative donor organs to prevent HCMV transmission following solid-organ transplantation is not practicable because of the limited supply of donor organs. Studies into the development of a vaccine for at-risk groups are underway, with HCMV gB and pp65 being potential components of a subunit vaccine (Pass *et al*, 1999). Meanwhile, antiviral therapy is the mainstay for the treatment of life-threatening HCMV disease in the immunocompromised. Since HCMV viral load is the primary risk factor for the development of HCMV disease, antiviral therapies should be deployed to prevent viral loads from rising above the level associated with disease.

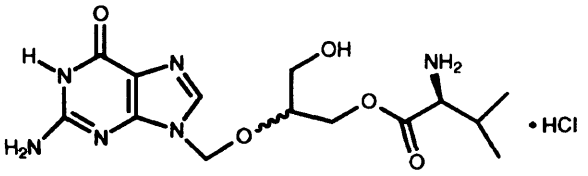
Antiviral therapy

Antiviral therapy can be employed through prophylactic or pre-emptive strategies, or by the treatment of established disease. In pre-emptive therapy antivirals are administered following the detection of systemic infection, i.e. viraemia, while prophylactic antiviral therapy is administered prior to the detection of virus. In the case of transplant recipients either pre-emptive therapy or prophylaxis are usually employed, although there are limitations associated with both approaches (Emery, 2001; Hart & Paya, 2001). At present, there are five antiviral agents licensed for use in the treatment of life-threatening HCMV infections in the immunocompromised; these being, ganciclovir, valganciclovir, foscarnet, cidofovir and fomivirsen (Figure 1.8) (De Clercq, 2003).

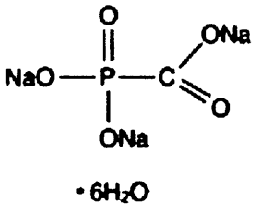
Ganciclovir



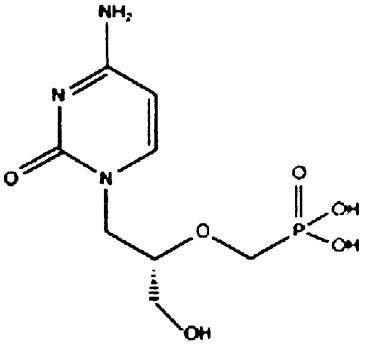
Valganciclovir



Foscarnet



Cidofovir



Fomivirsen

5'-d-G*C*G*T*T*T*G*C*T*C*T*T*C*T*T*C*T*T*G*C*G-3'

sodium salt

* = racemic phosphorothioate

Figure 1.8. Antiviral agents licensed for use in the treatment of HCMV infection.

Ganciclovir (GCV)

GCV is the first choice therapy for life-threatening or sight-threatening HCMV infections in the immunocompromised (British National Formulary (BNF), 2004). It is a competitive acyclic nucleoside analogue inhibitor of the viral DNA polymerase and requires initial mono-phosphorylation by the protein kinase encoded by HCMV UL97 (Figure 1.9). Following initial phosphorylation, GCV-MP is further phosphorylated by cellular kinases, and inhibits the viral DNA polymerase by competing with guanosine triphosphate (GTP) for incorporation into the DNA chain. Since GCV has a 3'-OH group it is not an absolute chain terminator, but because it is acyclic, elongation of the viral DNA becomes unstable and terminates.

Both GCV prophylactic and pre-emptive strategies are employed to prevent HCMV infection and disease in transplant recipients. Pre-emptive GCV therapy was shown to be effective in preventing HCMV disease in liver transplant recipients, with oral GCV more effective than intravenous treatment (Singh *et al*, 2000b), while, in a randomised, double-blind, placebo-controlled trial GCV prophylaxis reduced HCMV-induced illness in heart recipients (Merigan *et al*, 1992). Prolonged low-dose GCV prophylaxis following heart transplantation reduced HCMV disease following primary infection, but only delayed the onset of HCMV disease and reduced morbidity in seropositive recipients (Macdonald *et al*, 1995). In a more recent randomised, placebo-controlled study, GCV prophylaxis was found to be a safe and effective method for the prevention of HCMV disease after liver transplantation (Gane *et al*, 1997). In addition, valaciclovir prophylaxis was shown to be effective in preventing HCMV disease following renal transplantation, and interestingly showed a reduction in biopsy-proven graft rejection in those patients at greater risk of HCMV infection (Lowance *et al*, 1999).

The main side effect of GCV is bone marrow toxicity (myelosuppression), which results in neutropenia and thrombocytopenia (Dollery, 1999). In addition, poor oral bioavailability means that plasma concentrations are often too low to fully suppress HCMV replication, allowing the selection of resistant virus mutants. Mutations, including base substitutions, deletions or insertions, within the UL97 gene are the most common cause of this resistance, although secondary mutations may arise in the UL54 gene (Drew *et al*, 2001).

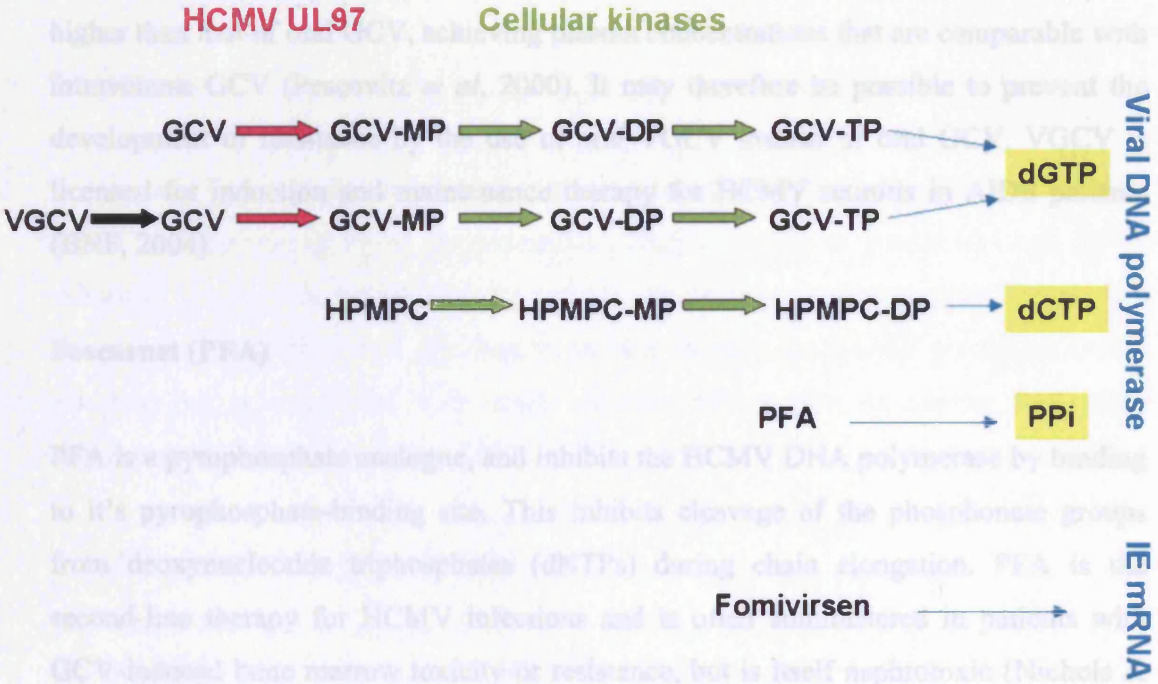


Figure 1.9. Mechanisms of action of antiviral agents licensed for use in the treatment of HCMV. Nucleotide analogues GCV and VGCV are first activated by HCMV UL97-encoded kinase, followed by phosphorylation by cellular kinases. Nucleotide analogue HPMPC is phosphorylated only by cellular kinases. These agents inhibit the viral DNA polymerase by competing with the native nucleoside triphosphates (dGTP and dCTP). PFA inhibits the viral DNA polymerase by competing with pyrophosphate (PPi), while fomivirsen inhibits translation of HCMV IE mRNA. MP, monophosphate; DP, diphosphate; TP triphosphate; dGTP deoxyguanosine triphosphate; dCTP deoxycytosine triphosphate.

Valganciclovir (VGCV)

VGCV is the valine ester, and pro-drug of GCV, and is rapidly metabolised into GCV following ingestion (Jung & Dorr, 1999). It has an oral bioavailability that is 10-fold higher than that of oral GCV, achieving plasma concentrations that are comparable with intravenous GCV (Pescovitz *et al*, 2000). It may therefore be possible to prevent the development of resistance by the use of oral VGCV instead of oral GCV. VGCV is licensed for induction and maintenance therapy for HCMV retinitis in AIDS patients (BNF, 2004).

Foscarnet (PFA)

PFA is a pyrophosphate analogue, and inhibits the HCMV DNA polymerase by binding to it's pyrophosphate-binding site. This inhibits cleavage of the phosphonate groups from deoxynucleotide triphosphates (dNTPs) during chain elongation. PFA is the second-line therapy for HCMV infections and is often administered in patients with GCV-induced bone marrow toxicity or resistance, but is itself nephrotoxic (Nichols & Boeckh, 2000). PFA also suffers from poor bioavailability and must be administered three times daily. PFA resistant virus has been documented, with resistance mapped to mutations within the UL54 DNA polymerase gene (Baldanti *et al*, 1996; Chou *et al*, 1998).

Cidofovir (HPMPC)

HPMPC is an acyclic nucleotide phosphonate analogue and a competitive inhibitor of the HCMV DNA polymerase. Unlike GCV, the presence of the phosphonate group means that HPMPC does not require initial phosphorylation by a virus-encoded kinase, and is further phosphorylated by cellular kinases (figure 1.9). The three HPMPC metabolites; HPMPC phosphate, diphosphate, and phosphate adduct, all have long intracellular half-lives of approximately 6, 17, and 48 hrs respectively (Ho *et al*, 1992), which means that the drug can be administered less frequently. The primary indication for HPMPC, is the treatment of HCMV retinitis in AIDS patients for whom GCV and PFA are inappropriate (BNF, 2004). In addition, primary HPMPC pre-emptive therapy, pre-emptive therapy for whom GCV or PFA had failed, and HPMPC treatment of

established disease, are all effective against HCMV disease in BMT patients (Ljungman *et al*, 2001). Side effects and limitations include nephrotoxicity, the fact that it must be administered intravenously, and the development of resistance, which is mapped to the UL54 DNA polymerase gene (De Clercq, 2003).

Fomivirsen

Fomivirsen is a phosphorothioate oligonucleotide which inhibits translation of HCMV IE proteins by binding to the complementary mRNA sequences (Anderson *et al*, 1996). Although it is specific for HCMV, the technology could be applied to other viruses. It is administered by intravitreal injection in salvage therapy for HCMV retinitis in AIDS patients, but is associated with ocular adverse effects such as uveitis and retinal detachment (Nichols & Boeckh, 2000).

1.10.5 Antiviral therapy for HHV-6 and -7

To date, there have been no controlled trials of antiviral therapy against HHV-6 and -7 infection *in vivo*, however, a number of studies have demonstrated the ability of currently licensed antiherpetic agents such as GCV, PFA and HPMPC, to suppress HHV-6 and -7 replication *in vitro* (Reymen *et al*, 1995; Takahashi *et al*, 1997; De Clercq *et al*, 2001). The U69 gene of HHV-6 is homologous to the UL97 gene of HCMV, and was shown to confer GCV susceptibility to a recombinant baculovirus in insect cells (Ansari & Emery, 1999). However, the phosphorylation of GCV by the HHV-6 U69 gene product appears to be limited, which may account for its modest antiviral activity against HHV-6 in certain cell culture systems (De Bolle *et al*, 2002). *In vivo*, case reports show that GCV and PFA were effective in the treatment of HHV-6 infection in a BMT patient (Johnston *et al*, 1999), while GCV also reduced HHV-6 and -7 viral loads in liver transplant patients (Mendez *et al*, 2001). In addition, high-dose ACV prophylaxis demonstrated activity against HHV-6 following bone marrow transplantation (Wang *et al*, 1996). Resistance of HHV-6 to antiherpetic agents has been mapped to the U38 and U69 genes (Manichanh *et al*, 2001). Mutations in the U38 DNA polymerase gene and the U69 phosophotransferase gene of HHV-6 developed *in vitro* and *in vivo*, following prolonged exposure to GCV, and resulted in a reduced susceptibility to GCV, HPMPC and, to a lesser extent, PFA.

1.11 Aims of the thesis

The overall aim of this thesis was to investigate porcine cytomegalovirus issues relevant to xenotransplantation, and is divided into four objectives:

1. To develop and optimise qualitative and fully quantitative competitive (QC) polymerase chain reaction (PCR) assays for PCMV.

2. To determine the prevalence, quantity and organ distribution of PCMV in immunocompetent and immunosuppressed pigs being bred for xenograft organs, to investigate when virus is acquired, and to examine methods to eliminate PCMV from these animals.

3. To clone and express the complete ORF of the glycoprotein B gene of PCMV and a truncated version excluding the proposed transmembrane regions, in insect cells, using a baculovirus expression system, and to develop a recombinant protein-based serological assay for PCMV.

4. To determine the susceptibility of PCMV to established antiherpetic agents *in vitro*.

Chapter 2: Materials and methods

2.1 The development of qualitative and quantitative-competitive (QC) PCR assays for PCMV

The methods in this section describe the development of nested qualitative and quantitative-competitive (QC) PCR assays to allow both the detection and measurement of PCMV DNA loads within porcine samples. The primers used in both of these assays amplify a region of the DNA polymerase gene of PCMV (PCMVpol), and were designed from a recently published sequence of the gene (Widen *et al*, 1999). This gene was selected as the target in the development of these assays since it is highly conserved among different strains of herpesviruses. Both PCMV PCR assays involve two rounds of amplification, using nested primers, in order to increase the specificity and yield of the product. The first round primers PCMVF1 and PCMVR1 amplify a 236-bp product, while primers PCMVF2 and PCMVR2 amplify a 184-bp product, and are shown in Table 2.1.

The QC PCR assay for PCMV involves the co-amplification of a PCMVpol control sequence alongside sample (wild-type) DNA, and is based on previous fully quantitative PCR assays for HCMV, HHV-6, HHV-7, HIV and HHV-8, developed in our department (Fox *et al*, 1992; Clark *et al*, 1996; Kidd *et al*, 1996; Atkins *et al*, 1996; Lock *et al*, 1997). This control sequence is identical to the wild-type target, with respect to primer-binding sites, length and G+C content, and differs only by the presence of a unique restriction site (*Sma* I), brought about by a 2-bp change in the PCMVpol sequence. This 2-bp alteration was introduced by PCR-mediated site-directed mutagenesis, and allows for the separation of wild-type and control amplicons by *Sma* I digestion after PCR amplification. Following the generation of wild-type and control PCMVpol sequences and their subsequent cloning into the pGEM-T Easy vector (Promega), the PCR parameters were altered to optimise the sensitivity and specificity of the PCMV PCR. Finally, the utility of the PCMVpol control sequence to accurately quantify PCMV DNA was assessed.

2.1.1 Amplification of the PCMVpol insert

First round primers PCMVF1 and PCMVR1 were used to amplify the 236-bp PCMVpol target region (wild-type sequence), from a sample of tissue culture supernatant (TCS) of

porcine alveolar macrophages infected with PCMV strain B6 (provided by Dr M. Banks, Veterinary Laboratories Agency, UK). The PCR mixture contained 100 ng of each primer, 2 mM $MgCl_2$ (Bioline), 200 µM of each dNTP (Promega), 1 U of *Taq* polymerase (Bioline), and either 1 µl, 5 µl, or 10 µl of neat TCS sample of PCMV, in $(NH_4)_2SO_4$ PCR buffer (Bioline). The cycle parameters used were initial denaturation at 95 °C for 6 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The final extension was increased to 10 min. The PCR was performed on a Hybaid Omnigene thermal cycler.

Table 2.1. PCR primer sequences used in this thesis.

Primer name	Primer sequence
PCMVF1	5'-CCTATGTTGGCACTGATACTTGAC-3'
PCMVR1	5'-CCCTGAAAATCACCGTCTGAGAGA-3'
PCMVF2	5'-AAGCAGCAGCTTGCCCTCAAGGTG-3'
PCMVR2	5'-ACTTCTCTGACACGTATTCTCTAG-3'
PCMVFM	5'-TAAGCATGTCCCGGGCTATGCTGG-3'
PCMVRM	5'-CCAGCATAGCCCGGGACATGCTTA-3'
PCMVFB	5'-ACGTGCAATGCGTTTTACGGCTTC-3'
PCMVgBF	5'-AACGCGTATGTTTGCGGTCTCTTGTC-3'
PCMVgBR	5'-CACGTCCTCGGTGGATAGCTGCT-3'
PCMVgBT	5'-CTCTAACGGCTTTATATTAAGGTGGATA-3'
Baculovirus forward	5'-TTTACTGTTTTCGTAACAGTTTTG-3'
Baculovirus reverse	5'-CAACAACGCACAGAATCTAGC-3'
PCMVpolF (Taqman)	5'-GCTGCCGTGTCTCCCTCTAG-3'
PCMVpolR (Taqman)	5'-ATTGTTGATAAAGTCACTCGTCTGC-3'
Taqman probe	5'-6FAM-CCATCACCAGCATAGGGCGGGAC-TAMRA-3'

2.1.2 Agarose gel electrophoresis

PCR products were analysed by electrophoresis on a 3 % agarose gel in TBE buffer (90 mM Tris-Borate, pH 8.3; 2 mM EDTA), alongside HyperLadder IV DNA markers

(Bioline). To prepare the 3 % agarose gel, 3 g of multipurpose agarose (Bioline) were added to 100 ml of TBE buffer in a 500 ml sterile Duran bottle, and heated in a microwave (850 W) until dissolved. The gel was cooled to ~50 °C, before the addition of 30 µg of ethidium bromide (Sigma), and poured into a gel frame containing a comb for loading samples. Ten microlitres of PCR product were mixed with 3 µl of loading dye (0.25 % bromophenol blue; 0.25 % xylene cyanol; 15 % ficoll type 400 (all Sigma) in sterile distilled water (SDW) (Baxter)), and loaded into each well. Gels were electrophoresed at 120 V for 30-45 min, and visualised using a UV transilluminator (302 nm) with a Baby Imager (Appligene-Oncor) and photographed using a video copy processor (Mitsubishi).

2.1.3 Purification of the PCMVpol PCR product and cloning into the pGEM-T Easy Vector

The 236-bp PCMVpol product was purified from the PCR mixture by separation on a 2 % low gelling temperature agarose (Sigma) gel, containing TAE buffer (40 mM Tris-acetate; 1 mM EDTA) and ethidium bromide (30 µg), followed by DNA extraction using the Wizard PCR Preps DNA Purification System (Promega). Briefly, following visualisation on a UV transilluminator, the 236-bp PCMVpol sequence was excised from the gel using a clean sterile scalpel. The agarose slice was transferred to a 1.5 ml eppendorf and incubated at 70 °C on a heating block until dissolved. One millilitre of DNA Purification Resin was added to the melted gel (~300 µl), mixed gently for 20 s, and loaded into a 2 ml syringe barrel attached to a Wizard Minicolumn. The syringe plunger was inserted slowly to push the mixture through the Minicolumn, which was then washed by plunging 2 ml of 80 % isopropanol through the column. The Minicolumn was detached from the syringe, placed in a 1.5 ml eppendorf and centrifuged at 10,000 ×g for 2 min to dry the resin. DNA was eluted by the addition of 50 µl of SDW and collection of the eluent into a 1.5 ml eppendorf following centrifugation at 10,000 ×g for 20 s.

The purified PCMVpol PCR product was then cloned into the pGEM-T Easy vector (Figure 2.1) to produce the 'wild-type plasmid'. This is a high copy number vector, which has single 3'-T overhangs at the multiple cloning site. This greatly improves the efficiency of ligation of the PCR product by preventing recircularisation of the vector

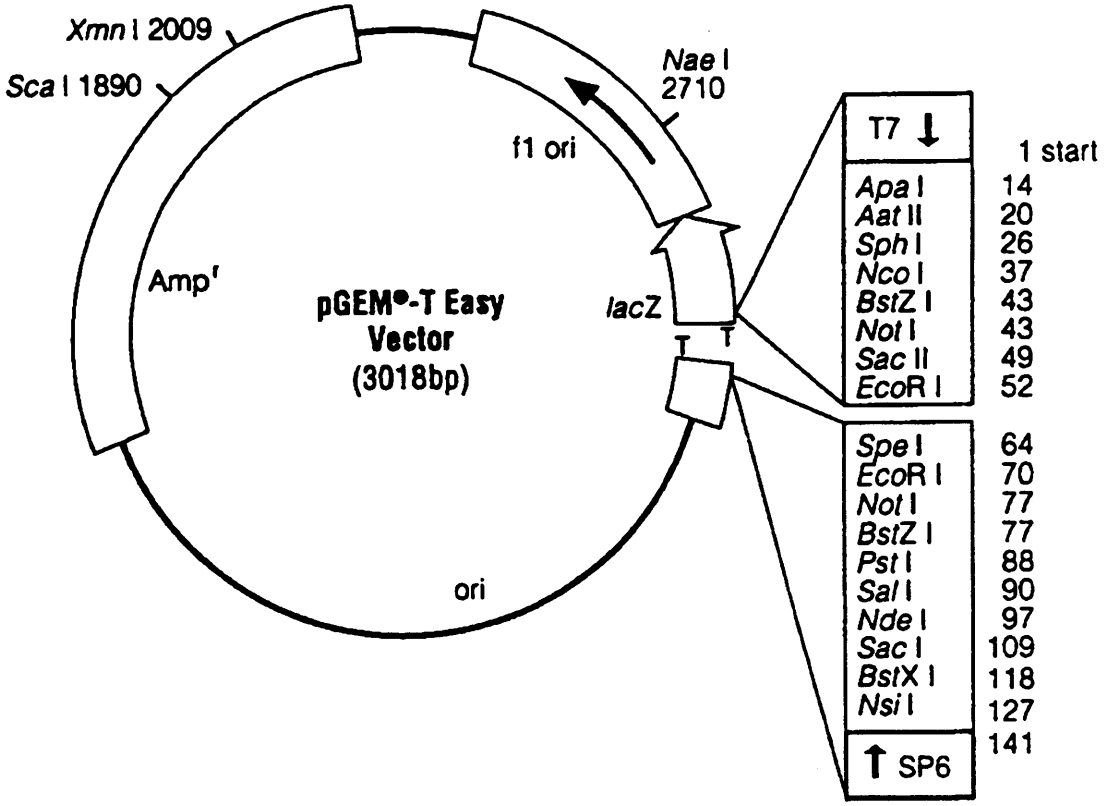


Figure 2.1. A diagram of the pGEM-T Easy vector (Promega). The pGEM-T Easy vector is a high copy number vector, with a multiple cloning site containing a number of restriction sites, located within the α-peptide coding region of the enzyme β-galactosidase. The vector has single 3'-T overhangs at the insertion site to allow direct cloning of PCR products generated by certain polymerases, and an ampicillin resistance gene for selection in *E. coli*.

and provides a compatible overhang for PCR products generated by *Taq* polymerase. The vector contains an ampicillin resistance gene and the α-peptide coding region of the β-galactosidase gene, allowing for selection and identification of recombinant clones.

The purified PCMVpol PCR product was ligated into the pGEM-T Easy vector according to the pGEM-T Easy Vector Systems protocol (Promega). Briefly, the PCMVpol PCR product and pGEM-T Easy vector were mixed in a 3:1 and 1:1 molar ratio, in a 1.5 ml eppendorf containing; 5 µl of 2× Rapid Ligation Buffer (60 mM Tris-HCl, pH 7.8; 20 mM $MgCl_2$; 20 mM DTT; 2 mM ATP; 10 % polyethylene glycol), 3 Weiss units of T4 DNA ligase and SDW to a final volume of 10 µl, and incubated overnight at 4 °C. Two microlitres of ligation products were transformed into 50 µl of competent JM109 *E. coli* cells on ice for 20 min, followed by heat-shock at 42 °C for 45 s. Transformed cells were returned to ice for 2 min, before the addition of 950 µl of SOC medium (Invitrogen), and incubation at 37 °C for 1 hr in a shaking incubator (~200 rpm). One hundred microlitres of transformed cells were plated onto antibiotic selection plates containing 2 % Luria Broth (LB) (Sigma), 1.5 % bacteriological agar (Oxoid), 100 µg/ml of ampicillin, 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and 100 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (all Sigma), and incubated overnight at 37 °C.

2.1.4 Analysis of recombinant clones

Putative recombinant clones were identified and analysed by picking three single white colonies and growing them in 6 ml cultures of 2 % LB containing 50 µg/ml of ampicillin. Cultures were incubated for 16 hrs at 37 °C in a shaking incubator (~200 rpm). Glycerol stocks were prepared from 800 µl of each culture containing 25 % glycerol (Sigma), snap frozen in a methanol and dry ice bath, and stored at -80 °C. Plasmid DNA was extracted from the remaining culture using the Wizard Plus SV Minipreps DNA Purification System (Promega). Each bacterial culture was harvested by centrifugation at 2000 ×g for 10 min. The pellet was resuspended in 250 µl of Cell Resuspension Solution (50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A), transferred to a clean 1.5 ml eppendorf, and lysed in 250 µl of Cell Lysis Solution (0.2 M NaOH; 1 % SDS) for 5 min. Lysed cells were incubated with 10 µl of Alkaline

Protease Solution for 5 min, before the addition of 350 µl of Neutralisation Solution (4.09 M guanidine-HCl; 0.759 M potassium acetate; 2.12 M glacial acetic acid; pH 4.2), to precipitate cellular proteins. The alkaline protease inactivates endonucleases and degrades other proteins. The lysate was cleared by centrifugation (14,000 ×g, 10 min), loaded onto a Spin Column and centrifuged (14,000 ×g) for 1 min. The column was washed twice with 750 µl, followed by 250 µl of Column Wash Solution (60 mM potassium acetate; 10 mM Tris-HCl, pH 7.5; 60 % ethanol), centrifuging at full speed (14,000 ×g) for 1 min each time. Plasmid DNA was eluted in 100 µl of Nuclease-Free Water (14,000 ×g, 1 min) and analysed by nested PCR using primers PCMVF1 and PCMVR1 in the first round PCR, and primers PCMVF2 and PCMVR2 in the nested round. PCR components and cycle parameters for both rounds were as described in section 2.1.1. One microlitre of each first round product was added to the nested round PCR. PCR products from both rounds were analysed by electrophoresis on a 3 % agarose gel, alongside HyperLadder IV DNA markers (Bioline), as in section 2.1.2.

2.1.5 Generation of the *Sma* I restriction site

A *Sma* I restriction endonuclease site was introduced into the 236-bp PCMVpol wild-type sequence by site-directed mutagenesis (Figure 2.2), to allow for separation of wild-type and control amplicons following PCMV QC PCR. A putative *Sma* I site was identified whereby substitution of two bases would produce the sequence CCCGGG as shown below. This site was situated just off-centre in the PCMVpol sequence, to allow for the visualisation of both *Sma* I-digested fragments by polyacrylamide gel electrophoresis. Mutagenic primers PCMVFM and PCMVRM were designed with a 2-bp mismatch to this region (Table 2.1).

——————PCMVRM——————→
GTCCA TCACC AGCAT AGGGC GGGAC ATGCT TAGGC AGACG
←——————PCMVFM——————

These mutagenic primers were used in two separate PCRs; primers PCMVF1 and PCMVFM were used in one PCR to amplify a 148-bp sequence, while primers PCMVR1 and PCMVRM were used in the other, to amplify a 112-bp sequence from 1, 5, and 10 µl of recombinant plasmid, purified in section 2.1.4. PCR components and

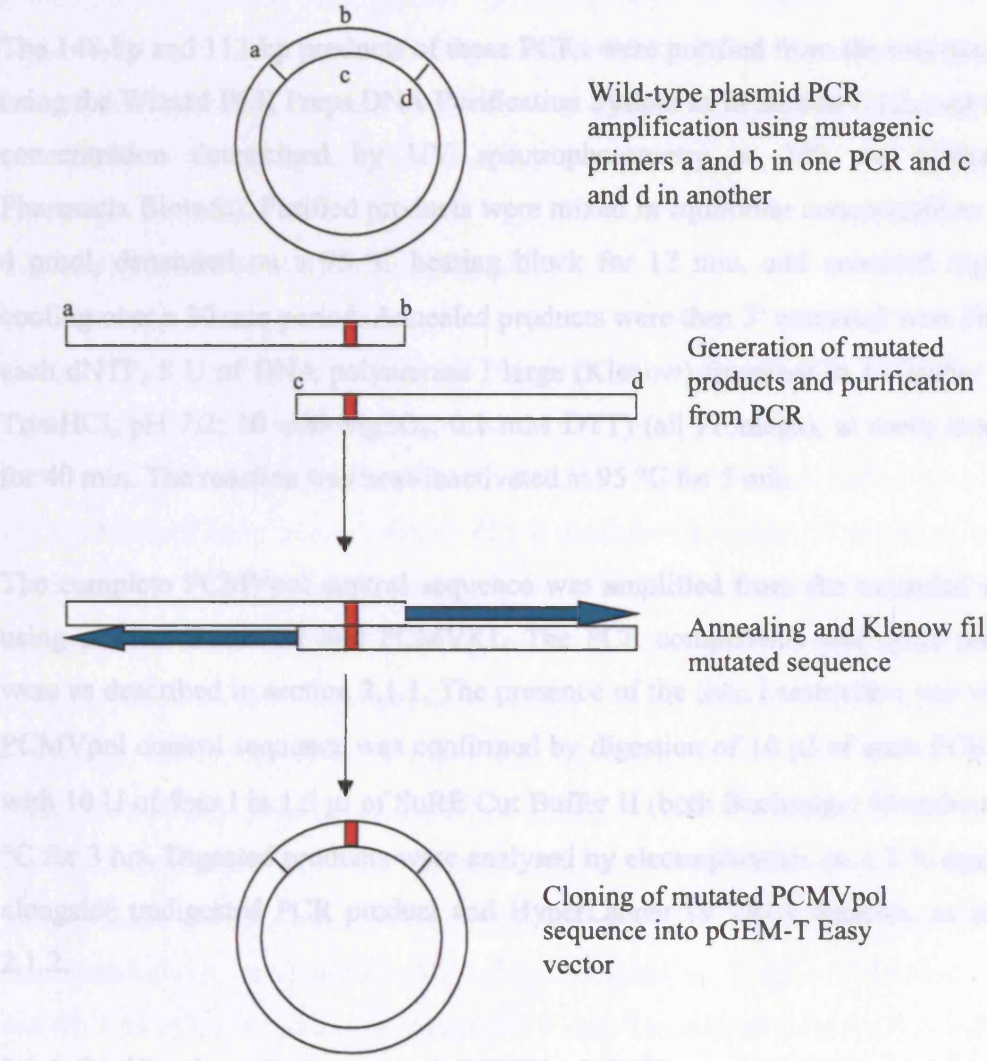


Figure 2.2. A schematic of the site-directed mutagenesis method used to introduce a *Sma* I restriction enzyme site into the PCMVpol wild-type plasmid to generate the PCMVpol control plasmid.

cycle parameters were as described in section 2.1.1. PCR products were analysed by electrophoresis on a 3 % agarose gel, alongside HyperLadder IV DNA markers, as in section 2.1.2.

The 148-bp and 112-bp products of these PCRs were purified from the reaction mixture using the Wizard PCR Preps DNA Purification System as in section 2.1.3, and the DNA concentration determined by UV spectrophotometry at 260 nm (GeneQuantII, Pharmacia Biotech). Purified products were mixed in equimolar concentrations of 1 and 4 pmol, denatured on a 95 °C heating block for 12 min, and annealed together by cooling over a 30 min period. Annealed products were then 3' extended with 200 µM of each dNTP, 5 U of DNA polymerase I large (Klenow) fragment in 1× buffer (50 mM Tris-HCl, pH 7.2; 10 mM $MgSO_4$; 0.1 mM DTT) (all Promega), at room temperature for 40 min. The reaction was heat-inactivated at 95 °C for 5 min.

The complete PCMVpol control sequence was amplified from the extended sequence using primers PCMVF1 and PCMVR1. The PCR components and cycle parameters were as described in section 2.1.1. The presence of the *Sma* I restriction site within the PCMVpol control sequence was confirmed by digestion of 10 µl of each PCR product with 10 U of *Sma* I in 1.5 µl of SuRE Cut Buffer H (both Boehringer Mannheim), at 28 °C for 3 hrs. Digested products were analysed by electrophoresis on a 3 % agarose gel, alongside undigested PCR product and HyperLadder IV DNA markers, as in section 2.1.2.

2.1.6 Purification of the control PCMVpol PCR product and cloning into the pGEM-T Easy vector

The PCMVpol control sequence was purified from the above PCR, ligated into the pGEM-T Easy vector to generate the 'control plasmid', and used to transform competent JM109 *E. coli* cells, as in section 2.1.3. Putative recombinant clones were identified and analysed by picking five single white colonies and growing them in 6 ml cultures as in section 2.1.4. Glycerol stocks were prepared from each overnight culture, and plasmid DNA extracted from the remaining culture as previously described (see section 2.1.4). The presence of the PCMVpol control sequence within the plasmid was confirmed by PCR using primers PCMVF1 and PCMVR1. PCR components and cycle

parameters were as described in section 1.2.1. The presence of the *Sma* I restriction site within the PCMVpol control sequence was confirmed by restriction enzyme digestion of the PCR product as in section 2.1.5. Digested products were analysed by electrophoresis on a 3% agarose gel, alongside undigested PCR product and HyperLadder IV DNA markers, as in section 2.1.2. The presence of the *Sma* I site within the PCMVpol control sequence was also confirmed by sequence analysis (conducted by Oswell Research Products Ltd.).

2.1.7 Large-scale preparation of plasmid DNA

Stocks of wild-type and control PCMVpol plasmids were prepared for use in subsequent experiments by the large-scale preparation of plasmid DNA. Recombinant clones containing each plasmid were freshly streaked onto selection plates from glycerol stocks. Five millilitre starter cultures of LB medium containing 50 µg/ml of ampicillin were prepared by picking a single colony from the freshly streaked selection plates, and incubated for 8 hrs in a 37 °C shaking incubator (~200 rpm). Two hundred microlitres of each starter culture was used to inoculate 100 ml of LB containing 50 µg/ml of ampicillin, and incubated for 16 hrs in a 37 °C shaking incubator (~200 rpm). Cultures were harvested by centrifugation (6000 ×g, 15 min) and plasmid DNA extracted using the QIAfilter Plasmid Maxi Kit (Qiagen). Briefly, the cell pellet was resuspended in 10 ml of Buffer P1 (50 mM Tris-HCl, pH 8; 10 mM EDTA; 100 µg/ml RNase A). Resuspended cells were mixed thoroughly and lysed in 10 ml of buffer P2 (200 mM NaOH; 1 % SDS) at room temperature for 5 min. Ten millilitres of chilled Buffer P3 (3 M potassium acetate, pH 5.5) were then added to the lysate, mixed gently, and loaded into the barrel of a QIAfilter cartridge. Following incubation for 10 min at room temperature, the syringe plunger was inserted gently and the cell lysate filtered into a previously equilibrated QIAGEN-tip 500. The cleared lysate was allowed to enter the resin by gravity flow, before the column was washed twice with 30 ml of Buffer QC (1 M NaCl; 50 mM MOPS, pH 7; 15 % isopropanol). Plasmid DNA was eluted from the column with 15 ml of Buffer QF (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15 % isopropanol) and precipitated in 10.5 ml of room temperature isopropanol. Precipitated DNA was pelleted by centrifugation at 15,000 ×g for 30 min (4 °C). The pellet was washed with 5 ml of 70 % ethanol, air-dried, and resuspended in 1 ml of SDW. The DNA concentration was determined by UV spectrophotometry at 260 nm.

The presence of the wild-type and control PCMVpol sequences within the purified plasmid DNA was confirmed by restriction enzyme analysis. The presence of the wild-type sequence was confirmed by a single digest with *Eco* RI, while the presence of the control sequence was confirmed by a double digest using *Sma* I and *Eco* RI. For the *Eco* RI single digest, 10 µl of purified plasmid were digested with 10 U of *Eco* RI in 1.5 µl of SuRE Cut Buffer H (both Boehringer Mannheim), made up to 15 µl with SDW. The reaction was incubated at 37 °C for 1.5 hrs. For the *Eco* RI and *Sma* I double digest, 10 µl of purified plasmid were digested with 10 U of *Sma* I in 1.5 µl of SuRE Cut Buffer A in SDW to a final volume of 15 µl for 3 hrs at 28 °C, after which 10 U of *Eco* RI was added and incubated at 37 °C for 1.5 hrs. Digested products were analysed by electrophoresis on a 3 % agarose gel, alongside HyperLadder IV DNA markers, as in section 2.1.2.

2.1.8 Dilution and aliquoting of wild-type and control PCMVpol plasmids

Wild-type and control plasmids were diluted to 10^{10} copies/µl for use in subsequent experiments where known amounts of plasmid are added to the PCR. The size of both wild-type and control plasmids was calculated as follows:

pGEM-T Easy plasmid	= 3019 bp (including extra T)
PCMVpol insert	= 236 bp
Total	= 3255 bp

The number of molecules of plasmid/µg of DNA was then calculated as follows:

As 1 µg of double-stranded DNA of 1-kb is equal to 1.52 pmoles

Number of pmoles of plasmid/µg of DNA $= 4.67 \times 10^{-13}$

Number of molecules of plasmid/µg of DNA = number of pmoles × Avogadro's constant (6.023×10^{23})

$= 2.81 \times 10^{11}$ molecules/µg of DNA

Plasmids prepared and purified in section 2.1.7 were diluted to 1×10^{10} copies/µl in SDW, aliquoted in 10 µl volumes, and stored at -80 °C until use.

2.1.9 Optimisation of the PCMV PCR

The annealing temperature, magnesium ion concentration, and cycle number were altered to optimise the sensitivity of the PCMV PCR. The specificity of a PCR can also be optimised by altering the annealing temperature and magnesium ion concentration.

The magnesium ion concentration and annealing temperature were optimised by nested PCR amplification of 50 copies of PCMVpol control plasmid, using either; 1, 1.5, or 2 mM of $MgCl_2$, at an annealing temperature of either 55 °C or 60 °C in both rounds, and performed in triplicate. Since primer PCMVF1 proved to be insensitive, primers PCMVF2 and PCMVR1 were used in the first round PCR to amplify a 212-bp amplicon, while primers PCMVFB (Table 2.1) and PCMVR2 were used in the nested round to amplify a 160-bp product. PCR components and cycle parameters were otherwise as previously described (see section 2.1.1). One microlitre of the first round PCR product was added to the nested round and amplified for 18 cycles. Nested round PCR products were analysed by electrophoresis on a 1 % agarose gel, alongside HyperLadder IV DNA markers, as in section 2.1.2. For a 1 % agarose gel, 1 g of multipurpose agarose was dissolved in 100 ml of TBE buffer.

The number of cycles to be used in the nested round of the QC PCR was optimised to a point where the product resulting from the amplification of 10 copies of PCMVpol control plasmid could be easily identified, but distinguished from the products resulting from the amplification of 10^2 and 10^3 copies of PCMVpol control plasmid, i.e. during the exponential phase of the PCR. Between 10 and 10^3 copies of PCMVpol control plasmid were amplified by nested PCR as in sections 2.1.1 and 2.1.4, using the new primer sets described above. Nested round PCR tubes were removed from the thermal cycler between cycles 10 and 15. PCR products were analysed by electrophoresis on a 1 % agarose gel, alongside HyperLadder IV DNA markers, as in section 2.1.2.

2.1.10 Investigating the sensitivity of the PCMV PCR

To investigate the sensitivity of the PCMV PCR, the PCMVpol control plasmid was diluted from between 10^3 and 1 copy, and amplified in triplicate by nested PCR using

the new primer sets described above. PCR components and cycle parameters of both rounds were as described in section 2.1.1, however in the first round an annealing temperature of 60 °C was used, while in the nested round, the annealing temperature was reduced to 55 °C and 15 cycles of amplification were used. One microlitre of first round PCR product was added to the nested round PCR. Nested round PCR products were analysed by electrophoresis on a 3 % agarose gel, alongside HyperLadder IV DNA markers, as in section 2.1.2.

2.1.11 Investigating the specificity of the PCMV PCR

The specificity of the PCMVpol primers was determined by nested PCR amplification of viral DNA from other betaherpesviruses; HHV-6, HHV-7, and HCMV, alongside PCMV. One microlitre of a TCS or DNA sample of each virus was added to the PCR in duplicate and amplified as in section 2.1.10. PCR products from both rounds were analysed by electrophoresis on a 3 % agarose gel, alongside HyperLadder IV DNA markers, as in section 2.1.2.

2.1.12 Characterisation of the PCMV QC PCR

To assess the utility of the PCMVpol control sequence in the QC PCR, a range of known copy numbers of wild-type and control plasmids were co-amplified in triplicate. Both plasmids were diluted from between 5×10^6 and 5 copies in SDW. A single round of PCR was performed when 5×10^6 to 10^3 copies were used, while a nested PCR was performed when 10^3 to 5 copies of plasmid were used. PCR components and cycle parameters were as otherwise described (see section 2.1.10). Following amplification, 10 µl of PCR product were digested with *Sma* I (as in section 2.1.5), and analysed on a 10 % (first round) or 12 % (nested round) polyacrylamide gel electrophoresis (PAGE) gel.

10 % PAGE gels were prepared using 2.5 ml of 40 % bis-acrylamide (Scotlab), 1 ml of 10× TBE, 70 µl of 10 % ammonium persulphate (APS) and 7 µl of N,N,N',N' tetramethylethylenediamine (TEMED) (both Sigma) in 6.5 ml of SDW. 12 % PAGE gels were prepared using 3 ml of 40 % bis-acrylamide, 1 ml of 10× TBE, 70 µl of 10 % APS and 7 µl of TEMED in 6 ml of SDW. Gels were allowed to set at room

temperature for 20 min. Ten microlitres of each PCR product were mixed with 3 µl of loading dye, and loaded into the wells. Gels were electrophoresed at 45 mA for 45 min and stained in 50 ml of TBE buffer containing 30 µg of ethidium bromide for 5 min. Gels were visualised as in section 2.1.2.

From the photographs of each gel, the intensities of the product bands for wild-type and control amplicons were determined using a computer analysis method of quantitation as previously described (Kidd *et al*, 2000). Briefly, QC PCR PAGE gel images were scanned at 200 pixels per inch using an Epsom scanner, and imported into the NIH Image program (version 1.63). A negative of the photograph was then obtained by inverting the image, and the lanes on the gel marked using the gel reading macros supplied. Electronic desitometric plots were generated, and the pixel area under each peak was calculated. The ratio of wild-type signal to control signal, multiplied by the input PCMVpol control sequence copy number, was used to calculate the input copy number of wild-type sequence. The range of quantitation for each control sequence copy number was 0.5 logunit, for example, with a control plasmid copy number of 100, between 50 and 500 copies of wild-type plasmid were tested. The mean result from three experiments was plotted against the known input copy numbers of wild-type sequence.

2.2 Investigating the prevalence, quantity and organ distribution of PCMV in immunocompetent and immunosuppressed pigs being bred for xenograft organs, investigating when virus is acquired, and examining methods to eliminate PCMV from these animals.

The qualitative and QC PCR assays developed in section 2.1 were used to detect and quantify PCMV DNA in a range of organs and clinical samples from both immunocompetent and immunosuppressed pigs being bred for xenograft organs. This section describes the methodologies used to extract total DNA from these porcine samples, followed by the PCMV PCR assays used to detect and quantify PCMV.

2.2.1 Porcine samples

A range of porcine organs and clinical samples (as shown in Chapter 4, section 4.2) were obtained from Imutran Ltd., principally in collaboration with Dr Dan Tucker, and were derived from Large White pigs, transgenic for the human decay accelerating factor (CD55) (Cozzi & White, 1995). This gene has been cloned into the genome of these pigs in order to prevent hyperacute xenograft rejection. Samples were collected using individual disposable sets of equipment to minimise the possibility of cross-contamination of DNA between tissues of the same animal, or between tissues of two separate animals. Samples were stored at -80 °C before shipment, on dry ice, to the Royal Free and University College Medical School, London, where they were stored at -80 °C prior to analysis.

The rearing of animals included in this study and all the associated procedures were undertaken according to the requirements of the United Kingdom Home Office Animal (Scientific Procedures) Act 1986, or the Ontario (Canada) Animals for Research Act, as appropriate.

To investigate the tissue distribution and PCMV viral loads in adult pigs and piglets, a range of organs and clinical samples were collected from 6 adult and 4 juvenile conventionally-reared pigs. All adult pigs were aged >15 months while juvenile pigs were aged between 19 and 34 days at the time of sampling. Animals were not directly related to each other but were reared identically in a shared airspace.

To investigate temporal acquisition of PCMV two sows and their litters were prospectively monitored from birth. The first litter and their dam (sow P49M), were blood sampled (1 ml of EDTA anti-coagulated blood) on days 1, 7, 14, 35, and 49 following birth. Both sow and litter were euthanised on day 82 when a final blood sample and spleen tissue sample was collected from each animal. The second litter and their dam (sow G72E) were blood sampled on days 1, 7, 17, and 36. All animals were reared under conventional farm conditions.

To investigate evidence for transplacental transmission of PCMV, two pregnant sows were euthanised at day 86 out of an average full-term gestation of 114 days, and spleen samples collected from both sows and their foetuses. The two sows were reared under conventional farm conditions.

