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Porcine Cytomegalovirus, studies on the viral genome and development of novel diagnostic techniques.

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Abstract

Porcine cytomegalovirus (PCMV) is a recognised pathogen in pigs causing reproductive failure, piglet mortality, rhinitis and possibly retarded growth. The virus is highly prevalent in pig populations worldwide. The introduction of PCMV in non-immune herds can cause substantial losses. The current interest in transplantation of tissues from pigs to humans (xenotransplantation) and the related risks for cross species virus infections and interactions constitute an important incentive for research on PCMV. In order to eliminate the risk of xenogeneic PCMV infections and recombinations, effective diagnostic techniques are needed. PCMV is a poorly characterised virus. It has previously been assigned to the subfamily *Betaherpesvirinae* based on its biological properties but no information regarding molecular characteristics was available. Very few studies have been performed on this highly prevalent virus. The main reason for this being the lack of practically usable and effective diagnostic techniques. This in turn is due to the biological properties of PCMV and the difficulties in finding a usable *in vitro* cultivation system.

In order to develop efficient diagnostic techniques for direct and indirect detection of PCMV and gain more information on the evolutionary relationship and taxonomical position of PCMV it was necessary to perform molecular characterisation. As a first step a PCR assay for PCMV was developed and a short fragment of the DNA polymerase (DPOL) gene was sequenced. This provided initial information regarding the evolutionary relationship to other members of the *Herpesviridae* family and served as a basis for further characterisation of the DPOL gene.

The complete sequencing of the DPOL gene provided information regarding the genetic stability of PCMV and identified conserved and variable regions thereby enabling the development of sensitive and reliable PCR assays. It also confirmed previous results regarding evolutionary relationship of PCMV to other viruses. The results demonstrated that PCMV is a betaherpesvirus and indicated that it belongs to the genus *Roseolovirus*. Also, the DPOL sequence served as a basis for further acquisition of genetic information. Thus, it was possible to sequence the complete glycoprotein B (gB) gene, one complete and two partial flanking genes, together with the DPOL gene comprising 7200 base pairs. In this manner it was possible to show that in PCMV exist a block of five genes that is conserved amongst betaherpesviruses. The sequencing of the gB gene provided further information regarding genetic stability of PCMV and a basis for the development of a serological assay.

Further characterisation of the gB gene and the deduced gB protein identified immunogenic regions. The cloning of a fragment encoding such a region and its expression in *E. coli* provided a highly immunogenic truncated protein. With the use of this truncated protein it was possible to perform initial studies of an ELISA detecting antibodies to PCMV. The ELISA was used to analyse 198 Swedish pig serum samples. 54% of the samples were classified as positive 37% as negative and 7% as inconclusive. The results should however be interpreted with caution since the assay has not yet been fully validated

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Contents

Appendix

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I Widén B.F., Lowings J.P., Belák S. and Banks M. 1999. Development of a PCR system for porcine cytomegalovirus detection and determination of the putative partial sequence of its DNA polymerase gene. Epidemiology and Infection 123, 177-180.

II Goltz M., Widén F., Banks M., Belák S. and Ehlers B. 2000. Characterisation of the DNA Polymerase loci of Porcine Cytomegalovirus from diverse geographic origins. Virus Genes 21, 249-255.

III Widén F., Goltz M., Wittenbrink N., Ehlers B., Banks M., Belák S. 2001. Identification and sequence analysis of the glycoprotein B gene of porcine cytomegalovirus. Virus Genes 23, 339-346.

IV Widén F., Banks M. And Belák S. 2002. Expression of truncated glycoprotein B from of PCMV and its application for a serological assay. Manuscript to be submitted.

Reprints of papers I, II and III are published with the kind permission from the publishers.