To investigate the prevalence of PCMV in SPF pigs, spleen samples were collected from a total of 9 sows and their hysterotomy-derived, barrier-reared piglets (total of 31 piglets). The sows were reared under conventional farm conditions. Piglets were derived from these sows by caesarean section at day 113 of gestation. Each litter (cohort) was raised in a gnotobiotic unit for the first 14 days of life, at the Onterio Veterinary College, before being transferred to a bioexclusion facility at the Toronto General Hospital. These rearing conditions are further described elsewhere (Tucker *et al*, 2002a). Spleen samples were collected from two or three piglets from 7 of the cohorts, which were taken as sentinel animals. These piglets had a median age of 9 weeks (range 4-16 weeks). In the remaining 2 cohorts, spleen samples were collected from all piglets (total of 8) which were taken as sentinel animals. These piglets had a median age of 18 weeks (range 5-21 weeks).

To investigate the effect of immunosuppression on PCMV viral loads, a range of tissue samples were collected from three groups of animals reared under different conditions. The first group were derived by hysterotomy, reared in a gnotobiotic unit, and aged 58 days at necropsy. The second group of SEW pigs were naturally farrowed, but weaned at 5 days of age, and reared in a gnotobiotic unit. These pigs were aged 57 days at necropsy. While the third group of pigs were naturally farrowed, weaned at 5 weeks of age, and reared under conventional farm conditions. These pigs were aged 52 days at

necropsy. All groups consisted of immunosuppressed and control animals. Immunosuppressed pigs received a daily regimen of cyclosporine A, azathioprine, and prednisolone from 7-12 days of age for a period of approximately 2 months, while control pigs did not receive any immunosuppressive treatment. Cyclosporin A was administered at 15 mg/kg *per os*, azathioprine at 1 mg/kg *per os*, and prednisolone at 1 mg/kg *per os*, reducing to 0.5 mg/kg in the second week, 0.25 mg/kg in the third week, and to 0.1 mg/kg thereafter.

2.2.2 Extraction of DNA from porcine samples

Extraction of total DNA from tissues samples

All porcine samples were handled in a Class II Microbiological Safety Cabinet. Porcine tissue samples were allowed to thaw at room temperature. Once thawed, a section of approximately 0.125 cm^3 was cut from each tissue sample and cut further into smaller pieces using disposable sterile scalpels and forceps. The tissue fragments were digested overnight in 500 µl of TNE buffer (55 mM Tris-HCl, pH 8; 110 mM NaCl; 1.1 mM EDTA, pH 8; 0.55 % SDS), and 4 µg/ml of Proteinase K (Boehringer Mannheim) at 65 °C. The proteinase K was inactivated by incubation at 95 °C for 5 min, and DNA extracted using phenol and chloroform, followed by ethanol precipitation. Five hundred microlitres of phenol (Sigma) were added to each tube and vortexed for 1 min. Samples were centrifuged at 13,000 ×g for 5 min to separate the lower organic layer from the upper aqueous layer containing the DNA. The top aqueous layer was transferred to a 1.5 ml eppendorf containing 250 µl of phenol and 250 µl of chloroform (BDH). Samples were mixed by vortexing and centrifuged at 13,000 ×g for 5 min. The top layer was again transferred to a 1.5 ml eppendorf containing 50 µl of 3 mM sodium acetate, pH 5.2, and mixed by vortexing. DNA was precipitated in 1 ml of ice-cold ethanol and incubated at -80 °C for 1 hr. The DNA was pelleted by centrifugation at 13,000 ×g for 5 min and washed in 600 µl of 70 % ethanol. The pellet was then air-dried for 10-15 min before resuspension in 100 µl of SDW. The concentration of the extracted DNA was determined by UV spectrophotometry at 260 nm and 1 µg was analysed by PCR.

Extraction of total DNA from whole blood and PBMC samples

DNA was extracted from both whole blood and PBMC samples using the Wizard Genomic DNA Purification Kit (Promega). DNA was extracted from PBMCs using the protocol for the Isolation of Genomic DNA from Tissue Culture Cells and Animal Tissue. Briefly, PBMCs were lysed by pipetting in 600 µl of Nuclei Lysis Solution. Three microlitres of RNase was added to the nuclear lysate and samples mixed by inversion, before incubation at 37 °C for 15-30 min. After cooling to room temperature, protein was precipitated by the addition of 200 µl of Protein Precipitation Solution and vortexed at high speed for 20 s. Samples were chilled on ice for 5 min to aid protein precipitation, before being centrifuged at 13,000 ×g for 4 min to pellet the protein. The supernatant was transferred to a 1.5 ml eppendorf containing 600 µl of isopropanol and mixed by inversion to precipitate the DNA. The DNA was pelleted by centrifugation at 13,000 ×g for 1 min and washed in 600 µl of 70 % ethanol. The pellet was air-dried for 10-15 min and resuspended in 50 µl of SDW.

DNA was extracted from 1 ml of EDTA anti-coagulated whole blood samples using the protocol for the Isolation of Genomic DNA from Whole Blood. Briefly, 3× 300 µl of each whole blood sample were added to 1.5 ml eppendorfs containing 900 µl of Cell Lysis Solution. Samples were mixed by inversion and incubated at room temperature for 10 min to lyse the red blood cells. White blood cells were pelleted by centrifugation at 13,000 ×g for 20 s, and resuspended in 10-20 µl of residual liquid. Cells were lysed by the addition of 300 µl of Nuclei Lysis Solution to each tube and pipetted until homogeneous. One and a half microlitres of RNase solution were added to samples and mixed by inversion, before incubation at 37 °C for 15 min. After cooling to room temperature, protein was precipitated by the addition of 100 µl of Protein Precipitation Solution and centrifugation at 13,000 ×g for 3 min. The supernatant was transferred to a 1.5 ml eppendorf containing 300 µl of isopropanol and mixed by inversion to precipitate the DNA. The DNA was pelleted by centrifugation for 1 min at 13,000 ×g and washed in 300 µl of 70 % ethanol. The pellet was air-dried for 10-15 min and resuspended in 50 µl of SDW. The concentration of the extracted DNA was determined by UV spectrophotometry and 1 µg was analysed by PCR.

Extraction of total DNA from serum and faeces samples

DNA was extracted from porcine serum and faeces samples using the QIAamp DNA Mini Kit (Qiagen). Serum samples were extracted using the Blood and Body Fluid Spin Protocol. Briefly, 200 µl of serum was transferred to a 1.5 ml eppendorf containing 20 µl of QIAGEN Protease (20 mg/ml). Two hundred microlitres of Buffer AL were added to each tube, mixed by vortexing, and incubated at 56 °C for 10 min. Two hundred microlitres of ethanol were added to each sample, and loaded onto a QIAamp Spin Column in a 2 ml collection tube, before centrifugation at 6,000 ×g for 1 min. Each column was washed with 500 µl of buffer AW1, centrifuging at 6,000 ×g for 1 min, followed by washing with 500 µl of buffer AW2 and centrifugation at 13,000 ×g for 3 min. DNA was eluted by the addition of 200 µl of SDW and centrifuging at 6,000 ×g for 1 min.

Approximately 1 ml of each faeces sample was resuspended in 4 ml of 0.89 % NaCl and clarified by centrifugation at 4,000 ×g for 20 minutes. The supernatant was filtered through a 0.2 µm filter (Sartorius), and 200 µl of each filtrate was extracted using the Blood and Body Fluid Spin Protocol (as above), without performing the QIAGEN Protease step. Five microlitres of each extracted serum and faeces sample were analysed directly by PCR.

Extraction of total DNA from nasal and saliva swab samples

Nasal and saliva swabs were transferred to 1.5 ml eppendorf tubes containing 200 µl of PBS and incubated at room temperature for 20 min. Swabs were then washed and squeezed against the side to the tube to remove excess sample, and discarded. Fifty microlitres of each swab sample were transferred to a 0.5 ml eppendorf, held in a 0.5 ml eppendorf box (TreffLab), and boiled for 10 min in a boiling water bath. Five microlitres of each sample were analysed directly by PCR.

Urine samples

Three microlitres of each urine sample were analysed directly by PCR.

2.2.3 PCR procedure for clinical samples

In order to prevent contamination of PCR reagents and individual reactions with PCMV DNA, the stages in setting up the PCR were performed in separate rooms using different sets of pippettes. The PCR mixture was prepared and aliquoted into tubes in a 'clean PCR' laboratory, following which, tubes were transferred to the 'PCR set-up' laboratory, where clinical samples were added. The actual PCR and analysis of products were performed in separate 'post-PCR' laboratories.

2.2.4 Detection of PCMV DNA by qualitative PCR

In the qualitative PCMV PCR, first round primers PCMVF2 and PCMVR1 amplify a 212-bp amplicon, while nested primers PCMVFB and PCMVR2 amplify a 160-bp product. Each PCR mixture contained 100 ng of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 1 U of *Taq* polymerase, in $(NH_4)_2SO_4$ PCR buffer. The cycle parameters used for both rounds were; initial denaturation at 95 °C for 6 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The final extension was increased to 10 min. In the nested round, the annealing temperature was reduced to 55 °C. One microlitre of first round product was added to the nested round PCR. Positive and negative controls were amplified by PCR alongside porcine samples, to ensure accuracy of the results. Positive controls consisted of 10^3 copies of PCMVpol control plasmid, while negative controls comprised SDW prepared throughout the addition of clinical samples and 1 μg of DNA extracted from known negative porcine tissues. Ten microlitres of nested round PCR product were analysed on a 3 % agarose gel, alongside HyperLadder IV DNA markers, as in section 2.1.2.

2.2.5 Quantification of PCMV DNA by QC PCR

QC PCR was used to determine the PCMV viral load in samples testing positive by qualitative PCR. Sample DNA was amplified alongside varying copy numbers of PCMVpol control plasmid. PCR components and cycle parameters were as used in the qualitative PCR (section 2.2.4), however, in the nested round the number of cycles of amplification was reduced to 15.

Positive and negative controls were also amplified by QC PCR alongside porcine samples, to ensure accuracy of the results. Positive controls consisted of an equivalent copy number of PCMVpol control plasmid only, as was used in the testing of samples, while negative controls comprised SDW.

Nested round PCR products were digested with *Sma* I (see section 2.1.5), and analysed by PAGE on 10 % (first round) or 12 % (nested round) gel (see section 2.1.12). The gels were stained with ethidium bromide and photographed. The intensities of the bands for sample (wild-type) and control amplicons were analysed as previously described (Kidd *et al*, 2000). The viral load of the sample was determined from the value where the wild-type-to-control-amplicon signal ratio was closest to equivalence. Viral loads below the threshold of quantification were given an arbitrary viral load of 1 genome copy/μg of DNA.

2.3 Cloning and expression of PCMV glycoprotein B in insect cells using a baculovirus expression system, and the development of a recombinant protein-based serological assay for PCMV

This section describes the methodologies used to clone and express the complete ORF of PCMV glycoprotein B (gB) in insect cells using a baculovirus expression system. In order to ease purification of the recombinant protein, a truncated sequence of glycoprotein B (gBT), excluding the proposed transmembrane regions, was amplified and cloned using the same system. Both sequences were initially cloned into a baculovirus transfer vector, before co-transfection into insect cells alongside baculovirus DNA, to generate recombinant baculovirus. Recombinant protein production in Sf9 and High Five insect cells was optimised, and used to develop a recombinant protein-based serological assay for the detection of PCMV-specific antibodies in pig serum.

2.3.1 PCR amplification of the complete and truncated PCMV gB gene

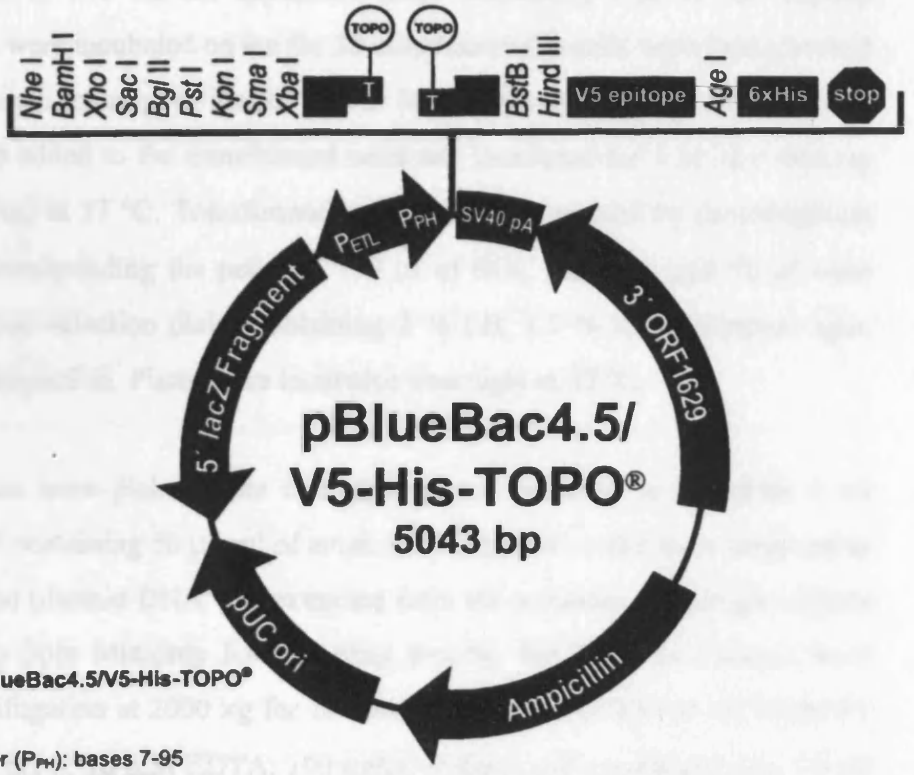
Primers PCMVgBF and PCMVgBR were used to amplify the complete ORF of PCMV gB, while primers PCMVgBF and PCMVgBT were used to amplify a truncated sequence of the gB gene. All primers were designed from the published sequence of the PCMV gB gene (Widen *et al*, 2001), and are shown in Table 2.1. The forward primer PCMVgBF, was designed to anneal 30 nucleotides upstream from the ATG initiation codon of PCMV gB, and out of frame with the mutated polyhedrin site in the baculovirus vector. The reverse primer PCMVgBR, was designed to anneal to the 3' end of the gene, just upstream from the stop codon at amino acid residue 859, and in frame with the carboxyl-terminal peptide of the baculovirus transfer vector containing the 6× histidine (His) tag, which enables detection and purification of recombinant protein in insect cells. The truncated primer PCMVgBT, was designed to anneal 33 nucleotides downstream from the HCMV AD-1-homologous region at amino acid residue 634, 213 nucleotides upstream from the predicted transmembrane regions of PCMV gB (Widen *et al*, 2001), and in frame with the carboxyl-terminal peptide of the transfer vector. Complete (2.6-kb) and truncated (1.93-kb) sequences were amplified from a TCS sample of porcine alveolar macrophages infected with PCMV strain B6 (provided by Dr M. Banks, Veterinary Laboratories Agency, UK). The PCR mixture

contained 100 ng of each primer, 2 mM of $MgCl_2$, 200 µM of each dNTP, 2 U of BIO-X-ACT DNA polymerase (Bioline), and 1 µl of PCMV TCS sample diluted 1/10 in SDW, in PCR Optibuffer (Bioline). BIO-X-ACT DNA polymerase was used to amplify PCMV gB and gBT as it has a 3'-5' proof-reading activity, which prevents the misincorporation of nucleotides during primer extension. The cycle parameters used were initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 2.5 min. The final extension was increased to 10 min. PCR products were analysed on a 0.8 % agarose gel alongside HyperLadder I DNA markers (Bioline), as in section 2.1.2.

2.3.2 Purification and cloning of the PCMV gB and gBT sequences into the baculovirus vector

The PCR amplified complete and truncated PCMV gB sequences were purified from the PCR mixture by separation on a 2 % low gelling temperature agarose gel in TAE buffer, followed by DNA extraction using the QIAquick Gel Extraction Kit (Qiagen). Briefly, the DNA fragment was excised from the gel using a sterile scalpel, and weighed in a 1.5 ml eppendorf tube. Three hundred microlitres of Buffer QC was added to each 100 mg of gel, and incubated at 50 °C for 10 min. One gel volume of isopropanol was then added, before the samples were loaded onto a QIAquick spin column in a 2 ml collection tube and centrifuged at 13,000 ×g for 1 min. The flow-through was discarded and 500 µl of Buffer QG were added to the column, before being centrifuged as before. The column was washed with 750 µl of Buffer PE, left to stand for 4 min, and again centrifuged for 1 min at 13,000 ×g. The QIAquick column was then transferred to a 1.5 ml eppendorf tube. To elute the DNA, 50 µl of SDW were added to the column, left to stand for 1 min and centrifuged at 13,000 ×g for 1 min.

Purified PCMV gB and gBT sequences were ligated into the baculovirus vector pBlueBac4.5/V5-His TOPO TA (Invitrogen) (Figure 2.3), to produce the recombinant transfer vector, and used to transform competent DH5α *E. coli* cells (Invitrogen). Each ligation reaction contained 1 µl of purified PCR product, 1 µl of salt solution, 3 µl of SDW and 1 µl of baculovirus vector in a 0.5 ml eppendorf tube. Ligation reactions were mixed gently and incubated for 30 min at room temperature, before returning to ice.



Comments for pBlueBac4.5/V5-His-TOPO®
5043 nucleotides

Polyhedrin promoter (P_{PH}): bases 7-95
Multiple cloning site: bases 132-205
TOPO® Cloning site: bases 187-188
V5 epitope: bases 212-253
6xHis tag: bases: 263-280
SV40 polyadenylation sequence: bases 305-432
3´ end of Ac-PH: bases 436-537
3´ end of ORF1629: bases 1445-567 (complementary)
Sequence homologous to ORF1629 in Bac-N-Blue DNA: bases 1447-649 (complementary)
Ampicillin resistance gene: bases 1833-2690
pUC origin: bases 2838-3511
5´ *lacZ* fragment: bases 4741-3629 (complementary)
Sequence homologous to *lacZ* sequence in Bac-N-Blue DNA: bases 4514-3629 (complementary)
Early to late promoter: bases 5043-4742 (complementary)

Figure 2.3. The pBlueBac4.5/V5-His-TOPO vector (Invitrogen), contains a multiple cloning site adjacent to the polyhedrin promoter (P_{PH}). An ampicillin resistance gene and the pUC origin allow for selection and replication of the recombinant vector in *E. coli*, while a C-terminal peptide encoding a 6× histidine (His) tag allows for detection and purification of recombinant protein in insect cells. The linearised vector also has single 3' thymidine (T) overhangs, which enable direct cloning of PCR products amplified using *Taq* DNA polymerases, such as BIO-X-ACT, which add a single deoxyadenosine (A) to the 3' ends of PCR products.

Two vials of competent DH5α *E. coli* cells were thawed on ice, and 50 µl were transferred to each of two 1.5 ml eppendorf tubes containing 2 µl of the ligation reactions. Samples were incubated on ice for 20 min, before the cells were heat-shocked at 42 °C for 20 s, then returned to ice for 2 min. Nine hundred and fifty microlitres of SOC medium were added to the transformed cells and incubated for 1 hr in a shaking incubator (~200 rpm) at 37 °C. Transformed cells were concentrated by centrifugation (70 ×g, 10 min), resuspending the pellet in 150 µl of SOC medium, and 50 µl were plated onto antibiotic selection plates containing 2 % LB, 1.5 % bacteriological agar, and 100 µg/ml of ampicillin. Plates were incubated overnight at 37 °C.

Recombinant clones were picked from overnight plates and used to inoculate 6 ml cultures of 2 % LB containing 50 µg/ml of ampicillin. Glycerol stocks were prepared as in section 2.1.4, and plasmid DNA was extracted from the remaining overnight culture using the QIAprep Spin Miniprep Kit (Qiagen). Briefly, the bacterial cultures were harvested by centrifugation at 2000 ×g for 10 min, resuspended in 250 µl of Buffer P1 (50 mM Tris-HCl, pH 8; 10 mM EDTA; 100 µg/ml RNase) and transferred to a 1.5 ml eppendorf tube. Cells were lysed in 250 µl of Buffer P2 (200 mM NaOH; 1 % SDS) by inverting 4-6 times, before the addition of 350 µl of Buffer N3 (3M potassium acetate, pH 5.5), and centrifuged at 13,000 ×g to pellet cellular protein. The supernatant was then applied to a QIAprep spin column in a 2 ml collection tube and centrifuged at 13,000 ×g for 1 min. The flow-through was discarded and the column washed with 750 µl of Buffer PE (Qiagen). To elute the DNA, 100 µl of SDW was added to the column, left for 1 min, and centrifuged at 13,000 ×g for 1 min. The presence of the PCMV gB and gBT sequences within the recombinant baculovirus vector was confirmed by digestion with *Bam* HI and *Hind* III restriction enzymes. Ten microlitres of purified plasmid were digested with 10 U of *Bam* HI and 10 U of *Hind* III in 1.5 µl of SuRE Cut Buffer B (all Boehringer Mannheim), in SDW to a final volume of 15 µl, for 1 hr 15 min at 37 °C. Digested products were analysed by electrophoresis on a 1 % agarose gel, alongside HyperLadder I DNA markers, as in section 2.1.2. In addition, in order to confirm that no mutations had been introduced into the PCMV gB and gBT sequences during the PCR, recombinant vector DNA was sent for sequencing at an in house facility (Windeyer Institute, UCL).

2.3.3 Preparation of high quality plasmid DNA for transfection

High quality plasmid DNA was needed for efficient transfection of insect cells. Recombinant PCMV gB and gBT clones were streaked onto ampicillin selection plates, from glycerol stocks. Five millilitre starter cultures of 2 % LB containing 50 μg/ml of ampicillin were prepared by picking a single colony from the freshly streaked selection plate, and incubated for 8 hrs in a 37 °C shaking incubator (~200 rpm). Two hundred microlitres of each starter culture were used to inoculate 100 ml of 2 % LB containing 50 μg/ml of ampicillin and incubated for 16 hrs in a 37 °C shaking incubator (~200 rpm). Cultures were harvested by centrifugation (6000 ×g, 15 min) and plasmid DNA was extracted using the Endofree Plasmid Maxi Kit (Qiagen). Briefly, the cell pellet was resuspended in 10 ml of Buffer P1 (50 mM Tris-HCl, pH 8; 10 mM EDTA; 100 μg/ml RNase), and lysed in 10 ml of Buffer P2 (200 mM NaOH; 1 % SDS), inverting several times over a 5 min period. Ten millilitres of chilled Buffer P3 (3M potassium acetate, pH 5.5) were added to the lysate, mixed by invertion, and then loaded into the barrel of a QIAfilter cartridge. Following incubation for 10 min at room temperature, the cell lysate was pushed through the column using a plunger, into a 50 ml collection tube. Two and a half millilitres of Buffer ER were added to the filtered lysate and incubated on ice for 30 min. Meanwhile, a QIAGEN-tip 500 was equilibrated by applying 10 ml of Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7; 15 % isopropanol; 0.15 % Triton X-100). The filtered lysate was then applied to the column, allowed to enter the resin by gravity flow, and the flow-through was discarded. The column was washed twice with 30 ml of Buffer QC (1 M NaCl; 50 mM MOPS, pH 7; 15 % isopropanol), and plasmid DNA eluted with 15 ml of Buffer QN (1.6 M NaCl; 50 mM MOPS, pH 7; 15 % isopropanol). DNA was precipitated by the addition of 10.5 ml of isopropanol, mixed and centrifuged at 15,000 ×g for 30 min (4 °C), to pellet the DNA. The DNA pellet was washed with 5 ml of endotoxin-free 70 % ethanol, air-dried, and resuspended in 100 μl of endotoxin-free Buffer TE (10 mM Tris-Cl, pH 8; 1 mM EDTA). The DNA concentration of the purified plasmid DNA was determined by UV spectrophotometry at 260 nm.

2.3.4 Insect cell culture

Sf9 insect cells derived from *Spodoptera frugiperda* and High Five (Tn5) insect cells derived from *Trichoplusia ni* were used throughout this investigation. Sf9 insect cells were used for the transfection, plaque purification and the generation of high-titre virus stocks, since their regular size makes them ideal for the formation of monolayers and plaques. High Five insect cells were investigated for the expression of recombinant PCMV gBT since they have been reported to increase the yield of certain secreted proteins (Wickham & Nemerow, 1993).

Sf9 insect cells were maintained in Sf-900II SFM medium (Invitrogen), supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin (both Sigma) and 10 % FCS (Labtech International), at 28 °C, and split at a ratio of 1:3, every 2-3 days. High Five insect cells were maintained in Express Five medium (Invitrogen), supplemented with 20 U/ml of penicillin, 20 µg/ml of streptomycin and 17 mM of L-Glutamine (Invitrogen), at 28 °C, and split at a ratio of 1:4, every 2-3 days.

2.3.5 Transfection of Sf9 insect cells

Transfection of recombinant baculovirus into insect cells was carried out using the Bac-N-Blue Transfection Kit (Invitrogen), according to the manufacturer's instructions. Sf9 insect cells (2×10^6) were seeded in two 60 mm cell culture dishes in 4 ml of serum-free Sf-900II medium, and allowed to attach at room temperature for 30 min. Meanwhile, 4 µl (4 µg) of endofree-purified recombinant transfer vector, 20 µl of Cellfectin reagent (Invitrogen) and 1 ml of serum-free Sf-900II medium were added to a 1.5 ml tube containing 10 µl (0.5 µg) of Bac-N-Blue DNA (Invitrogen), and mixed gently by flicking. Once the insect cells had attached to the cell culture dishes, medium was removed from the cells, and the transfection mixture was added drop-wise onto one 60 mm dish, while 1 ml of serum-free Sf-900II media was added to the other dish as a cells-only control. Cell culture dishes were then incubated at room temperature for 4 hrs with gentle rocking, before the serum-free medium was replaced with complete Sf-900II medium, and returned to the 28 °C incubator. Culture medium was harvested 72 hrs posttransfection by centrifugation (400 ×g, 6 min) and labelled as transfection virus

stock, while the cells were incubated at 28 °C for a further 4-7 days for visual confirmation of successful transfection by light microscopy.

2.3.6 Purification of recombinant virus by plaque assay

Purification of recombinant virus from uncut or non-recombinant (wild-type) baculovirus DNA was important to prevent dilution of recombinant virus, since wild-type virus replicates more efficiently than recombinant virus (Bac-N-Blue Transfection and Expression Guide, 2002). Recombinant virus was purified by plaque assay according to the Bac-N-Blue Transfection Kit. Briefly, 1 ml dilutions of 10^{-1}, 10^{-2} and 10^{-3} of transfection virus stock (see section 2.3.5), harvested 72 hrs posttransfection, were prepared and used to infect a monolayer of 5 $\times 10^6$ Sf9 insect cells seeded in 100 mm cell culture dishes. Infected cells were incubated for 1 hr at room temperature with gentle rocking. The cells were then covered with 10 ml of an overlay of complete Sf-900II medium containing 0.625 % low gelling temperature agarose, and incubated at 28 °C on paper towels dampened with 5 mM EDTA, in a sealed plastic bag. Five days postinfection, 1 ml of complete Sf-900II medium containing 75 µg/ml of X-Gal was added to each plate and incubated overnight at 28 °C. Cells were screened for the formation of viral plaques 6 dpi. Viral plaques appeared as areas of lysed cells and were identified as distinct blue spots on a clear background of cells. Plaques were cut out of the agarose overlay using a wide-ended pastette and stored in 1.5 ml eppendorfs containing 1 ml of complete Sf-900II medium at 4 °C.

2.3.7 PCR analysis of recombinant virus

The presence of recombinant virus was confirmed by PCR analysis of each viral plaque. Four hundred microlitres of complete Sf-900II medium containing each recombinant virus agarose plug was used to infect 5 $\times 10^6$ Sf9 insect cells in 25 cm^2 cell culture flasks, and incubated at 28 °C for 6 days. Infected cells were harvested and separated from the culture medium by centrifugation (400 ×g, 6 min). The supernatant was labelled as P1 virus stock and stored at 4 °C, while viral DNA was isolated from the cell pellet using the Puregene DNA Extraction Kit (Gentra). Briefly, the cell pellet was lysed in 600 µl of Cell Lysis Solution and transferred to a 1.5 ml eppendorf tube. Two hundred microlitres of Protein Precipitation Solution were added to samples, mixed by

vortexing and centrifuged at 13,000 ×g for 5 min. The supernatant was transferred to a 1.5 ml eppendorf tube containing 600 µl of isopropanol, inverted ~50 times, and incubated on ice for 3-4 min to aid DNA precipitation. The DNA was pelleted by centrifugation (13,000 ×g, 5 min), washed with 600 µl of 70 % ethanol, and air-dried for 20 s. The DNA pellet was resuspended in 200 µl of DNA Rehydration Solution and incubated at 65 °C for 5 min to aid resuspension.

Both PCMV gB- and baculovirus-specific primers (Table 2.1), were used to amplify recombinant viral DNA from the extracted insect cells. A positive control comprising of recombinant transfer vector, and negative controls comprising wild-type baculovirus (provided by Dr Hassan-Walker, Centre for Virology, UCL), DNA extracted from uninfected Sf9 insect cells, and SDW, were also amplified by PCR. Each PCR mixture using baculovirus primers contained 100 ng of each primer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, 2.5 U of HotStarTaq polymerase (Qiagen), and 1 µg of extracted DNA, in PCR Buffer (Qiagen), to a final volume of 50 µl. The cycle parameters used were initial denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and extension at 72 °C for 3 min. The final extension was increased to 10 min. The PCR mixture using PCMV gB-specific primers contained 100 ng of each primer, 2 mM of $MgCl_2$, 200 µM of each dNTP, 2 U of BIO-X-ACT polymerase, and 1 µg of extracted DNA, in PCR Optibuffer, to a final volume of 50 µl. The cycle parameters used were initial denaturation at 95 °C for 2 min, followed by 41 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 2.5 min. The final extension was increased to 10 min. PCR products were analysed by electrophoresis on a 1 % agarose gel, alongside HyperLadder I DNA markers, as in section 2.1.2.

2.3.8 Preparation of high-titre virus stocks and determination of virus titre

High-titre virus stocks of recombinant PCMV gB and gBT baculoviruses were prepared for the production of recombinant proteins. Sf9 insect cells (2×10^6), seeded in 25 cm^2 cell culture flasks, were infected with 20 µl of the P1 virus stock harvested in section 2.3.7, and incubated at 28 °C for 7 days. The culture medium was harvested by centrifugation (400 ×g, 6 min) 7 dpi, and the supernatant labelled as P2 virus stock. Two

hundred microlitres of this P2 stock were used to infect 1.5×10^7 Sf9 insect cells in 175 cm^2 cell culture flasks. The culture medium from these infected cells was harvested by centrifugation (400 ×g, 6 min) 6 dpi and the supernatant labelled as P3 virus stock.

The titre of the P3 virus stock was determined by plaque assay. Sf9 insect cells (5×10^6) were seeded in 100 mm cell culture dishes, and infected with 1 ml of 10^{-5}, 10^{-6} and 10^{-7} dilutions of the P3 virus stock, in triplicate. Infected cells were covered with an overlay as in section 2.3.6. Six days postinfection plates were covered with 1.5 ml of a second overlay of complete Sf-900II medium containing 0.41 % low gelling temperature agarose and 0.25 mg/ml of Neutral Red Solution (Sigma), and incubated overnight at room temperature. Plaques were identified as pale pink spots on a red background of cells and counted on each of the dilution plates. Virus titre, measured in plaque forming units per ml (pfu/ml), was determined from the plate containing 50-100 plaques, according to the formula:

$$\text{pfu/ml} = (1/\text{dilution}) \times \text{number of plaques}$$

2.3.9 Determining optimal recombinant protein expression

Time-course for recombinant protein expression in High Five insect cells

High Five insect cells (2.5×10^6) were seeded in 25 cm^2 cell culture flasks, and infected with 500 µl of recombinant PCMV gB and gBT baculovirus P3 stocks at a multiplicity of infection (MOI) of 10. Cultures were harvested 1, 2, 3, and 4 dpi, by sloughing and centrifugation (400 ×g, 6 min). Cell pellets were washed twice in 5 ml of sterile PBS, resuspended in 750 µl of sterile PBS, and stored at -80 °C for further analysis.

Comparison of Sf9 vs. High Five insect cells for the expression of recombinant protein

Sf9 and High Five insect cells (2.5×10^6) were each seeded in 25 cm^2 cell culture flasks, and infected with 500 µl of recombinant PCMV gB and gBT baculovirus P3 stocks at an MOI of 10. Cultures were harvested 2 dpi, by sloughing and centrifugation (400 ×g,

6 min). Cell pellets were washed twice in 5 ml of sterile PBS, resuspended in 750 µl of sterile PBS, and stored at -80 °C for further analysis.

Investigating the secretion of recombinant PCMV gBT in the culture supernatant of High Five insect cells 2 and 3 dpi

High Five insect cells (5×10^6) were seeded in 75 cm^2 cell culture flasks, and infected with 1 ml of recombinant PCMV gBT baculovirus P3 stock at an MOI of 10. Cultures were harvested 2 and 3 dpi, by sloughing and centrifugation (400 ×g, 6 min). An aliquot of the centrifuged culture supernatant was stored at -80 °C for further analysis. Meanwhile, cell pellets were washed twice in 10 ml of sterile PBS, resuspended in 150 µl of sterile PBS, and stored at -80 °C for further analysis.

Investigating the secretion of recombinant PCMV gBT in the culture supernatant of Sf9 and High Five insect cells 4 dpi

Sf9 and High Five insect cells (5×10^6) were each seeded in 75 cm^2 cell culture flasks, and infected with 1 ml of recombinant PCMV gBT baculovirus P3 stock at an MOI of 10. Cultures were harvested 4 dpi, by sloughing and centrifugation (400 ×g, 6 min). The centrifuged culture supernatants were concentrated using Amicon Ultra-15 Centrifugal Filter Devices (Millipore) with a 10-kDa molecular weight cut-off. Briefly, 15 ml of culture supernatant was centrifuged at 4000 ×g in a swinging bucket rotor for 20 min. The retentate was stored at -80 °C for further analysis. Meanwhile, cell pellets were washed twice in 10 ml of sterile PBS, resuspended in 150 µl of sterile PBS, and stored at -80 °C for further analysis.

2.3.10 Analysis of recombinant protein by SDS-PAGE

PCMV gB- and gBT-infected and uninfected cell pellet samples were lysed in 4× NuPAGE LDS Sample Buffer (Invitrogen). Culture supernatant samples were also diluted in 4× NuPAGE LDS Sample Buffer. All samples were heated to 70 °C for 10 min and 20 µl of each sample were loaded into the wells of each of two 4-12% NuPAGE Novex Bis-Tris gels (Invitrogen), alongside 10 µl of RPN 756 Rainbow

markers (Amersham Pharmacia) and 5 µl of 6×His Protein Ladder (Qiagen). Gels were run in 1× NuPAGE MOPS SDS Running Buffer (Invitrogen) at 200 V for ~50 min.

2.3.11 Coomassie staining of SDS-PAGE gels

Following SDS-PAGE, one gel was soaked in 100 ml of fixing solution (70 % (v/v) deionised (d) H_2O; 20 % (v/v) methanol; 10 % (v/v) glacial acetic acid (both BDH)) for 5-10 min, and stained in 100 ml of Coomassie staining solution (50 % (v/v) dH_2O; 40 % (v/v) methanol; 10 % (v/v) glacial acetic acid; 0.05 % (w/v) Coomassie Brilliant Blue R-250 (Sigma)), for 2.5 hrs, on a rocking platform. Gels were then rinsed in several changes of destain (50 % (v/v) dH_2O; 40 % (v/v) methanol; 10 % (v/v) glacial acetic acid) until no more colour was removed.

2.3.12 Western blot analysis of recombinant protein

Recombinant proteins were transferred from the second SDS-PAGE gel to a polyvinylidene difluoride (PVDF) membrane by western blot. Filter paper, blotting pads and the Hybond-P PVDF Transfer Membrane (Amersham Pharmacia) were cut to the size of the gel and assembled as in Figure 2.4. The PVDF membrane was pre-soaked in methanol for 30 s, rinsed with dH_2O, and then soaked in 1× NuPAGE Transfer Buffer (Invitrogen) for 5 min. The blotting pads and filter paper were also soaked in 1× NuPAGE Transfer Buffer for 5 min before assembly of the blot. Proteins were transferred using a Trans-Blot SD Semi-dry transfer cell (Biorad), at 30 V for 1 hr.

Following the transfer of proteins, the PVDF membrane was washed twice in 100 ml of TBS buffer (10 mM Tris-HCl; 150 mM NaCl; pH 7.5) for 10 min each on a rocking platform. The remaining protein-free sites on the membrane were blocked in 20 ml of Anti-His horseradish peroxidase (HRP) Conjugate blocking buffer (Qiagen), for 1 hr on a rocking platform. Following blocking, the membrane was washed twice in 100 ml of TBS-Tween/Triton Buffer (20 mM Tris-HCl; 500 mM NaCl; 0.05 % (v/v) Tween 20; 0.2 % (v/v) Triton X-100; pH 7.5), and once in 100 ml of TBS buffer (10 min each), on a rocking platform. Hybridised 6×His-tagged proteins were detected by incubation with Penta-His HRP conjugate mouse IgG1 antibodies, diluted 1/2000 in 10 ml of Anti-His HRP Conjugate blocking buffer (both Qiagen), on a rocking platform for 1 hr.

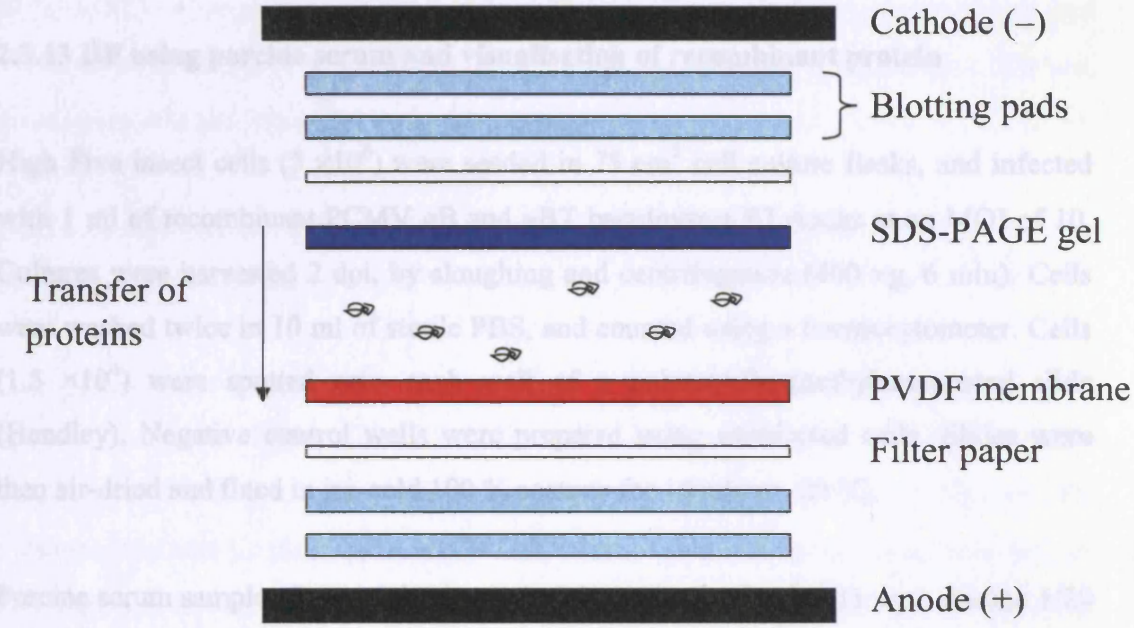


Figure 2.4. Assembly of the western blot. The PVDF membrane was pre-soaked in methanol for 30 s and rinsed with dH_2O. The membrane, blotting pads, and filter paper were then soaked in 1× NuPAGE Transfer Buffer for 5 min before assembly of the blot. SDS-coated, negatively charged proteins were transferred from the SDS-PAGE gel to the PVDF membrane using a Trans-Blot SD Semi-dry transfer cell, at 30 V for 1 hr.

The membrane was washed as before, and incubated in 3,3'-diaminobenzidine (DAB) substrate (6 drops of Buffer Stock Solution; 12 drops of DAB Stock Solution; 6 drops of Hydrogen Peroxide Solution; 6 drops of Nickel Solution in 15 ml of SDW) (Vector Laboratories), for 15 min without shaking. Finally, the membrane was rinsed in dH_2O for 5 min and air-dried.

2.3.13 IIF using porcine serum and visualisation of recombinant protein

High Five insect cells (5×10^6) were seeded in 75 cm^2 cell culture flasks, and infected with 1 ml of recombinant PCMV gB and gBT baculovirus P3 stocks at an MOI of 10. Cultures were harvested 2 dpi, by sloughing and centrifugation (400 ×g, 6 min). Cells were washed twice in 10 ml of sterile PBS, and counted using a haemocytometer. Cells (1.5×10^4) were spotted onto each well of a polytetrafluoroethylene-coated slide (Hendley). Negative control wells were prepared using uninfected cells. Slides were then air-dried and fixed in ice-cold 100 % acetone for 10 min at -20 °C.

Porcine serum samples from adult pigs and piglets (see section 2.2.1) were diluted 1/20 and 1/40 in sterile PBS, and 10 µl of each dilution were spotted onto the slides prepared above, and incubated in a humidified chamber at 37 °C for 40 min. PBS was added to wells containing infected and uninfected cells as a negative control. Slides were then washed, once in 400 ml of PBS containing 1 % BSA (Sigma), and twice in 400 ml of PBS alone for 5 min each. Non-well areas of the slides were dried, and 10 µl of fluorescein isothiocyanate isomer 1 (FITC)-Conjugated Rabbit Anti-Swine IgG Antibody (Dako-Cytomation), diluted 1/40 in sterile PBS, were added to each well and incubated at 37 °C for 40 min. Following incubation, slides were washed and dried as before, mounted in Vectashield fluorescence preservative (Vector Laboratories), and visualised under an Olympus BX60 fluorescence microscope. Photographs were taken using an Olympus DPII digital camera.

Cross-reactivity of mouse anti-HCMV gB monoclonal antibodies with recombinant PCMV gB, and PCMV-seropositive serum with recombinant HCMV gB was investigated. Porcine serum from a PCMV-seropositive and seronegative pig, was diluted 1/20 in sterile PBS, while mouse anti-HCMV gB monoclonal antibodies (provided by K Lawson, Centre for Virology, UCL) were diluted 1/10 in sterile PBS.

Ten microlitres of each dilution were spotted onto slides prepared from Sf9 insect cells infected at an MOI of 10 with either recombinant PCMV gB or HCMV gB (provided by K Lawson) baculovirus, and incubated in a humidified chamber at 37 °C for 40 min. Slides were washed and dried as before, and incubated with 10 µl of either FITC-Conjugated Rabbit Anti-Swine IgG Antibody (Dako-Cytomation), diluted 1/40 in sterile PBS, or FITC-Conjugated Goat Anti-Mouse IgG Antibody (Dako-Cytomation), diluted 1/100 in sterile PBS, at 37 °C for 40 min. Following incubation, slides were washed, dried, mounted, and visualised as before.

2.3.14 Analysis of cell lysis methods for the preparation of recombinant protein for use in a PCMV ELISA

Cell pellets from PCMV gBT-infected High Five insect cells harvested 2 dpi (see section 2.3.9), were lysed in 1 ml of TNE buffer (10 mM Tris-HCl pH 7.2; 100 mM NaCl; 1 mM EDTA; 1 % NP40) with or without the addition of 10 µl of General Use Protease Inhibitor Cocktail (Sigma), followed by four freeze-thaw cycles in a dry ice and methanol bath, and a 37 °C water bath. Cell lysates were clarified by centrifugation at 13,000 ×g for 30 min (4 °C) and the supernatant was transferred to a 1.5 ml eppendorf tube. In addition, PCMV gBT-infected High Five insect cells were lysed in 1 ml of TNE buffer followed by sonication, at a peak-to-peak power of 8, 10 and 12 microns for 10-20 s. The effectiveness of each method for the preparation of ELISA antigen was determined by SDS-PAGE and western blot as in sections 2.3.10-2.3.12.

2.3.15 Preparation of ELISA antigen and determination of protein concentration

Cell lysates were prepared from PCMV gBT-infected and uninfected High Five insect cells 2 dpi (see section 2.3.9), by lysis in 1 ml of TNE buffer containing 10 µl of General Use Protease Inhibitor Cocktail, followed by sonication, twice, at a peak-to-peak power of 10 microns for 10 s each time. Five hundred microlitres of each lysate sample was filtered through a 0.8 µm filter (Sartorius) to, in part, clarify the sample.

The protein concentration of each filtered and non-filtered lysate sample was determined using the BCA Protein Assay Reagent Kit (Pierce). Briefly, the BSA standard supplied with the kit was serially diluted from 0-250 µg/ml in TNE buffer,

while each protein lysate was diluted 1/5 and 1/10 in TNE buffer. BCA Working Reagent (WR) was prepared by mixing BCA Reagents A and B, at a ratio of 50:1. One millilitre of WR was added to 1.5 ml eppendorf tubes containing 50 µl of each BSA or lysate dilution, and mixed by vortexing. The reactions were then incubated in a 60 °C water bath for 30 min, before cooling to room temperature, and measuring the absorbance at 562 nm using a UV spectrophotometer (Camspec). A standard curve of the absorbance measurements of the BSA standard dilution reactions, plotted against their concentration, was used to determine the protein concentration of each unknown lysate sample.

2.3.16 Adsorption of protein onto 96-well plates and preliminary PCMV ELISA

Filtered and non-filtered protein lysate samples from PCMV gBT-infected and uninfected (control) High Five insect cells were diluted to 5 µg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9.6 (Sigma), and 100 µl were used to coat each well of a 96-well Maxisorp immuno-plate (Nalge Nunc International) at 37 °C for 2 hrs. Negative control wells were comprised of 100 µl of 0.1 M carbonate-bicarbonate buffer only. Following washing of each well, with six volumes of 200 µl of sterile PBS using an EL_X50 Auto Strip plate washer (Bio-Tek Instruments Inc.), wells were incubated overnight at 4 °C, with 200 µl of blocking buffer (2.5 % BSA; 0.01 % Tween in PBS). One hundred microlitres of PCMV-positive and negative pooled porcine serum samples, serially diluted to 1/50, 1/100 and 1/1000 in sterile PBS, were added to each well and incubated at room temperature for 1 hr. High-titre HCMV-positive and negative pooled human serum, diluted 1/5000 in sterile PBS, were used as negative controls. After washing three times with 200 µl of 0.05 % Tween in PBS, each well was incubated with 200 µl of HRP-Conjugated Rabbit Anti-Swine IgG Antibody (Dako-Cytomation), diluted 1/7000 in sterile PBS, at 37 °C for 1 hr. Wells were washed four times with 200 µl of 0.05 % Tween in PBS, before incubating with 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) for 30 min. The reaction was stopped by the addition of 50 µl of 2M H_2SO_4 and the absorbance measured at 450 and 630 nm using an EL_X800 plate reader (Bio-Tek Instruments Inc.).

2.3.17 Optimisation of the PCMV ELISA

The concentrations of the protein lysate antigen and HRP-conjugated antibody, and the washing procedure were altered to optimise the sensitivity and reduce non-specific reactions in the PCMV ELISA. To optimise the protein lysate concentration, lysate samples diluted to 20, 10, 1, 0.5 and 0.1 μg/ml were added to each well of a 96-well plate. Porcine serum was tested at dilutions of 1/50, 1/100, 1/500 and 1/1000, while HRP-conjugated antibody was used at a dilution of 1/7000. To optimise the HRP conjugate concentration, 0.1 μg/ml of each protein lysate was added to each well, and incubated with porcine serum diluted to 1/50, 1/100, 1/500 and 1/1000. HRP-conjugated antibody was tested at dilutions of 1/5000, 1/10000 and 1/15000. To optimise the washing procedure, again 0.1 μg/ml of each protein lysate was added to each well, and, following incubation with porcine serum diluted to 1/50, 1/100, 1/500 and 1/1000, wells were washed either three or six times with either 0.05 % or 0.1 % Tween in PBS. In this experiment, HRP-conjugated antibody was used at a dilution of 1/10000. The remaining assay conditions in the PCMV ELISA were as otherwise described in section 2.3.16.

2.4 The susceptibility of PCMV to established antiherpetic agents *in vitro*

This section describes the methodologies used to investigate the *in vitro* susceptibility of PCMV to antiviral agents, currently licensed for use in the prophylaxis and/or treatment of human herpesvirus infections. PCMV infection was established and characterised in pig fallopian tube (PFT) cells, an adherent, fibroblast-like cell line, although a porcine T cell line, L45, was also investigated for susceptibility to PCMV infection. The antiviral inhibition of PCMV in chronically-infected PFT cells was subsequently determined by real-time PCR and IIF.

2.4.1 PFT cell culture

PFT cells (kindly provided by Dr Clive Patience, Immerge BioTherapeutics Inc.) were maintained in minimum essential medium (MEM) (Invitrogen), supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin and 10 % FCS, in a 5 % CO_2 humidified incubator at 37 °C. Once confluent, cells were passaged every 5-6 days at a ratio of 1:4. On these days medium was removed and the cells washed with 5 ml of versene (Invitrogen), and removed from the flask by incubation in 4 ml of trypsin (Invitrogen) at 37 °C for no more than 5 min.

2.4.2 Establishing PCMV infection in PFT cells

PCMV-infected PFT cells (kindly provided by Dr Clive Patience, Immerge BioTherapeutics Inc.) were mixed with 1 ml of freshly trypsinised uninfected PFT cells in 8 ml of complete MEM and seeded in a 25 cm^2 cell culture flask (day 0). An aliquot of TCS was harvested from the culture at 3, 4, 7 and 8 days following mixing of cells, and stored at -80 °C for PCR analysis, prior to total medium replacement. The cells were passaged at a ratio of 1:3 into three 25 cm^2 flasks on day 4. Productive infection in the PFT-PCMV culture was confirmed by nested qualitative PCMV PCR of TCS harvested on days 3, 4, 7, and 8, by RT PCR of RNA extracted from one 25 cm^2 flask at day 8, and by PCMV IIF assay using slides prepared from a second 25 cm^2 flask of PFT-PCMV cells at day 10, following mixing of cells. The remaining 25 cm^2 flask of PFT-PCMV cells was maintained at 37 °C.

2.4.3 Extraction of RNA from PFT-PCMV cells

Following removal of the culture medium, 2.5 ml of TRIzol (Invitrogen) was added to one 25 cm^2 flask of PFT-PCMV cells 8 dpi, and pipetted up and down to dislodge and lyse the cells. Lysed cells were transferred to a 15 ml tube on ice. Five hundred microlitres of chloroform was added, mixed for 15 s and incubated at room temperature for 2-3 min. Cells were centrifuged at 2000 ×g for 15 min and the upper, clear layer transferred to a clean 15 ml tube. The RNA was precipitated in 1.25 ml of isopropanol at room temperature for 10 min and pelleted by centrifugation at 2000 ×g for 10 min. The RNA pellet was washed with 2.5 ml of 75 % ethanol, air-dried, and resuspended in 50 µl of RNase-free water (Promega). The concentration of the RNA was determined by UV spectrophotometry at 260 nm. Extracted RNA was then treated with RNase-Free DNase (Promega) to remove contaminating DNA. Three microlitres of 10× DNase Reaction Buffer, 6.6 µl of RNase-Free DNase and 16.5 µl (0.4 µg/µl) of RNA, were added to 3.9 µl of RNase-free water (all Promega) in a 1.5 ml RNase-free tube, and incubated on a 37 °C heating block for 30 min. The reaction was stopped by incubation with 3 µl of DNase Stop Solution at 65 °C for 10 min.

2.4.4 Confirmation of PCMV productive infection by RT and qualitative PCMV PCR

An RT PCR for PCMV was performed on 10 µl of DNase-treated RNA extracted from PFT-PCMV cells 8 dpi, using the Omniscript Reverse Transcriptase Kit (Qiagen). The RT reaction was prepared on ice and contained; 0.5 mM of each dNTP (Qiagen), 1 µM of Oligo-dT primer (Roche), 10 U of RNase inhibitor (Roche), 4 U of Omniscript Reverse Transcriptase (Qiagen) and 10 µl of DNase-treated RNA, in RT Buffer (Qiagen) in a 0.5 ml RNase-free tube. The RT reaction was incubated for 60 min on a PCR block at 37 °C, and inactivated by heating to 93 °C for 5 min. Ten microlitres of cDNA, 5 µl of DNase-treated RNA, and 2 µl of each TCS sample harvested from PFT-PCMV cells 3, 4, 7, and 8 dpi, were amplified by nested qualitative PCMV PCR as in section 2.2.4. One thousand copies of PCMVpol control plasmid were amplified as a positive control, while SDW was used as a negative control.

2.4.5 Confirmation of PCMV productive infection in PFT cells by IIF assay

PCMV infection was also confirmed by IIF of PFT-PCMV cells harvested 10 dpi. Cells were harvested from one 25 cm^2 flask using trypsin, transferred to a 15 ml tube containing 6 ml of complete MEM, and pelleted at 400 ×g for 6 min. The cell pellet was washed twice in 5 ml of sterile PBS, centrifuging at 400 ×g for 6 min each time, and resuspended in 1 ml of sterile PBS. An aliquot of cells was counted in 20 % paraformaldehyde using a haemocytometer and diluted to 1 $\times 10^6$ cells/ml in sterile PBS. IIF slides were then prepared as in section 2.3.13. Uninfected PFT cells were added to slides as a negative control. The IIF assay was performed using serum from PCMV seropositive pigs, diluted 1/20 and 1/40 in sterile PBS. In addition, sterile PBS was added to control wells.

2.4.6 The effect of TPA on the growth of PCMV in PFT cells

The effect of TPA on the growth of PCMV in PFT cells *in vitro* was determined by monitoring PFT-PCMV cells 3, 7, and 11 days following incubation either with or without 5 ng/ml of TPA (Sigma). Immunofluorescence slides were prepared from PFT-PCMV cells incubated with and without TPA as in section 2.3.13, and the IIF assay was performed using serum from PCMV-seropositive pigs, diluted 1/20 and 1/40 in sterile PBS. Sterile PBS was added to control wells. In addition, the effect of TPA on uninfected PFT cells was determined by comparing cultures under a light microscope, 3 days following incubation either with or without 0, 1, 2, or 5 ng/ml of TPA.

2.4.7 Preparation of PCMV inoculum

In order to determine the optimum method of infecting PFT cells with PCMV, two inoculums derived from TCS and lysed cells were prepared from a flask of PFT-PCMV cells. A TCS inoculum was prepared by harvesting TCS, followed by centrifugation at 400 ×g for 6 min to remove floating cells, and filtering through a 0.45 μm filter (Sartorius). Meanwhile, cells were harvested from the flask by trypsinisation, pelleted (400 ×g, 6 min), resuspended in 1 ml of complete MEM, and lysed by three cycles of freeze-thawing for 1 min in liquid nitrogen followed by 5 min in a 37 °C water bath. The cell lysate was clarified by centrifugation at 14,000 ×g for 5 min. One millilitre of

each inoculum was used to infect semi-confluent PFT cells in two 25 cm^2 cell culture flasks in a 37 °C incubator for 1 hr, rocking occasionally. Following infection, the inoculum was replaced with 8 ml of complete MEM, with or without 5 ng/ml of TPA, and returned to the 37 °C incubator. An IIF assay was performed using slides prepared from PFT cells 21 dpi with each PCMV inoculum as in section 2.3.13. The PCMV DNA load of each inoculum was also determined by QC PCR as in section 2.2.5, with 2 µl of neat inoculum, and inoculum diluted to 1/10 and 1/100, added to each PCR.

The virus titre of each PCMV inoculum was assessed by plaque assay using the method previously described by Kawamura *et al* (Kawamura *et al*, 1992). Approximately 8×10^5 PFT cells were seeded in each well of a 6-well plate and incubated overnight at 37 °C in a 5 % CO_2 humidified incubator. Serial tenfold dilutions (10^{-2} to 10^{-5}) of TCS and cell lysate inoculums were prepared in complete MEM. Once PFT cells were 80-90 % confluent, 200 µl of each virus inoculum dilution were used to infect PFT cells in triplicate. Following virus adsorption at 37 °C for 1 hr, the inoculum was removed and each monolayer was overlaid with 2.5 ml of 0.8 % bacteriological agar in complete MEM, and returned to the humidified incubator. Fresh overlay was added to plates every 5 dpi. Following 10 and 15 days of culture an overlay containing 0.005 % Neutral Red Solution was added to each well. Plaques were observed up to 20 dpi under a light microscope.

The virus titre of TCS and cell lysate inoculums were also assessed using an alternative plaque assay method using a methyl cellulose overlay. PFT cells (5×10^4) were seeded in each well of a 48-well plate and incubated overnight at 37 °C. Serial tenfold dilutions ($10^{-2.5}$ to $10^{-5.5}$) of TCS and cell lysate inoculum were prepared in complete MEM medium. Once PFT cells were 80-90 % confluent, 100 µl of each virus inoculum dilution were used to infect PFT cells in triplicate. Meanwhile, 100 ml of methyl cellulose overlay were prepared by adding 10 ml of 10× MEM, 30 ml of Leibovitz's L-15 medium (both Invitrogen), 5 ml of FCS and 3 ml of 7.5 % sodium bicarbonate (Invitrogen), supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin, to 51 ml of 2 % methyl cellulose. Following virus adsorption at 37 °C for 1 hr, the inoculum was removed and each monolayer was overlaid with 0.5 ml of methyl cellulose overlay and returned to the humidified incubator. Thirteen days postinfection, 1 ml of formal saline (140 mM NaCl; 10 % formaldehyde (37 %) (both Sigma)) was

added to each well and incubated at room temperature for 45 min. Plates were washed ten times in dH_2O, dried, and incubated with 0.5 ml of 0.03 % methylene blue (Sigma)/well for 45 min. Wells were washed and dried as before and observed under a light microscope.

In addition, the effect of using freshly harvested PCMV TCS vs. TCS which had been stored at -80 °C, on the resulting PCMV infection in PFT cells was investigated. TCS was harvested from chronically-infected PFT-PCMV cells, centrifuged (400 ×g, 6 min) to remove floating cells and filtered through a 0.45 µm filter. TCS was then either stored at -80 °C overnight or used directly to infect PFT cells. One millilitre of each TCS inoculum was used to infect semi-confluent PFT cells in two 25 cm^2 cell culture flasks for 1 hr in a 37 °C incubator. Following virus adsorption, the inoculum was removed and replaced with 8 ml of complete MEM. The effect of freezing TCS inoculum on PCMV virus titre was then determined by observing the development of CPE under a light microscope, and by the preparation of IIF slides from cultures 17 and 27 dpi. The IIF assay was performed as in section 2.3.13. In addition, the PCMV DNA load of each inoculum was determined by QC PCR as in section 2.2.5, with 2 µl of neat inoculum, and inoculum diluted to 1/10 and 1/100, added to each PCR.

2.4.8 Investigating the use of 70 % sorbitol to preserve virus titre during freezing

The effect of using 70 % sorbitol to preserve PCMV virus titre when freezing PCMV TCS inoculum was assessed. TCS was harvested from chronically-infected PFT-PCMV cells, centrifuged (400 ×g, 6 min) to remove floating cells and filtered through a 0.45 µm filter. TCS was then stored at -80 °C overnight in either, an equal volume of 70 % sorbitol (BDH), an equal volume of complete MEM, or undiluted. One millilitre of each inoculum was then used to infect semi-confluent PFT cells in three 25 cm^2 cell culture flasks for 1 hr in a 37 °C incubator. Following virus adsorption, the inoculum was removed and replaced with 8 ml of complete MEM. The effect of using 70 % sorbitol to preserve PCMV virus titre was determined by observing the development of CPE under a light microscope, and by the preparation of IIF slides from cultures 14 dpi. The IIF assay was performed as in section 2.3.13.

2.4.9 Establishing PCMV infection in L45 cells

In order to investigate alternative cell lines in which to determine PCMV antiviral susceptibility, a suspension porcine T cell line, L45, was infected with TCS inoculum from PFT-PCMV cells. A growing culture of L45 cells (purchased from the European Collection of Cell Cultures) was maintained in 8 ml of RPMI medium (Invitrogen), supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, 1 mM of sodium pyruvate, 0.05 mM of 2-mercaptoethanol (both Invitrogen), and 10 % FCS, in 25 cm^2 cell culture flasks at 37 °C (5 % CO_2). TCS was harvested from chronically-infected PFT-PCMV cells, centrifuged (400 ×g, 6 min) to remove floating cells and filtered through a 0.45 µm filter. Two millilitres of L45 cells were incubated with 1 ml of PCMV TCS inoculum for 1 hr prior to the addition of 7 ml of complete RPMI medium. Cultures were then incubated at 37 °C and passaged at a ratio of 1:5 every 5 days. IIF slides were prepared from L45-PCMV cells harvested 10, 20 and 30 dpi, and an IIF assay was performed as in section 2.3.13.

2.4.10 Establishing chronically-infected PFT-PCMV cultures to investigate antiviral susceptibility

Chronic PCMV infection of PFT cells was established by limiting passaging of cells. PFT cells (1×10^6) were seeded in 25 cm^2 cell culture flasks. Following incubation overnight (37 °C, 5 % CO_2), sub-confluent (90 %) PFT cells were inoculated with 1 ml of fresh PCMV TCS (filtered, 0.45 µm) harvested from PFT-PCMV cells, for 1 hr, rocking occasionally. Following virus adsorption at 37 °C for 1 hr, the inoculum was removed and replaced with 8 ml of complete MEM. Following the appearance of CPE approximately 5-10 dpi, cultures were transferred to 75 cm^2 cell culture flasks at 14 dpi, and incubated for a further 7 days, with the culture medium replaced weekly. The development of CPE was additionally recorded in PFT-PCMV cultures incubated up to 20 dpi without passaging, and photographed using an Olympus DPII digital camera attached to a light microscope.

2.4.11 Visualisation of PCMV virus particles by electron microscopy

PCMV TCS was harvested from chronically-infected PFT-PCMV cells and ultracentrifuged at 40,000 rpm in a Beckman L-80 Ultracentrifuge (SW60 rotor) for 30 min. The viral pellet was resuspended in 3 % phototungstic acid, pH 6.3, EM stain. Visualisation of virus particles using the electron microscope was then performed by a member of the diagnostic staff in the department, and photographs were developed on site.

2.4.12 Investigating antiviral susceptibility in TCS and PFT-PCMV cells

Chronically-infected PFT-PCMV cells were divided between four 25 cm^2 cell culture flasks; one flask for each of three concentrations of antiviral drug tested, and one control flask containing no antiviral drug (day -2). Unattached cells were removed from cultures after 2 days by total medium replacement (day 0), and real-time PCMV PCR (see section 2.4.13) of the culture supernatant, harvested on day 2, was used to confirm that equal numbers of PFT-PCMV cells were present in each flask. The effect of each antiviral drug on PCMV replication was investigated by monitoring PCMV viral load in the supernatant every two days from day 2 (the first day that antiviral drug was added) until day 14. On these days, the supernatant was collected prior to total medium replacement including the appropriate antiviral drug concentration. Ganciclovir (Roche) and cidofovir (Moravek Biochemicals) were diluted to a final concentration of 1, 5 and 10 µg/ml, while foscarnet (AstraZeneca) was diluted to a final concentration of 25, 50 and 100 µg/ml. Aciclovir (Foulding) was diluted to a final concentration of 5, 10 and 25 µg/ml. Supernatant at each time point was centrifuged (400 ×g, 6 min), to remove detached cells and 5 µl of cell-free supernatant was tested by real-time PCR. On day 14, all cultures were harvested for the preparation of IIF slides (as in section 2.3.13) and DNA extraction. Following IIF, the average number of fluorescing, PFT-PCMV cells, in each well of the IIF slide was calculated by counting the total number of cells in each of four wells on the IIF slide. DNA was extracted from cells using the Wizard Genomic DNA Purification Kit, using the protocol for the Isolation of Genomic DNA from Tissues Culture Cells and Animal Tissue (see section 2.2.2). One hundred nanograms of DNA were analysed by real-time PCR, with uninfected PFT cellular DNA tested as a negative control.

2.4.13 Real-time PCR for PCMV DNA

PCMV target sequences were quantified by real-time PCR using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers PCMVpolF and PCMVpolR, and Taqman probe (table 2.1), were designed using Primer Express software (Applied Biosystems), from a region of the DNA polymerase gene of PCMV (Widen *et al*, 1999). The sensitivity of each primer to amplify the wild-type PCMVpol plasmid (generated in sections 2.1.1-2.1.4) was initially assessed by testing 10^6-0.1 copies of wild-type plasmid per reaction. Wild-type PCMVpol plasmid was diluted in 10 mM Tris buffer (pH 8.5), containing 150 ng/µl of salmon sperm DNA (both Sigma) as a carrier. Each reaction contained 100 ng of each primer, 5 pmol of probe, 200 µM of each dNTP, 2.5 U of HotStarTaq and target DNA, in PCR buffer (containing 1.5 mM $MgCl_2$) (Qiagen), in a final volume of 25 µl. Reagents were aliquoted into each well of a 96-well optical reaction plate (Applied Biosystems). Cycle parameters were; an initial cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C denaturation for 15 s and 60 °C annealing/extension for 1 min. Real-time PCR products were analysed using Taqman software and additionally run on a 3 % agarose gel to visualise results. Subsequently, target sequences were amplified alongside 10^9-10^3 copies of PCMV standard (wild-type PCMVpol plasmid), with all samples being tested in triplicate. The threshold cycle values for the PCR amplification of the standards were used to generate a standard curve for the quantitation of target DNA, and the mean of three viral loads was calculated.

2.4.14 Determining the cytotoxicity of antiviral agents

The cytotoxic effect of each antiviral on PFT-PCMV cells was determined by counting the total number of remaining adherent cells, harvested from each culture at day 14, following removal of culture supernatant. An aliquot of cells was diluted in an equal volume of 0.4 % trypan blue (Sigma) and 37 % formaldehyde (1:1), and counted using a haemocytometer. In a parallel study to that in section 2.4.12, cytotoxicity was determined using cellular DNA extracted at day 14 from uninfected adherent cells, cultured with each concentration of drug, as a marker of the total cell count. In addition, DNA was extracted from detached cells removed in the culture supernatant over the 14

day period. DNA was extracted from uninfected cells using the Wizard Genomic DNA Purification Kit, using the protocol for the Isolation of Genomic DNA from Tissues Culture Cells and Animal Tissue (see section 2.2.2), and the DNA concentration was determined by UV spectrophotometry at 260 nm.

2.4.15 Investigating the dynamics of PCMV replication *in vivo*

In order to estimate the doubling time of PCMV replication *in vivo*, PCMV viral loads were determined in PCMV-positive blood samples from piglets derived from sow P49M, which had been prospectively monitored from birth (see section 2.2.1). DNA (0.5 μg) extracted from whole blood samples in section 2.2.2 was analysed by real-time PCR as in section 2.4.13. Each sample was tested in triplicate and the mean of each result was calculated.

Chapter 3: The development of qualitative and quantitative competitive (QC) PCR assays for PCMV

3.1 Introduction

Evidence of PCMV infection is most often detected by IIF or ELISA of porcine sera, or by the presence of intranuclear inclusions within infected cells (Edington, 1999). However, these methods are time consuming and suffer from limited sensitivity. More recently, PCR assays for the detection of PCMV DNA have been developed (Hamel *et al*, 1999; Widen *et al*, 1999). Both of these assays are qualitative, thereby detecting the presence or absence of the virus within a sample, and use primers specific for the DNA polymerase gene of PCMV.

PCR is a powerful diagnostic tool, which has revolutionised the detection of many viruses. Advantages of PCR are that it is reliable, fast, versatile, cost effective, and sensitive. Specificity is brought about by primers which are designed to bind only to a specific target sequence under stringent conditions. The choice of target gene also influences the specificity of the PCR. The DNA polymerase gene is a common target for the detection of herpesviral DNA by PCR, since the gene is highly conserved. Specificity and sensitivity can be further increased by performing two rounds of amplification using nested primers, which bind internally to the first round primers.

Viral load measurements are important in understanding the pathogenesis of viruses, and have significant prognostic implications (Mellors *et al*, 1996; Spector *et al*, 1998). In particular, in renal transplant recipients, the quantity of HCMV DNA detected in urine, was significantly increased in those patients with clinically apparent disease. In fact, high viral load has been identified as the primary risk factor for the development of HCMV disease in transplant recipients (Cope *et al*, 1997a; Hassan-Walker *et al*, 1999). There are currently no methods available to quantify PCMV DNA.

At the time of conducting this work, the main PCR methods of quantifying viral DNA within clinical samples include co-amplification of the sample with genes such as β-globin (Pang *et al*, 1990), and limiting dilution of the sample, followed by qualitative PCR (Simmonds *et al*, 1990), however, the latter method is very time-consuming and inappropriate for the analysis of a large number of samples. At the Centre for Virology, at the Royal Free and University College Medical School, an alternative approach has been developed, and used to accurately quantify viruses including HCMV, HHV-6, -7,

and -8, and HIV (Fox *et al*, 1992; Clark *et al*, 1996; Kidd *et al*, 1996; Lock *et al*, 1997; Atkins *et al*, 1996). These methods are based on the co-amplification of 'wild-type' DNA extracted from a clinical sample alongside a control sequence of known copy number. This control sequence is identical, in length, primer binding sequences and G+C content, to the actual wild-type sequence apart from the presence of a unique restriction endonuclease site, which allows for the separation of control and wild-type amplicons following PCR (Figure 3.1). Comparison of wild-type and control amplicons by electrophoresis and densitometric analysis, allows for the calculation of the number of wild-type molecules within the original sample (Kidd *et al*, 2000). Advantages of this method are that it compensates for local variation between PCRs, and controls for the presence of inhibitory substances within specific clinical samples, since the control sequence is added with the sample DNA. In addition, since both wild-type and control sequences are identical in size, base composition, and possess identical primer binding sites, the kinetics of the amplification of both sequences is the same. A nested QC PCR is used to quantify viral DNA present in low copy numbers.

The following chapter describes the development and optimisation of a qualitative and QC PCR assay for PCMV, using primers specific for a region of the viral DNA polymerase gene PCMVpol. The target PCMVpol region was initially amplified and cloned into the pGEM-T Easy vector to generate the PCMVpol wild-type plasmid. A *Sma* I restriction endonuclease site was then introduced into the PCMVpol region by PCR-mediated, site-directed mutagenesis (Fox *et al*, 1992), to produce the PCMVpol control sequence. The sensitivity of the PCMV PCR was optimised by altering the PCR conditions. The utility of the PCMVpol control plasmid to accurately quantify PCMVpol wild-type DNA was then determined in a series of co-amplification experiments.

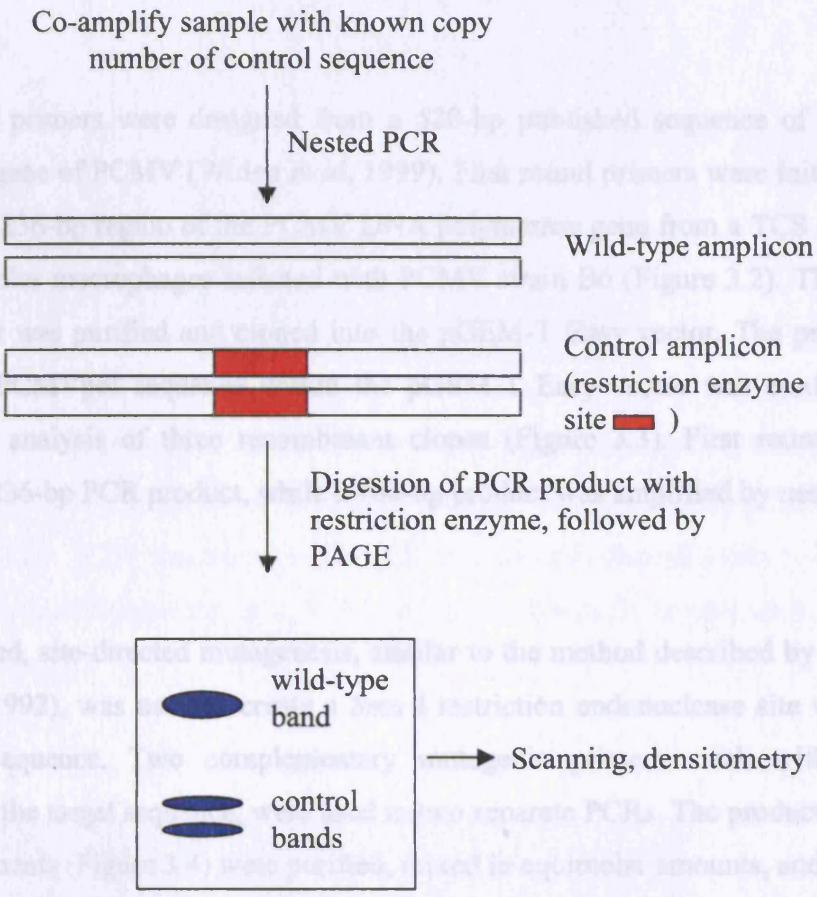


Figure 3.1. Quantitative competitive (QC) PCR. Following co-amplification of sample DNA with a known copy number of control sequence, control amplicons are digested with a restriction enzyme and separated from wild-type amplicons by PAGE. Comparison of wild-type and control amplicons by densitometric analysis, allows for the calculation of the number of wild-type molecules within the original sample.

3.2 Results

3.2.1 Generation of the PCMVpol control plasmid as an internal standard for the QC PCR

Nested PCR primers were designed from a 520-bp published sequence of the DNA polymerase gene of PCMV (Widen *et al*, 1999). First round primers were initially used to amplify a 236-bp region of the PCMV DNA polymerase gene from a TCS sample of porcine alveolar macrophages infected with PCMV strain B6 (Figure 3.2). The 236-bp PCR product was purified and cloned into the pGEM-T Easy vector. The presence of the 236-bp PCMVpol sequence within the pGEM-T Easy vector was confirmed by nested PCR analysis of three recombinant clones (Figure 3.3). First round primers amplified a 236-bp PCR product, while a 184-bp product was amplified by nested round primers.

PCR-mediated, site-directed mutagenesis, similar to the method described by Fox *et al* (Fox *et al*, 1992), was used to create a *Sma* I restriction endonuclease site within the PCMVpol sequence. Two complementary mutagenic primers, each with a 2-bp mismatch to the target sequence, were used in two separate PCRs. The products of these PCR experiments (Figure 3.4) were purified, mixed in equimolar amounts, and annealed together. The annealed products were 3' extended to produce the PCMVpol control sequence, and used as the template for amplification with first round PCMV PCR primers. The presence of the *Sma* I site within the control sequence was confirmed by restriction enzyme digestion of the PCR product (Figure 3.5). The PCMVpol control sequence was then cloned into the pGEM-T Easy vector, and used to transform competent *E. coli* cells. Five recombinant clones were analysed for the presence of the PCMVpol control sequence by PCMV PCR and *Sma* I digestion (Figure 3.6). The presence of the *Sma* I restriction site was also confirmed by sequence analysis of the PCMVpol control plasmid (Figure 3.7). Stocks of wild-type and control plasmids were produced by large-scale plasmid preparation, diluted to 10^{10} copies/µl, and stored at -80 °C.

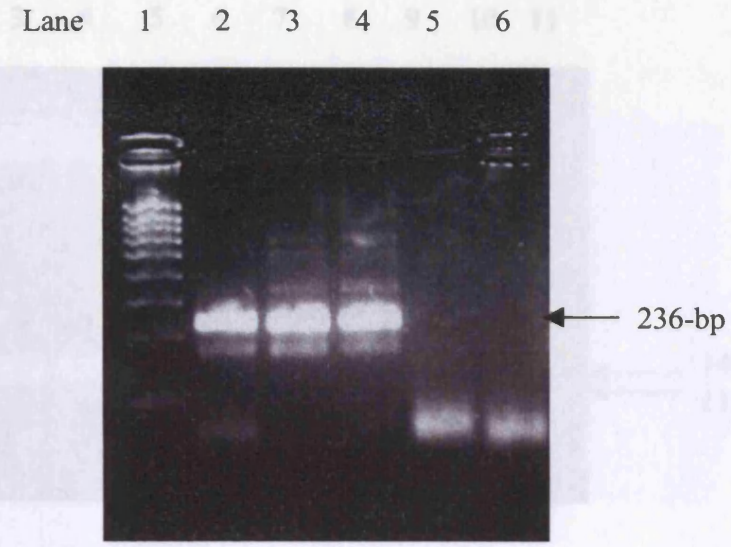


Figure 3.2. PCR amplification of the 236-bp PCMVpol sequence from 1 µl (lane 2), 5 µl (lane 3), and 10 µl (lane 4) of TCS from alveolar lung macrophages infected with PCMV, strain B6. SDW was added to the PCR as a negative control (lanes 5 & 6). PCR products were electrophoresed on a 3 % agarose gel alongside HyperLadder IV DNA markers (lane 1).

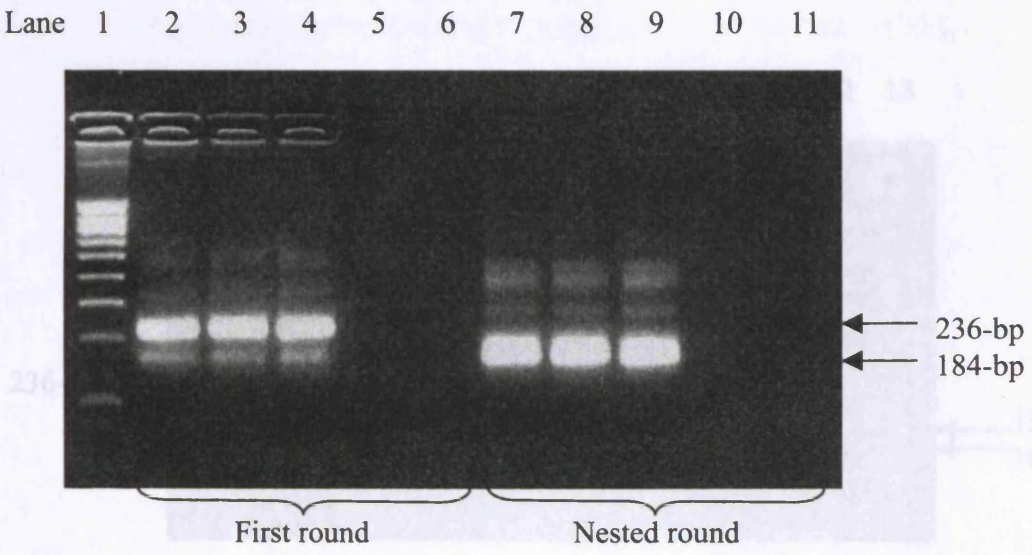


Figure 3.3. Nested PCR analysis of three recombinant clones (lanes 2-4 & 7-9), following cloning of the 236-bp PCMVpol sequence into the pGEM-T Easy vector and transformation of competent *E. coli*. SDW was added to the PCR as a negative control (lanes 5,6,10 & 11). PCR products were electrophoresed on a 3 % agarose gel alongside HyperLadder IV DNA markers (lane 1).

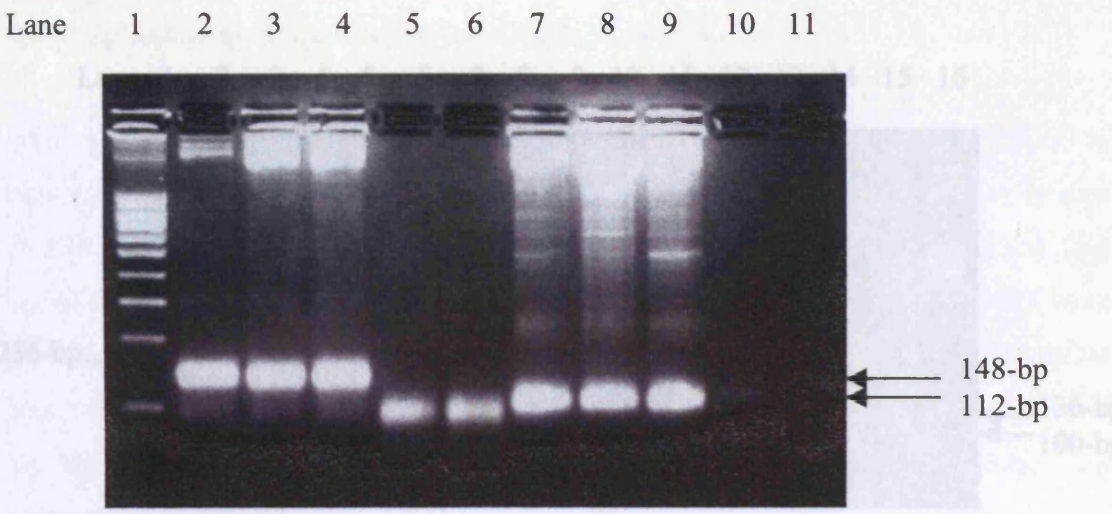


Figure 3.4. Site-directed mutagenesis products generated by PCR. A 148-bp sequence was amplified from 1, 5, and 10 µl of recombinant plasmid, using primers PCMVF1 and PCMVFM in one PCR (lanes 2-4). In a separate PCR, a 112-bp sequence was amplified from 1, 5 and 10 µl of recombinant plasmid, using primers PCMVR1 and PCMVRM (lanes 7-9). SDW was added to each PCR as a negative control (lanes 5, 6, 10 & 11). PCR products were electrophoresed on a 3 % agarose gel alongside HyperLadder IV DNA markers (lane 1).

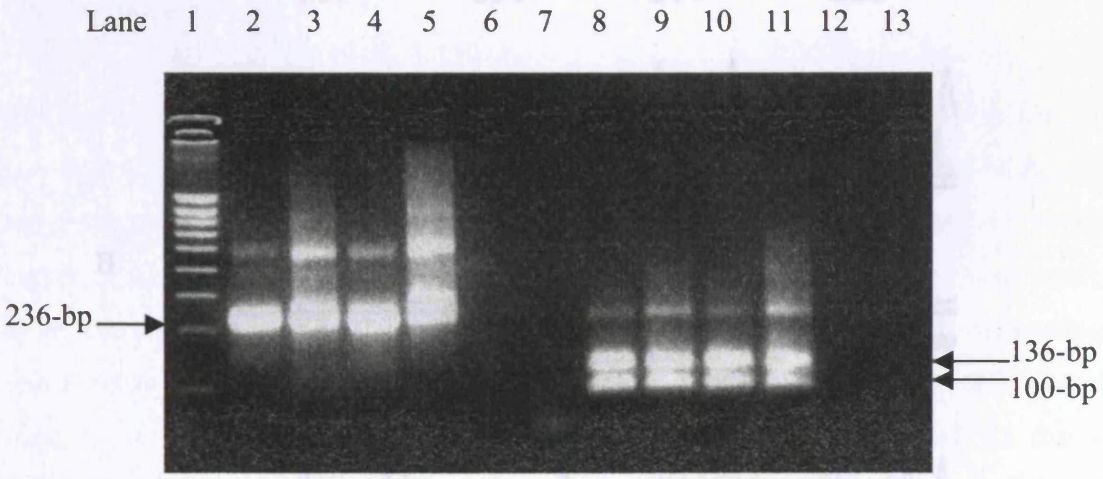


Figure 3.5. *Sma* I digestion of the PCMVpol control sequence. The PCMVpol control sequence (lanes 2-5) was PCR-amplified from the Klenow-filled mutated PCMVpol sequence using primers PCMVF1 and PCMVR1. SDW was added to the PCR as a negative control (lanes 6 & 7). The presence of the *Sma* I restriction enzyme site was confirmed by *Sma* I digestion of PCR products (lanes 8-13). All samples were run on a 3 % agarose gel alongside HyperLadder IV DNA markers (lane 1).

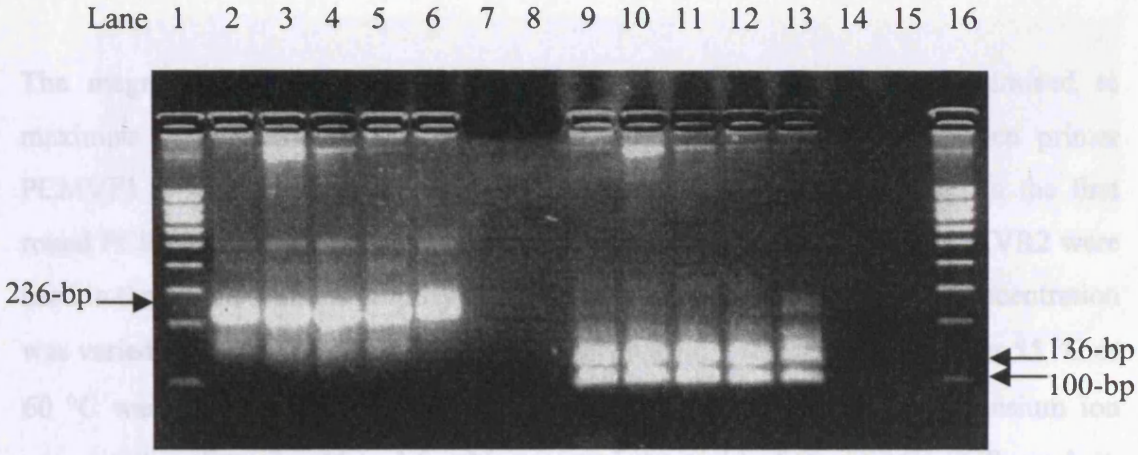


Figure 3.6. PCR analysis of five PCMVpol control plasmid recombinant clones (lanes 2-6). SDW was added to the PCR as a negative control (lanes 7 & 8). The presence of the *Sma* I restriction enzyme site was confirmed by *Sma* I digestion of PCR products (lanes 9-15). All samples were run on a 3 % agarose gel alongside HyperLadder IV DNA markers (lanes 1 & 16).

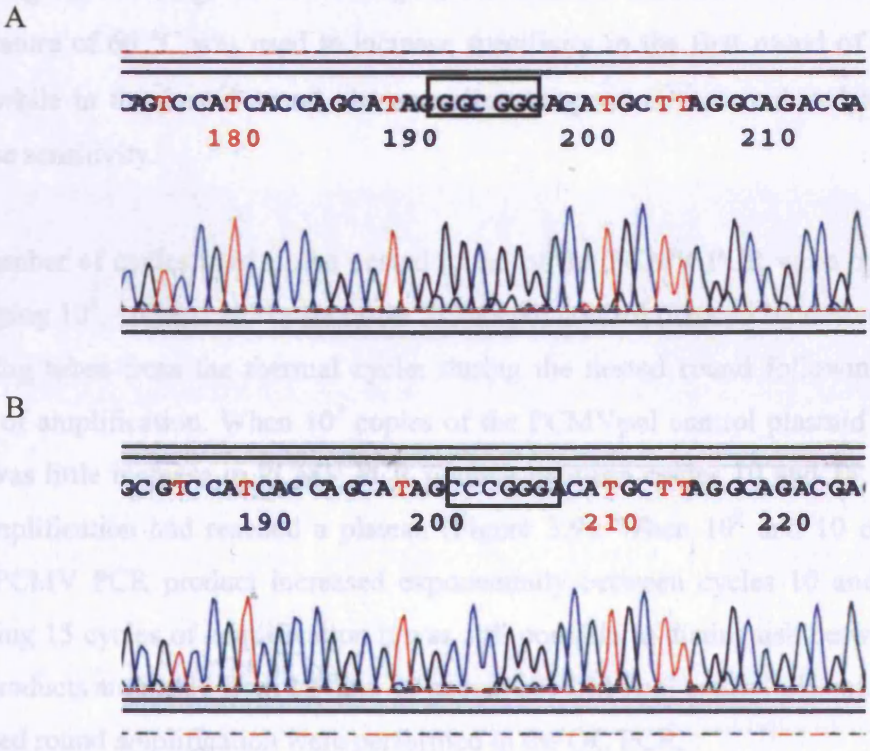


Figure 3.7. Sequence analysis of wild-type and control PCMVpol plasmids. Panel A shows a section of the wild-type PCMVpol sequence, showing the putative *Sma* I restriction site. Panel B shows a section of the control PCMVpol sequence, showing the introduced *Sma* I restriction enzyme site.

3.2.2 Optimisation of qualitative and QC PCR assays

The magnesium ion concentration and annealing temperature were optimised to maximise the sensitivity and specificity of the PCMV PCR assays. Since primer PCMVF1 proved insensitive, primers PCMVF2 and PCMVR1 were used in the first round PCR to amplify a 212-bp amplicon, while primers PCMVFB and PCMVR2 were used in the nested round to amplify a 160-bp product. The magnesium ion concentration was varied between 1 mM and 2 mM, while an annealing temperature of either 55 °C or 60 °C was used. The results in Figure 3.8 show that increasing the magnesium ion concentration from 1 mM to 1.5 mM increased the yield of the PCMV PCR product, while increasing the magnesium ion concentration to 2 mM did not lead to any further increase in yield of the PCMV PCR product. At a magnesium ion concentration of 1 mM the yield of the PCMV PCR product was greater when an annealing temperature of 55 °C was used. Whilst at a magnesium ion concentration of 1.5 mM, there was no difference in yield of the PCMV PCR product at either annealing temperature. Following these findings 1.5 mM of $MgCl_2$ was added to the PCMV PCR. An annealing temperature of 60 °C was used to increase specificity in the first round of the PCMV PCR, while in the nested round, the annealing temperature was reduced to 55 °C to increase sensitivity.

The number of cycles used in the nested round of the PCMV PCR were optimised by amplifying 10^3, 10^2 and 10 copies of the PCMVpol control plasmid by nested PCR, and removing tubes from the thermal cycler during the nested round following 10 to 15 cycles of amplification. When 10^3 copies of the PCMVpol control plasmid were used, there was little increase in PCMV PCR product between cycles 10 and 15, suggesting that amplification had reached a plateau (Figure 3.9). When 10^2 and 10 copies were used, PCMV PCR product increased exponentially between cycles 10 and 15. Since following 15 cycles of amplification it was still possible to distinguish between PCMV PCR products amplified from 10^2 and 10 copies of PCMVpol control plasmid, 15 cycles of nested round amplification were performed in the QC PCR.

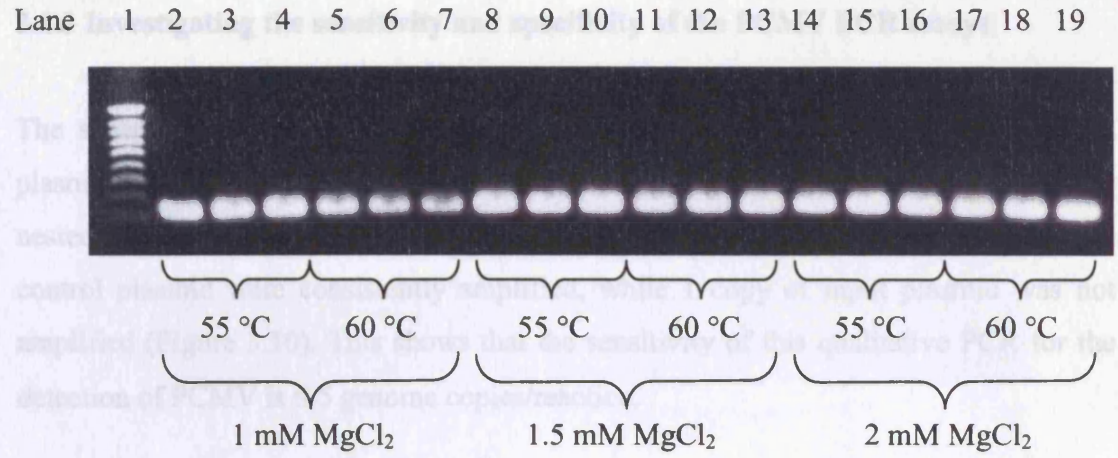


Figure 3.8. Optimisation of magnesium ion concentration and annealing temperature in the PCMV PCR. Fifty copies of PCMVpol control plasmid were amplified by nested PCR, using either; 1, 1.5, or 1 mM of $MgCl_2$, at an annealing temperature of either 55 °C or 60 °C. One microlitre of each first round PCR product was added to the nested round and amplified for 18 cycles. Nested round products were run on a 1 % agarose gel alongside HyperLadder IV DNA markers (lane 1).