Abbreviations and definitions

BCMV:	Baboon cytomegalovirus
BSA:	Bovine serum albumine
DMSO:	Dimethyl sulfoxide
DPOL:	DNA Polymerase
EBV:	Epstein Barr virus
EBI:	European Bioinformatics Institute
EM:	Electromicrscopy
gB:	Glycoprotein B
HCMV:	Human cytomegalovirus
HHV-6:	Human herpesvirus 6
HHV-7:	Human herpesvirus 7
HRP:	Horse radish peroxidase
HSV:	Herpes simplex virus
IF:	Immunofluorescence
IFAT:	Immunofluorescence antibody assay
Kbp:	kilobase pairs
LAT:	Latency associated transcripts
MCP:	Major capsid protein
mRNA:	Messenger RNA
nm:	nanometer
nt:	nucleotide
O.D.:	optical density
ORF:	Open reading frame
p.i.:	post infection
PAM:	Porcine alveolar macrophages
PBST:	Phosphate buffered saline with Tween 20
PCMV:	Porcine cytomegalovirus
PCR:	polymerase chain reaction
PRV:	Pseudorabies virus
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	Poly acrylamid gel electrophoresis with SDS
Taq:	*Thermostabilus aquaticus*
tRNA:	Transfer RNA

Open reading frames (ORF) are named according to the corresponding genes in Human herpesvirus 6 (HHV-6, Dominguez et al., 1999), except for in paper I, where the nomenclature of Herpes simplex Virus 1 (HSV-1, Roizman and Sears, 1996) is used.

INTRODUCTION

Background

Porcine cytomegalovirus (PCMV) is a poorly characterised herpesvirus of pigs that is capable of causing various disease forms and mortality in young pigs and reproductive failure in sows and gilts. The virus probably has a worldwide distribution in the swine populations. For example, a very high prevalence of over 90% has been demonstrated in The Netherlands, USA, Germany and in the United Kingdom (Kanitz and Woodruff, 1976; Plowright et al., 1976; Marquardt, 1979; Rondhuis, 1980; Edington, 1992). The introduction of PCMV in non-immune herds can be associated with significant economical losses. However, the economical importance of the virus for the pig industry as a whole is disputed. The importance of this virus as an immunomodulator and the possible interaction with other porcine viruses, for example porcine circovirus 2, has not been studied.

It is a paradox that although PCMV seems to be very common worldwide, very few studies on this herpesvirus have been published.

This is mainly due to the following factors:
- PCMV has not been considered as an economically important pathogen of swine; thus, very limited research has been carried out to study this virus;
- In principle no effective methods are available for PCMV detection and characterisation. Concerning **direct detection** of viruses, the classical methods are hardly usable. Virus isolation is very difficult, because the host range *in vitro* of PCMV is extremely restricted (see below) and replication is very slow and restricted by the limited lifetime of permissive cell cultures. Other methods, like electron microscopy, Southern blots, immunofluorescence, antigen-ELISA are either to insensitive, very slow or not yet developed for the detection of this virus. Immunofluorescence has been the standard method, but is very slow and insensitive. The **indirect methods** of virus detection, like serumneutralisation, antibody-ELISA and immunofluorescence are also hampered by the strictly limited *in vitro* host range of the virus. Thus, the routine diagnostic laboratories practically do not have any tools today to diagnose PCMV infections;

With reference to these shortages, the aim of the present *studies was to develop novel methods of PCMV detection and to start the preliminary genomic characterisation* of this virus. The main reasons to initiate these studies were the followings:

- Observations indicate that PCMV is able to cause various disease forms in swine, as a primary agent;
- PCMV may act as immunomodulator, influencing the effect of vaccinations, the establishment of other infections, weight-gain and many other important factors in the swine industry;

- The current interest in transplantation of tissues and organs from pigs to humans (xenotransplantation) and the related risks for cross species virus infections and interactions constitute an important incentive for research on PCMV;
- From a general scientific point of view, it is of interest to clarify the evolutionary relationship between PCMV and other herpesviruses;
- The occurrence of PCMV in Sweden has not been studied;

The studies presented here focus on the development of effective diagnostic tools and on selected aspects of PCMV molecular biology. I believe that it is important to put the results from this study in relation to the inherent nature of herpesvirus biology and pathogenesis. Therefore, a brief synopsis of the *Herpesviridae* family and the comparative aspects of the *Betaherpesvirinae* subfamily, is warranted.

The Herpesviridae family

This very vast family contains approximately 120 known members (Minson et al., 2000). Herpesviruses have been isolated from almost all species of mammals and birds that have been investigated as well as from reptiles, insects, molluscs and amphibians (Roizman, 1996; Murphy, 1999).