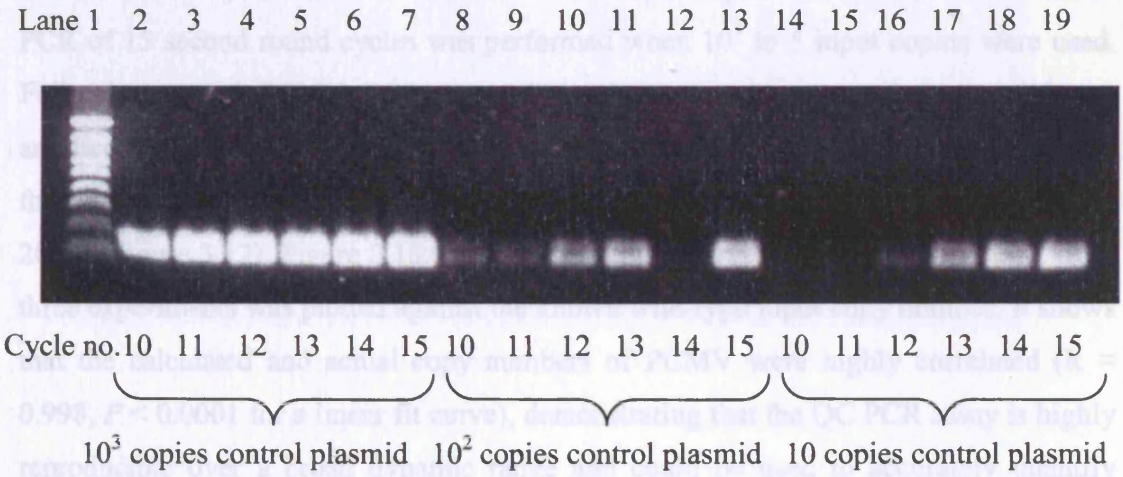


Figure 3.9. Optimisation of the nested round cycle number in the PCMV PCR. Between 10 and 10^3 copies of PCMVpol control plasmid were amplified by nested PCR. Nested round PCR tubes were removed from the thermal cycler between cycles 10 and 15 and PCR products were electrophoresed on a 1 % agarose gel alongside HyperLadder IV DNA markers (lane 1).

3.2.3 Investigating the sensitivity and specificity of the PCMV PCR assays

The sensitivity of the PCMV PCR was investigated by diluting PCMVpol control plasmid from 10^3 to 1 copy. Control plasmid dilutions were amplified in triplicate by nested qualitative PCR. The results show that 10^3, 10^2, 10, and 5 copies of PCMVpol control plasmid were consistently amplified, while 1 copy of input plasmid was not amplified (Figure 3.10). This shows that the sensitivity of this qualitative PCR for the detection of PCMV is ≤ 5 genome copies/reaction.

The specificity of the PCMV PCR assay was tested by amplifying DNA from other betaherpesviruses; HHV-6, HHV-7, and HCMV alongside PCMV. Figure 3.11 shows that none of these samples yielded detectable amplification products.

3.2.4 Characterisation of the PCMV QC PCR

To assess the utility of the PCMVpol control sequence in the QC PCR, a range of known copy numbers of wild-type and control plasmids were co-amplified. A single round PCR was performed when 5×10^6 to 10^3 input copies were used, while a nested PCR of 15 second round cycles was performed when 10^3 to 5 input copies were used. Following nested PCMV PCR, control amplicons were separated from wild-type amplicons by *Sma* I digestion and PAGE. Gels were stained with ethidium bromide and the input wild-type plasmid copy number calculated as previously described (Kidd *et al*, 2000) (Figure 3.12). Figure 3.13 shows a calibration curve where the mean result from three experiments was plotted against the known wild-type input copy number. It shows that the calculated and actual copy numbers of PCMV were highly correlated ($R = 0.998$, $P < 0.0001$ for a linear fit curve), demonstrating that the QC PCR assay is highly reproducible over a broad dynamic range and could be used to accurately quantify PCMV DNA.

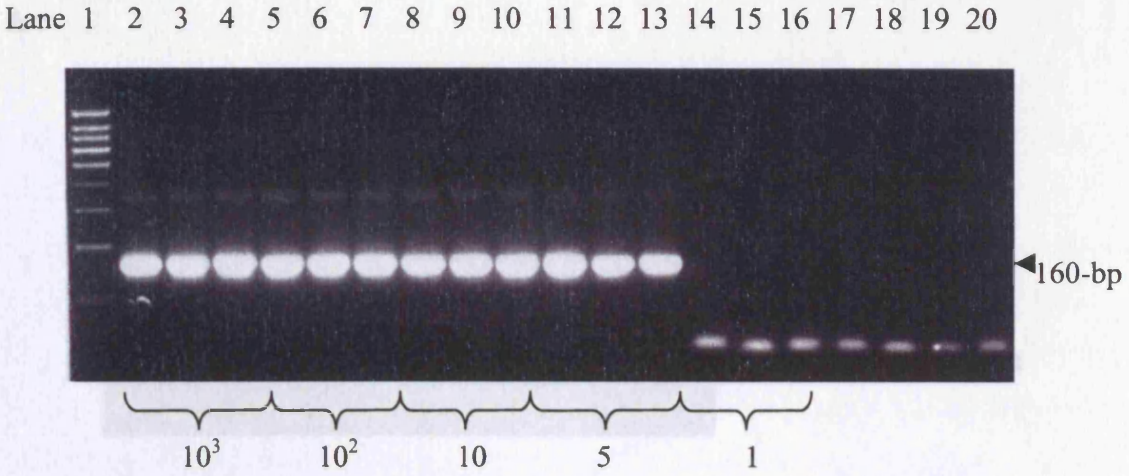


Figure 3.10. Sensitivity of the PCMV PCR. One thousand (lanes 2-4), 10^2 (lanes 5-7), 10 (lanes 8-10), 5 (lanes 11-13), and 1 (lanes 14-16) copy of PCMVpol control plasmid were amplified by nested PCR in triplicate, and 10 µl of nested products were electrophoresed on a 3 % agarose gel, alongside HyperLadder IV DNA markers (lane 1). SDW was used added to the PCR as a negative control (lanes 17-20).

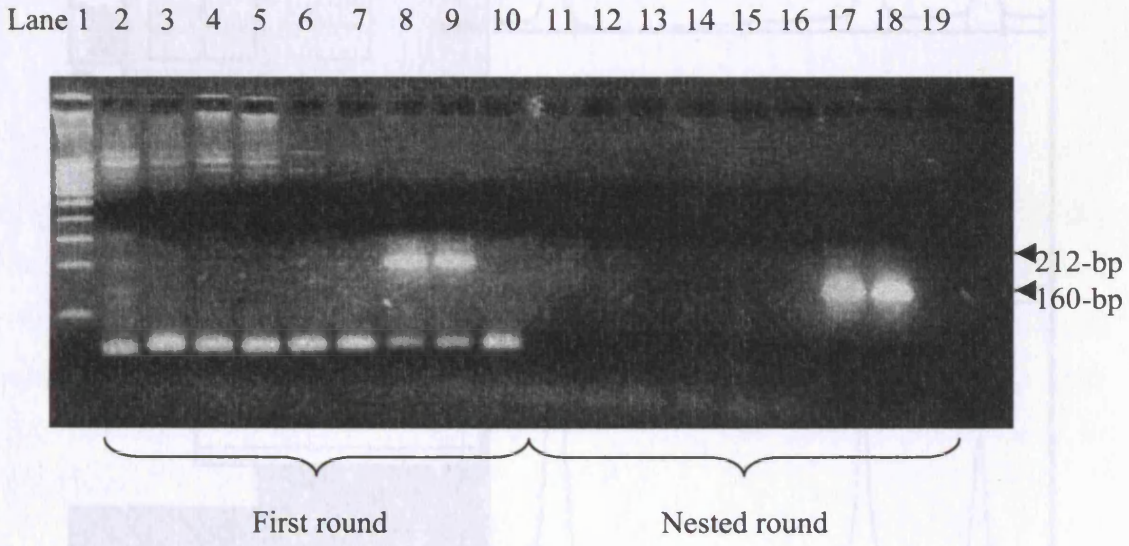
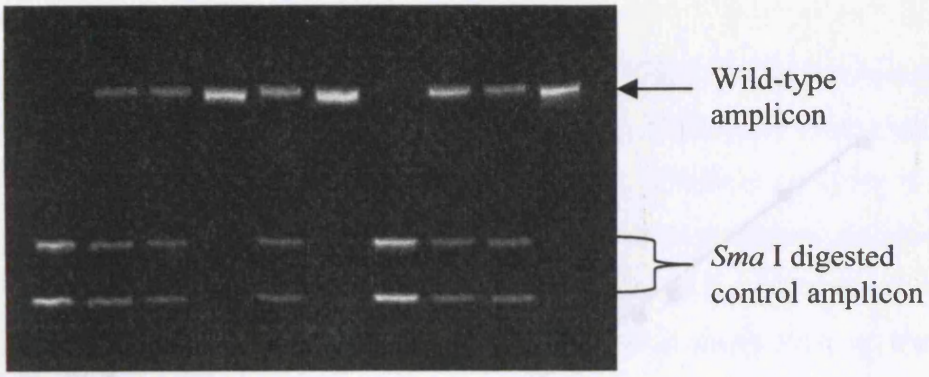


Figure 3.11. Specificity of the PCMV PCR. Viral DNA from HHV-6 (lanes 2, 3, 11 & 12), HHV-7 (lanes 4, 5, 13 & 14), and HCMV (lanes 6, 7, 15 & 16), was not amplified by nested PCMV PCR. PCMV (lanes 8, 9, 17 & 18), and SDW (lanes 10 & 19), were used as positive and negative controls respectively. First round (lanes 2-10) and nested round (lanes 11-19) PCR products were electrophoresed on a 3 % agarose gel, alongside HyperLadder IV DNA markers (lane 1).

A



B

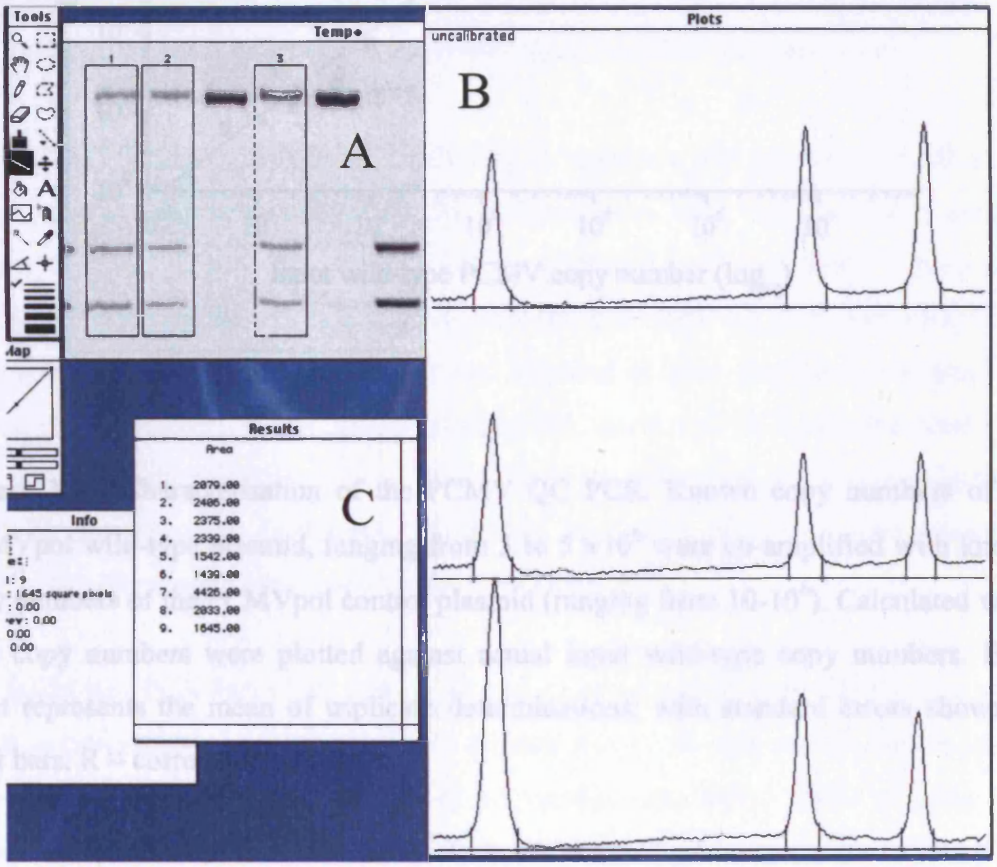


Figure 3.12. An example of the densitometric analysis performed in the PCMV QC PCR. QC PCR PAGE images (A) were scanned at 200 pixels per inch using an Epsom scanner. The scanned image was imported into the NIH Image program (version 1.63), for densitometric analysis (B). The scanned image was inverted (screen window A), and the lanes were marked using the gel reading macros supplied. Electronic densitometric plots were generated (screen window B), and the pixel area under each peak was calculated (screen window C).

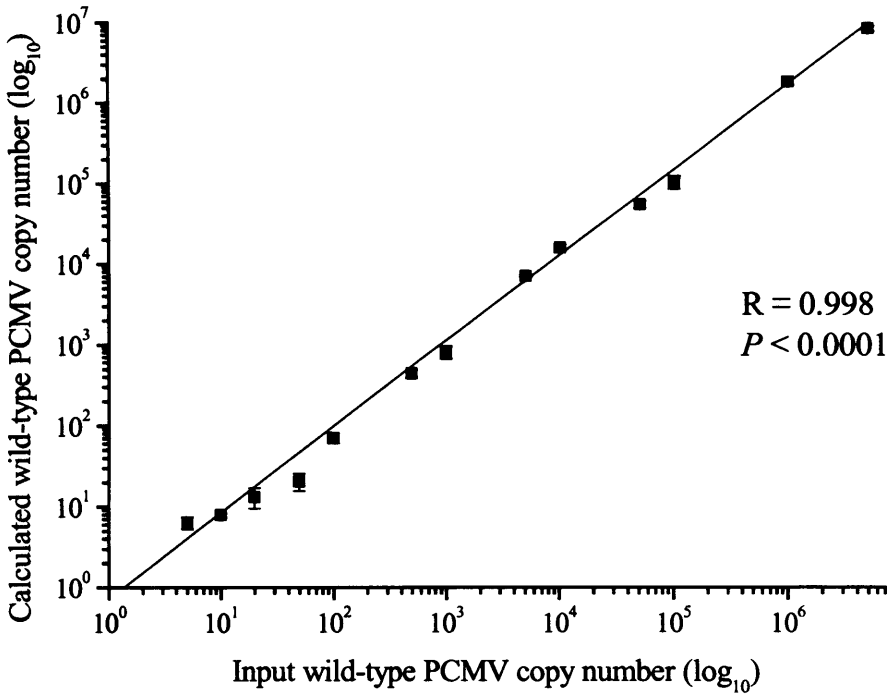


Figure 3.13. Characterisation of the PCMV QC PCR. Known copy numbers of the PCMVpol wild-type plasmid, ranging from 5 to 5 $\times 10^6$ were co-amplified with known copy numbers of the PCMVpol control plasmid (ranging from 10-10^6). Calculated wild-type copy numbers were plotted against actual input wild-type copy numbers. Each point represents the mean of triplicate determinations, with standard errors shown in error bars. R = correlation coefficient.

3.3 Discussion

This chapter describes the development of qualitative and fully quantitative competitive PCR assays for PCMV (Fryer *et al*, 2001). Although there have been reports of qualitative PCR assays for the detection of PCMV DNA (Widen *et al*, 1999; Hamel *et al*, 1999), this was the first quantitative PCR for PCMV to be published. As previously described, viral load measurements can provide information on the pathogenesis of viral infections, are important for the diagnosis of disease in patients such as transplant recipients, and are therefore useful in the treatment of these patients (Manez *et al*, 1996; Mutimer *et al*, 1997). In the context of xenotransplantation, it will be equally important to be able to measure PCMV viral loads in pigs being bred for xenograft organs, and also potentially in human xenograft recipients should clinical trials proceed.

The QC PCR method developed for PCMV is based on that previously used in our department to accurately and efficiently measure viral loads within clinical samples (Fox *et al*, 1992; Clark *et al*, 1996; Kidd *et al*, 1996; Lock *et al*, 1997; Atkins *et al*, 1996). Compared with other quantitative methods, it controls for sample-to-sample and sample-to-well variations, since target and identical control sequences are amplified within the same tube. At the time of starting this thesis, QC PCR was the method of choice for the quantification of viral loads. However, in later stages, key developments in PCR quantification facilitated the change from this relatively time-consuming method to a more rapid real-time PCR assay (Chapter 6).

The qualitative PCR for PCMV was developed using primers specific for a region of the DNA polymerase gene of PCMV. Since initially one of the first round primers proved insensitive, primers pairs were changed to increase sensitivity. These primers were shown by BLAST analysis to share 100 % identity with sequence data for the DNA polymerase gene of all three strains of PCMV; B6, OF-1, and 55b, suggesting their ability to amplify different isolates. In addition, results obtained in this chapter show that these primers do not amplify HCMV, HHV-6 or HHV-7 DNA sequences, and are sensitive to $\leq$ 5 PCMV genome copies/PCR reaction. The same primers were used to develop a QC PCR assay for the quantification of PCMV DNA. This assay was shown to be accurate and reproducible over a broad dynamic range ($5\text{-}5\times10^6$ PCMV genome copies).

The PCMV PCR assays developed in this chapter will be useful in further studying the pathogenesis of PCMV, and will enable the detection and accurate quantification of PCMV in porcine organs and tissues from pigs being bred for use in xenotransplantation (Chapter 4).

Chapter 4: Investigating the prevalence, quantity and organ distribution of PCMV in immunocompetent and immunosuppressed pigs being bred for xenograft organs, investigating when virus is acquired, and examining methods to eliminate PCMV from these animals

4.1 Introduction

Xenotransplantation raises considerable safety issues including the potential transmission of porcine infectious agents, particularly viruses, to humans (Murphy, 1996; Brown *et al*, 1998). Experience from human allotransplantation indicates that members of the retrovirus and herpesvirus families may be transmitted in the graft (Fishman & Rubin, 1998). In particular, since HCMV is frequently transmitted to human allograft recipients, where it reactivates and is associated with end-organ disease and graft rejection, the porcine equivalent, PCMV, may pose a risk to human recipients of porcine xenografts. This is highlighted by the fact that BCMV is the only virus to date, which has been transmitted to humans following xenotransplantation (Michaels *et al*, 2001).

Serological evidence indicates that approximately 90 % of pig herds in the UK are infected with PCMV (Edington, 1999). A high seroprevalence of PCMV has also been reported in pigs from the Netherlands and Japan (93 % and 99.4 % respectively) (Rondhuis *et al*, 1980; Tajima *et al*, 1993). The prevalence of PCMV in lung tissue and nasal scrapings from Canadian pigs, as detected by PCR, was lower (59 % of pigs tested PCMV PCR-positive) (Hamel *et al*, 1999).

PCMV infection is characterised by rhinitis, pneumonia and poor weight gain in young pigs, and is associated with a significant loss of newborn piglets, while infection in adults is generally silent (Edington, 1999). In commercial pig herds, infection occurs between 5 and 8 weeks of age, coinciding with the mixing of litters in the early post-weaning period (Plowright *et al*, 1976). PCMV causes fatal generalised disease, characterised by piglet death and poor weight gain, in young piglets of less than two weeks of age, with viraemia detected 5-19 dpi (Edington *et al*, 1976b), and characteristic PCMV intranuclear inclusion bodies detected in many tissues including lung macrophages, liver, spleen, lymph nodes, and adrenal glands (Edington *et al*, 1976a). In older piglets, PCMV causes asymptomatic generalised infection, with viraemia detected 14-16 dpi (Edington *et al*, 1976b), and inclusion bodies detected primarily in epithelial cells of the nasal mucosa and kidney tubules (Edington *et al*, 1976a). Virus is most commonly transmitted via nasal secretions (Plowright *et al*, 1976), but also through saliva and contaminated urine. *In utero* infection has been

demonstrated following experimental infection of pregnant sows at various stages of gestation, resulting in significant losses of foetuses and neonates (Edington *et al*, 1977; Edington *et al*, 1988a; Edington *et al*, 1988b). However, the frequency of *in utero* infection in a conventional setting has yet to be reported.

As pigs are the most likely source for organs in xenotransplantation, it is important to gain a greater understanding of the pathogenesis of PCMV infection, and to investigate whether transmission of virus can be interrupted. In previous epidemiological studies, evidence of PCMV infection was detected by serology, virus isolation or by the identification of inclusion bodies. These methods are generally not sufficiently sensitive for the detection of low-level viral infection, in particular, high HCMV loads are required before inclusion bodies can be visualised in histopathological samples (Mattes *et al*, 2000). In the era of PCR, it is now possible to re-examine the prevalence of PCMV, it's organ distribution and temporal acquisition.

Since xenograft recipients are likely to be subject to immunosuppressive treatment to prevent rejection, it is also important to investigate the effect that this immunosuppression might have on PCMV replication. As a herpesvirus, PCMV has the ability to remain life-long in the host following primary infection, including a latent phase, and may reactivate periodically to produce infectious virus and potentially cause disease. Herpesviruses, such as HCMV are frequently reactivated from latency following human allotransplantation (Fishman & Rubin, 1998). Meanwhile, reactivation of PCMV has been reported following the administration of corticosteroids (Edington *et al*, 1976b; Narita *et al*, 1985). PCMV reactivation in pigs can otherwise occur when new breeding stock is introduced or when routine is disturbed (Edington, 1999).

The following chapter describes the use of qualitative and QC PCR assays, developed in Chapter 3, to investigate the prevalence, quantity and organ distribution of PCMV in immunocompetent and immunosuppressed pigs being bred for xenotransplantation. The chapter also describes the investigations carried out to determine the temporal acquisition of virus and methods for it's control (Clark *et al*, 2003). Porcine samples were obtained from Imutran Ltd., and were derived from Large White pigs (Figure 4.1), transgenic for the human decay accelerating factor (CD55). The expression of this protein on porcine xenografts used in non-human primate models of xenotransplantation

has been shown to prevent HAR (Bhatti *et al*, 1999; Cozzi *et al*, 2000; Schuurman *et al*, 2002).

Figure 4.1. Large White pig (source *JSR Healthbreed*).

4.2 Results

4.2.1 Tissue distribution and PCMV viral loads in adult pigs and piglets

PCMV DNA was detected in a range of organs and clinical samples from six adult and four juvenile pigs by qualitative PCR, and viral loads of PCR-positive samples were determined by QC PCR (Tables 4.1 and 4.2). All pigs had been conventionally reared.

All six adult pigs tested positive for PCMV DNA in at least one tissue (median of 4 tissues, range 1-6). The spleen tested positive in all adult pigs, with a median viral load of 27 PCMV genomes/μg of DNA (range 1-151). Of potential xenograft organs, three liver samples and one heart and kidney sample tested positive. In general, viral loads were low (median of 6 PCMV genomes/μg of DNA, range 1-151), indicating either latent or low-level active infection. No excretion of virus was detected in urine, faeces, or nasal and saliva swab samples.

In contrast to the adult pigs, PCMV was widely disseminated in 3-5 week-old weaned piglets, with a median of 13 PCR-positive tissues (range 12-15). This was significantly increased compared to adult pigs (Mann Whitney U test, $P < 0.05$). In addition, viral loads in the piglets (median of 667 genomes/μg of DNA, range 1-5.5 $\times 10^6$) were significantly higher than those of adult pigs (Mann Whitney U test, $P < 0.0001$). These results suggest either recent or ongoing active PCMV infection in the piglets. Only 3 out of the 4 piglets tested positive for PCMV by PCR, although all were reared identically. In addition, there was evidence for excretion of virus in the urine, nasal and saliva swab samples of one of the piglets, with high viral loads detected in these samples.

4.2.2 Investigating the temporal acquisition of PCMV

To investigate when virus is acquired, two sows and their respective litters were prospectively monitored in blood, from the time of birth (day 1), for a period of 82 and 36 days. In the first litter examined, both sow and piglets tested PCR-negative for PCMV in blood for the first 14 days (Table 4.3). At day 35, five of the ten surviving piglets tested positive for PCMV DNA by PCR. By day 49, all piglets tested PCMV-positive and remained positive at day 82. Significantly, at day 28, the piglets were

Table 4.1. Tissue distribution and PCMV viral loads in adult pigs (aged >15 months). Numerical values represent PCMV viral load (genomes/μg of DNA). Viral loads below the threshold of quantitation were given an arbitrary viral load of 1 genome copy/μg of DNA.

Pig	Heart	Large intestine	Small intestine	Kidney	Liver	Lung	Pancreas	Salivary gland	Spleen	Tonsil	Serum	PBMC	Urine	Faeces	Nasal swab	Saliva swab
D681	-[a]	1	1	-	-	-	-	-	8	-	-	6	-	-	-	-
E765	-	-	-	-	-	-	-	-	8	-	-	-	-	-	-	-
F800	-	-	-	-	-	-	-	-	1	-	-	1	-	-	-	-
F894	28	-	5	-	1	147	-	6	86	-	-	nt	-	-	-	-
F934	-	-	-	-	1	15	-	-	45	-	-	1	-	-	-	-
G473	-	-	-	4	1	40	-	-	151	-	-	-	-	-	-	-

[a] samples tested negative by qualitative PCMV PCR.

nt = sample not tested owing to insufficient DNA.

Table 4.2. Tissue distribution and PCMV viral loads in 3-5 week old piglets. Numerical values represent PCMV viral load (genomes/µg of DNA). Viral loads below the threshold of quantitation were given an arbitrary viral load of 1 genome copy/µg of DNA.

Pig	Heart	Large intestine	Small intestine	Kidney	Liver	Lung	Pancreas	Salivary gland	Spleen	Tonsil	Serum[1]	PBMC	Urine[1]	Faeces	Nasal swab[2]	Saliva swab[2]	Cerebrum
J147	460	1538	425	19674	513	8282	1	87	11552	7407	-	126	5.4×10^6	-	5.5×10^6	4.3×10^5	767
J159	1381	126	241	72	1304	8116	-	27	3298	66	2800	536	-	-	-	-	567
J176	-[a]	-	-	-	-	-	-	-	-	-	-	nt	nt	-	-	-	-
J184	3975	468	838	1831	390	2922	22	302	10576	128	200	848	-	-	-	-	145

[a] samples tested negative by qualitative PCMV PCR.

[1] PCMV genomes/ml sample, [2] PCMV genomes/swab, nt = sample not tested owing to insufficient DNA.

Table 4.3. Temporal acquisition of PCMV in sow P49M and litter. At day 28 the piglets were moved to a building for weaning with shared airspace but no direct contact with other pigs.

	Sow	Piglets										
Day	P49M	J443	J444	J445	J446	J447	J448	J449*	J450	J451	J452	J453
1	-[a]	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	na	-	-	-	-
14	-	-	-	-	-	-	-	na	-	-	-	-
35	na	+[b]	-	-	+	+	+	na	+	-	-	-
49	na	+	+	+	+	+	+	na	+	+	+	+
82	na	+	+	+	nt	+	nt	na	nt	+	+	+
Spleen[1]	na	969	439	3518	7346	107	110	na	3329	1653	4671	9205

[a] blood samples tested negative by qualitative PCMV PCR, [b] samples tested positive by qualitative PCMV PCR.

[1] PCMV viral load (genomes/μg of DNA) in spleen harvested at day 82.

na = sample not available, nt = sample not tested owing to insufficient DNA, * piglet died at day 1.

moved to a building for weaning, with shared airspace but no direct contact with other pigs. These results indicate horizontal transmission of the virus, which may have occurred after mixing of the litter with other pigs at day 28. In addition, spleen samples harvested from the litter at day 82 all tested PCMV-positive by PCR with a median viral load of 2491 genomes/μg of DNA (range 107-9205) (Table 4.3).

In the second litter examined, both sow and piglets tested PCR-negative for PCMV during the first 7 days (Table 4.4). At day 17, one out of eleven piglets tested positive for PCMV, and by day 36 all twelve piglets tested PCMV positive by PCR. These results again indicate horizontal transmission of the virus.

Our results in both adult pigs and piglets suggest the spleen is an appropriate tissue to sample for PCR analysis of PCMV DNA.

4.2.3 Investigating transplacental transmission of PCMV

In order to investigate whether PCMV is transmitted *in utero*, spleen samples were tested from two conventionally-reared sows and their corresponding foetuses (total of 22), at day 86 from an average full-term gestation time of 114 days. Both sows tested PCR-positive for PCMV in the spleen, with viral loads of 61 and 75 genomes/μg of DNA. Meanwhile, all 22 foetuses tested PCR-negative for PCMV in the spleen, even after re-testing using 2.5 μg of input DNA in the PCR. These results show that no transplacental transmission of PCMV had taken place in the first three-quarters of full-term gestation.

In addition, spleen samples were tested from nine sows and their hysterotomy-derived, barrier-reared piglets. The rearing of these piglets is further described in chapter 2, section 2.2.1. Piglets comprised two or three sentinel piglets from seven sows, and all piglets from two further sows. All sows were conventionally reared. Six of the nine sows tested positive for PCMV using 1 μg of input DNA in the PCR (median viral load of 34 PCMV genomes/μg of DNA, range 9-81) (Table 4.5). On re-testing with 2.5 μg of input DNA in the PCR, two further sows tested PCMV-positive. One sow remained PCMV-negative even after re-testing with 5 μg of input DNA in the PCR. Meanwhile, all 31 hysterotomy-derived, barrier reared piglets (median age of 15 weeks, range 4-21),

Table 4.4. Temporal acquisition of PCMV in sow G72E and litter. At day 28 the piglets were moved to a building for weaning with shared airspace but no direct contact with other pigs.

	Sow	Piglets											
Day	G72E	J479	J480	J481	J482	J483	J484	J485	J486	J487	J488	J489	J490
1	-[a]	-	-	-	-	-	-	-	-	-	-	nt	-
7	na	-	-	-	-	-	-	-	-	-	-	-	-
17	na	-	-	-	-	-	-	-	nt	-	+	-	-
36	na	+[b]	+	+	+	+	+	+	+	+	+	+	+

[a] blood samples tested negative by qualitative PCMV PCR, [b] samples tested positive by qualitative PCMV PCR.

na = sample not available, nt = sample not tested owing to insufficient DNA.

Table 4.5. Prevalence of PCMV in spleen samples from SPF piglets and sows.

Sow	Sow spleen PCMV viral load/µg DNA	Number of piglets PCR positive/total tested
593A	81	0/2
671A	+[a]	0/2
004A	-[b]	0/2
45704	9	0/8
476B	37	0/8
616A	36	0/2
423B	+	0/2
744A	32	0/3
595A	12	0/2
No. positive/Total	8/9	0/31

[a] indicates positive for PCMV DNA by PCR when 2.5 µg of DNA were used, negative at 1µg of DNA input.

[b] indicates PCR negative when tested at 1, 2.5, and 5 µg of DNA input.

tested PCR-negative for PCMV, even after re-testing with 2.5 µg of input DNA in the PCR. These results show that piglets delivered by caesarean section and subsequently barrier-reared did not acquire PCMV. In addition, despite eight of the sows testing PCMV-positive in the spleen, there was no transplacental or perinatal transmission of PCMV to their offspring.

4.2.4 Prevalence of PCMV and viral loads in immunosuppressed pigs

Qualitative and QC PCR assays were used to detect and quantify PCMV DNA in a range of organs from three groups of pigs comprising immunosuppressed and control animals. Pigs in group 1 (aged 58 days at necropsy), were delivered by caesarean section and reared in a gnotobiotic unit. Pigs in group 2 (aged 57 days at necropsy), were weaned at 5 days of age and reared in a gnotobiotic unit. While pigs in group 3 (aged 52 days at necropsy), were weaned from 5 weeks of age and reared under conventional farm conditions. Immunosuppressed pigs received a daily regimen of cyclosporin A, azathioprine and prednisolone, from 7-12 days of age, for a period of approximately 2 months, while control pigs received no treatment.

In group 1, all tissue samples from both immunosuppressed and control pigs tested PCMV-negative by qualitative PCR (Table 4.6). It was therefore not possible to compare the effect of immunosuppression on PCMV viral load in these gnotobiotic pigs. Meanwhile, all tissues from the conventionally-reared sow, from which these group 1 pigs were derived, tested PCMV-positive by PCR (median viral load of 160 genomes/µg of DNA, range 57-284). These results further support the findings obtained in section 4.3.3, that piglets delivered by caesarean section and subsequently barrier-reared do not acquire PCMV.

In group 2, again, all tissue samples from both immunosuppressed and control pigs tested PCMV-negative by qualitative PCR (Table 4.7). As with results obtained in group 1 pigs, it was not possible to compare the effect of immunosuppression on PCMV in this group of piglets.

In group 3, both immunosuppressed and control piglets tested PCMV-positive by qualitative PCR (median 5 tissues, range 3-5) (Table 4.8). Viral loads varied between

Table 4.6. Group 1: Prevalence of PCMV in immunosuppressed and control gnotobiotic pigs.

	Immunosuppressed pigs				Control pigs			
Sample	192	193	194	195	191	196	197	Sow
Heart	-[a]	-	-	-	-	-	-	160
Kidney	-	-	-	-	-	-	-	57
Liver	-	-	-	-	-	-	-	104
Lung	-	-	-	-	-	-	-	218
Spleen	-	-	-	-	-	-	-	284

[a] DNA extracted from each tissue sample tested negative by qualitative PCMV PCR. Numerical values represent PCMV viral load (genomes/μg of DNA).

Table 4.7. Group 2: Prevalence of PCMV in immunosuppressed and control SEW pigs.

	Immunosuppressed pigs					Control pigs		
Sample	253	239	244	270	275	238	243	271
Heart	-[a]	-	-	-	-	-	-	-
Kidney	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	-

[a] DNA extracted from each tissue sample tested negative by qualitative PCMV PCR.

Table 4.8. Group 3: Prevalence of PCMV in immunosuppressed and control conventionally-reared pigs.

	Immunosuppressed pigs				Control pigs	
Sample	F312	F314	F315	F321	F313	F324
Heart	153	11277	770	-[a]	344	4164
Kidney	378	7	26	-	77	9881
Liver	261	29	23	25	57	28
Lung	290	883	2407	202	94	597
Spleen	7953	984	2870	32	2676	3986

[a] DNA extracted from each tissue sample tested negative by qualitative PCMV PCR. Numerical values represent PCMV viral load (genomes/μg of DNA).

different tissues in each piglet, and between different piglets in the group. There was however, no noticeable difference in the viral loads between immunosuppressed and control pigs (median viral load of 276 genomes/μg of DNA in immunosuppressed pigs (range 7-11277) vs. median viral load of 471 genomes/μg of DNA in control pigs (range 28-9881)). Therefore, immunosuppression did not appear to affect the PCMV viral load in these pigs.

4.3 Discussion

The use of pig organs in xenotransplantation is associated with a risk of transmission of porcine infectious agents, particularly viruses, to the xenograft recipient. Since in human allotransplantation, HCMV is frequently transmitted via the donor organ, where it reactivates and is associated with end-organ disease and graft rejection (Fishman & Rubin, 1998), there is a possibility that the porcine equivalent, PCMV, may be transmitted to and cause disease in human xenograft recipients.

The aim of this study was therefore to gain a greater understanding of the epidemiology of PCMV, to investigate how virus is acquired, and to investigate whether PCMV transmission could be interrupted. Qualitative and QC PCR assays developed in chapter 3, were used to detect and quantify PCMV in transgenic pigs being bred for xenograft organs.

This is the first molecular study examining PCMV prevalence, organ distribution, temporal acquisition and methods to control its transmission (Clark *et al*, 2003). The results obtained in this study confirm that PCMV is a common infection in transgenic pigs reared under conventional farm conditions. The virus was widely disseminated in young 3-5 week-old piglets, which is consistent with previous serological findings of the epidemiology of PCMV in commercial pig herds (Plowright *et al*, 1976). Virus persisted in adult pigs (aged >15 months) in potential xenograft organs including the heart, liver and kidney. The spleen was consistently positive, and in fact tested positive in all but one of the adult pigs tested throughout the whole study. This important finding highlights the potential for this organ to be monitored for the PCMV status of the donor or sentinel animal, if clinical trials proceed. Viral loads in the piglets were significantly higher than in the adult pigs ($P < 0.0001$), indicative of recent or ongoing active infection in the piglets.

In a previous PCR analysis of porcine blood and tissue samples from four European countries, 50-100 % of lung and spleen samples from domestic pigs of Germany (total of 54 samples), Great Britain (total of 8 samples) and Spain (total of 9 samples), tested PCMV-positive, while only 4-23 % of PBMC samples from domestic pigs of Germany

(total of 52 samples) and the Netherlands (total of 34 samples), tested PCMV-positive (Goltz *et al*, 2000).

Prospective monitoring of piglets from birth showed that virus was acquired in the first few weeks of life. This is again consistent with previous findings in commercial pig herds (Plowright *et al*, 1976). Although the exact source of virus was not determined, it is likely that virus was transmitted horizontally, perhaps from other piglets during the post-weaning mixing of litters.

In the context of xenotransplantation, it is essential to determine how PCMV is transmitted, and whether it can be eliminated from pigs being bred for xenograft organs. Previous studies have shown that *in utero* infection can occur following experimental infection of pregnant sows (Edington *et al*, 1977; Edington *et al*, 1988a; Edington *et al*, 1988b), however, the frequency of *in utero* transmission in a conventional setting has not been reported. Results obtained in this study only identified post-natal transmission of virus. *In utero* infection was not detected in either of two foetal litters, in the first three-quarters of full-term gestation, suggesting that it may be uncommon.

The absence of *in utero* infection in this investigation may have been a result of the immune status of the sows studied. Although transplacental transmission of PCMV has been shown to take place despite the presence of pre-existing immunity, following experimental infection of pregnant sows, it occurred more frequently as a result of primary infection in the pregnant sow; 56.6 % congenital infection in seronegative sows (Edington *et al*, 1977) vs. 33.3 % in seropositive sows (Edington *et al*, 1988a). HCMV congenital infection occurs by primary infection or reactivation, with the latter being more common (Griffiths, 2004).

Although transplacental transmission of PCMV is common following experimental infection of pregnant sows, it may be much rarer in a conventional setting. In humans, epidemiological evidence suggests that *in utero* infection with HCMV occurs in 30-40% of pregnant women with primary infection and in 1 % of seropositive women (Stagno *et al*, 1982; Stagno *et al*, 1986). In pigs this incidence may in fact be much lower due to differences in placental structure between humans and pigs. In particular, the epitheliochorial structure of the pig placenta may provide a more protective barrier

against *in utero* infection than the hemomonochorial human placenta, by preventing invasion of infected trophoblast cells (Leiser & Kaufmann, 1994).

Until recently, pig-to-nonhuman primate models of xenotransplantation were not examined for the presence of PCMV, and to date PCMV has not been excluded from pig xenografts used in these studies. Reactivation of PCMV and associated disease has since been demonstrated following pig-to-baboon xenotransplantation (Mueller *et al*, 2002). Six baboons received pig thymokidney grafts and underwent intense immunosuppression. Activation of PCMV, as measured by quantitative PCR, was demonstrated in 5/6 xenografts, and was associated with ureteric necrosis in one case. This xenograft did not show evidence of graft rejection suggesting that PCMV infection was indeed the cause of disease. In addition, BCMV reactivation was demonstrated in 3/6 baboons resulting in CMV disease in one animal. In both cases, reactivation of virus and disease was restricted to the native host species of the virus. However, low levels of PCMV were detected in baboon tissue and vice verse, and were most likely a result of microchimerism. In a further study by the same group, activation of PCMV was detected in 6/7 pig kidney-to-baboon, 2/3 pig thymus-to-baboon, and 3/6 pig heart-to-baboon xenografts (Mueller *et al*, 2004).

PCMV reactivation in non-human primate models of xenotransplantation has also been associated with consumptive coagulopathy seen in these animals (Gollackner *et al*, 2003). Consumptive coagulopathy (CC) is defined as defective blood clotting, caused by decreasing levels of fibrinogen and platelets, which leads to increased haemorrhage. CC and thrombotic microangiopathy have been described in human allograft recipients with disseminated primary HCMV infection (Jeejeebhoy & Zaltzman, 1998). It is frequently observed in pig-to-baboon renal xenografts, in particular in 5/9 baboon recipients of PCMV-infected pig kidney xenografts (Gollackner *et al*, 2003), and is potentially fatal unless the xenograft is removed. The cause is not definitively known, but it may be initiated by endothelial activation, immunologic injury, infection or molecular incompatibilities. These recent findings highlight the risks of PCMV in xenotransplantation; not only that it may be transmitted to the xenograft recipient, but that it could reactivate and result in graft rejection or direct damage to the organ.

The most significant finding of our study was that there was no transmission of virus to hysterotomy-derived barrier-reared piglets, suggesting that this approach could be used to eliminate PCMV from pigs being bred for xenograft organs. These piglets were delivered by caesarean section and reared for two weeks in isolator tanks under gnotobiotic conditions (Miniats & Jol, 1978). Gnotobiotic pigs were then transferred to a bioexclusion, barrier-rearing facility, in which all air, water and feed was sterilised (Tucker *et al*, 2002a). Such SPF breeding stocks could potentially be established using α1,3GT knock-out animals, and the SPF status quality controlled by monitoring saliva or urine from the herd, and by testing the spleen from individual donor animals. Transmission of PCMV to xenograft recipients should then be preventable, first in non-human primate models, and also in humans should clinical trials proceed.

Since xenograft recipients are likely to be subject to immunosuppressive therapy to prevent rejection, the effect of immunosuppression on PCMV viral load in pigs was investigated. This immunosuppressive treatment was based on that used to prevent allograft rejection. Corticosteroids such as prednisolone have previously been shown to reactivate PCMV (Edington *et al*, 1976b; Narita *et al*, 1985). However, in this study, immunosuppression did not appear to affect viral load of PCMV in tissues from conventionally-reared pigs. In future investigations, it would be useful to look at a greater number of pigs and to prospectively monitor them during the period of immunosuppression, since in this study, any reactivation of PCMV may have occurred within the two month period before pigs were euthanised and samples collected. However, the results from the gnotobiotic group examined support the findings earlier in the study that PCMV can be eliminated by SPF breeding.

In summary, PCMV is a common infection in pigs being bred for xenograft organs and is acquired in the first few weeks of life, most probably following mixing of litters in the early post-weaning period. In contrast with previous experimental results, transplacental transmission was not common. Importantly, animals that were hysterotomy-derived and barrier-reared did not acquire PCMV, suggesting that this approach could be used to generate PCMV-free pigs for use in xenotransplantation. Finally, these results show that the PCMV-free status of these pigs could be quality controlled by monitoring the saliva or urine from the herd, and by testing the spleen from individual sentinel animals.

Chapter 5: Cloning and expression of PCMV glycoprotein B in insect cells using a baculovirus expression system and the development of a recombinant protein-based serological assay for PCMV

5.1 Introduction

If pigs are to be used as source animals in xenotransplantation they will need to be free from a wide range of porcine infectious agents. The results obtained in Chapter 4 show that PCMV can be eliminated from pigs being bred for xenograft organs by caesarian delivery and barrier-rearing. Pigs bred using these methods will need to be comprehensively screened to ensure that they are free from potential zoonotic agents, and to achieve this, reliable and sensitive diagnostic tests are needed. The results obtained in chapter 4 suggest that the PCMV status of pig herds being bred for xenograft organs can be monitored by testing spleen samples from sentinel or individual donor animals by PCMV PCR, since this organ tested consistently PCMV DNA positive in infected animals. However, the results also showed that PCMV DNA was not always detected in readily available samples, such as blood or serum, from pigs with latent or low-level active infection, highlighting the importance of a serological assay for this virus. Serological assays such as IIF and ELISA, are important tools for the detection of prior exposure to a virus. Serological assays that have previously been described for PCMV are time consuming, since they require *in vitro* culturing of PCMV (Plowright *et al*, 1976; Rondhuis *et al*, 1980; Tajima *et al*, 1993). In the IIF assay described by Rondhuis *et al*, antigen is prepared by *in vitro* culturing of PCMV in porcine lung macrophages for 10-12 days. Since lung macrophages do not replicate, primary cultures must be used. These are difficult to obtain and potentially compromised by other infectious agents. In addition, different batches of macrophages can vary considerably in their quality and sensitivity to detect virus (Plowright *et al*, 1976). In the ELISA described by Tajima *et al*, antigen was prepared from 19-PFT-F cells 2-10 dpi with PCMV (Tajima *et al*, 1993).

Problems associated with *in vitro* cultivation systems might be overcome by using immunogenic PCMV proteins produced using a recombinant expression system. Glycoprotein B (gB) (UL55) is the major antigenic protein of HCMV, and has been shown to be a common target of neutralising antibodies (Britt *et al*, 1990), with 40-70 % of total serum neutralising antibodies being directed against this protein. The UL55 ORF of HCMV encodes a 906-907 amino acid protein (Figure 5.1A) (Mocarski & Courcelle, 2001). The HCMV gB precursor (105 kDa) is modified to a protein of 150 kDa by N-linked glycosylation in the endoplasmic reticulum (ER), and is further

glycosylated to a 165-170 kDa glycoprotein as it is transported through the Golgi network (Britt & Vugler, 1989). The cellular endoprotease furin cleaves the mature HCMV gB in the Golgi (Vey *et al*, 1995), into a 116 kDa amino-terminal fragment and a 55 kDa carboxyl-terminal fragment, which associate on the virion envelope as a disulphide-linked heterodimer (Britt & Vugler, 1989). A phosphorylation site, situated at the carboxyl-terminus (Norais *et al*, 1996), is thought to determine the cellular distribution of HCMV gB (Fish *et al*, 1998). HCMV gB contains two distinct antigenic domains containing neutralising epitopes including AD-1, located in the gp55 component (amino acids 552-635), and AD-2 located in the gp116 component (amino acids 68-77) (Griffiths & Emery, 2002). However, the major neutralising domain maps to the AD-1 region within the carboxyl-terminal end of the protein (Banks *et al*, 1989).

Glycoprotein B is one of the herpesvirus core genes and is highly conserved across all subfamilies of herpesviruses (Roizman & Pellet, 2001). The gB genes of five PCMV strains and isolates were recently identified and sequenced by genome walking (Widen *et al*, 2001). The gB gene of PCMV strain B6 and isolate #55 (Spanish isolate) were found to have an ORF of 2580 nucleotides, encoding a protein of 860 amino acids. The Japanese strain OF-1 and isolates #489 (German isolate) and #1469 (Swedish isolate) have an ORF of 2583 nucleotides encoding a protein of 861 amino acids. The amino acid sequences of these proteins showed highest identities with the corresponding sequences of the betaherpesviruses, in particular HHV-6 & -7 (43.5 % and 42 % identity, respectively), and less so with HCMV (35.1 % identity). They also showed sequence similarity with members of the gammaherpesvirus subfamily including rhesus rhadinovirus (32.4 % identity), bovine herpesvirus 4 and HHV-8 (both 31.1 % identity), and EBV (30.4 % identity). The deduced protein sequence of PCMV gB shows positional conservation of 10/11 cysteine residues and 4-5/15 N-linked glycosylation motifs, with HHV-6 and -7 gB proteins. A potential cleavage site (residues 439-441), signal peptide sequence (residues 7-19) and transmembrane anchor sequences (residues 706-726 and 730-747) were also identified (Figure 5.1B). In addition, the region between residues 521 and 623 was found to be 45.6 % identical to the immunodominant AD-1 region of HCMV gB strain Ad169, suggesting that this protein could be used as a source of viral antigen in IIF or an ELISA. The five strains and isolates of PCMV gB exhibited pair differences of 0.6-1.7 % on the nucleotide level and 3.4 % on the amino acid level, with most of these differences attributed to the UK strain B6.

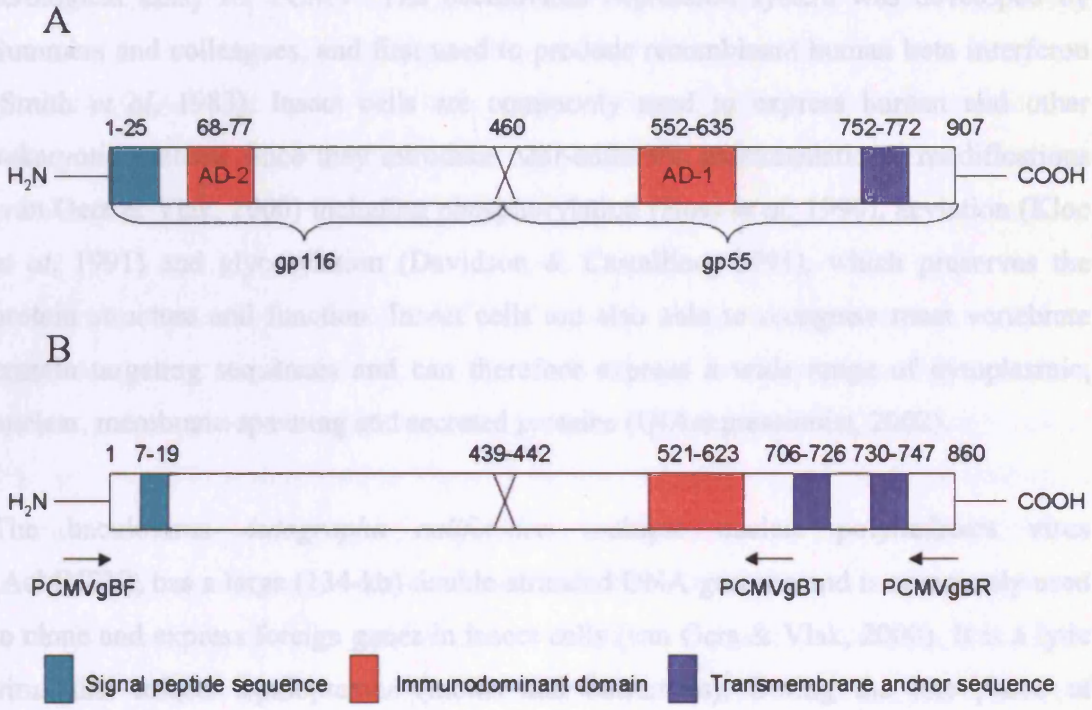


Figure 5.1. A schematic of HCMV gB (A) and PCMV gB (B) proteins (Griffiths & Emery, 2002; Widen *et al*, 2001). Numbers represent amino acid codons. The furin protease cleavage site is located at amino acid residue 460 in HCMV gB, and between residues 439-442 in PCMV gB. Primers PCMVgBF and PCMVgBR were used to amplify the complete PCMV gB sequence, while primers PCMVgBF and PCMVgBT were used to amplify a truncated sequence.

The following chapter describes the expression of PCMV gB in insect cells, using a baculovirus expression system, for the development of a recombinant protein-based serological assay for PCMV. The baculovirus expression system was developed by Summers and colleagues, and first used to produce recombinant human beta interferon (Smith *et al*, 1983). Insect cells are commonly used to express human and other eukaryotic proteins since they introduce near-authentic posttranslational modifications (van Oers & Vlak, 2000) including phosphorylation (Hoss *et al*, 1990), acylation (Kloc *et al*, 1991) and glycosylation (Davidson & Castellino, 1991), which preserves the protein structure and function. Insect cells are also able to recognise most vertebrate protein targeting sequences and can therefore express a wide range of cytoplasmic, nuclear, membrane-spanning and secreted proteins (QIAexpressionist, 2002).

The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), has a large (134-kb) double-stranded DNA genome and is commonly used to clone and express foreign genes in insect cells (van Oers & Vlak, 2000). It is a lytic virus that infects lepidopterans (moths and butterflies). During the late phase of baculovirus infection occluded virus particles encased in polyhedrin aggregate form intranuclear inclusions (Kidd & Emery, 1993). This polyhedrin protein is the most abundant protein produced by baculoviruses, making up 30-50 % of the total cell protein (Ansari & Emery, 1998). The baculovirus expression system takes advantage of the fact that this protein is expressed at very high levels and is dispensable for growth in cell culture, such that the polyhedrin gene is replaced with a foreign gene. To generate a recombinant baculovirus the gene of interest is initially cloned into an appropriate transfer vector, under the control of the polyhedrin promoter, and flanked by baculovirus-derived sequences. This is then co-transfected into insect cells with baculovirus DNA using cationic liposomes. Site-specific recombination between the viral DNA and the transfer vector, results in insertion of the gene of interest into the baculovirus genome (van Oers & Vlak, 2000). Recombinant virus can then be isolated and amplified for large-scale recombinant protein production.

Important applications of the baculovirus expression system include the production of antigens for use in serological assays (Brown *et al*, 1991), and the development of subunit vaccines, for example, against classical swine fever virus (Hulst *et al*, 1993). The baculovirus production of recombinant glycoproteins from human and animal

herpesviruses, and their use in serological assays and vaccines, has previously been described (Wells *et al*, 1990; Abdelmagid *et al*, 1998; Schleiss & Jensen, 2003). In addition, baculovirus expression of a truncated version of HCMV gB (residues 1-692), that was secreted into the culture medium, thus aiding purification, has also been reported (Carlson *et al*, 1997). This secreted version of HCMV gB retained its functional activity in that it was dimeric, properly folded and able to bind to a heparin affinity matrix.

In order to clone the complete PCMV gB gene, a forward primer was designed to anneal upstream from, and in frame with the PCMV gB initiation codon ATG, while a reverse primer was designed to anneal just upstream from the PCMV gB stop codon. Primer binding sites are shown in Figure 5.1B. Since the complete PCMV gB contains a transmembrane anchor sequence it should be expressed on the surface of infected insect cells. In addition, a truncated version of PCMV gB (PCMV gBT) was cloned using the same forward primer, and a reverse primer designed to anneal in-between the AD-1-homologous region and the predicted transmembrane anchor sequences of PCMV gB. PCMV gBT should be secreted into the culture medium of infected insect cells from where it can be purified for use in the development of an ELISA for PCMV. The expression of PCMV gB and gBT was optimised in Sf9 and High Five insect cells. Insect cells expressing recombinant PCMV gB proteins were subsequently used as a substrate in the development of an IIF assay, such as has been used to measure antibody titres to HCMV gB (Deayton *et al*, 2002). Recombinant PCMV gBT was also used as a viral antigen in the development of an ELISA for PCMV.

5.2 Results

5.2.1 PCR amplification of the complete and truncated PCMV gB ORFs and cloning into the baculovirus vector

Primers specific for the 3' and 5' ends of the PCMV gB gene were used to amplify the complete 2.6-kb ORF of PCMV gB (excluding the stop codon), from a TCS sample of PCMV strain B6-infected porcine alveolar macrophages (Figure 5.2, panel A). In addition, a 1.93-kb truncated sequence was amplified from the same sample (Figure 5.2, panel B). Both sequences were purified, and cloned into the baculovirus transfer vector pBlueBac4.5/V5 His TOPO TA, in frame with the carboxyl-terminal peptide. The presence of the both sequences, in the correct orientation within the vector, was confirmed by restriction enzyme analysis (Figure 5.3). Sequence analysis also confirmed that no mutations had been introduced into the gB and gBT sequences during PCR amplification with the proof-reading enzyme BIO-X-ACT DNA polymerase (data not shown).

5.2.2 Transfection of Sf9 insect cells and PCR analysis of recombinant baculovirus

Recombinant transfer vectors and Bac-N-Blue DNA were combined, and used to co-transfect Sf9 insect cells. Recombinant baculovirus in the culture medium was harvested 3 dpi, while subsequent lysis and detachment of infected cells from the cell culture dish was observed 4-7 dpi. Recombinant baculovirus was purified from wild-type virus by plaque assay and screening for recombinant blue plaques following the addition of the chromogenic substrate X-Gal. Recombination between the 5' portion of the *lac*Z gene in the transfer vector, and the 3' portion of the *lac*Z gene in the Bac-N-Blue DNA, resulted in the expression of β-galactosidase, converting X-Gal to a blue product. Blue plaques were identified 6-7 dpi and used to generate a P1 virus stock.

Purification of recombinant virus from wild-type virus was confirmed by PCR analysis of DNA extracted from Sf9 insect cells 6 dpi with Sf900II medium containing recombinant plaques. Viral DNA was amplified by PCR using baculovirus and PCMV gB-specific primers. The results in Figure 5.4 show that a sequence of approximately 3-kb in size was amplified from viral DNA from each of four recombinant plaques using

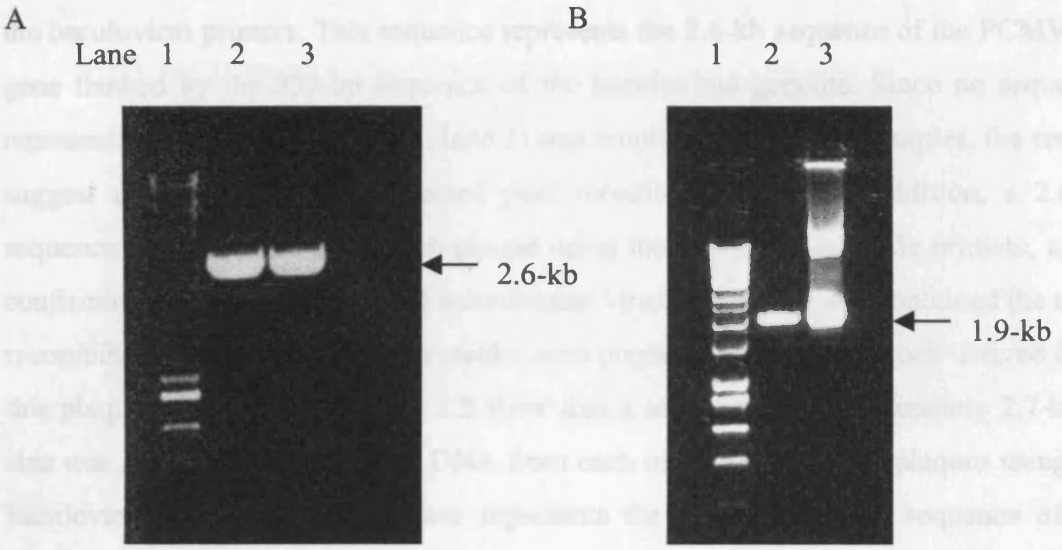


Figure 5.2. PCR amplification of the complete and truncated ORFs of PCMV gB from a TCS sample of porcine alveolar macrophages infected with PCMV strain B6. Panel A shows the 2.6-kb full length PCMV gB sequence amplified from 1 µl of 1/10 (lane 2) and 1/20 (lane 3) dilutions of PCMV TCS, alongside HyperLadder I DNA markers (lane 1). Panel B shows the 1.9-kb PCMV gBT sequence amplified from 1 µl of a 1/10 dilution of PCMV TCS (lane 2), and 2 µg of the recombinant pBlueBac4.5/V5-His-TOPO transfer vector containing the complete PCMV gB ORF (lane 3), alongside HyperLadder I DNA markers (lane 1).

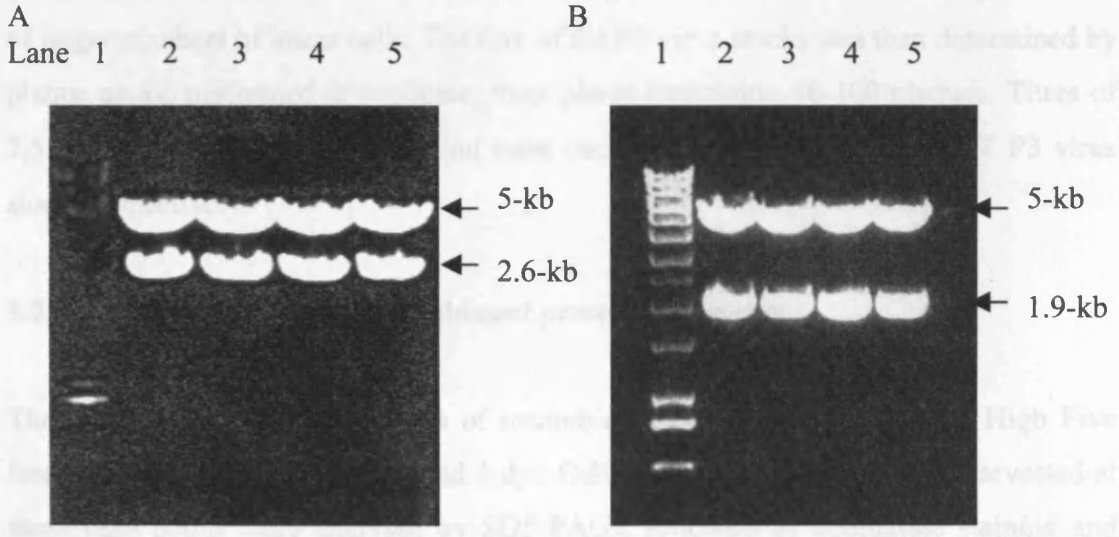


Figure 5.3. *Bam* HI and *Hind* III restriction enzyme analysis of recombinant PCMV gB (A) and gBT (B) transfer vector DNA extracted from four recombinant clones (lanes 2-5). Ten microlitres of each digestion reaction were run on a 1 % agarose gel alongside HyperLadder I DNA markers (lane 1).

the baculovirus primers. This sequence represents the 2.6-kb sequence of the PCMV gB gene flanked by the 839-bp sequence of the baculovirus genome. Since no sequence representing wild-type virus (as in lane 2) was amplified from these samples, the results suggest that these plaques contained pure recombinant virus. In addition, a 2.6-kb sequence was amplified from each plaque using the PCMV gB-specific primers, again confirming that plaques contained recombinant virus. Since plaque 1 contained the most recombinant virus, high-titre virus stocks were prepared from the P1 stock derived from this plaque. The results in Figure 5.5 show that a sequence of approximately 2.7-kb in size was amplified from the viral DNA from each of ten recombinant plaques using the baculovirus primers. This sequence represents the 1.9-kb truncated sequence of the PCMV gB gene flanked by the 839-bp sequence of the baculovirus genome. Since no sequence representing wild-type virus was amplified from these samples, the results suggest that these plaques contained pure recombinant virus. High-titre stocks of PCMV gBT recombinant baculovirus were prepared from the P1 stock derived from recombinant plaque 1.

5.2.3 Preparation of high-titre virus stocks and calculation of virus titre

The small-scale, low-titre P1 virus stocks were used to generate large-scale, high-titre P2 and P3 virus stocks of recombinant PCMV gB and gBT baculoviruses, by infection of larger numbers of insect cells. The titre of the P3 virus stocks was then determined by plaque assay, performed in triplicate, from plates containing 50-100 plaques. Titres of 7.5×10^7 pfu/ml and 9.8×10^7 pfu/ml were obtained for PCMV gB and gBT P3 virus stocks respectively.

5.2.4 Determining optimal recombinant protein expression

The time-course of the expression of recombinant PCMV gB and gBT in High Five insect cells was analysed 1, 2, 3 and 4 dpi. Cell lysates from infected cells harvested at these time points were analysed by SDS-PAGE followed by coomassie staining and western blotting. The results in Figure 5.6, show that maximal recombinant protein expression in High Five insect cells was achieved 2-4 dpi, with minimal recombinant protein production at 1 dpi. The western blot results show that recombinant PCMV gB comprised a large amount of protein of approximately 100-150 kDa in size and a

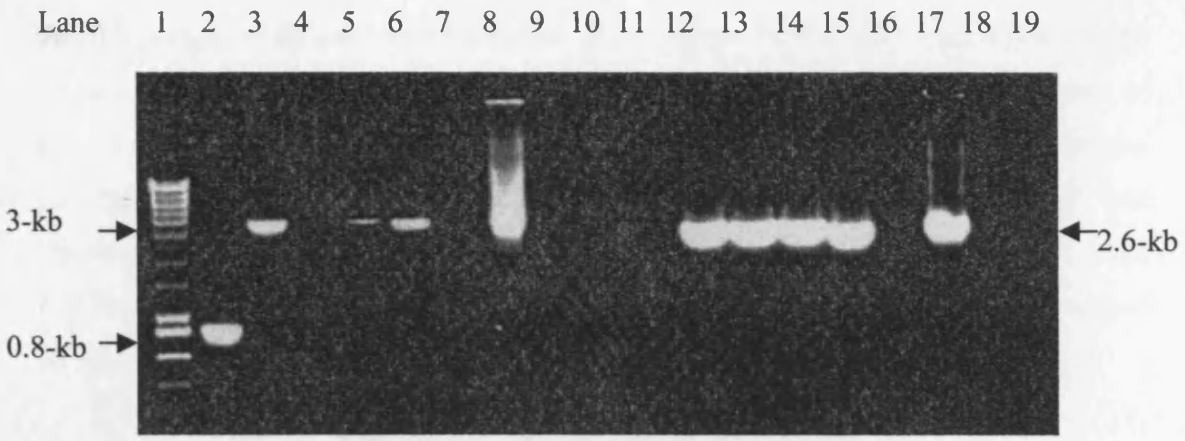


Figure 5.4. PCR analysis of recombinant PCMV gB baculovirus following purification by plaque assay, using baculovirus (lanes 2-10) and PCMV gB-specific primers (lanes 11-19). Analysis of four recombinant plaques 1-4 (lanes 3-6 & 12-15) shows recombinant baculovirus was purified from wild-type virus (lane 2). DNA extracted from uninfected Sf9 insect cells (lanes 7 & 16), and SDW (lanes 9, 10, 18 & 19), were used as negative controls, while recombinant transfer vector DNA was used as a positive control (lanes 8 & 17).

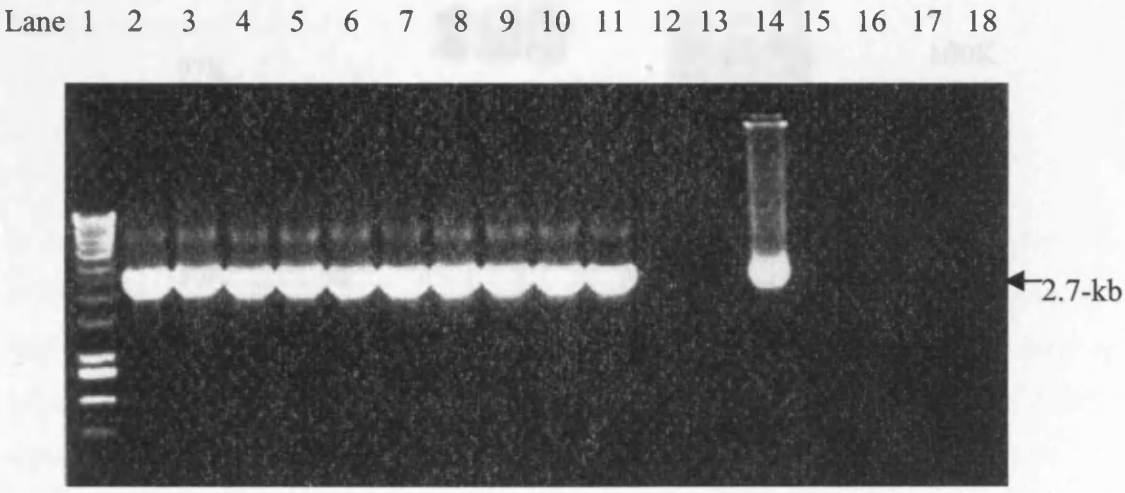


Figure 5.5. PCR analysis of recombinant PCMV gBT baculovirus following purification by plaque assay, using baculovirus primers. Analysis of ten recombinant plaques 1-10 (lanes 2-11) shows recombinant baculovirus was purified from wild-type virus. DNA extracted from uninfected Sf9 insect cells (lanes 12 & 13), and SDW (lanes 15-18), were used as negative controls, while recombinant transfer vector DNA was used as a positive control (lane 14).

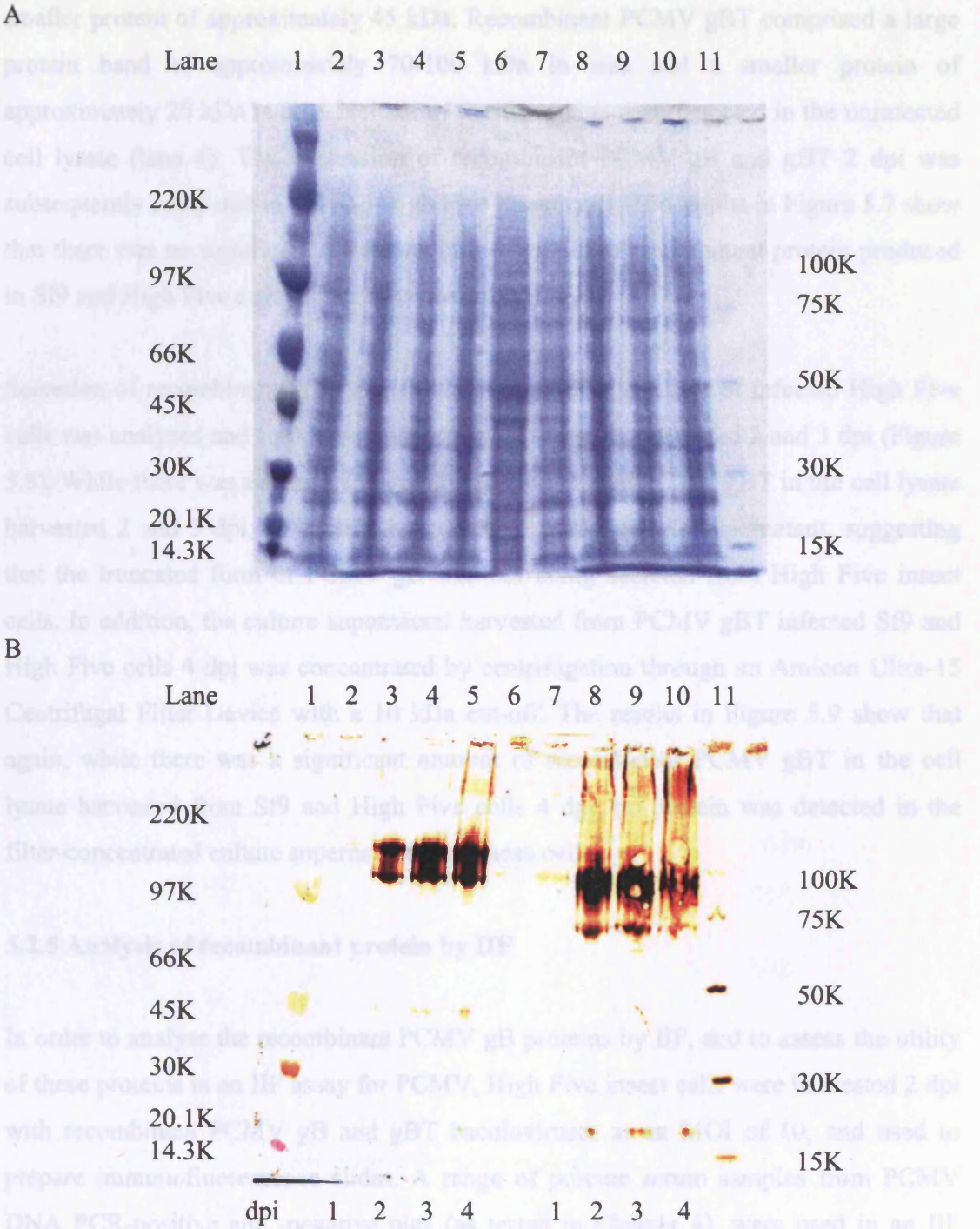


Figure 5.6. Coomassie stain (A) and western blot (B) showing the time-course of recombinant protein production in High Five insect cells. Cell lysates from PCMV gB- (lanes 2-5) and PCMV gBT- (lanes 7-10) infected High Five cells harvested 1-4 dpi were analysed by SDS-PAGE, alongside uninfected High Five cells (lane 6), Rainbow markers (lane 1) and 6×His Protein Ladder (lane 11) using non-reducing conditions.

smaller protein of approximately 45 kDa. Recombinant PCMV gBT comprised a large protein band of approximately 70-100 kDa in size and a smaller protein of approximately 20 kDa in size. Neither of these proteins were detected in the uninfected cell lysate (lane 6). The expression of recombinant PCMV gB and gBT 2 dpi was subsequently compared in Sf9 and High Five insect cells. The results in Figure 5.7 show that there was no significant difference in the amount of recombinant protein produced in Sf9 and High Five cells at this time point.

Secretion of recombinant PCMV gBT in the culture supernatant of infected High Five cells was analysed and compared with cell lysate samples harvested 2 and 3 dpi (Figure 5.8). While there was a significant amount of recombinant PCMV gBT in the cell lysate harvested 2 and 3 dpi, no protein was detected in the culture supernatant, suggesting that the truncated form of PCMV gB was not being secreted from High Five insect cells. In addition, the culture supernatant harvested from PCMV gBT infected Sf9 and High Five cells 4 dpi was concentrated by centrifugation through an Amicon Ultra-15 Centrifugal Filter Device with a 10 kDa cut-off. The results in Figure 5.9 show that again, while there was a significant amount of recombinant PCMV gBT in the cell lysate harvested from Sf9 and High Five cells 4 dpi, no protein was detected in the filter-concentrated culture supernatant from these cells.

5.2.5 Analysis of recombinant protein by IIF

In order to analyse the recombinant PCMV gB proteins by IIF, and to assess the utility of these proteins in an IIF assay for PCMV, High Five insect cells were harvested 2 dpi with recombinant PCMV gB and gBT baculoviruses at an MOI of 10, and used to prepare immunofluorescence slides. A range of porcine serum samples from PCMV DNA PCR-positive and -negative pigs (as tested in Chapter 4), were used in an IIF assay to detect PCMV gB-specific antibodies. PCMV gB-specific antibodies bound to recombinant PCMV gB proteins expressed in infected High Five cells, were detected using FITC-Conjugated Rabbit Anti-Swine IgG antibodies.

The immunofluorescence of recombinant baculovirus PCMV gB and gBT-infected High Five insect cells was significantly increased when compared with uninfected cells (Figure 5.10). Since glycoproteins are membrane-bound proteins, recombinant PCMV

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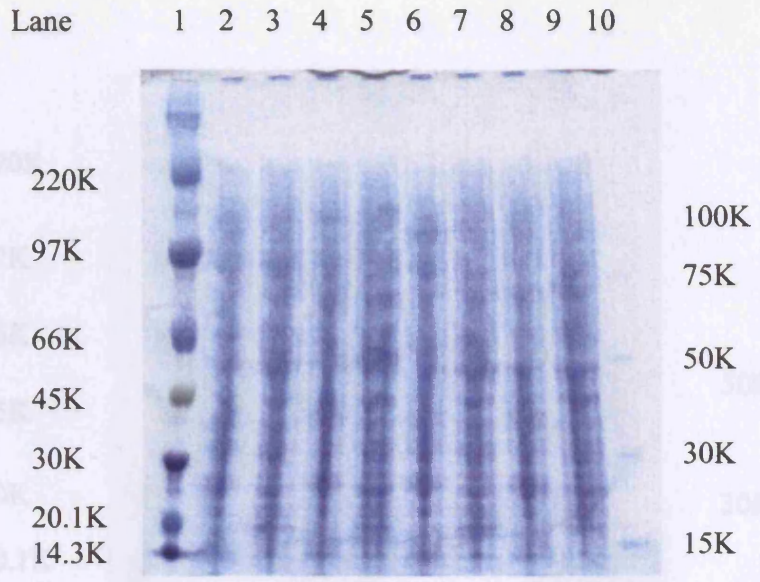

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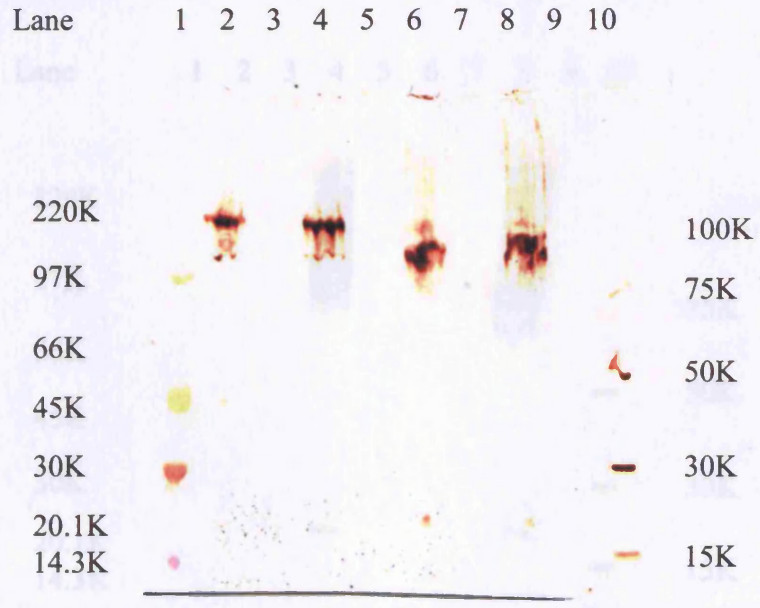

Figure 5.7. Coomassie stain (A) and western blot (B) showing the comparison of recombinant protein production in High Five vs. Sf9 insect cells 2 dpi. Cell lysates from PCMV gB- (lane 2) and PCMV gBT- (lane 6) infected Sf9 cells, and PCMV gB- (lane 4) and PCMV gBT- (lane 8) infected High Five insect cells, were analysed by SDS-PAGE, alongside uninfected Sf9 (lanes 3 & 7) and High Five cells (lanes 5 & 9) using non-reducing conditions. Rainbow markers (lane 1) and 6×His Protein Ladder (lane 11) were also run on the gel.

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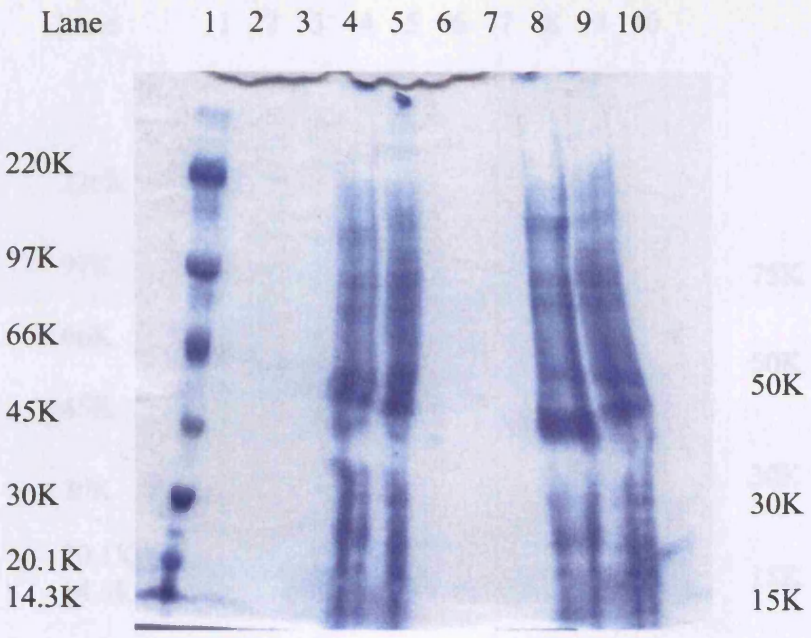

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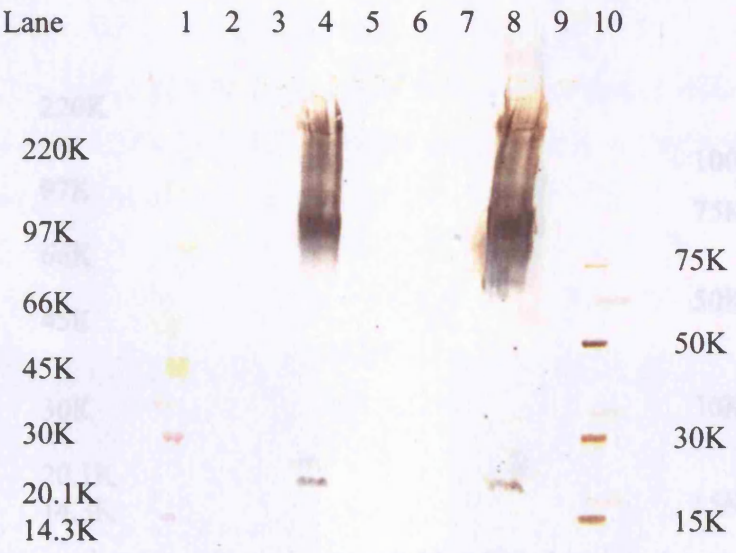

Figure 5.8. Coomassie stain (A) and western blot (B) showing the analysis of supernatant and cell lysate samples from PCMV gBT-infected High Five insect cells harvested 2 and 3 dpi following SDS-PAGE, using non-reducing conditions. Culture supernatant samples from PCMV gBT-infected High Five cells were run in lanes 2 (2 dpi) and 6 (3 dpi), while supernatant samples from uninfected cells were run in lanes 3 and 7. Cell lysate samples from PCMV gBT-infected High Five cells were run in lanes 4 (2 dpi) and 8 (3 dpi), while cells lysate samples from uninfected cells were run in lanes 5 and 9. Rainbow markers (lane 1) and 6×His Protein Ladder (lane 10) were also run on the gel.

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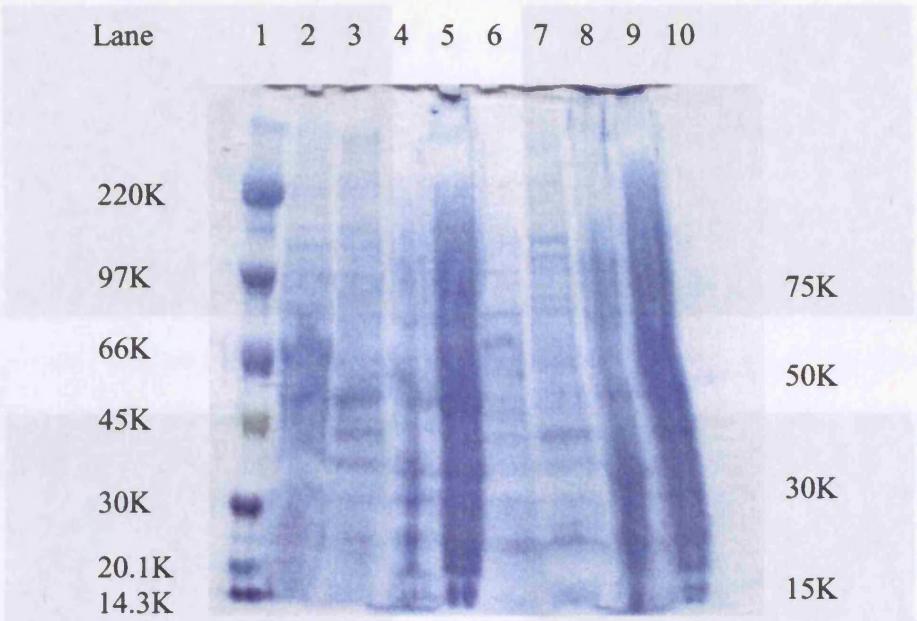

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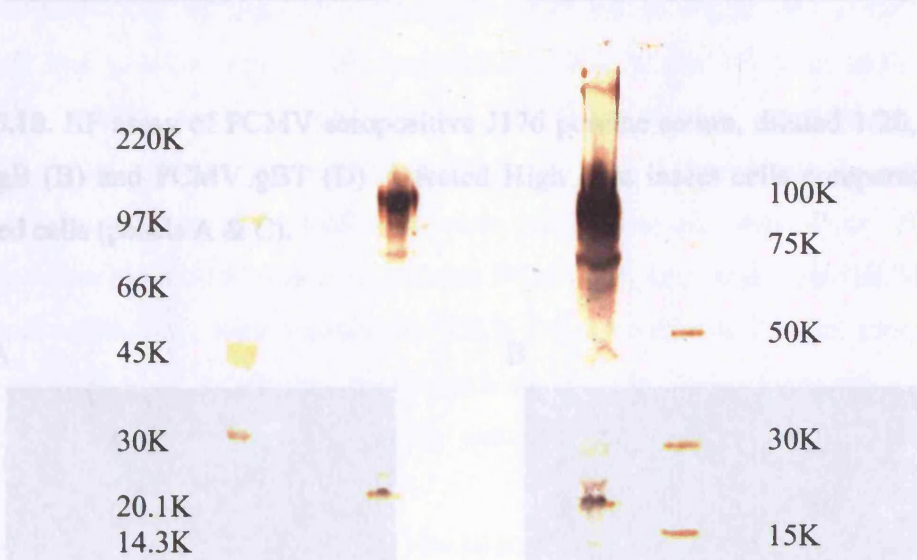

Figure 5.9. Coomassie stain (A) and western blot (B) showing the analysis of filter concentrated supernatant and cell lysate samples from PCMV gBT-infected Sf9 and High Five cells 4 dpi, by SDS-PAGE, using non-reducing conditions. Concentrated supernatant samples from PCMV gBT-infected Sf9 and High Five cells were in run lanes 2 & 6 respectively, while concentrated supernatant samples from uninfected cells were run in lanes 3 & 7. Cell lysate samples from PCMV gBT-infected Sf9 and High Five cells were run in lanes 4 & 8 respectively, while cells lysate samples from uninfected cells were run in lanes 5 & 9. Rainbow markers (lane 1) and 6×His Protein Ladder (lane 10) were also run on the gel.

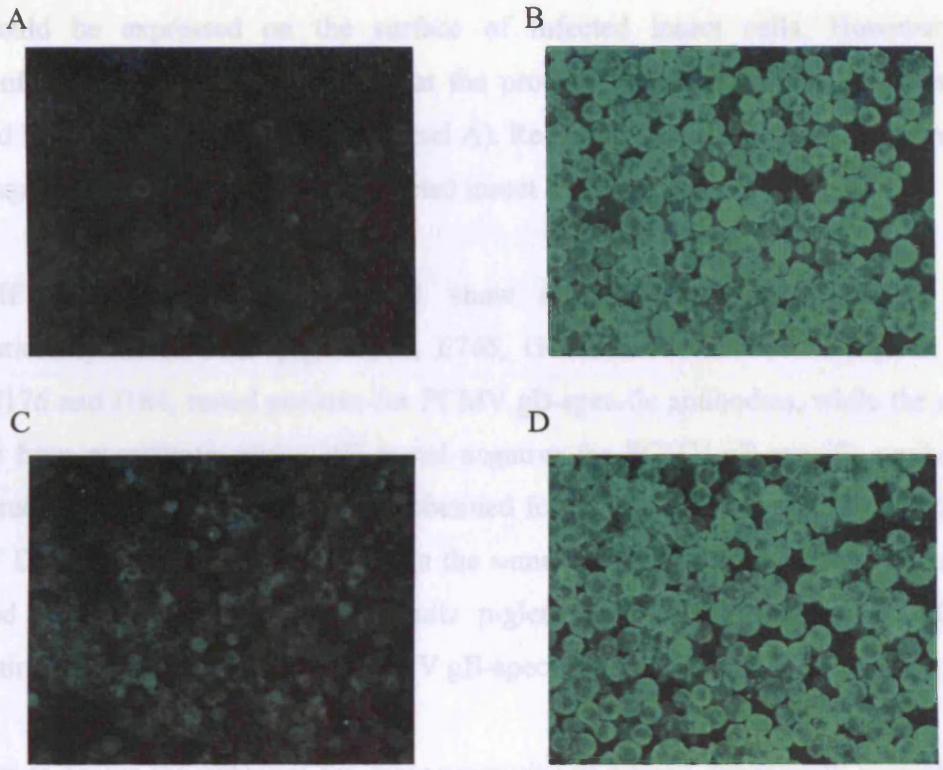

Figure 5.10. IIF assay of PCMV seropositive J176 porcine serum, diluted 1/20, using PCMV gB (B) and PCMV gBT (D) -infected High Five insect cells compared with uninfected cells (panels A & C).

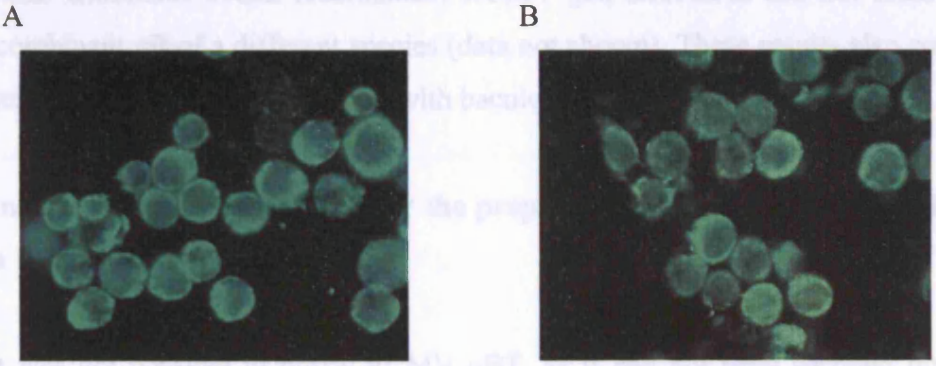

Figure 5.11. Recombinant PCMV gB (A) and PCMV gBT (B) proteins are expressed in the cytoplasm of infected High Five insect cells. The IIF assay was performed using serum from piglet J176, diluted 1/20. Cells were fixed in 100 % acetone.

gB should be expressed on the surface of infected insect cells. However, the immunofluorescence results showed that the protein was found in the cytoplasm of infected High Five cells (Figure 5.11, panel A). Recombinant PCMV gBT also appeared to be expressed in the cytoplasm of infected insect cells (Figure 5.11, panel B).

The IIF assay results in Table 5.1 show that porcine serum samples from conventionally-reared adult pigs D681, E765, G473, F800, F934, and piglets J147, J159, J176 and J184, tested positive for PCMV gB-specific antibodies, while the serum sample from gnotobiotic piglet 193 tested negative for PCMV gB-specific antibodies. These results are consistent with those obtained following qualitative PCR detection of PCMV DNA in a range of samples from the same animals (see Chapter 4, Tables 4.1, 4.2 and 4.6). However, in those results piglet J176 tested negative for PCMV, suggesting that maternally-derived PCMV gB-specific antibodies were detected by IIF.