Characteristics of the herpesvirus virion and genome

The virion

The viral particles of this family are large, spherical, enveloped and carry linear double stranded DNA in the core, wrapped up with histones in the form of a torus. The capsid, measuring approximately 100-125 nm in diameter surrounds the core. Around the capsid is the tegument consisting of an amorphous structure of variable thickness and distribution. This in turn is surrounded by a bi- or trilaminar envelope composed of lipids with at least ten, sometimes more, virally-encoded glycoproteins embedded (Roizman, 1996; Minson, 2000; Davison, 2002). The glycoproteins protrude from the envelope as spikes, also called peplomers, having carbohydrates covalently linked to them. By a modifying effect on the cell membrane, they play a role for virus entry into and spread between cells, as well as syncytia formation (Murphy, 1999; Minson, 2000). The mechanisms behind these effects are complex and not well understood and several glycoproteins may act in concert. Studies on HCMV indicate that the glycoproteins gB, gH and gL together induce cell fusion (Kinzler and Comton, 2002). Furthermore, these spikes also carry important immunological functions.

The whole virion has a diameter varying between 120 and 300 nm. The thickness of the tegument and state of the envelope most probably account for the observed variation in virion diameter (Roizman, 1996; Minson, 2000).

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Herpesvirus genome

The DNA genome is linear and double stranded. However when released from the capsid, to enter the nuclei of the infected cell, the DNA acquires a circular shape.

The virion contains at least 30 structural polypeptides. The size of herpesvirus DNA varies between 120 and 245 kilobase pairs (kbp) and the GC content between 31% and 75%, depending on species (Roizman, 1996; Minson, 2000; Davison, 2002). There is also a small (up to 10 kbp) variation in size of DNA within a species depending on the number of terminal or internal repeats. Another cause of variation in size is the occurrence of spontaneous deletions (Roizman, 1996).

Complete nucleotide sequences are now available for at least 26 herpesviruses (Davison, 2002). The number of open reading frames varies between 70 to more than 200 (Mocarski, 1994; Sample, 1994; Minson, 2000).

The proteins encoded by the herpesvirus genome are divided in the following three groups:
1) Proteins involved in viral replication (encoded by immediate early and early genes);
2) Structural proteins (encoded by late genes);
3) A heterogeneous and variable group of non-essential proteins.

All herpesviruses encode enzymes and other molecules involved in the replication of nucleic acids (Roizman, 1996). Analyses of open reading frames (ORFs) have revealed 43 core genes, grouped in seven blocks, which are conserved among all mammalian herpesviruses. The core genes code for capsid proteins, glycoproteins, and enzymes required for DNA replication etc. (Dominguez et al., 1999; Davison, 2002).

The number and position of terminal and internal repeats in the genome have been used for classification of the herpesviruses into six groups, termed A-F (Roizman, 1996; Murphy, 1999). The terminal repeats can change position and direction and for example HCMV exist in several isomeric forms.

Host range and replication

Host range and effect on the infected cell

The members of *Herpesviridae* are grouped into the subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. In the text that follows, the vernacular names will be used with the exception for the section on taxonomy.

Alphaherpesviruses are characterised by a variable, sometimes broad host range *in vivo* and *in vitro*, short replication cycle and rapid spread in cell culture, causing destruction of the cell monolayer and the ability to establish latent infection in sensory ganglia (possibly also other tissues). Aujeszky's disease virus of pigs

(pseudorabies virus) and Herpes simplex virus of man can exemplify the Alphaherpesvirus genus.

Betaherpesviruses are characterised by restricted host range *in vivo* and *in vitro*, infecting only one specific cell type in most cases. Another feature is the long replication cycle and the slow progress of infection in cell culture, often causing enlargement of infected cells (cytomegaly). Latent or persistent infections can be established in a range of tissues. Betaherpesvirus can be exemplified by Murine cytomegalovirus in mice, Porcine cytomegalovirus (Suid herpesvirus 2) in pigs and Human cytomegalovirus (HCMV) in humans.

Gammaherpesviruses are mostly but not always restricted to the host's family or order. These viruses may cause lysis of certain infected cell types such as fibroblasts *in vitro*, while replication in lymphoblastoid cells rarely results in the production of infectious progeny. Specificity for either B or T-lymphocytes is a common feature. These viruses may cause lymphoproliferative disease in their host and latent infections can be established in lymphoid tissues. Gammaherpesvirus can be exemplified by Equine herpesvirus 2 in horses and by Epstein Barr virus in humans.

It has been estimated that these subfamilies arose 180 to 220 million years ago (McGeoch et al., 1995).