In order to assess the potential for cross-reactivity of human serum with recombinant PCMV gB and porcine serum with recombinant HCMV gB, IIF was additionally performed using mouse anti-HCMV gB monoclonal antibodies and immunofluorescence slides prepared from Sf9 insect cells infected with recombinant HCMV gB baculovirus. Immunofluorescence results showed that while PCMV-seropositive porcine serum bound recombinant PCMV gB, and mouse anti-HCMV gB monoclonal antibodies bound recombinant HCMV gB, antibodies did not cross-react with recombinant gB of a different species (data not shown). These results also confirm that porcine serum does not cross-react with baculovirus proteins

5.2.6 Analysis of cell lysis methods for the preparation of recombinant protein for use in a PCMV ELISA

Since it was not possible to purify PCMV gBT, as it had not been secreted into the culture medium of infected insect cells, an ELISA was set up using a crude lysate preparation of PCMV gBT-infected High Five insect cells. In order to determine the best method for preparing this lysate, PCMV gBT-infected High Five cell pellets were resuspended in 1 ml of TNE Buffer and lysed by either four cycles of freeze-thawing, or by sonication on ice. Lysates were then clarified by centrifugation to pellet the cell debris, and the supernatant was transferred to a clean tube. Centrifuged and non-

Table 5.1. Qualitative PCMV IIF assay results of porcine serum samples, using slides prepared from PCMV gB-infected High Five insect cells.

Porcine serum		IIF result	PCMV DNA PCR result (spleen)
Adult pig D681 (conventionally reared)		+	+
	E765	+	+
	G473	+	+
	F800	+	+
	F894	+	+
	F934	+	+
Piglet	J147	+	+
	J159	+	+
	J176	+	-
	J184	+	+
Adult pig 193 (gnotobiotic)		-	-

centrifuged cell lysate samples were analysed by SDS-PAGE and western blot. The results in Figures 5.12 and 5.13 show that little or no recombinant protein was detected in the clarified cell lysate samples prepared by either freeze-thawing or sonication. Recombinant protein was however detected in all non-centrifuged cell lysate samples, suggesting that either PCMV gBT-infected High Five cells were not properly lysed, or that the recombinant protein was insoluble.

5.2.7 Preliminary PCMV ELISA

Since clarified lysates prepared from PCMV gBT-infected High Five cells did not contain PCMV gBT, whole and filtered lysate samples from PCMVgBT-infected High Five cells were tested in an ELISA format for reactivity with porcine serum. Protein lysates from PCMV gBT-infected (2 dpi) and uninfected (control) High Five insect cells were prepared by lysis in TNE buffer, followed by sonication. A portion of each cell lysate sample was partially clarified by filtration through a 0.8 μm filter, and 5 μg/ml of filtered and non-filtered samples were adsorbed onto each well of a 96-well Maxisorp immuno-plate. Following blocking, wells were incubated with serial dilutions of pooled sera from PCMV-positive and negative pigs, and pooled high-titre HCMV-positive and negative human serum, diluted 1/5000. HRP conjugate antibody, diluted 1/7000, followed by TMB substrate, were added to wells and the absorbance determined at 450 and 630 nm. As shown in Figure 5.14, PCMV-negative porcine serum, and both HCMV-positive and negative human serum gave absorbance values below 0.4 for all protein lysates tested. PCMV-positive porcine serum gave high absorbance values (within range 1.9-2.4 when diluted 1/50) to both filtered and non-filtered lysates prepared from PCMV gBT-infected and control High Five cells, but a much lower absorbance in the wells containing buffer only. These results suggest non-specific reactions between porcine antibodies in PCMV-positive serum and proteins within the insect cell lysate.

5.2.8 Optimisation of the PCMV ELISA

Checker-board titration experiments were performed to optimise the PCMV ELISA and to try to reduce the non-specific reactions described above. Protein lysates were adsorbed onto plates at concentrations of between 0.1 and 20 μg/ml, and the ELISA

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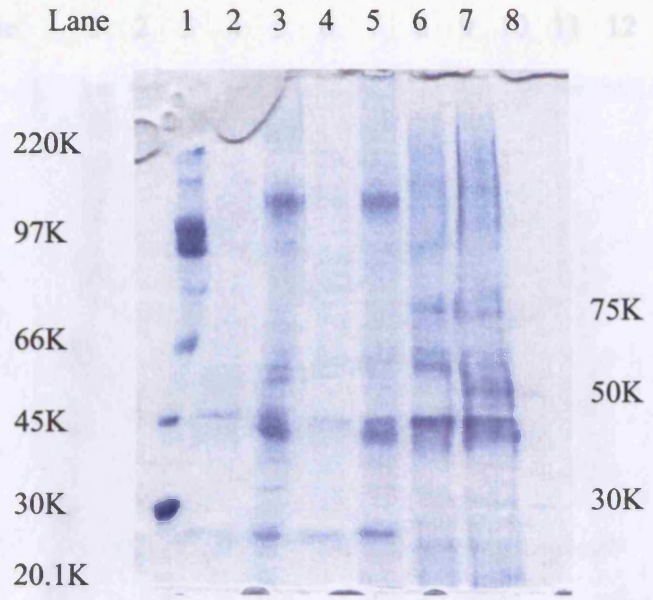

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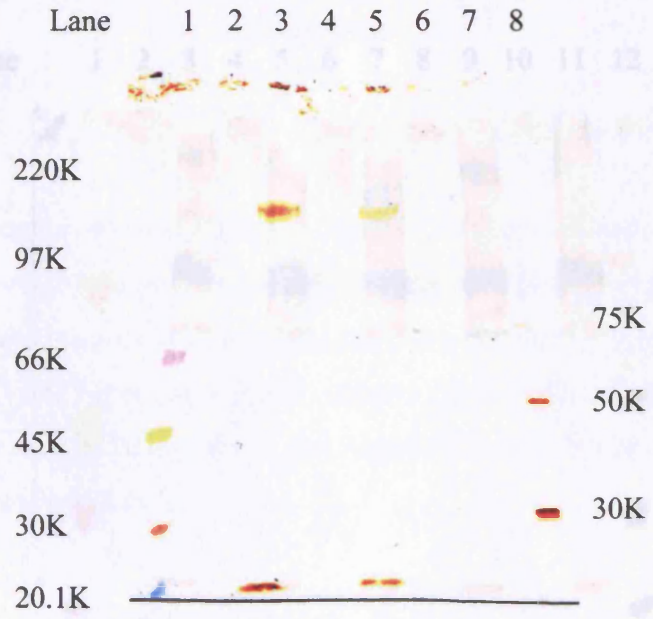
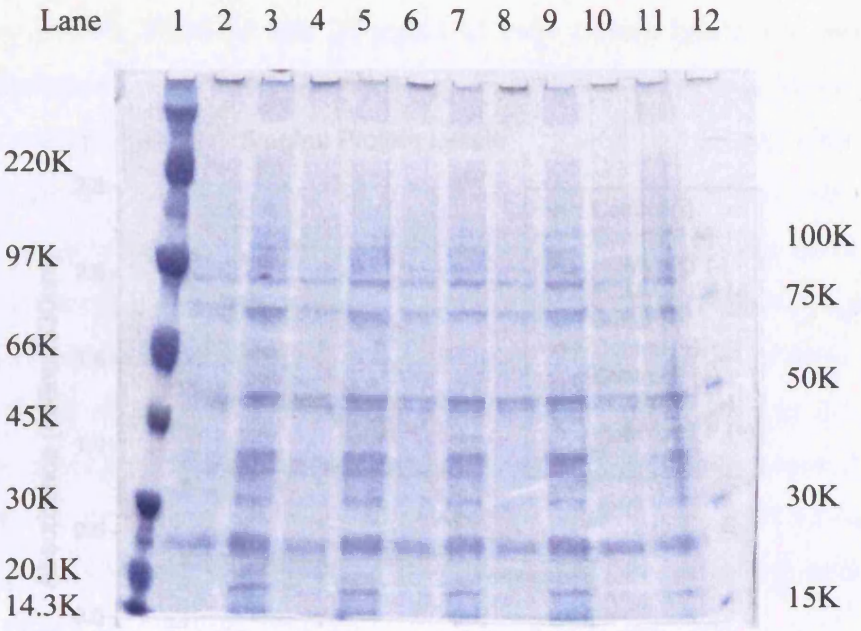


Figure 5.12. Coomassie stain (A) and western blot (B) of PCMV gBT-infected and uninfected High Five insect cells lysed in TNE buffer by four freeze-thaw cycles. PCMV gBT-infected cell lysates with and without protease inhibitor were run in lanes 3 & 5 respectively. Uninfected cell lysate containing protease inhibitor was run in lane 7. Cell lysates, cleared by centrifugation at 15,000 ×g for 30 min, were run in lanes 2, 4, and 6 respectively. Rainbow markers (lane 1) and 6×His protein ladder (lane 8) were also run on the gel.

A



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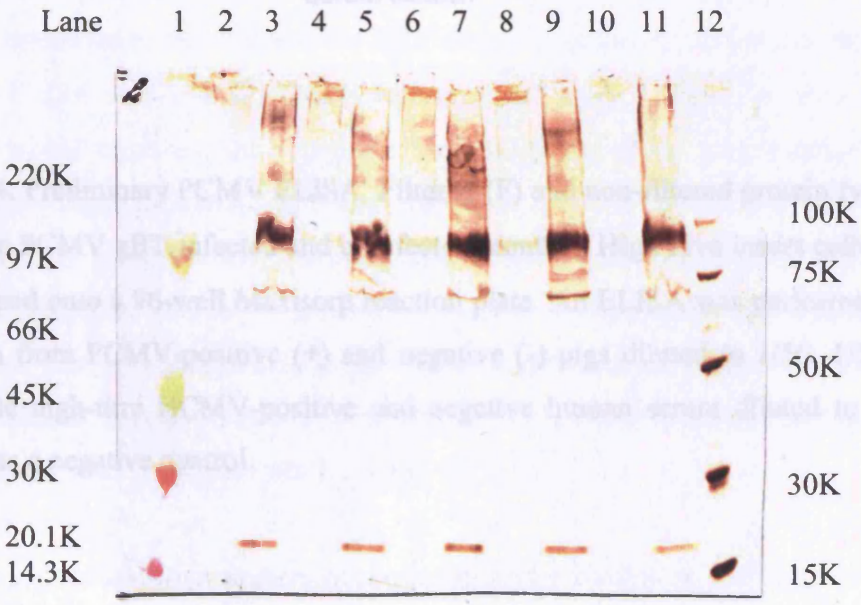


Figure 5.13. Coomassie stain (A) and western blot (B) analysis of PCMV gBT-infected High Five insect cells lysed in TNE buffer by increasing levels of sonication, following SDS-PAGE using non-reducing conditions. Cells were sonicated at a peak-to-peak power of 8 microns for 10 s (lane 3) and 20 s (lane 5), a peak-to-peak power 10 microns for 10 s (lane 7), and 12 microns for 10 s (lane 9), while the cell lysate in lane 11 was not sonicated. Each cell lysate was then cleared by centrifugation at 15,000 ×g for 30 min and run in lanes 2, 4, 6, 8 and 10 respectively. Rainbow markers (lane 1) and 6×His protein ladder (lane 12) were also run on the gel.

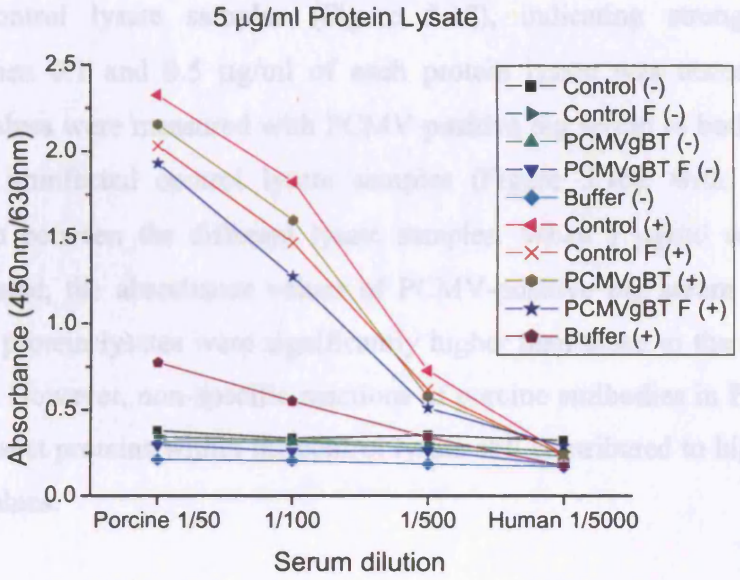


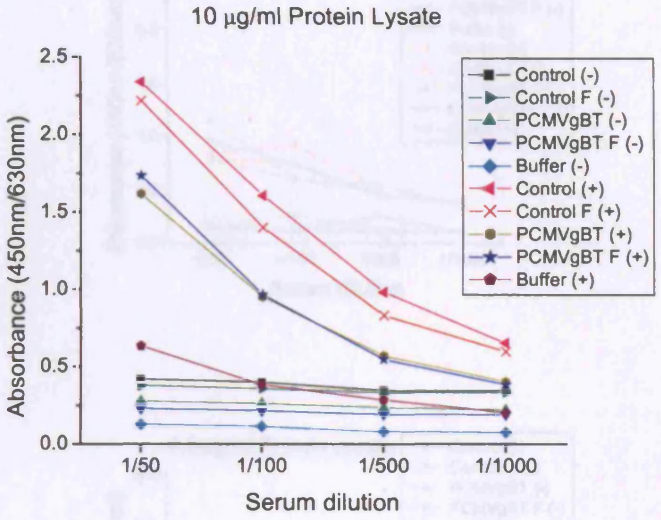
Figure 5.14. Preliminary PCMV ELISA. Filtered (F) and non-filtered protein lysates (5 µg/ml) from PCMV gBT-infected and uninfected (control) High Five insect cells, 2 dpi, were adsorped onto a 96-well Maxisorp reaction plate. An ELISA was performed using pooled sera from PCMV-positive (+) and negative (-) pigs diluted to 1/50, 1/100 and 1/500, while high-titre HCMV-positive and negative human serum diluted to 1/5000 was tested as a negative control.

performed using pooled PCMV-positive and negative porcine serum serially diluted from 1/50 to 1/1000. When 10 and 20 μg/ml of each protein lysate was tested, the greatest absorbance values were measured with PCMV-positive pig serum to the uninfected control lysate samples (Figure 5.15), indicating strong non-specific reactions. When 0.1 and 0.5 μg/ml of each protein lysate was tested, the greatest absorbance values were measured with PCMV-positive pig serum to both PCMV gBT-infected and uninfected control lysate samples (Figure 5.16), with no significant discrimination between the different lysate samples. When 1 μg/ml of each protein lysate was tested, the absorbance values of PCMV-positive pig serum to the PCMV gBT-infected protein lysates were significantly higher than those to the control lysates (Figure 5.16). However, non-specific reactions of porcine antibodies in PCMV-positive serum with insect proteins within the control lysate still contributed to high background absorbance values.

In a further optimisation experiment, the HRP conjugate antibody concentration used in the PCMV ELISA was altered. Plates were prepared using 1 μg/ml of each protein lysate sample, and following the removal of porcine serum, plates were incubated with HRP conjugate antibody diluted to either 1/5000, 1/10000 or 1/15000 in sterile PBS. Varying the HRP conjugate concentration used in the PCMV ELISA, did not reduce non-specific reactions, but simply altered the degree of absorbance measured in each reaction, such that high absorbance values were measured at the highest concentration of HRP conjugate tested, while low absorbance values were measured at the lowest concentration of HRP conjugate tested (Figure 5.17).

Finally, the plate washing procedure between the serum incubation and HRP conjugate antibody incubation steps was altered to reduce the non-specific reactions observed in the PCMV ELISA. Plates were washed 3 or 6 times, using either 0.05 % or 0.1 % Tween in PBS on an automated plate washer. As shown in Figure 5.18, the most significant reduction in non-specific reactions was measured in the plate washed 3 times with 0.05 % Tween in PBS. Therefore, increasing the number of washes, and the concentration of Tween 20 in the wash, did not prevent non-specific reactions as might have been expected.

A



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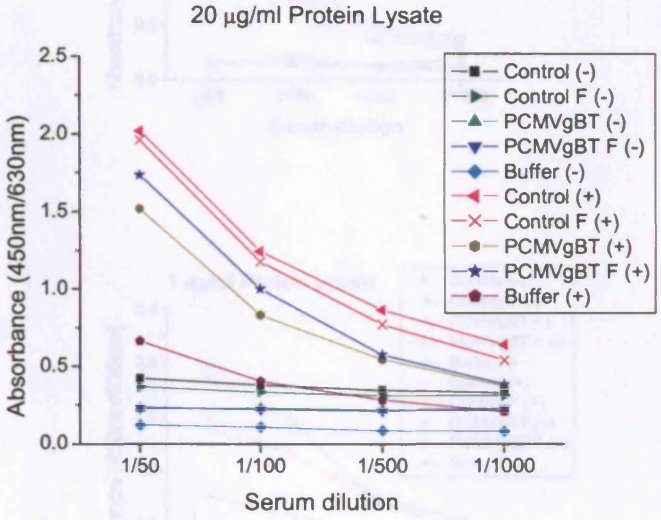
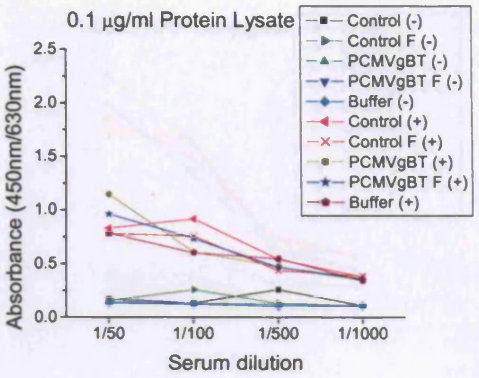


Figure 5.15. The effect of increasing the concentration of protein lysate in the PCMV ELISA. Filtered (F) and non-filtered protein lysates from PCMV gBT-infected and uninfected (control) High Five insect cells, 2 dpi, were diluted to 10 µg/ml (A) and 20 µg/ml (B), and were adsorped onto a 96-well Maxisorp reaction plate. An ELISA was performed using pooled sera from PCMV-positive (+) and negative (-) pigs, serially diluted from 1/50 to 1/1000.

A



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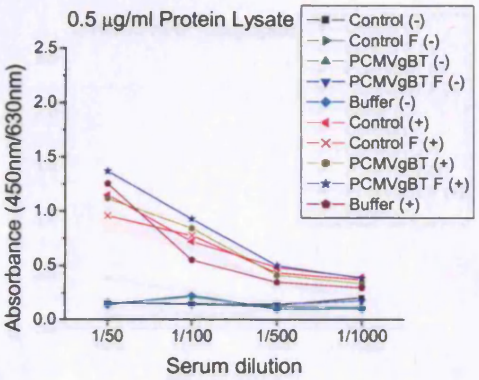


C

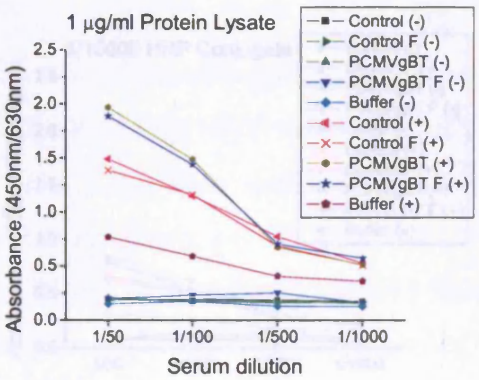
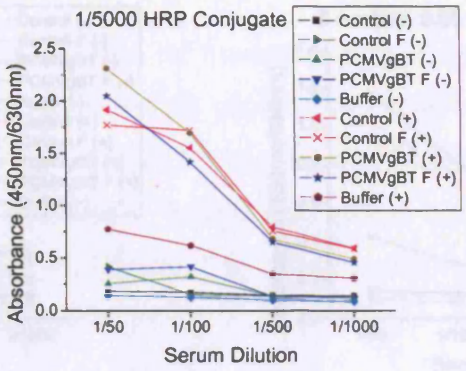


Figure 5.16. The effect of decreasing the concentration of protein lysate in the PCMV ELISA. Filtered (F) and non-filtered protein lysates from PCMV gBT-infected and uninfected (control) High Five insect cells, 2 dpi, were diluted to 0.1 µg/ml (A), 0.5 µg/ml (B) and 1 µg/ml (C), and were adsorped onto a 96-well Maxisorp reaction plate. An ELISA was performed using pooled sera from PCMV-positive (+) and negative (-) pigs, serially diluted from 1/50 to 1/1000.

A



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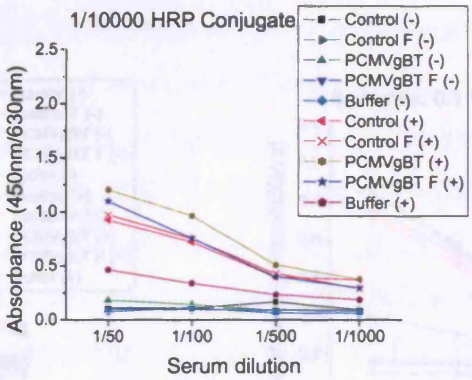


C

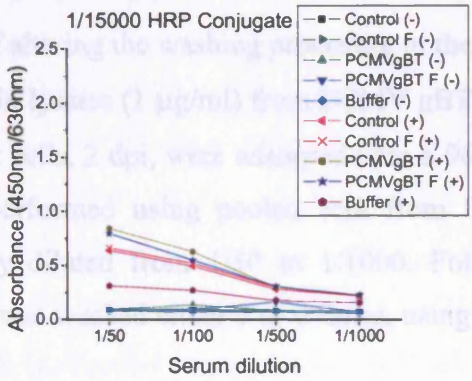


Figure 5.17. The effect of altering the concentration of HRP conjugate in the PCMV ELISA. Filtered (F) and non-filtered protein lysates (1 µg/ml), from PCMV gBT-infected and uninfected (control) High Five insect cells, 2 dpi, were adsorped onto a 96-well Maxisorp reaction plate. An ELISA was performed using pooled sera from PCMV-positive (+) and negative (-) pigs, serially diluted from 1/50 to 1/1000, while HRP congugate was diluted to 1/5000 (A), 1/10000 (B) and 1/15000 (C).

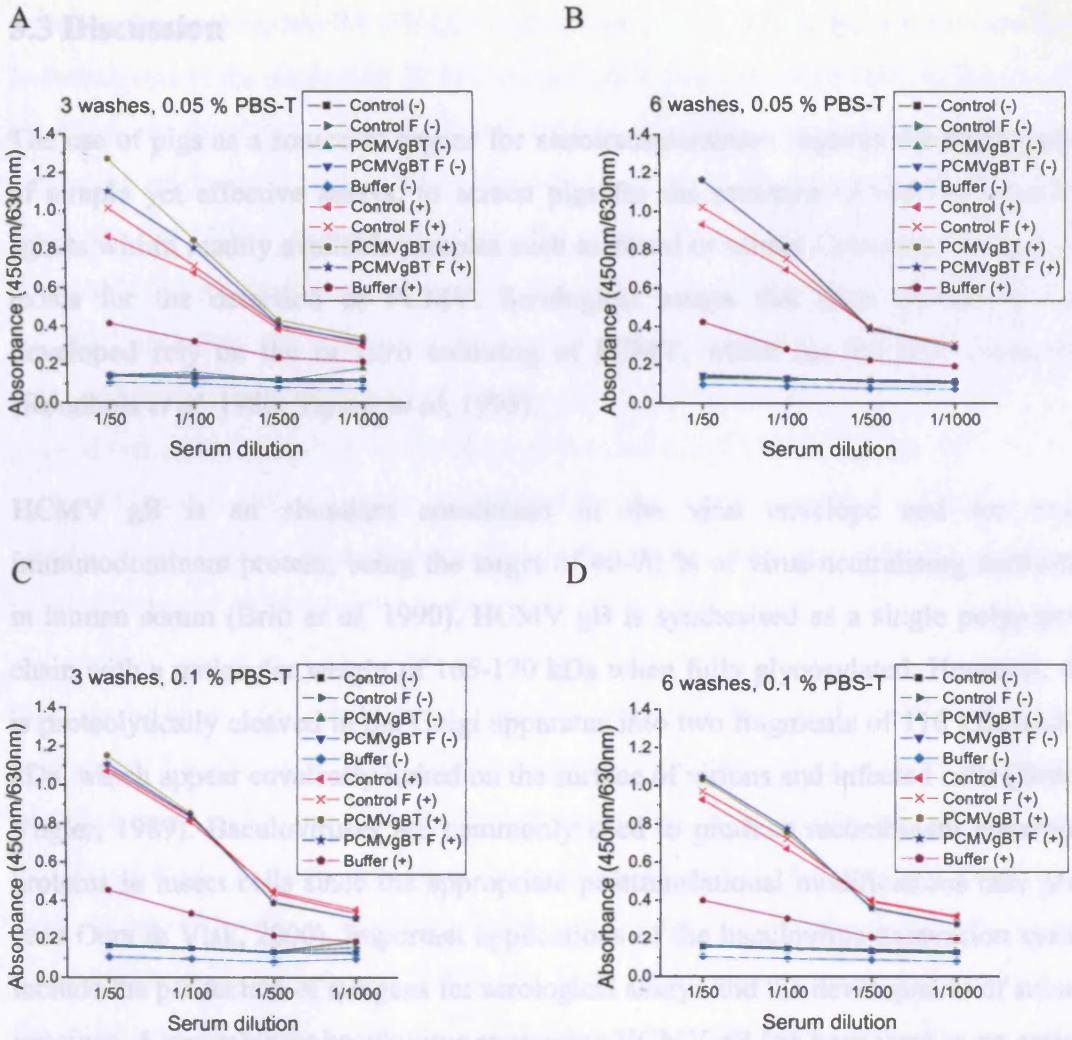


Figure 5.18. The effect of altering the washing procedure in the PCMV ELISA. Filtered (F) and non-filtered protein lysates (1 µg/ml) from PCMV gBT-infected and uninfected (control) High Five insect cells, 2 dpi, were adsorped onto a 96-well Maxisorp reaction plate. An ELISA was performed using pooled sera from PCMV-positive (+) and negative (-) pigs, serially diluted from 1/50 to 1/1000. Following incubation with porcine serum, each well was washed either 3 or 6 times, using 0.05 % or 0.1 % Tween in PBS (PBS-T).

5.3 Discussion

The use of pigs as a source of organs for xenotransplantation requires the development of simple yet effective assays, to screen pigs for the presence of porcine infectious agents within readily available samples such as blood or serum. Currently, no such test exists for the detection of PCMV. Serological assays that have previously been developed rely on the *in vitro* culturing of PCMV, which can be time consuming (Rondhuis *et al*, 1980; Tajima *et al*, 1993).

HCMV gB is an abundant constituent of the viral envelope and the major immunodominant protein, being the target of 40-70 % of virus-neutralising antibodies in human serum (Britt *et al*, 1990). HCMV gB is synthesised as a single polypeptide chain with a molecular weight of 165-170 kDa when fully glycosylated. However, this is proteolytically cleaved in the Golgi apparatus into two fragments of 116 kDa and 55 kDa, which appear covalently linked on the surface of virions and infected cells (Britt & Vugler, 1989). Baculoviruses are commonly used to produce recombinant eukaryotic proteins in insect cells since the appropriate posttranslational modifications take place (van Oers & Vlak, 2000). Important applications of the baculovirus expression system include the production of antigens for serological assays and the development of subunit vaccines. A recombinant baculovirus expressing HCMV gB has been used as an antigen in IIF to detect and measure antibody titres to HCMV gB (Deayton *et al*, 2002), while a baculovirus-expressed recombinant guinea pig CMV gB vaccine has been shown to protect against congenital CMV infection and disease in guinea pigs (Schleiss *et al*, 2004).

This chapter describes the development of a recombinant protein-based serological assay for PCMV, using the viral protein glycoprotein B as an antigenic target for the detection of PCMV-specific antibodies in porcine serum. The complete ORF of PCMV gB and a truncated sequence excluding the predicted transmembrane regions were cloned and expressed in Sf9 insect cells using a baculovirus expression system. Western blot analysis confirmed the expression of the recombinant proteins in Sf9 and High Five insect cells. The western blot of PCMV gB-infected insect cells identified His-tagged recombinant proteins of approximately 100-150 kDa and 45 kDa in size, while the western blot of PCMV gBT-infected insect cells identified His-tagged recombinant

proteins of approximately 70-100 kDa and 20 kDa in size. The larger proteins are likely to correspond to the uncleaved PCMV gB and gBT proteins, while the smaller proteins are likely to correspond to the proteolytically-cleaved carboxyl-terminal His-tagged fragments of these proteins. These visualised proteins correspond well with those predicted for PCMV gB and gBT proteins using the internet-based protein analysis website ExPASy (http://ca.expasy.org). Theoretical molecular weights for PCMV gB and gBT, including the proteolytically-cleaved carboxyl-terminal His-tagged fragments, were calculated by the addition of the average isotopic masses of amino acids in the protein sequence. The predicted masses of the uncleaved PCMV gB and gBT proteins were 101 kDa and 76 kDa respectively, while the predicted masses of the proteolytically-cleaved carboxyl-terminal His-tagged PCMV gB and gBT fragments were 50 kDa and 25 kDa respectively.

The time-course for recombinant protein expression showed maximum protein expression 2-4 dpi, with little protein expressed at 1 dpi. This was expected since polydera-derived virus particles are normally produced in the nucleus of infected cells at ~ 18 hrs postinfection and continue to accumulate as late as 72 hrs postinfection, or until the cells lyse (Murphy *et al*, 1997). There was no significant difference in the yield of recombinant proteins produced in Sf9 and High Five insect cells, despite the fact that the expression of certain secreted proteins has been reported to be up to 28-fold greater in Tn5 (High Five) insect cells compared with Sf9 cells (Wickham & Nemerow, 1993).

The truncation of recombinant baculovirus-expressed HCMV gB at amino acid 692, to remove the transmembrane anchor region, was reported to result in the secretion of the recombinant protein into the culture medium of infected Tn 5 (High Five) insect cells (Carlson *et al*, 1997). A similar truncation of PCMV gB at amino acid 634 did not result in secretion of the recombinant protein into the culture supernatant of infected Sf9 or High Five insect cells. This may have been due to problems in the secretory pathways from the ER to the cell surface. A region of the carboxyl-terminal hydrophobic sequence of HCMV gB (amino acids 714-750) was shown to be involved in transporting HCMV gB through the exocytic pathway, while amino acids 751-771 were found to function as a membrane anchor. Mutated recombinant HCMV gB proteins with deletions in either amino acids 717-747 or a substitution at amino acid 748 were reported to be blocked from cell surface expression, while wild-type protein was not

(Zheng *et al*, 1996). Additionally, these mutated proteins were found to form stable complexes with some molecular chaperones, causing their retention in the ER. However, in the same study a HCMV gB mutant with a deletion in the entire region from amino acids 717 to 772 was secreted into the culture medium.

The IIF assay of recombinant PCMV gB and gBT-infected High Five insect cells demonstrated the immunogenic nature of PCMV gB and again confirmed expression of the recombinant proteins. IIF showed recombinant proteins to be only located in the cytoplasm of infected High Five cells, suggesting that PCMV gB especially, was not being targeted to the appropriate destination within the cell. This may additionally be a result of improper posttranslational modifications or folding which may affect transport within the cell. N-glycosylation is sometimes required for the transport of recombinant glycoproteins through the Sf9 secretory pathway (Jarvis *et al*, 1990). Lepidopteran insect cells have been shown to add mannose, fucose and probably N-acetylglucose groups onto native glycoproteins, but not galactose or sialic acid groups as in mammalian cells (Jarvis & Finn, 1995). Recently, a new transgenic insect cell line, expressing N-acetylglucosaminyltransferase II has been produced, and could be used to produce more authentic recombinant glycoproteins by baculovirus expression vectors (Hollister *et al*, 2002).

The IIF assay of porcine serum samples showed that the expression of recombinant PCMV gB in insect cells could effectively be used to detect antibody to PCMV. The positive IIF result of piglet J176 was misleading about the true PCMV status of that piglet, since all samples (total of 15) obtained from that piglet, including spleen, tested negative for PCMV DNA by PCR. PCMV-specific antibodies are likely to have been passed from the sow to her offspring in milk secretions. Piglets are born with almost no serum antibody and absorb IgG, IgM and IgA from sow colostrum, with subsequent milk from 3 days of age until the end of lactation containing predominantly IgA immunoglobulins (Roth, 1999). All three classes of immunoglobulins are absorbed from the colostrum into the circulation of newborn pigs, however, IgA is absorbed less frequently than the other classes of antibody (Roth, 1999). Since pigs being bred for xenograft organs will not receive colostrum or milk from the sow (Onions *et al*, 2000), false-positive results such as this are unlikely to occur during pre-transplant screening of herds.

An ELISA using purified PCMV gBT could not be established since this truncated protein was not secreted from infected insect cells. Therefore, an attempt was made to set up an ELISA using a crude lysate preparation of PCMV gBT-infected High Five insect cells. Control lysates were prepared from uninfected High Five insect cells. Since lysate samples clarified by centrifugation did not contain PCMV gBT, possibly because it was insoluble and removed in the cell pellet, whole lysates and those partially clarified by filtration were adsorbed onto immuno-plates. Despite altering lysate and HRP conjugate concentrations, and altering the washing step between serum and HRP conjugate incubations, non-specific reactions between PCMV-positive pig sera and insect cell proteins, remained a frequent problem in this PCMV ELISA. These non-specific reactions are an inherent problem with using crude lysates, since they contain a large number of non-specific targets. Another problem is that a significantly greater number of insect cell proteins were likely to be present within the uninfected control lysate, compared with the PCMV gBT-infected lysate. Since the proportion of recombinant protein within the PCMV gBT-infected lysate was unknown, it was not possible to ensure that equal quantities of control insect cell proteins were present in infected and uninfected lysates. For example, if recombinant PCMV gBT made up 50 % of the total protein content of infected cell lysates, and 1 μg/ml of each lysate was added to wells, then twice the amount of insect proteins were present in control wells, than in infected wells, which would undoubtedly have resulted in increased background absorbance in the control well. The only feasible method of preventing non-specific reactions would be to use purified recombinant protein in the assay, however, time constraints did not allow for the purification of recombinant PCMV gBT from infected lysate samples.

A further problem with this PCMV ELISA was the absence of an appropriate negative control porcine serum. The only PCMV-negative porcine serum samples available were from gnotobiotic pigs which have lower serum antibody levels, compared with conventionally-reared pigs (Tucker *et al*, 2002b). The use of such a serum as a negative control in a clinical ELISA may give rise to false-positive results, since, a PCMV-negative serum from a conventionally-reared pig might be expected to produce a higher non-specific absorbance, than the gnotobiotic serum, and could be confused with a positive result for PCMV.

In summary, this chapter describes the production of recombinant PCMV gB using a baculovirus expression system, and the development of a recombinant PCMV gB IIF assay for the detection of PCMV-specific antibodies. It is hoped that this assay will provide a useful tool for the detection of PCMV in pigs being bred for xenograft organs. Recombinant PCMV gB could also potentially be used in the development of a subunit vaccine for PCMV. Clinical trials using a HCMV gB subunit vaccine combined with MF59 adjuvent, developed by CHIRON, are underway. Phase 1 studies showed that the immunisation of seronegative individuals with the HCMV gB/MF59 vaccine resulted in high levels of neutralising antibodies (Pass *et al*, 1999; Frey *et al*, 1999).

Chapter 6: The susceptibility of PCMV to established antiherpetic agents *in vitro*

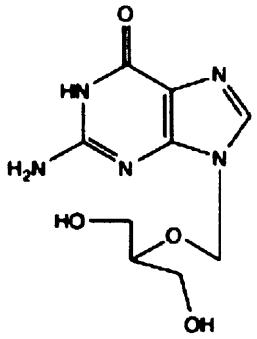
6.1 Introduction

The results obtained in Chapter 4 show that PCMV can be eliminated from pigs by caesarian delivery and barrier-rearing. These methods could therefore be used to generate PCMV-free pig herds for use in xenotransplantation. The SPF status of these herds would need to be quality controlled by testing spleen samples from sentinel animals, and from individual donor animals at the time of harvesting of the xenograft. The transmission of PCMV to human xenograft recipients should thus be preventable, however, knowledge of the antiviral susceptibility of PCMV is also important as it would provide therapeutic options should this screening procedure fail.

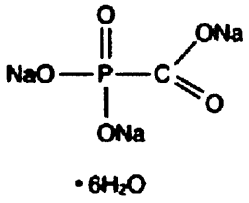
This chapter describes the investigations into the *in vitro* effect on PCMV replication of antiviral drugs (Fryer *et al*, 2004), including ganciclovir (GCV), foscarnet (PFA), cidofovir (HPMPC) and aciclovir (ACV) (Figure 6.1), currently licensed for use in the treatment and/or prophylaxis of human herpesvirus infections. GCV is the first choice therapy for life-threatening or sight-threatening HCMV infections in the immunocompromised including AIDS and transplant patients, while foscarnet and cidofovir are used to treat CMV retinitis in AIDS patients (BNF, 2004). ACV is predominantly used for the treatment of HSV and VZV infections, but has also demonstrated activity against HCMV (Meyers *et al*, 1988; Prentice *et al*, 1994). These antiviral agents are all nucleoside or nucleotide analogue inhibitors of the viral DNA polymerase, apart from foscarnet, which is a pyrophosphate analogue.

Antiviral susceptibility is conventionally determined by plaque reduction assay relying on the development of CPE. Currently, there is very little information on the ability to culture PCMV *in vitro*. The virus is slow growing in pig fallopian tube (PFT) cells, an adherent fibroblast-like cell line, replicating to produce CPE approximately 11 days post infection (Kawamura *et al*, 1992). It has otherwise only been shown to grow in porcine alveolar macrophages, which are difficult to obtain and potentially compromised by other infectious agents.

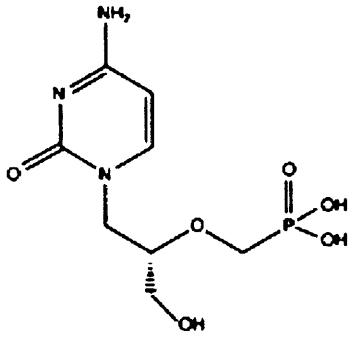
Ganciclovir (GCV)



Foscarnet (PFA)



Cidofovir (HPMPC)



Aciclovir (ACV)

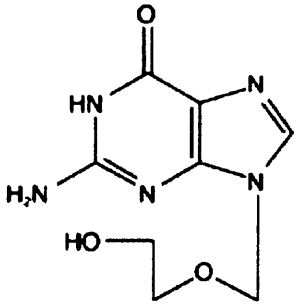


Figure 6.1. Chemical structures of some of the antiviral agents currently licensed for use in the treatment and/or prophylaxis of human herpesvirus infections.

The first aim of this chapter was to establish PCMV infection in PFT cells, although a porcine peripheral blood T cell line, L45, was also investigated for it's susceptibility to PCMV infection. PCMV (laboratory adapted strain) and the PFT cell line were kindly provided by Dr Clive Patience (Immerge BioTherapeutics Inc.). The effect of TPA on the growth of PCMV in PFT cells was also investigated since PCMV has previously been shown to replicate more rapidly in medium containing 5-60 ng/ml of TPA, with CPE observed 2 dpi (Kawamura & Matsuzaki, 1996). TPA is a mitogen which induces cell replication, particularly nuclear division. It has also been shown to induce EBV reactivation via nuclear factor (NF) -κB and activator protein (AP) -1 *in vitro* (Gao *et al*, 2001).

As an alternative to the use of plaque reduction assays, the antiviral inhibition of PCMV DNA production by PFT cells was determined by quantification of viral DNA in the cell culture supernatant using real-time PCR. Real-time PCR provides an alternative and favourable method for quantifying DNA compared to QC PCR, by the detection and quantitation of a fluorescent reporter. A dual-labelled probe, containing a 5' fluorescent dye and a 3' quenching dye, is designed to anneal to an internal region of the PCR product. The close proximity of the quencher to the fluorescent dye prevents any emission of fluorescence. During the PCR, the DNA polymerase cleaves the fluorescent dye from the probe, resulting in an increased emission of fluorescence with the formation of PCR product. This fluorescence is then quantified and used to determine the quantity of DNA target present in the original sample. For the PCMV real-time PCR, primers and probe were designed using Primer Express software from a region of the DNA polymerase gene of PCMV (Widen *et al*, 1999). The primers and probe were shown by BLAST analysis to share 100 % nucleotide identity with sequence data for the DNA polymerase of all three PCMV strains (55b, B6, and OF-1), deposited in GenBank, suggesting their ability to amplify different isolates. A similar real-time PCR approach was recently used to measure the antiviral susceptibility of HSV (Stranska *et al*, 2002). In addition, PCMV IIF was used to determine the effect of each antiviral on PCMV-infected cells.

6.2 Results

6.2.1 Establishing PCMV infection in PFT cells

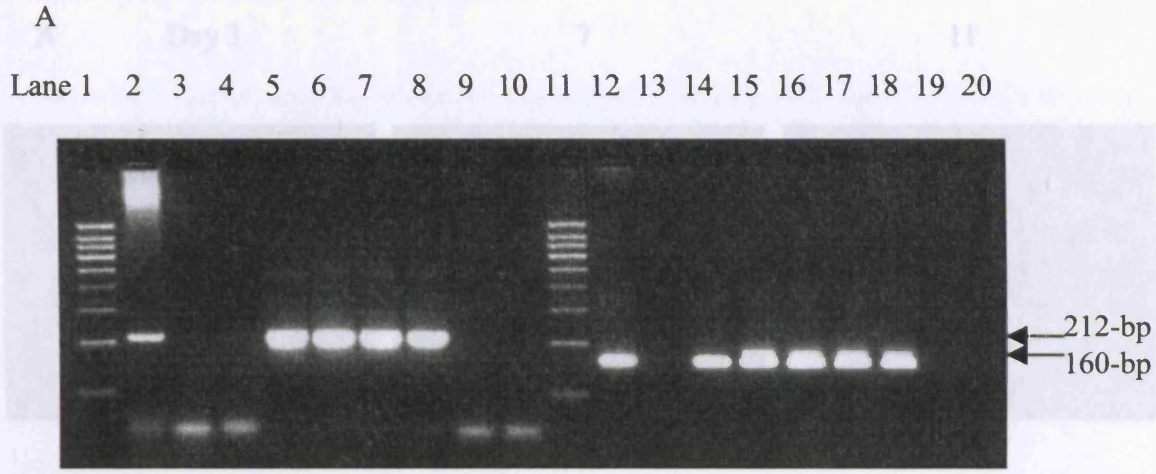
PCMV infection of PFT cells was initially established by mixing infected and uninfected cells and culturing in complete MEM. Propagation and replication of PCMV in PFT cells was confirmed by RT PCR of total RNA extracted from PFT-PCMV cells following 8 days of culture, and by nested qualitative PCMV PCR of TCS harvested from PFT-PCMV cultures at 3, 4, 7, and 8 days following initial mixing of cells. The results in Figure 6.2, panel A, show that PCMV mRNA was detected in PFT-PCMV cells following 8 days of culture, while PCMV DNA was detected in TCS harvested from PFT-PCMV cells up to 8 days following culture, despite several complete changes of medium. Propagation and replication of PCMV was also confirmed by IIF assay of slides prepared from PFT-PCMV cells following 10 days of culture (Figure 6.2, panel B). The photographs of a section of a well on the IIF slide show a number of fluorescing, PCMV-infected cells, present in the culture.

6.2.2 The effect of TPA on the growth of PCMV in PFT cells

The effect of TPA on the growth of PCMV in PFT cells was investigated by IIF of PFT-PCMV cells following 3, 7 and 11 days of culture, with or without incubation with 5 ng/ml of TPA. The IIF assay results (Figure 6.3) show that the number of fluorescing, PCMV-infected PFT cells increased following culture with 5 ng/ml of TPA (panel B), compared to the culture containing no TPA (panel A), such that by day 11 there was a dramatic increase in the number of fluorescing PFT-PCMV cells seen by IIF.

In addition, the effect of various concentrations of TPA on uninfected PFT cells was assessed by observing cells under a light microscope following 3 days of incubation with 0, 1, 2, and 5 ng/ml of TPA. It was noted that at the highest concentration of TPA tested, cells were less confluent and there were significantly more floating cells compared to those incubated with no TPA. At the lower concentrations of TPA tested fewer floating cells were seen and cells looked more confluent, however, the morphology of the cells was altered from those incubated with no TPA (data not shown).

A



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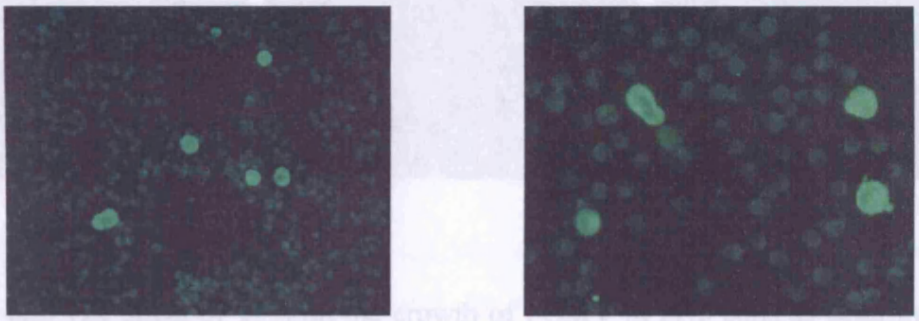


Figure 6.2. Confirmation of PCMV infection in PFT cells. Panel A shows first round (lanes 2-10), and nested round (lanes 12-20), qualitative PCMV PCR products of the RT reaction of RNA harvested 8 dpi, and TCS, harvested from PCMV-infected PFT cells. Positive PCMV PCR controls comprised 10^3 copies of PCMVpol control plasmid (lanes 4 and 14), while negative controls comprised SDW (lanes 9-10 and 19-20). PCR products were electrophoresed on a 3 % agarose gel, alongside HyperLadder IV DNA markers. PCMVpol sequences were amplified from the RT reaction (lanes 2 and 12), but not from the RNA control (lanes 3 and 13) showing the absence of contaminating DNA in the purified RNA. Meanwhile, PCMVpol sequences were amplified from TCS samples harvested 3, 4, 7 and 8 dpi (lanes 5-8 and 15-18), following several changes of medium. Panel B shows fluorescing PFT-PCMV cells harvested from the culture 10 dpi.

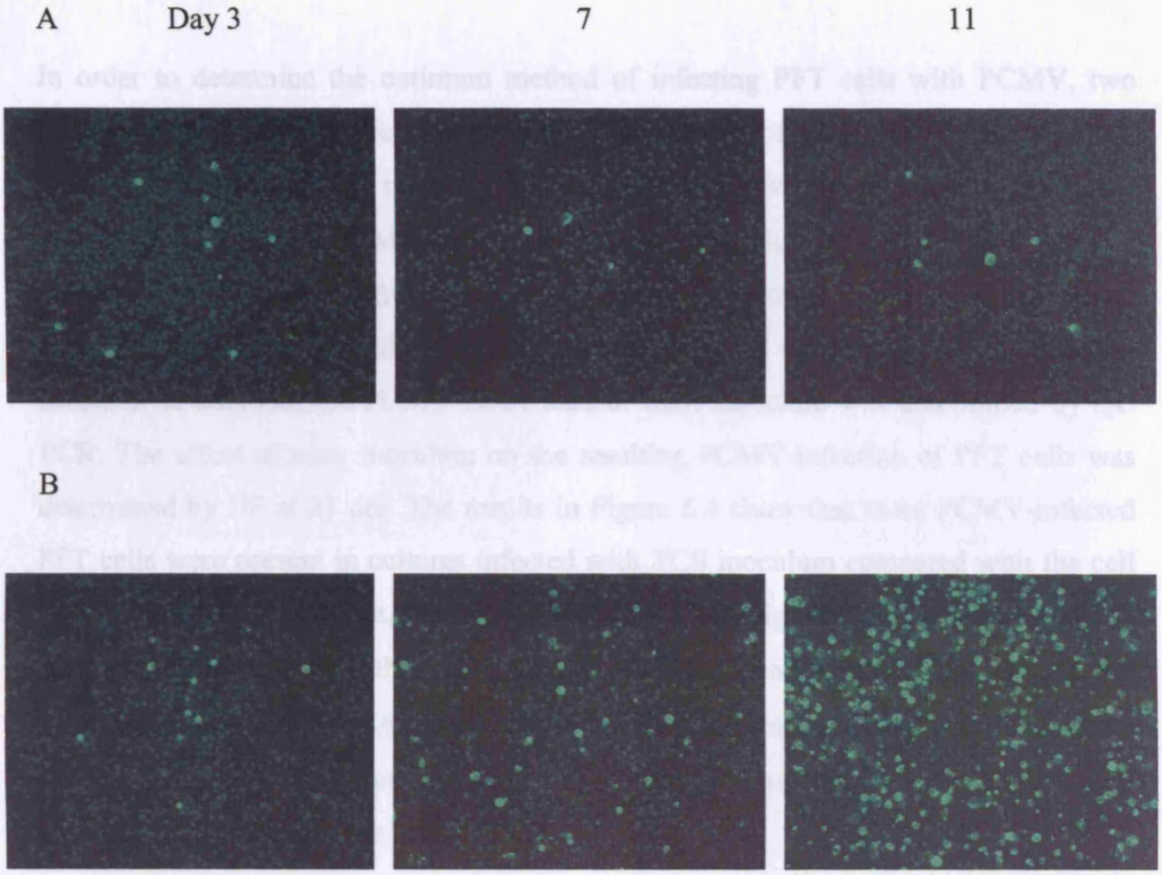


Figure 6.3. The effect of TPA on the growth of PCMV in PFT cells as determined by IIF. Panel A shows little or no increase in the number of fluorescing PFT-PCMV cells following 3, 7 and 11 days of incubation without TPA, whereas panel B shows a dramatic increase in the number of fluorescing PFT-PCMV cells following 3, 7 and 11 days of incubation with 5 ng/ml of TPA.

6.2.3 Preparation of PCMV inoculum

In order to determine the optimum method of infecting PFT cells with PCMV, two PCMV inoculums were prepared from a single culture of PFT-PCMV cells. A TCS inoculum was prepared by centrifugation of TCS to remove floating cells, and filtered through a 0.45 μm filter. Meanwhile, a cell lysate inoculum was prepared by freeze-thawing of cells harvested from the culture flask. An aliquot of each inoculum was then used to infect PFT cells with or without the presence of 5 ng/ml of TPA in the culture medium. In addition, the PCMV DNA load of each inoculum was determined by QC PCR. The effect of each inoculum on the resulting PCMV-infection of PFT cells was determined by IIF at 21 dpi. The results in Figure 6.4 show that more PCMV-infected PFT cells were present in cultures infected with TCS inoculum compared with the cell lysate inoculum. In contrast, the PCMV DNA load was significantly higher in the cell lysate inoculum (PCMV DNA viral load of 3.4×10^7 genome copies/μl), than in the TCS inoculum (PCMV DNA viral load of 1.32×10^6 genome copies/μl), suggesting that although more viral DNA was detected, less infectious virus was present in PFT-PCMV cells than was present in the TCS.

The effect of using freshly harvested TCS compared with TCS inoculum that had been stored at -80 °C, to infect PFT cells was also investigated by IIF at 17 and 27 dpi. Figure 6.5 shows that at both 17 and 27 dpi there were more PCMV-infected PFT cells present in cultures infected with fresh compared to frozen TCS inoculum. These results were found despite the fact that the PCMV DNA load was higher in the TCS inoculum that had been stored at -80 °C (PCMV DNA viral load of 9.6×10^5 genome copies/μl), compared with the PCMV DNA load in the TCS that was freshly harvested (PCMV DNA viral load of 1.24×10^5 genome copies/μl). These results suggest that the PCMV infectious titre was reduced by storing TCS inoculum at -80 °C.

It was next investigated whether 70 % sorbitol could be used to preserve virus titre when storing PCMV inoculum at -80 °C. TCS inoculum was prepared from PFT-PCMV cells, and stored at -80 °C in either an equal volume of 70 % sorbitol (inoculum 1), an equal volume of complete MEM (inoculum 2), or neat (inoculum 3). Each inoculum was subsequently used to infect PFT cells and IIF was performed at 14 dpi. The effect of each inoculum on subsequent PCMV infection in PFT cells was determined by

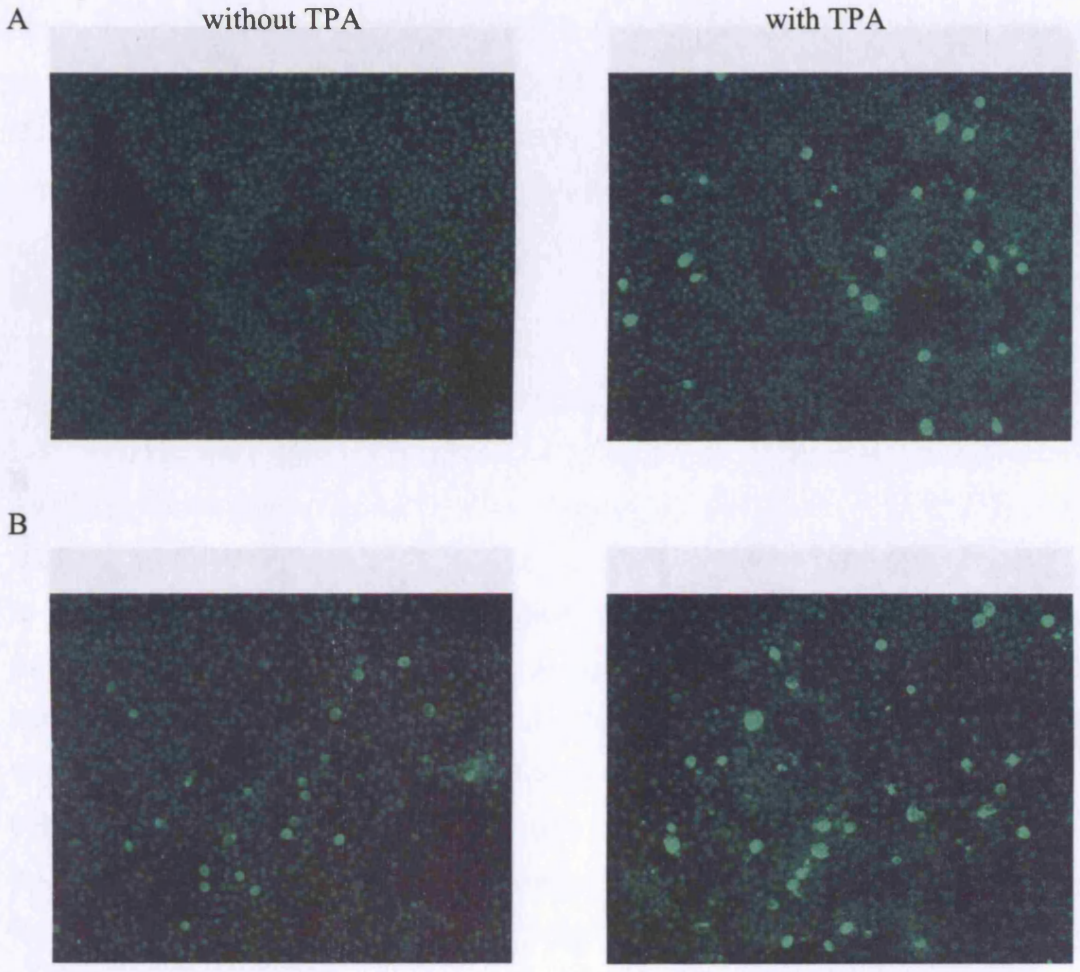


Figure 6.4. IIF of PFT-PCMV cells 21 dpi with 1 ml of either cell lysate inoculum (PCMV DNA viral load of 3.4×10^7 genome copies/µl) (A), or TCS inoculum (PCMV DNA viral load of 1.32×10^6 genome copies/µl) (B), with or without incubation with 5 ng/ml of TPA.

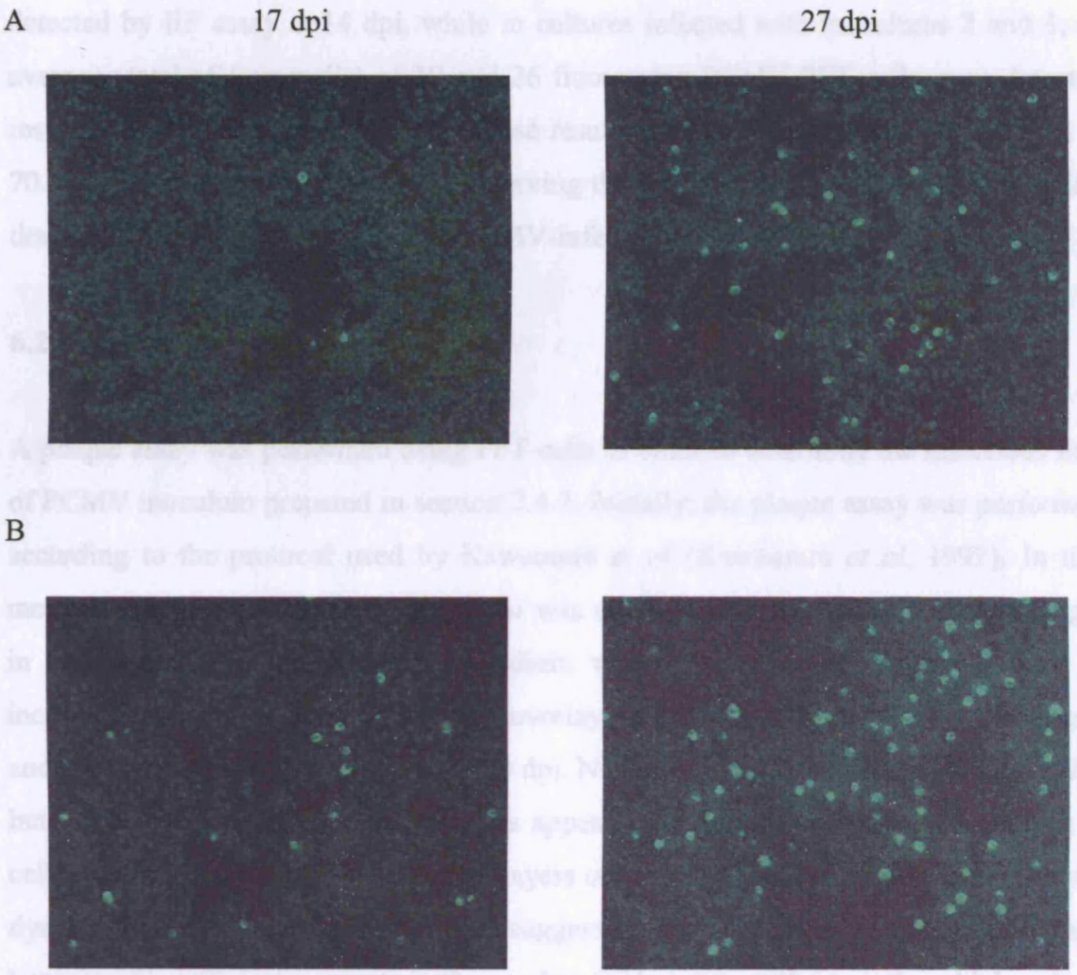


Figure 6.5. Comparison of using fresh vs. frozen PCMV TCS to infect PFT cells as determined by IIF. Panel A shows PFT-PCMV cells infected with PCMV TCS inoculum that had been stored at -80 °C (PCMV DNA viral load of 9.6 $\times 10^5$ genome copies/µl) 17 and 27 dpi. Panel B shows PFT-PCMV cells infected with freshly harvested PCMV TCS inoculum (PCMV DNA viral load of 1.24 $\times 10^5$ genome copies/µl) 17 and 27 dpi.

counting the total number of fluorescing, PCMV-infected PFT cells in each well of the IIF slide. In cultures infected with inoculum 1 no fluorescing PCMV-PFT cells were detected by IIF assay at 14 dpi, while in cultures infected with inoculums 2 and 3, an average (total of four wells) of 19 and 26 fluorescing PCMV-PFT cells were detected respectively by IIF assay at 14 dpi. These results indicate that freezing PCMV TCS in 70 % sorbitol was not effective in preserving the titre of infectious PCMV, and in fact dramatically reduced the number of PCMV-infected cells seen by IIF.

6.2.4 The PCMV plaque assay

A plaque assay was performed using PFT cells in order to determine the infectious titre of PCMV inoculum prepared in section 2.4.7. Initially, the plaque assay was performed according to the protocol used by Kawamura *et al* (Kawamura *et al*, 1992). In this method, the infected PFT cell monolayer was overlaid with 0.8 % bacteriological agar in complete MEM. Fresh overlay medium was added following every 5 days of incubation, with a 0.005 % neutral red overlay added after 10 and 15 days of culture, and plates examined for plaques up to 20 dpi. Neutral red dye is taken up by living cells, but not by dead cells, such that plaques appear as clear areas on a red monolayer of cells. However in this study, PFT monolayers only partially stained red with neutral red dye even in uninfected control wells, suggesting that the cells were dying, perhaps because of insufficient nutrients in the overlay. In the areas of living cells, which stained red with neutral red dye, no plaques were identified.

An alternative method was then used in order to determine the infectious titre of the PCMV inoculum. In this method, the infected PFT cell monolayer was overlaid with methyl cellulose containing concentrated nutrients. This method has previously been used in our department to determine the titre of HCMV virus stocks using human embryonic lung fibroblast cells. Thirteen days postinfection PFT cells were stained with methylene blue which stains dead cells but not living cells. Although monolayers remained healthy in all wells (i.e. did not stain blue) no plaques were visible.

6.2.5 Experimental PCMV infection of L45 cells

The continuous cell line, L45, a porcine T-cell lineage, grown in suspension culture, was experimentally infected with PCMV TCS inoculum in order to determine whether they could be better used, rather than PFT cells, to determine the susceptibility of PCMV to antiviral agents. IIF of L45 cells 10, 20 and 30 dpi with PCMV TCS showed that only a small proportion of cells (approximately 1-10 cells/well, <1 % of total cells) were infected with PCMV (Figure 6.6). These results suggest that although L45 cells may be permissive for PCMV infection, it is likely that the nature of PCMV replication in these cells and the fact that these cells were grown in suspension meant that few infected cells were detected by IIF. L45 cells are grown in suspension culture and need to be passaged at a ratio of 1:5 every 5 days. Since PCMV may have been slow growing in these cells, regular passaging like this would mean that PCMV-infected cells would be diluted out, hindering virus spread.

6.2.6 Establishing chronic PCMV infection in PFT cells

Since PCMV was found to be slow growing in PFT cells when passaged approximately every 5-6 days, the potential for establishing chronic infection by not passaging cells was investigated. PCMV CPE in PFT cells was first detected 7 dpi with PCMV TCS, while the development of CPE in unpassaged cells was observed under a light microscope up to 20 dpi (Figure 6.8), and suggests that virus is spread between adjacent cells. The proportion of PCMV-infected cells determined by IIF was comparable to the proportion of cells exhibiting CPE (data not shown).

Electron microscopy was used to directly visualise PCMV in TCS harvested from chronically-infected PFT cells. PCMV virus particles were pelleted by ultracentrifugation and resuspended in EM stain. Figure 6.7, panel A, shows the typical CMV 'fried-egg' appearance of a negatively stained PCMV dense body, with visible clusters of viral glycoproteins protruding from the viral envelope, while Figure 6.7, panel B, shows the viral capsids.

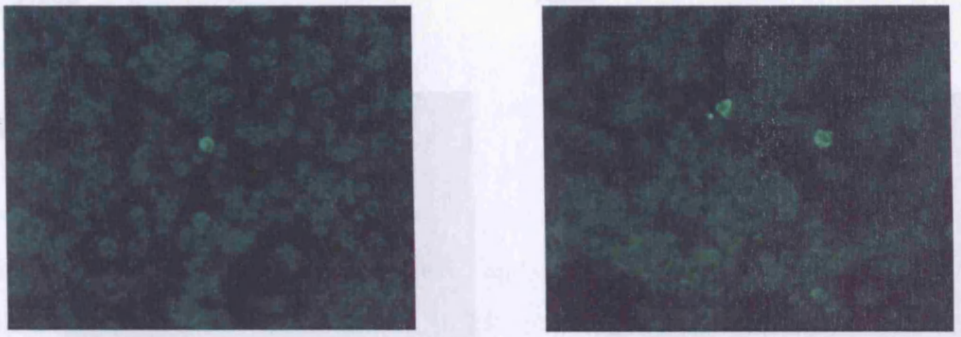
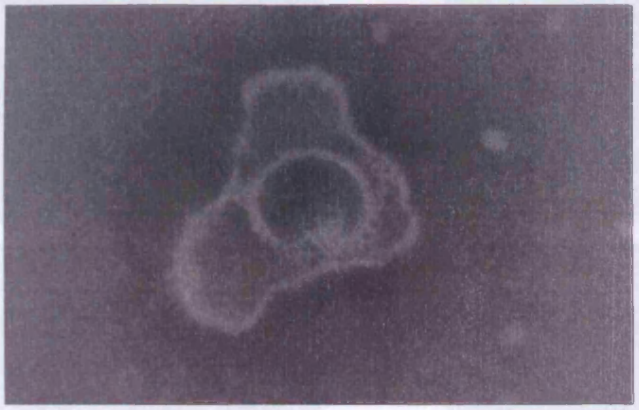


Figure 6.6. IIF of L45 cells 20 dpi with PCMV TCS.

A



B

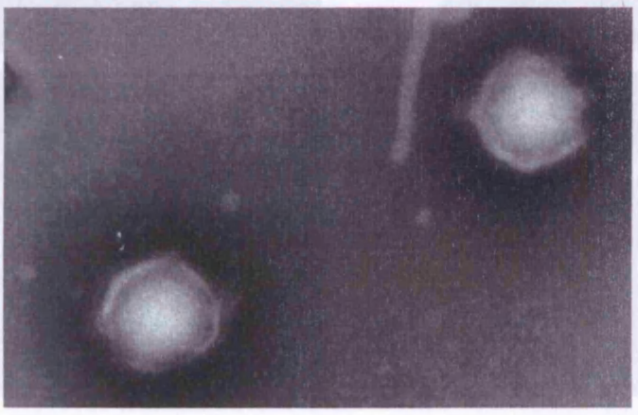


Figure 6.7. Electron microscopy of PCMV. Panel A shows the typical CMV 'fried-egg' appearance of a negatively stained dense body, with visible clusters of viral glycoproteins protruding from the viral envelope. Panel B shows PCMV viral capsids.

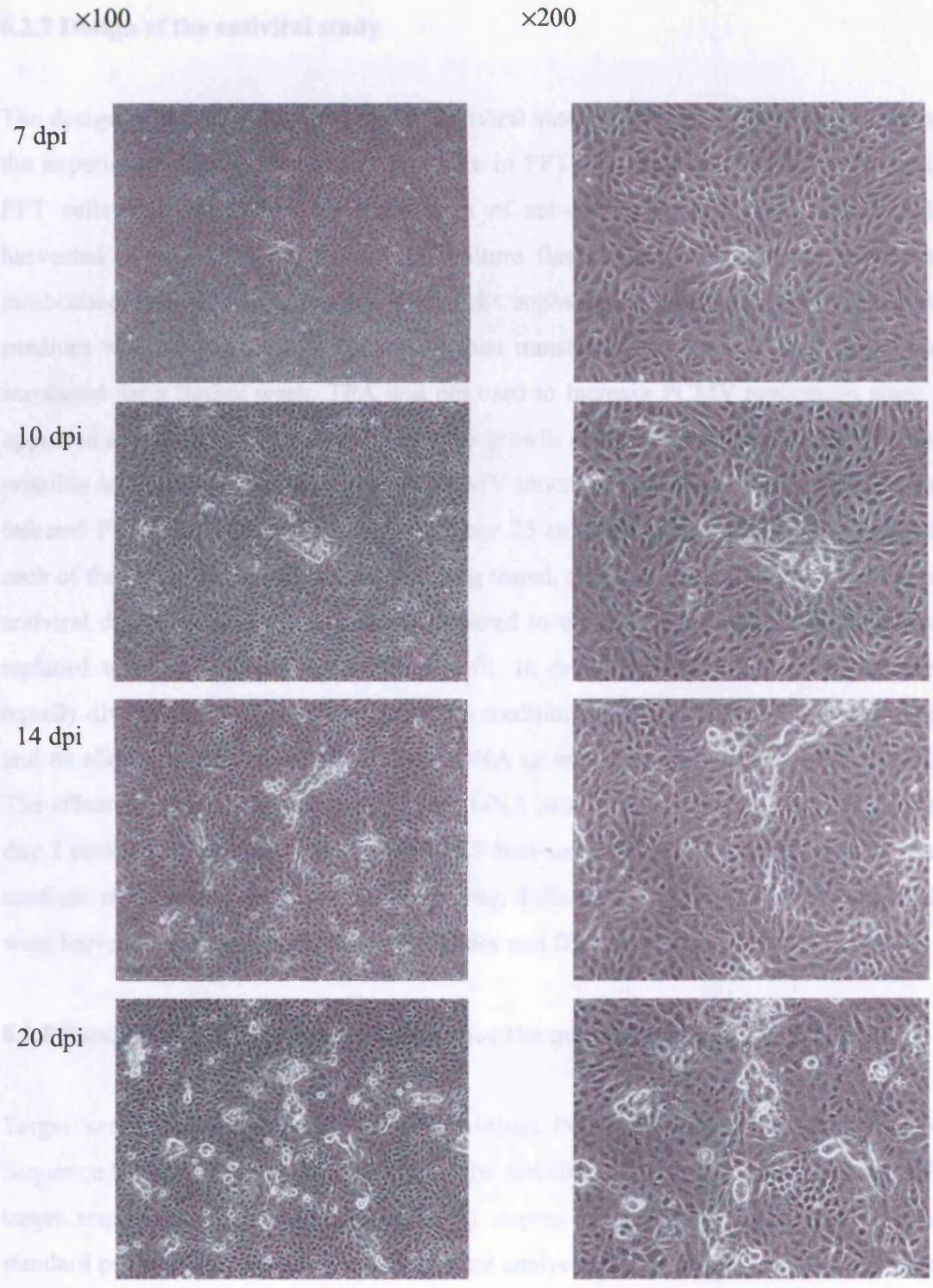


Figure 6.8. The development of CPE in PFT cells over 20 days following infection with PCMV TCS. Chronic infection was established in PFT cells by culturing PCMV for 20 days without passaging of cells. CPE was observed by phase-contrast microscopy.

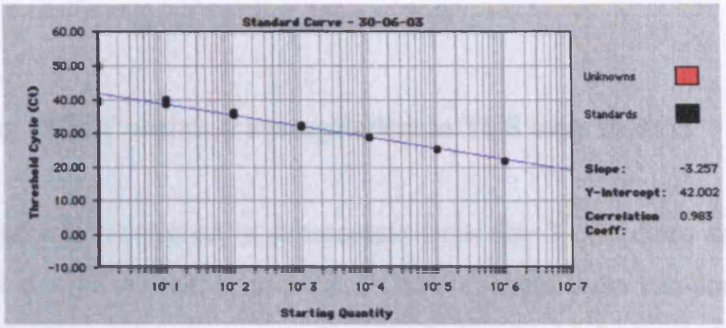
6.2.7 Design of the antiviral study

The design of the investigation into the antiviral susceptibility of PCMV was based on the experiences learned from PCMV culture in PFT cells thus far. PCMV infection in PFT cells was established by inoculation of sub-confluent PFT cells with freshly harvested PCMV TCS in 25 cm^2 cell culture flasks. Chronic infections were then established by incubating cells for two weeks unpassaged, whilst changing the culture medium weekly. PFT-PCMV cells were then transferred to larger 75 cm^2 flasks and incubated for a further week. TPA was not used to increase PCMV replication since it appeared to have a detrimental effect on the growth of PFT cells. Since it had not been possible to determine the virus titre of PCMV inoculum by plaque assay, chronically-infected PFT cells were divided between four 25 cm^2 cell culture flasks; one flask for each of three concentrations of antiviral drug tested, and one control flask containing no antiviral drug (day -2). Once cells had adhered to the flasks, the culture medium was replaced to remove unattached cells (day 0). In order to ensure that cells had been equally divided between the four flasks, the medium was again replaced two days later and an aliquot of TCS stored for PCMV DNA quantitation by real-time PCR (day 2). The effect of each antiviral drug on PCMV DNA load in TCS was then monitored from day 2 until day 14 by real-time PCR of TCS harvested from each culture prior to total medium replacement including antiviral drug. Following this two week period, cells were harvested for the preparation of IIF slides and DNA extraction.

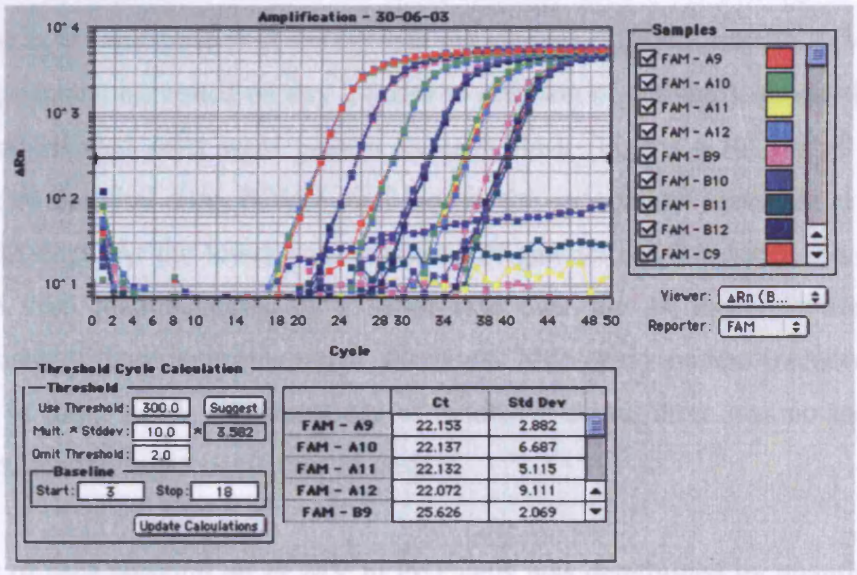
6.2.8 Establishing a real-time PCR assay for the quantification of PCMV DNA

Target sequences were quantified by real-time PCR using an ABI PRISM 7700 Sequence Detection System. The ability of the real-time PCMV PCR primers to amplify target sequences was tested using 10^6-0.1 copies of wild-type PCMVpol plasmid standard per reaction. Reaction products were analysed using Taqman software (Figure 6.9, panels A and B), and in addition, were run on 3 % agarose to show that real-time PCMV PCR primers were consistently able to amplify 10 copies of target sequence (Figure 6.9, panel C). In addition, Figure 6.9, panel C, shows that 0.1 copies of wild-type PCMVpol plasmid were not amplified, indicating correct dilution of the standards. Figure 6.9, panels A and B, show the standard curve and amplification plot of the PCMV standards as analysed using Taqman software. Subsequently, samples were

A



B



C

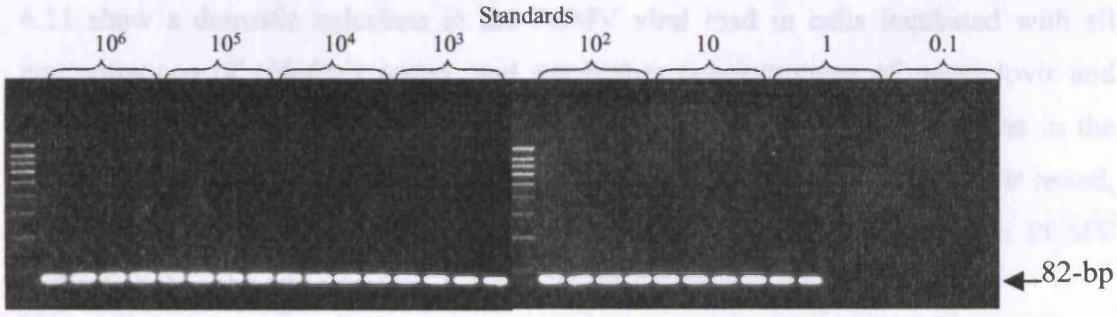


Figure 6.9. Standard curve (A) and amplification plot (B) of 10^6-0.1 copies of wild-type PCMVpol standard amplified by real-time PCMV PCR. PCR products were electrophoresed on a 3 % agarose gel, alongside HyperLadder IV DNA markers, to confirm the sensitivity of the real-time PCMV PCR (C).

amplified alongside 10^9-10^3 copies of standard, since PCMV DNA loads fell within this range.

6.2.9 Monitoring PCMV antiviral susceptibility in TCS over 14 days

PCMV DNA load was monitored in supernatant from day 2 of culture and then every second day until day 14 by real-time PCR. PCMV cultures were sub-confluent at the start of the experiment, replicating to confluency during the 14 day period. Antivirals at the highest concentrations used in this study did not inhibit the activity of the *Taq* polymerase in the real-time PCR assay (data not shown). The real-time PCMV PCR of culture supernatant harvested on day 2 (prior to addition of antiviral), shows that equal numbers of infected cells were present in each flask (Figure 6.10). In all cultures containing no antiviral drug, PCMV viral load in the supernatant increased at a steady rate over 14 days. At the lower concentrations of ganciclovir, foscarnet and aciclovir tested, the viral load increased at a lesser rate over the 14 days. At the highest concentrations of these antivirals tested, there was little or no evident increase in viral load over 14 days. At all concentrations of cidofovir tested, there was no increase in PCMV viral load in supernatant over 14 days.

The effect of each antiviral on PCMV in PFT cells was determined by quantifying the viral load in DNA extracted from cells after the 14 day period. The results in Figure 6.11 show a dramatic reduction in the PCMV viral load in cells incubated with all concentrations of cidofovir tested, and the higher concentrations of ganciclovir and foscarnet tested, compared with no drug controls. There was a slight reduction in the PCMV viral load in cells incubated with the higher concentrations of aciclovir tested, however, overall aciclovir and foscarnet had a much less dramatic effect on PCMV replication than the other drugs.

Figure 6.12 shows the percentage reduction in PCMV viral load in PFT-PCMV cells following incubation with antiviral agents. Antiviral susceptibility is often represented as the 50 % effective concentration (EC_{50}), or the concentration of antiviral which inhibits virus replication by 50 %. The antiviral susceptibilities of PCMV in PFT cells were calculated from the real-time PCR results of DNA extracted from PFT-PCMV cells following the 14 day period and are shown in Table 6.1. Ganciclovir and cidofovir

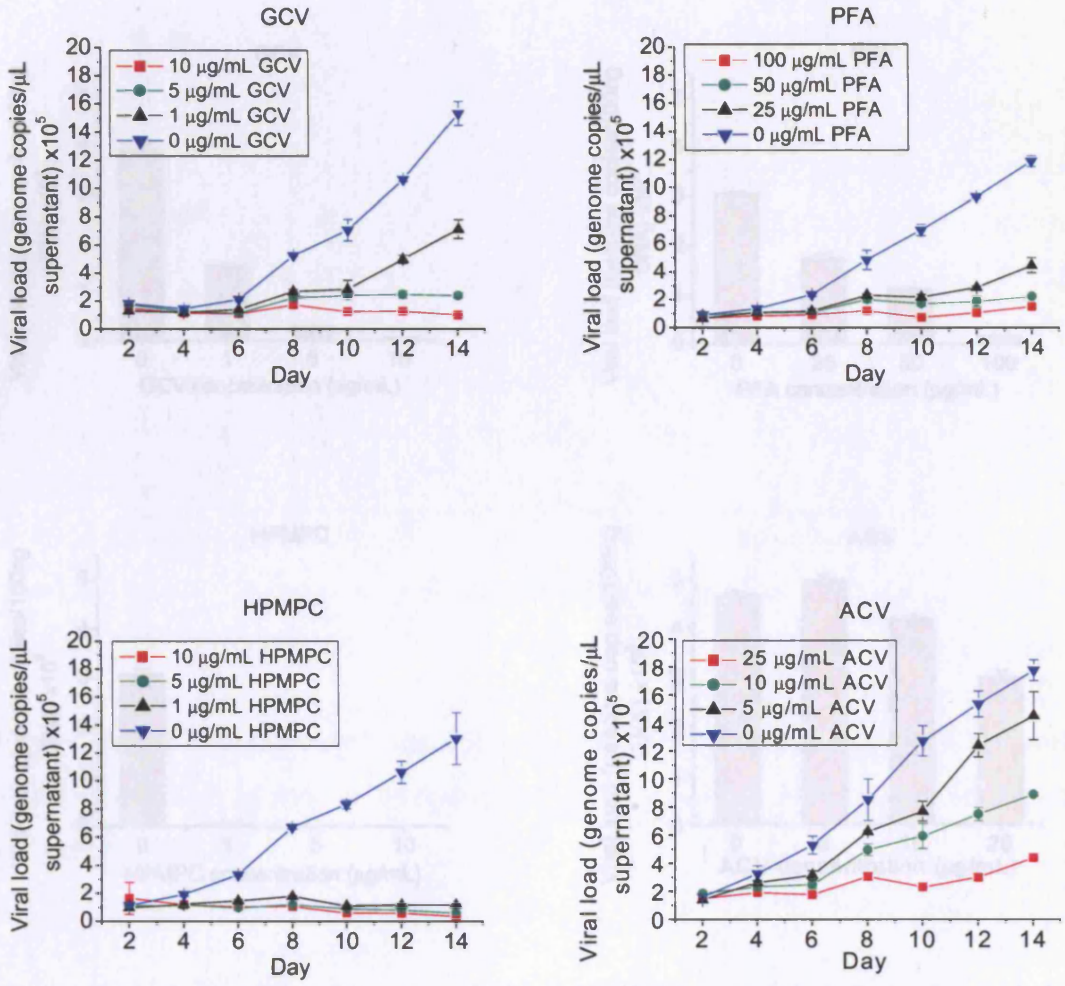


Figure 6.10. Investigating the susceptibility of PCMV to antiviral drugs, by monitoring cell-free virus in supernatant, over a 14 day period. Supernatant was collected every 2 days from day 2 until day 14, prior to total medium replacement including the appropriate concentration of antiviral drug. The viral load in each supernatant sample was quantified in triplicate by real-time PCR. Each value represents the mean of three results with the standard deviation of the mean shown in error bars.

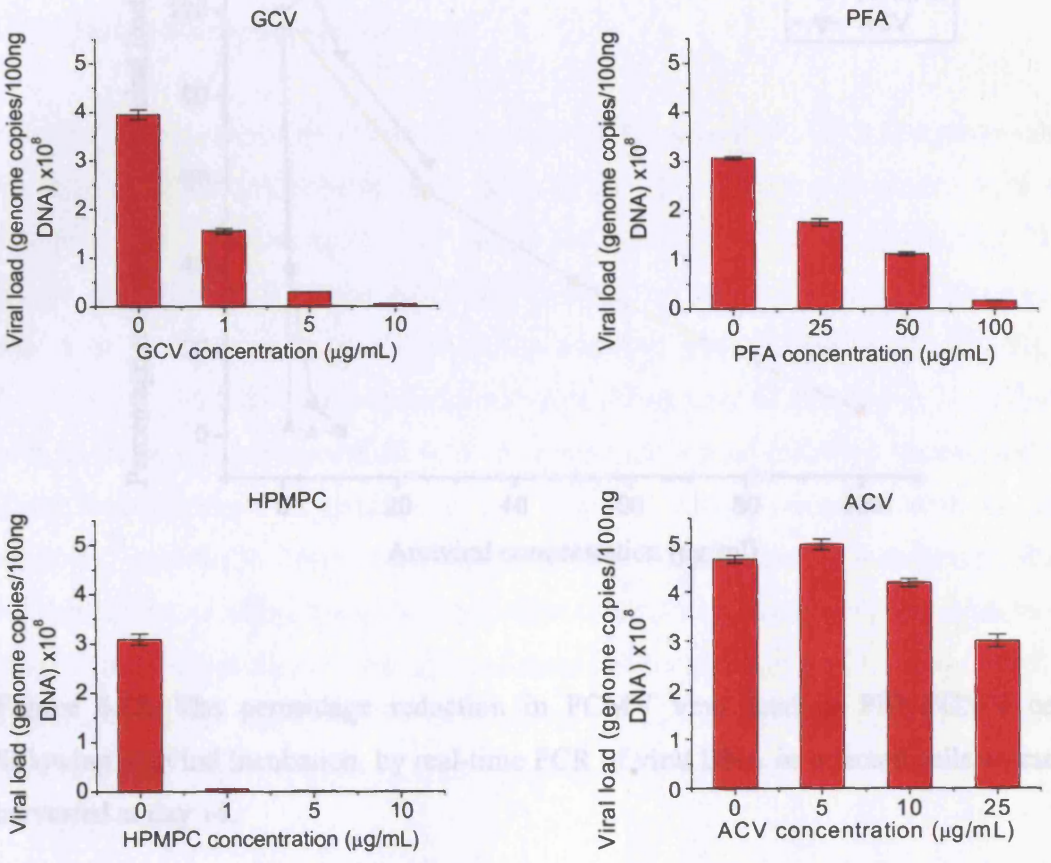


Figure 6.11. Determining the susceptibility of PCMV to antiviral drugs, by real-time PCR of viral DNA in infected cell extracts, harvested at day 14.

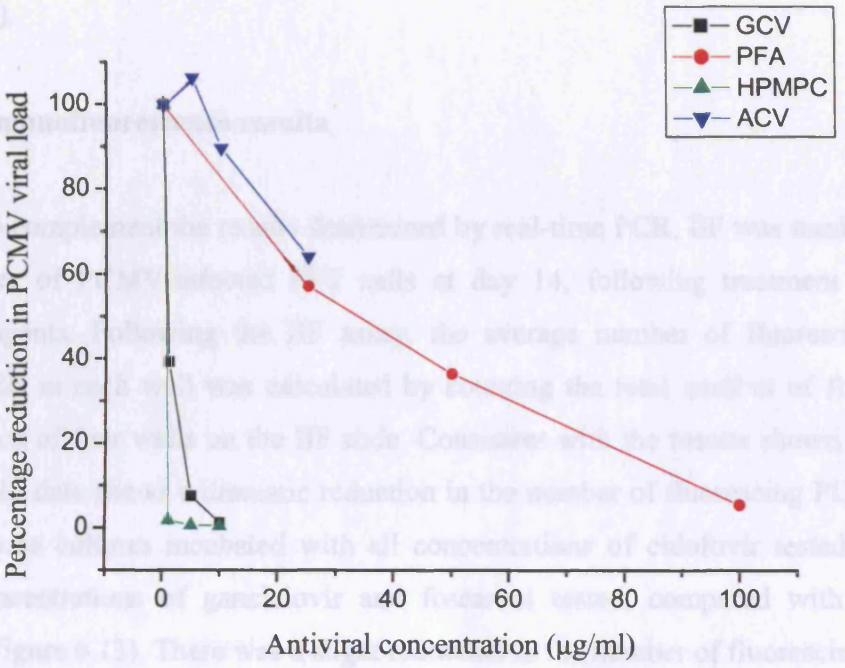


Figure 6.12. The percentage reduction in PCMV viral load in PFT-PCMV cells following antiviral incubation, by real-time PCR of viral DNA in infected cells extracts, harvested at day 14.

Table 6.1. Antiviral susceptibilities of PCMV in PFT cells. EC_{50} values were calculated from the real-time PCR results of DNA extracted from PFT-PCMV cells at day 14.

Antiviral	EC_{50} (µg/ml)	EC_{50} (µM)
GCV	<1	<4
PFA	25-50	83-167
HPMPC	<1	<4
ACV	>25	>111

both had EC_{50} values of <1 μg/ml (<4 μM), while foscarnet had an EC_{50} value within the range of 25-50 μg/ml (83-167 μM). Aciclovir had an EC_{50} value of >25 μg/ml (>111 μM).

6.2.10 Immunofluorescence results

In order to complement the results determined by real-time PCR, IIF was used to assess the number of PCMV-infected PFT cells at day 14, following treatment with the antiviral agents. Following the IIF assay, the average number of fluorescing PFT-PCMV cells in each well was calculated by counting the total number of fluorescing cells in each of four wells on the IIF slide. Consistent with the results shown in Figure 6.11, the IIF data shows a dramatic reduction in the number of fluorescing PCMV-PFT cells in those cultures incubated with all concentrations of cidofovir tested, and the higher concentrations of ganciclovir and foscarnet tested, compared with no drug controls (Figure 6.13). There was a slight reduction in the number of fluorescing cells in cultures incubated with varying concentrations of aciclovir tested, compared with the no drug control. Representative examples of these results are shown in Figures 6.14-6.17. These photographs show sections of wells on IIF slides, prepared using PFT-PCMV cells incubated with varying concentrations of antiviral agent.

6.2.11 Determining the cytotoxicity of antiviral agents

The toxicity of the antiviral drugs used in this study to PFT-PCMV cells was determined by counting the total number of remaining adherent cells harvested from cultures at day 14. Less than 5 % of adherent cells stained with trypan blue. A significant reduction (~50 %) in the total cell count was only found in the culture of PFT-PCMV cells incubated with 10 μg/ml of cidofovir (Table 6.2). No significant cytotoxicity was noted at the lower concentrations of cidofovir tested, or at any concentration of the other antivirals tested.

In a parallel study, the toxicity of the antiviral drugs to uninfected PFT cells was determined, using cellular DNA extracted from the remaining adherent uninfected cells at day 14, as a marker of the total cell count. Again, the results in Table 6.3 show that evidence for cytotoxicity was only observed in PFT cells incubated with the highest

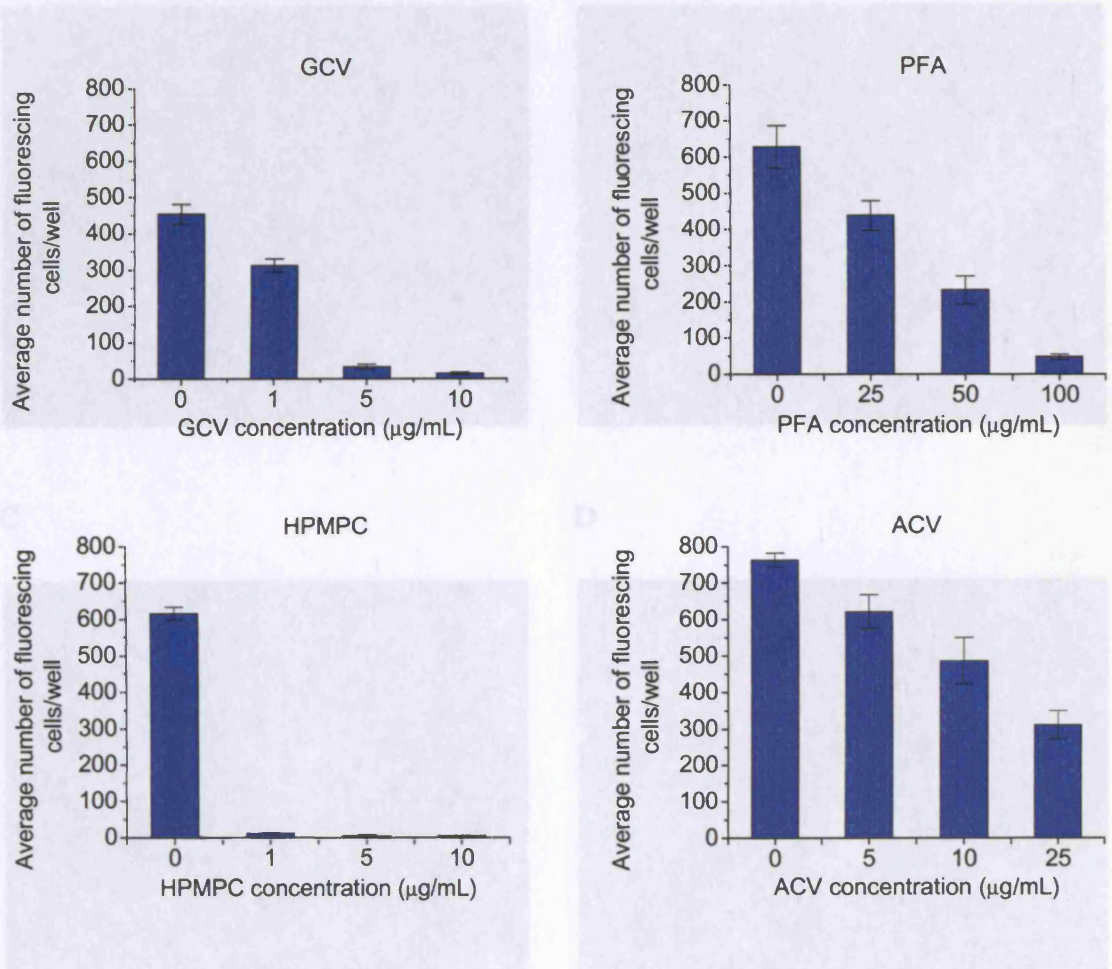


Figure 6.13. Determining the susceptibility of PCMV to antiviral drugs by IIF assay, following a 14 day period. Values represent the average number of fluorescing (PFT-PCMV) cells counted in each of four wells on the IIF slide.