The Channel catfish herpesvirus is the only known member of a recently formed subfamily called the Icalurid herpesviruses.

Finally, there are herpesviruses that have not yet been assigned to a subfamily (including many herpesviruses of molluscs and amphibians), as well as herpesviruses that have been assigned to a new sub family in the light of emerging genetic information, e.g. Mareks disease virus (Gallid herpesvirus 2) (Roizman, 1996; Minson, 2000).

Herpesvirus replication

The patterns of replication vary between the various members of the family. For example, the following outline is characteristic for the replication of HSV (Roizman and Sears, 1996; Davison and Clements, 1997).

The virions enter the cells by attachment of the viral glycoprotein spikes to receptors on the cell surface, followed by the penetration of the nucleocapsid. Penetration occurs by either fusion of the envelope with the cell membrane or through phagocytosis in a vacuole. The exact nature of the attachment has not been clarified. A recent study indicates that in HSV 1 glycoprotein D interacts with nectin, Hve A or Heparan sulphate (Cocchi et al., 2002). Other recent studies implicates that lipid rafts play an important role in HSV and PRV entry (Bender et al. 2002; Favoreel et al. 2002). The nucleocapsid with tegument proteins, but without membrane, migrates through the cytoplasm, to the nuclear membrane through which virus DNA and some associated proteins enter the nucleus.

A common feature for all herpesviruses is that viral DNA synthesis as well as formation of capsids is carried out in the nucleus (Roizman et al., 1992; Roizman, 1996; Murphy, 1999). The synthesis of host cell protein cells is inhibited, a

notable exception being HCMV wich stimulates, and alters, the cells own DNA synthesis (Gibson, 1994; Mocarski, 1996). Transcription of immediate early genes is initiated by cellular RNA polymerase II without prior protein synthesis (Roizman, 1996; Minson, 2000). The protein arising from transcription of immediate early genes into mRNA and its further translation starts expression of other viral genes. These genes can be divided into delayed early and late genes.

The replication of virus DNA and the nucleotide metabolism is directed by the delayed early genes while the structural proteins to a large extent are controlled by late genes (Wagner, 1985; Roizman, 1996). The proteins encoded by delayed early genes exert a negative feedback mechanism on the transcription of immediate early genes.

In the nucleus, newly synthesised DNA is cleaved and packaged into capsids. The capsids fuse with the nuclear membrane and bud into the cytoplasm, acquiring envelopes simultaneously. This envelope may be retained or, possibly, a new envelope is acquired during the egress through the cell membrane.

Mature virions accumulate in cytoplasmatic vacuoles, before being released from the cell by either exocytosis or cytolysis. The exact nature of envelopment and release from host cells remains unresolved. Production of virus progeny invariably results in cell death in a time course that can vary between hours and days (Roizman, 1996; Minson 2000). However if infection of a cell is established as a latent infection (see below) this does not result in cell death.

Latency
The ability to cause latent or persistent infections is another feature common to all herpesviruses.
In the latent state, the virus DNA has a circular form and gene expression is minimal (Roizman, 1996; Murphy, 1999). Transcription of the genome may be restricted to the production of latency-associated transcripts (LAT's). These *mRNA* molecules are not translated into proteins.

However, this is not an absolute rule for all herpesviruses. For example, in the case of EBV, nine proteins are expressed during latency in B-lymphocytes (Sample, 1994; Roizman, 1996; Murphy 1999).

It is not known whether members of the betaherpesviruses attain a true latent stage or just persist at a low level of replication (Mocarski, 1996).

Latent infection can be turned into productive infection by appropriate stimulis, like stress, disease or hormonal alterations.

Alphaherpesviruses establish latent infections predominantly in specific sites like bulbus olfactorius, tonsils, sensory nerve ganglia etc. while Gammaherpesviruses are thought to establish latency in lymphocytes. Betaherpesviruses are thought to establish latent or persistent infections in an array of different tissues and cell

types like, secretory glands, kidneys, peripheral blood monocytes, granulocyte-macrophage progenitor cells, lymphoreticular cells or haematopoietic stem cells Other tissues may also be implicated but the exact location of latency remains unresolved.

BETAHERPESVIRINAE

Members of the *Betaherpesvirinae* subfamily are ubiquitous and have been isolated from a wide array of animal hosts. Each virus species has a narrow host range both *in vivo* and *in vitro* (Staczek, 1990 Roizman, 1996; Murphy, 1999).