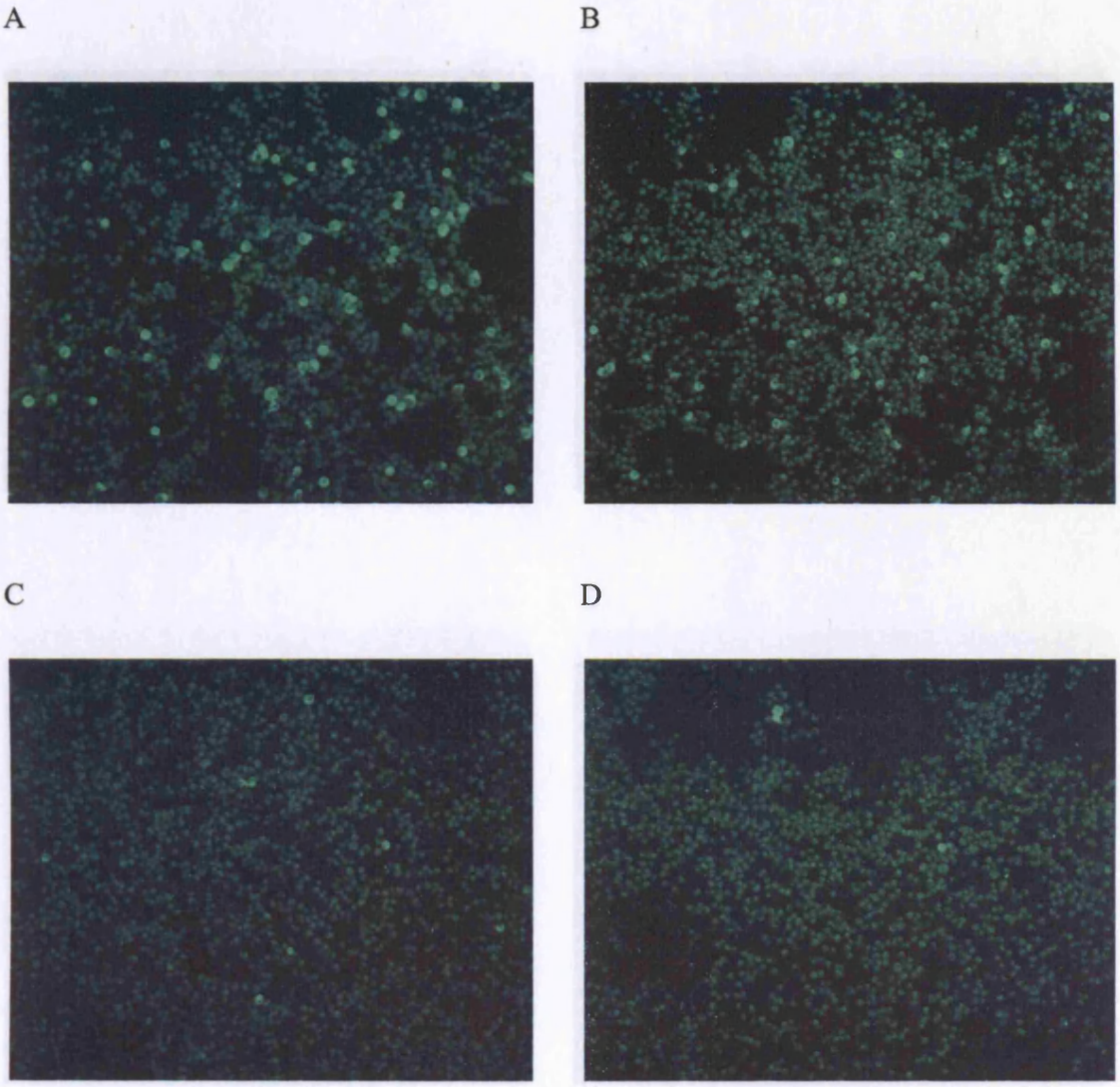


Figure 6.14. The effect of ganciclovir (GCV) on PFT-PCMV cells by IIF assay, following a 14 day period. Panels A-D show the centre of a well on an IIF slide, prepared using PFT-PCMV cells incubated with no GCV (A), 1 µg/ml of GCV (B), 5 µg/ml of GCV (C), and 10 µg/ml of GCV (D).

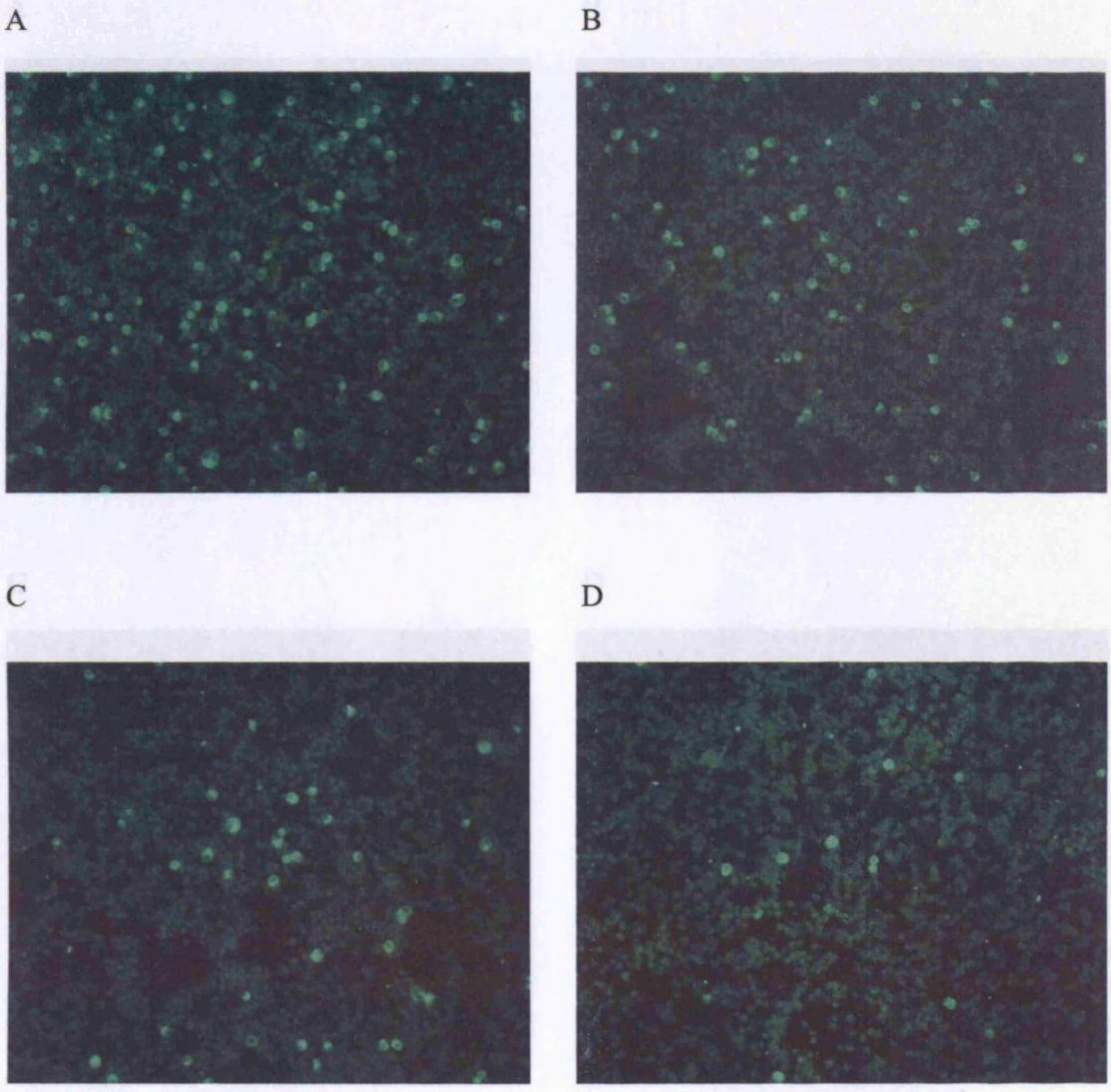


Figure 6.15. The effect of foscarnet (PFA) on PFT-PCMV cells by IIF assay, following a 14 day period. Panels A-D show the centre of a well on an IIF slide, prepared using PFT-PCMV cells incubated with no PFA (A), 25 of µg/ml PFA (B), 50 µg/ml of PFA (C), and 100 µg/ml of PFA (D).

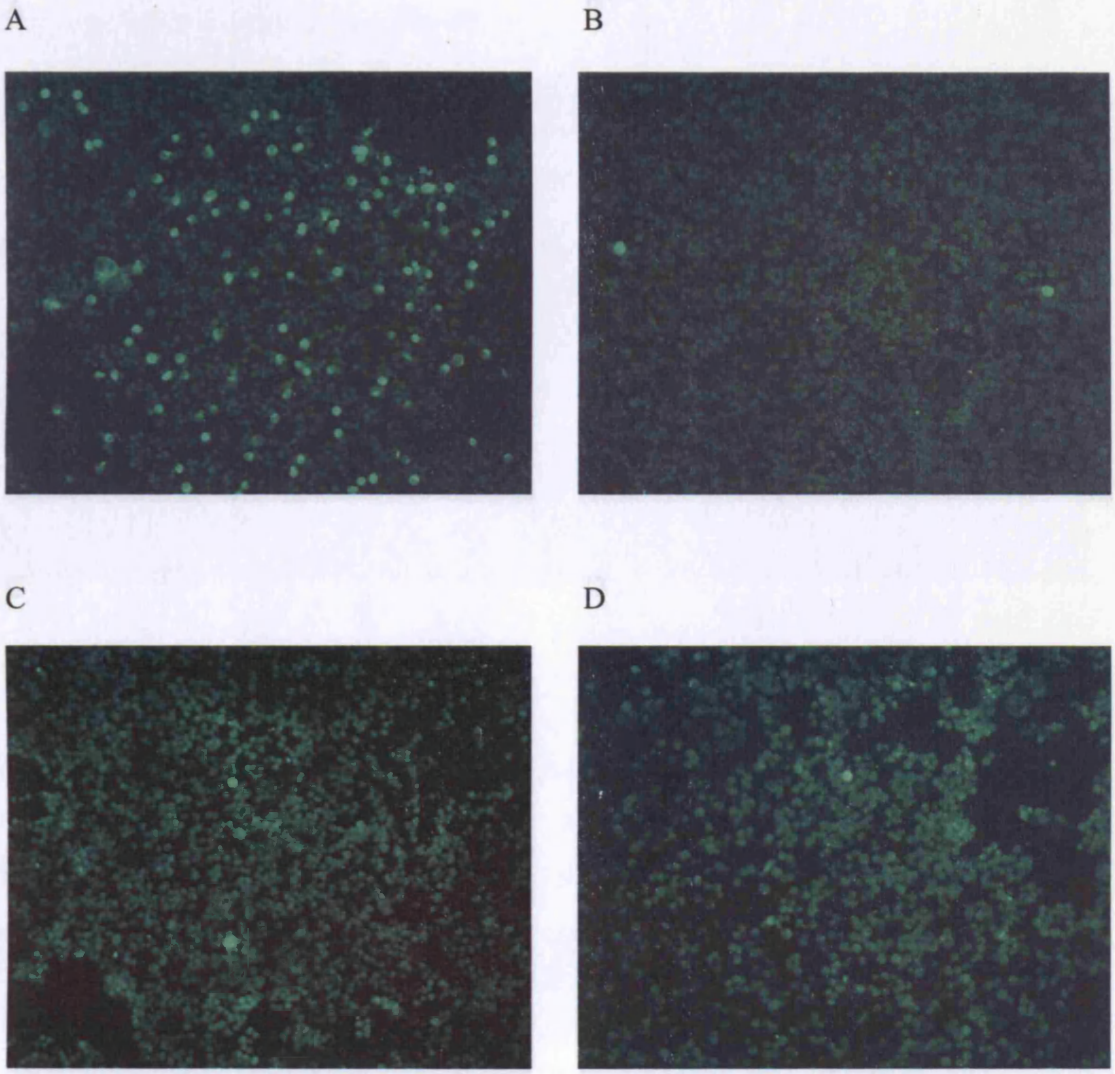


Figure 6.16. The effect of cidofovir (HPMPC) on PFT-PCMV cells by IIF assay, following a 14 day period. Panels A-D show the centre of a well on an IIF slide, prepared using PFT-PCMV cells incubated with no HPMPC (A), 1 µg/ml of HPMPC (B), 5 µg/ml of HPMPC (C), and 10 µg/ml of HPMPC (D).

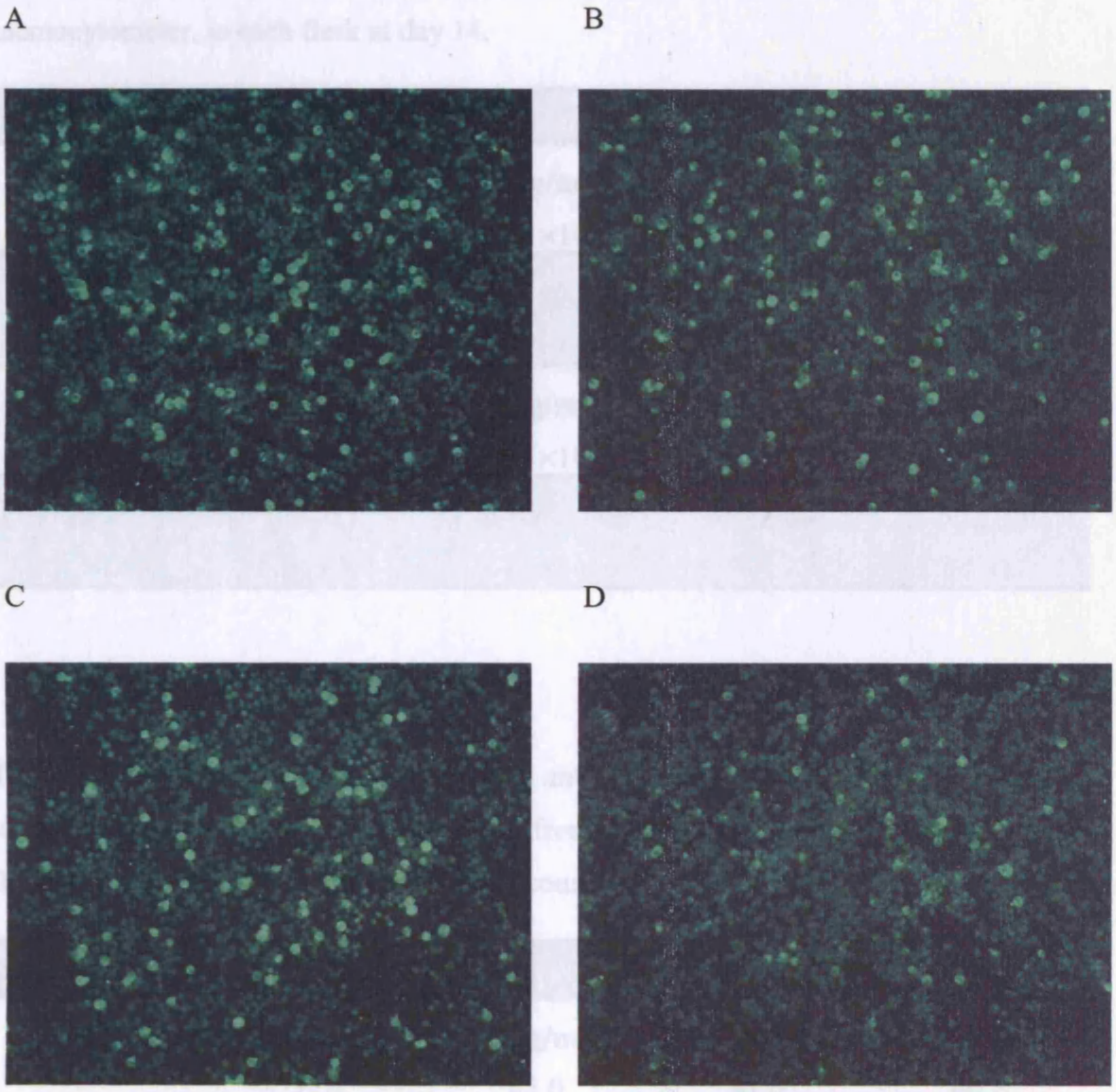


Figure 6.17. The effect of aciclovir (ACV) on PFT-PCMV cells by IIF assay, following a 14 day period. Panels A-D show the centre of a well on an IIF slide, prepared using PFT-PCMV cells incubated with no ACV (A), 5 µg/ml of ACV (B), 10 µg/ml of ACV (C), and 25 µg/ml of ACV (D).

Table 6.2. Determining the toxicity of each antiviral to PFT-PCMV cells. Numbers represent the total number of remaining adherent cells, counted using a haemocytometer, in each flask at day 14.

Antiviral	Total number of cells in each flask			
GCV	**0 μg/ml**	**1 μg/ml**	**5 μg/ml**	**10 μg/ml**
	5.84×10^6	5.32×10^6	6.08×10^6	6.48×10^6
PFA	**0 μg/ml**	**25 μg/ml**	**50 μg/ml**	**100 μg/ml**
	3.92×10^6	3.92×10^6	5.28×10^6	4.52×10^6
HPMPC	**0 μg/ml**	**1 μg/ml**	**5 μg/ml**	**10 μg/ml**
	5.44×10^6	5.60×10^6	4.44×10^6	2.49×10^6
ACV	**0 μg/ml**	**5 μg/ml**	**10 μg/ml**	**25 μg/ml**
	5.24×10^6	5.40×10^6	5.56×10^6	5.84×10^6

Table 6.3. Determining the toxicity of each antiviral to uninfected PFT cells. Numbers represent the total cellular DNA harvested from remaining adherent PFT cells in each flask at day 14, as a marker of the total cell count.

Antiviral	Total DNA (μg) in each flask			
GCV	**0 μg/ml**	**1 μg/ml**	**5 μg/ml**	**10 μg/ml**
	37.2	42.0	41.0	40.2
PFA	**0 μg/ml**	**25 μg/ml**	**50 μg/ml**	**100 μg/ml**
	32.4	37.8	41.1	37.3
HPMPC	**0 μg/ml**	**1 μg/ml**	**5 μg/ml**	**10 μg/ml**
	51.0	46.2	41.5	23.3
ACV	**0 μg/ml**	**5 μg/ml**	**10 μg/ml**	**25 μg/ml**
	38.4	37.7	43.6	43.3

concentration of cidofovir tested. In addition, only the cell extract of detached cells removed in the culture medium from PFT cells incubated with 10 μg/ml of cidofovir yielded a detectable quantity of DNA (data not shown).

6.2.12 Investigating the dynamics of PCMV replication *in vivo*

In order to estimate the doubling time of PCMV in pigs, PCMV viral load was determined in blood collected from piglets, derived from sow P49M, on days 35, 49 and 82 after birth, by real-time PCR. The results in Figure 6.18 show that between days 35 and 49, the PCMV viral load in the blood of the majority of piglets examined had reached a plateau, while between days 49 and 82, virus was being cleared in all piglets. From these results it was not possible to determine the doubling time of PCMV replication since samples were not taken sufficiently early or frequently.

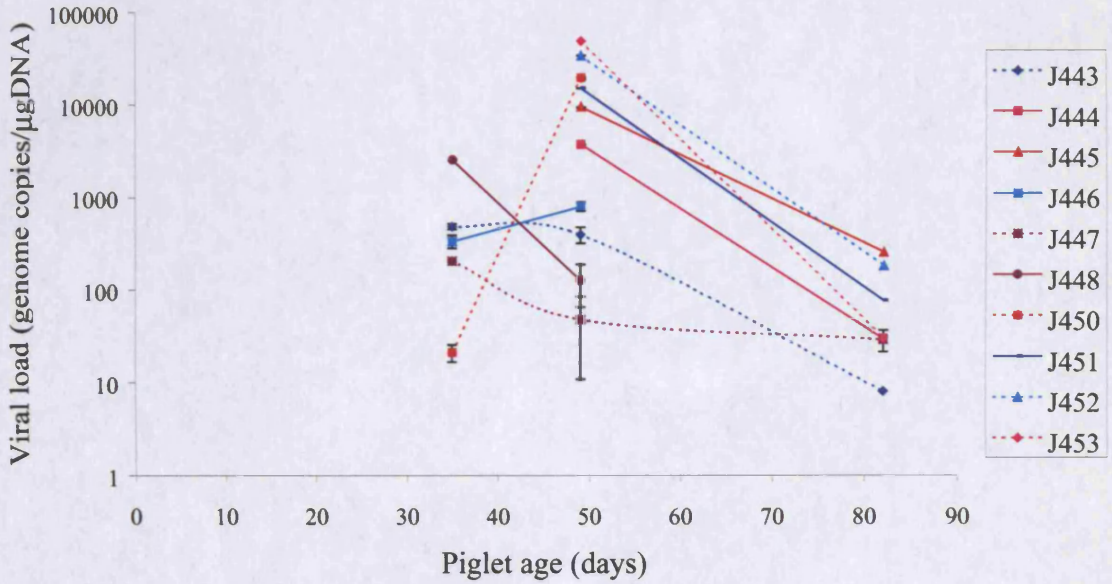


Figure 6.18. Investigating the dynamics of PCMV replication *in vivo* by monitoring PCMV viral loads in the blood of piglets (J443-J453) derived from sow P49M. The viral load in each blood sample was quantified in triplicate by real-time PCR. Each value represents the mean of three results with the standard deviation of the mean shown in error bars.

6.3 Discussion

HCMV is an important pathogen in the human allograft recipient, where viral transmission from the donor and reactivation are common occurrences, and a major cause of disease and graft failure. Similarly, in xenotransplantation, reactivation of PCMV is a concern, as it has been shown to cause invasive disease and possibly contribute to consumptive coagulopathy in pig-to-baboon models (Mueller *et al*, 2002; Gollackner *et al*, 2003). Whether PCMV would be transmitted to human cells outside the xenograft is unclear, as previous studies have failed to provide evidence for PCMV infection of human cells *in vitro* (Tucker *et al*, 1999), although only two cell lines were tested, while no *in vivo* studies have been performed to date. In order to avoid the potential zoonotic transmission of porcine viruses in xenotransplantation, it is ideal to eliminate as many potential pathogens as possible from pigs being bred for xenograft organs. For this, defined breeding methods to eliminate infectious agents from pigs and stringent testing of donor animals are required. It is possible to exclude most pathogens by caesarian delivery and barrier-rearing of donor animals (Tucker *et al*, 2002a). For the first time, the results obtained in Chapter 4 show that PCMV can be eliminated from pigs by these methods. If zoonotic transmission could not be prevented, through the failure of screening methods, knowledge of the antiviral susceptibility of PCMV would be essential, as PCMV would undoubtedly be reactivated under the strong immunosuppressive conditions (Mueller *et al*, 2002; Gollackner *et al*, 2003; Mueller *et al*, 2003).

In this chapter, the efficacy of anti-herpetic agents currently licensed for use in the treatment of human herpesvirus infections to prevent PCMV replication *in vitro* was determined. PCMV infection was established in PFT cells, an adherent, fibroblast-like cell line. PCMV was very slow growing in these cells with CPE detected 7 dpi with PCMV TCS. In addition, the continuous porcine T cell line, L45, was also investigated for susceptibility to PCMV infection, since PCMV is closely related to HHV-6 and -7, which are T cell-tropic viruses. Although L45 cells were susceptible to PCMV infection, chronic infection could not be established due to the slow growth of PCMV in this suspension culture. Since virus titre could not be determined by plaque assay using PFT cells, chronically-infected PFT-PCMV cells were divided between culture flasks and incubated with each concentration of antiviral tested. Real-time PCMV PCR was

used to calculate the effect of each antiviral, at three different concentrations, on PCMV viral load in supernatant over a 14 day period, and in DNA extracted from cells at day 14. In this cell culture system, ganciclovir and cidofovir (both EC_{50} <1 μg/ml) were the most effective against PCMV replication, however, some cytotoxicity was associated with the highest concentration of cidofovir tested. Foscarnet (EC_{50} within range 25-50 μg/ml) and aciclovir (EC_{50} >25 μl/ml) were the least effective antivirals.

The activity of ganciclovir and to a lesser extent aciclovir suggests the presence of a HCMV UL97 gene homologue in the PCMV genome, as these drugs require initial phosphorylation by viral-encoded protein kinases. Such a gene has yet to be identified and characterised in PCMV.

It is possible that the long intracellular half-life of cidofovir contributed to its antiviral activity against PCMV in this study. The long half-life of cidofovir (Ho *et al*, 1992) permits the drug to be administered once weekly in induction therapy, and twice weekly for maintenance therapy for HCMV infection. In this study, the replacement of cidofovir in the cell culture flasks every two days meant that high levels of the drug may have built up in the cells thus exaggerating its antiviral effect, and potentially contributing to the cytotoxicity found at the highest concentration tested. Ganciclovir and aciclovir have much shorter intracellular half-lives of approximately 1 day and 1-2 hours respectively (Lietman & Laskin, 1997), and are therefore less likely to accumulate within the cells.

In a recent study, similar antiviral susceptibilities for PCMV were reported to those identified in this chapter (Mueller *et al*, 2003). In that preliminary investigation, the level of PCMV infection in the culture system was presented only as a percentage reduction in viral load. The results obtained in this chapter confirm and extend those from this previous study.

Comparison of the antiviral EC_{50} values for PCMV determined in this study, with those previously obtained for HCMV is difficult, as differing methodologies have been used. Several studies have tried to compare the antiviral susceptibilities of different viruses obtained by plaque reduction assays and modern molecular methods (Underwood *et al*,

1998; Stranska *et al*, 2002), but have produced varying results suggesting that there is not a consistent relationship between the two methods.

In the case of antiviral agents that interfere with DNA replication, there is a delicate balance between cytotoxicity and efficacy. Cytotoxicity *in vitro* is often determined by trypan blue exclusion. However in this study, since PFT cells are adherent, and the culture medium was replaced at regular intervals throughout the study, this method was not feasible. Cytotoxicity was therefore best determined by counting the total number of remaining, adherent PFT-PCMV cells harvested following incubation with the antiviral agents. Trypan blue staining of cells showed that most adherent cells (>95 %) were living. Cytotoxicity results were confirmed in a parallel study, using cellular DNA extracted at day 14 from uninfected adherent cells, cultured with each concentration of antiviral, as a marker of the total cell count.

Although only one isolate of PCMV was tested, it is highly probable that it is representative of the antiviral activity of agents against PCMV. In particular, the DNA polymerase genes of PCMV strains B6 and OF-1, and isolate 55b are >99.3 % identical at the amino acid level (Goltz *et al*, 2000).

Following retrospective PCMV DNA real-time PCR analysis of the viral load in the blood of piglets monitored from birth, it was not possible to determine the doubling time of PCMV replication *in vivo* due to late and infrequent sampling. The dynamics of HCMV replication *in vivo* has previously been determined in our laboratory (Emery *et al*, 1999). In this study, a direct estimate of the doubling time of HCMV in BMT recipients was obtained by measuring the viral load in blood, sampled on average once per week. Previously, HCMV had been described as a slowly replicating virus, based on the slow development of CPE in virus culture, however, the results of this study demonstrated that the doubling time of HCMV in blood during active infection is ~1 day, when frequent samples are collected, showing that HCMV DNA replication *in vivo* is in fact a highly dynamic process. These results have significant implications for the treatment of HCMV infections. Similarly, should PCMV be transmitted to humans following xenotransplantation, knowledge of the dynamics of PCMV replication *in vivo* would have implications for it's treatment in these patients.

In solid-organ allograft recipients either ganciclovir prophylaxis or pre-emptive therapy is used to treat HCMV infections posttransplant. Although *in vivo* studies in a porcine model are needed to confirm the results obtained in this chapter, they suggest that ganciclovir may also be used to treat any potential PCMV infections that may arise following xenotransplantation, with cidofovir providing an alternative drug for management.

Chapter 7: General discussion

Xenotransplantation of porcine tissues and organs has been proposed to alleviate the current shortfall in human donor organs available for transplant. For clinical xenotransplantation to be feasible, several significant immunological hurdles need to be overcome. One of these is HAR, mediated by antibodies against glycoproteins containing terminal αGal sugar residues, which activate complement and lead to vascular graft loss within hours. Molecular approaches used to abrogate this response include the genetic modification of pig endothelium to express human complement control factors CD55 (Bhatti *et al*, 1999; Cozzi *et al*, 2000), CD46 (Diamond *et al*, 2001; Adams *et al*, 2001) and CD59 (Fodor *et al*, 1994; Diamond *et al*, 1996). Porcine xenografts expressing these proteins have been shown to prevent HAR in nonhuman primate models of xenotransplantation. In addition, it has recently been possible to knockout the α1,3GT gene, which adds these terminal αGal sugars onto the surface of porcine cells, in pigs (Phelps *et al*, 2003), however, extensive studies of these knockout animals to reduce donor immunogenicity have yet to be reported.

While advances have been made in overcoming immune rejection, xenotransplantation has raised considerable safety concerns regarding the potential for the transmission of porcine infectious agents, particularly viruses, to humans, and pigs being bred for use in xenotransplantation will need to be free from a long list of porcine infectious agents (Onions *et al*, 2000). Viruses of particular concern include those transmitted in the germline or *in utero*, such as members of the retrovirus or herpesvirus families (Magre *et al*, 2003), since they will potentially be difficult to eliminate from pigs being bred for xenograft organs. Much of the recent research in this area has focused on the porcine endogenous retroviruses which have been shown to be capable of infecting human cells *in vitro* (Patience *et al*, 1997a; Wilson *et al*, 1998; Martin *et al*, 1998; Martin *et al*, 2000), and crossing the species barrier *in vivo* into mice (van der Laan *et al*, 2000). However, retrospective analysis of human recipients of porcine xenografts, has revealed no evidence of PERV transmission to humans, despite long-term microchimerism in some patients (Heneine *et al*, 1998; Patience *et al*, 1998; Paradis *et al*, 1999; Pitkin & Mullon, 1999; Schumacher *et al*, 2000; Dinsmore *et al*, 2000; Levy *et al*, 2000). These patients were however, never tested for the presence of other porcine infectious agents such as herpesviruses. So far, five herpesviruses have been identified in pigs: these being PRV, PCMV, and three recently identified PLHVs, -1, -2 and -3 (Ehlers *et al*, 1999; Chmielewicz *et al*, 2003b).

PCMV is a betaherpesvirus which is endemic in the world pig population, with over 90 % of pigs being seropositive in the UK, many of which are from well-managed high health status farms (Edington, 1999). Although PCMV was originally classified as a betaherpesvirus, genus *Cytomegalovirus*, based on the formation of characteristic cytomegalic inclusions in infected cells, recent sequence analysis of the DNA polymerase gene (Goltz *et al*, 2000), the major capsid protein gene (Rupasinghe *et al*, 2001), and the glycoprotein B gene (Widen *et al*, 2001), has revealed that the virus is in fact more closely related to HHV-6 and -7, which are classified into the genus *Roseolovirus*. Since PCMV does not have a major economic impact on pig farming, much of what we know about the epidemiology of PCMV is based on studies carried out by Edington *et al* in the 1970s. PCMV causes generalised infection in newborn piglets, leading to foetal and piglet deaths, runting, rhinitis and pneumonia, while infection in adults is generally silent. Virus was thought to be transmitted horizontally and *in utero*, with a common mode of transmission being through infected nasal droplets (Plowright *et al*, 1976).

PCMV represents a potential zoonotic risk in xenotransplantation since its human counterpart, HCMV, is frequently transmitted from the donor organ and reactivates posttransplant where it is associated with end-organ disease and graft rejection (Fishman & Rubin, 1998). In addition, BCMV is the only infectious virus which has been transmitted to humans following xenotransplantation, when it was isolated from the peripheral blood of a human recipient of a baboon liver (Michaels *et al*, 2001). As a herpesvirus, PCMV has the ability to remain in the host life-long, following primary infection, including a latent phase, and may reactivate periodically to produce infectious virus and potentially cause disease. Indeed, reactivation of latent PCMV has been demonstrated following the administration of immunosuppressive agents in pigs (Edington *et al*, 1976b; Narita *et al*, 1985). The major theoretical concern associated with PCMV in the xenotransplantation setting is the possibility that PCMV, transplanted in a latent form within the xenograft, might reactivate and result in graft rejection or direct damage to the organ. Recent evidence has shown that PCMV is often reactivated and is associated with invasive disease in porcine organs transplanted into baboons, although reactivation appears to be limited to the porcine organ (Mueller *et al*, 2002; Mueller *et al*, 2004). PCMV reactivation in the xenograft may also contribute to

consumptive coagulopathy observed in pig-to-nonhuman primate xenotransplantation, a condition that could only be reversed by removal of the xenograft (Gollackner *et al*, 2003). Whether PCMV would subsequently spread to human cells outside the xenograft is unclear as previous studies have failed to provide evidence for PCMV infection of human cells *in vitro* (Tucker *et al*, 1999), although only two cell lines have been tested, while no *in vivo* studies have been performed to date. Co-culture of PCMV-infected macrophages with human B cells (Raji cells) and HEK293 cells, did not facilitate transmission. However, these *in vitro* cell culture experiments do not provide the same degree of chronic cell-to-cell contact that would be present following xenotransplantation, which includes intimate connection of the graft to the human vascular system, coupled with inflammatory up-regulation of multiple genes including those of herpesviruses. Even in the absence of transmission to the human recipient, the results of Mueller *et al* suggest that reactivation of PCMV in the xenograft could potentially compromise graft function and survival.

The initial aim of this thesis was to develop qualitative and QC PCR assays for the detection and quantification of PCMV DNA. Primers were designed from a region of the DNA polymerase gene of PCMV (Widen *et al*, 1999), and were shown by BLAST analysis to share 100 % nucleotide identity with sequence data for the DNA polymerase gene of all three PCMV strains (55b, B6 and OF-1), deposited in Genbank, suggesting their ability to amplify different isolates. The qualitative PCR was found to be sensitive to 5 genome copies per reaction and did not amplify HCMV, HHV-6 or -7 sequences. The QC PCR is based on the coamplification of the test sample with an internal competitor, which differs from the wild-type sequence by the presence of a unique restriction endonuclease site in the middle of the amplicon. The *Sma* I restriction endonuclease site was introduced into the middle of the PCMV amplicon by PCR-mediated, site-directed mutagenesis, similar to the method described by Fox *et al* (Fox *et al*, 1992). The PCMV QC PCR was shown to be accurate and reproducible over a broad dynamic range (Fryer *et al*, 2001).

These assays were subsequently used to investigate the epidemiology of PCMV in pigs being bred for xenograft organs at Imutran Ltd, and in particular, to investigate the prevalence, quantity and organ distribution of PCMV in immunocompetent and immunosuppressed pigs, to investigate when virus is acquired and examine methods to

eliminate PCMV from these animals (Clark *et al*, 2003). A range of porcine organs and clinical samples were tested from conventionally-reared CD55 transgenic Large White pigs. The results showed that the virus was persistent in a range of organs from adult pigs aged > 15 months, including those intended as potential xenografts, namely the heart, kidney and liver. Viral loads in these tissues were low indicating either latent or low-level active infection. Meanwhile, in 3-5 week old weaned piglets infection was widely disseminated, with high viral loads detected in tissues. This suggested that infection was occurring early in life possibly in the early post-weaning period following mixing of animals.

The next aim was to further investigate when virus is acquired, by testing foetal spleen samples from PCMV-positive sows and by monitoring piglets for PCMV viraemia following birth. The results showed that piglets monitored from birth acquired PCMV post-natally at 2-5 weeks of age. Although the precise source of virus was not determined in the two litters examined, the timing of infection correlated with their movement to a building with shared air space with other pigs. In addition, there was no evidence of infection *in utero* when foetal spleen was sampled at day 86 out of an average full-term gestation of 114 days. These results suggest that acquisition of the virus predominantly occurred in the post-natal period rather than *in utero*. These results were in agreement with the epidemiology of PCMV in commercial pig herds (Plowright *et al*, 1976). Importantly, the results showed that hysterotomy-derived, barrier-reared animals did not acquire PCMV in the post-natal period, up to the age of 21 weeks, and that transplacental infection from PCMV-positive sows did not occur. These results suggest that although transplacental transmission of PCMV can occur following the experimental infection of pregnant sows with or without the presence of circulating antibody (Edington *et al*, 1977; Edington *et al*, 1988a; Edington *et al*, 1988b), it is however uncommon in the conventional setting, if it occurs at all. These findings suggest that PCMV can realistically be targeted for eradication from pigs being bred for xenograft organs.

In order to determine the potential for PCMV reactivation in the transplant setting, the effect of standard immunosuppressive therapy on PCMV viral loads in treated and control pigs was investigated. Gnotobiotic, SEW and conventionally-reared pigs (with appropriate control groups) were subjected to a 2 month period of oral

immunosuppression including Cyclosporin A, azathioprine and prednisolone. In this study, such immunosuppression did not appear to affect the PCMV viral load in the tissues investigated, although the study was limited by the small number of pigs tested, and by the fact that the pigs were not examined throughout the period of immunosuppression.

The proposed method for the large-scale production of pigs for xenotransplantation involves caesarian delivery followed by barrier-rearing of source animals in closed cohorts (Onions *et al*, 2000). The results from this thesis suggest that PCMV could be eliminated from pigs being bred for xenograft organs by these methods. In addition, these methods have recently been demonstrated to exclude a number of other porcine infectious agents such as porcine circoviruses (Tucker *et al*, 2003). However, the prevalence of PLHV was only reduced by SPF breeding, suggesting that PLHV transfer *in utero* is rare. It should therefore be feasible to derive animals that are free from PLHVs and porcine circoviruses.

If xenotransplantation is to proceed, PCMV-free breeding stocks will need to be established in α1,3GT knockout pigs. The transmission of PCMV should then be preventable, first in nonhuman primate models of xenotransplantation, and then in humans if clinical trials proceed. Routine monitoring of these source pig cohorts for the presence of specific porcine infectious agents such as PCMV will be necessary at intervals throughout the life of the source pig. This will include extensive analysis of sentinel animals, representative of the entire cohort, at two months of age and prior to approval of the organs from the cohort for human clinical use (Onions *et al*, 2000). The results obtained in this thesis suggest that the spleen of these sentinel animals should be tested by PCMV PCR, meanwhile, at the time of transplant rapid PCR testing of this non-xenograft organ, should be performed in order to confirm the SPF status of the individual donor animal, with an appropriate quantity of input DNA added.

The fact that PCMV was not always detected in readily available samples such as blood or serum from seropositive pigs in chapter 4, highlights the need for a reliable serological assay for PCMV. Serological assays that have been developed for PCMV rely on the *in vitro* culturing of the virus in either primary alveolar macrophages or pig fallopian tube cells (Plowright *et al*, 1976; Rhonduis *et al*, 1980; Tajima *et al*, 1993).

Since the virus is slow growing in these cells, these assays can be time consuming. The use of baculovirus expression systems to produce recombinant viral immunogenic proteins in insect cells provides an alternative approach to these *in vitro* cultivation systems. Since glycoprotein B is the major immunodominant protein of HCMV, being the target of 40-70 % of virus-neutralising antibodies (Britt *et al*, 1990), PCMV gB was cloned and expressed in insect cells using a baculovirus expression system, and used to develop a recombinant protein-based serological assay for PCMV. In addition to the full-length ORF of PCMV gB, a truncated version of the protein, excluding the proposed transmembrane anchor regions, was cloned into the baculovirus vector. Both PCMV gB proteins were found to be expressed in Sf9 and High Five insect cells 2-4 dpi, however, the truncated protein was not secreted into the culture medium as expected. Both proteins were found to be immunogenic by PCMV IIF assay, and did not cross-react with anti-HCMV gB monoclonal antibodies. This fact is important when screening human recipients of porcine xenografts for the presence of PCMV, although the cross-reactivity of PCMV gB with anti-HCMV, HHV-6 and -7 polyclonal antibodies remains to be determined. The IIF assay of porcine serum samples from adult pigs and piglets from Imutran Ltd, corresponded well with the PCMV PCR results of DNA extracted from a range of organs and clinical samples from these animals in chapter 4. These results highlight the potential use of recombinant PCMV gB in a serological assay for PCMV. Although time constraints did not allow further development of the PCMV ELISA using recombinant PCMV gB, this assay would allow the screening of a large number of samples.

If zoonotic transmission of PCMV in xenotransplantation could not be prevented, presumably through the failure of screening methods, then knowledge of the antiviral susceptibility of PCMV would be essential, as the virus would undoubtedly be reactivated under the strong immunosuppressive conditions (Mueller *et al*, 2002; Mueller *et al*, 2004). Therefore, the final aim of this thesis was to determine the efficacy of anti-herpetic agents, currently licensed for use in the treatment and/or prophylaxis of human herpesvirus infections, to prevent PCMV replication *in vitro* (Fryer *et al*, 2004). The antiviral agents used in this study, included ganciclovir, foscarnet, cidofovir and aciclovir, which are all nucleoside/nucleotide analogue inhibitors of the viral DNA polymerase, apart from foscarnet which is a pyrophosphate analogue. Ganciclovir is the first choice therapy for HCMV infections in the immunocompromised, and in solid-

organ allograft recipients, with either prophylactic or pre-emptive strategies employed. It has also been used in the treatment of suspected HHV-6-associated diseases (Johnston *et al*, 1999). PCMV infection was established in PFT cells, an adherent, fibroblast-like cell line, in which it has previously been shown to be slow-growing, producing CPE approximately 14 dpi (Kawamura *et al*, 1992). Real-time PCR was used to calculate the effect of each antiviral, at three different concentrations, on the PCMV viral load in supernatant over a 14 day period, and in DNA extracted from cells at day 14. In addition, PCMV IIF was used to determine the effect of each antiviral on PCMV-infected cells. In this cell culture system, ganciclovir and cidofovir (both EC_{50} < 1 μg/ml) were the most effective against PCMV replication; however, some toxicity was associated with the highest concentration of cidofovir tested. Foscarnet (EC_{50} within range 25-50 μg/ml) and aciclovir (EC_{50} > 25 μg/ml) were the least effective antivirals. The antiviral activity of ganciclovir, and to a lesser extent aciclovir, suggests the presence of a HCMV UL97 gene homologue in the genome of PCMV. This may need to be further investigated in order to determine the mechanism of action of ganciclovir against PCMV.

The proposed breeding methods for producing pigs for xenotransplantation should eliminate the majority of porcine infectious agents, however, certain viruses such as the porcine endogenous viruses will prove more difficult to eliminate. Recent research suggests that pigs possess few copies of the HTRC PERVs, and that these could potentially be removed through selective breeding and knockout technologies. If they could not be removed, siRNA technology has been suggested as a means of preventing PERV expression in xenograft recipients (Karlas *et al*, 2004). In addition, knowledge of the susceptibility of porcine viruses to available antiviral therapies would be useful as it would provide therapeutic options should the screening procedure of xenografts fail. The results in this thesis show that should it prove necessary, ganciclovir should be considered as the first-line therapy for any PCMV infections that may arise following xenotransplantation, and that cidofovir provides an alternative drug for management. Other studies have suggested that PERVs are sensitive to AZT and ddGTP (Qari *et al*, 2001; Wilhelm *et al*, 2002).

Recently, five novel herpesviruses were identified in suid species from Africa and South-East Asia (Ehlers & Lowden, 2004), highlighting the risk that previously

unidentified viruses may pose in xenotransplantation. Three of these viruses were closely related to known porcine beta and gammaherpesviruses, while two species were more closely related to bovine herpesvirus 4. Although these results suggest that there are likely to be unidentified porcine herpesviruses present in pig species worldwide, pigs being bred for xenograft organs will most likely be derived from well characterised, inbred herds. The screening of commercial, experimental and wild pigs from Germany, Spain, Sweden, France and the USA by pan-herpes PCR failed to identify further porcine herpesviruses (Chmielewicz *et al*, 2003a).

Whether xenotransplantation becomes a clinical reality is unknown at present. The ultimate decision will depend on weighing the potential for new human pathogens against the benefit of an unlimited supply of organs.

Chapter 8: Bibliography

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Appendix A

List of publications

1. Fryer JF, Griffiths PD, Fishman JA, Emery VC, Clark DA. Quantitation of porcine cytomegalovirus in pig tissues by PCR. J Clin Microbiol 2001; 39(3):1155-1156.

2. Clark DA, Fryer JF, Tucker AW, McArdle PD, Hughes AE, Emery VC et al. Porcine cytomegalovirus in pigs being bred for xenograft organs: progress towards control. Xenotransplantation 2003; 10(2):142-148.

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