Taxonomic considerations

The subfamily consists of three genera, *Cytomegalovirus*, *Muromegalovirus* and *Roseolovirus*.

A typical member of the *Cytomegalovirus* genus is HHV-5 (HCMV).

Typical members of the *Muromegalovirus* genus are Mouse cytomegalovirus and Rat cytomegalovirus.

The only assigned members of the *Roseolovirus* genus are Human herpesvirus 6 and Human herpesvirus 7.

There is also an extensive list of species in the *Betaherpesvirinae* subfamily that has not yet been assigned to a specific genus. Porcine cytomegalovirus belongs to this list.

Genome and effects on the host cell

Genome

The Betaherpesvirus genome is significantly larger than that of other herpesviruses and spans between 180 and 240 kbp (Mocarski, 1996). Exceptions are the genomes of HHV-6 and HHV-7, which are restricted to approximately 150 kbp similarly to HSV-1 (Gompels et al., 1995; Nicholas, 1996).

Effects on the host cell

A characteristic feature of Betaherpesvirus replication *in vivo* and often also *in vitro* is the enlargement of infected cells often featuring a prominent and strongly basophilic inclusion body in the nucleus and sometimes in the cytoplasm (Done, 1955; Rac, 1961; Staczek, 1990; Britt, 1996;).

PORCINE CYTOMEGALOVIRUS (PCMV)

Characteristics of the virion and the genome

Virion Structure

By EM PCMV has a structure that is similar to other herpesviruses. The whole virion measures 120-170 nm in diameter. The core may have the shape of one or two electron dense toroids, that can be penetrated by a less dense cylindrical mass or have the shape of a cylinder with electron dense material spirally wound around, all of these structures connected, from both ends, to the inner surface of the capsid. Spherical or ring like cores have also been observed. Some authors reported that in electron microscopy preparations the nucleocapsid has an electron dense coat possibly representing the tegument seen in other herpesviruses, separated from the single or double unit membrane by a halo. However, this is a matter of controversy. The envelope is covered by external projections of about 10 nm in size (Duncan et al., 1965; Valicek and Smid, 1979; Shirai et al., 1985).

Genome

Genetic characterisation of PCMV has, to the best of my knowledge, not been published prior to 1999. A Japanese group have recently published the DNA sequence for the Major capsid protein (MCP), including some flanking sequences, and a short sequence from the DNA Polymerase gene of PCMV (Rupashinge et al., 1999; Widén et al., 1999).

Host range and replication

Host range

The *in vivo* host range of PCMV is restricted to pigs. The *in vitro* host range is also very narrow, because the cell cultures, which are usually applied for isolation of viruses from pigs (like swine kidney, testicles, etc, do not support the replication of PCMV. Thus, special cultures of porcine alveolar macrophages (PAM) have to be prepared for PCMV isolation. Interestingly, the replication is slow even in PAM and titres usually do not reach higher than 2-2.5 $TCID_{50}$ (Faithfull, 2001). Titres up to 5.5 $TCID_{50}$ were the highest reported in the literature (Edington, 1992). The slow replication of PCMV coincides with a limited life span of PAM. The effect of this is lower titres and poor sensitivity of diagnostic techniques based on this system. Furthermore, the presence of PCMV antibodies results in a markedly slower virus replication.

Since PAM do not undergo cell division, a constant supply of new cells is necessary. Because of the very high prevalence of PCMV, it is necessary to collect PAM from specific PCMV free herds, established for the purpose. At the moment, there are very few such herds in Europe and the availability is very restricted. A considerable batch-to-batch variation in terms of permissiveness to PCMV replication has also been reported (Plowright, 1976).

The use of a porcine fallopian tube cells (PFT) for replication of PCMV has been reported (Bouillant et al, 1975; Kawamura, 1992). However, failure to replicate

PCMV in these cells has also been reported (Edington, 1989). The author's attempts to replicate PCMV in PFT cells have been inconclusive.

In conclusion, *in vitro* replication of PCMV is hampered by major obstacles, is prone to substantial variation and generally inefficient.

Effects on the host cell

PCMV causes marked swelling (cytomegaly) of salivary gland epithelial cells. In these cells both the cytoplasm and nucleus are markedly enlarged. The cytoplasmic swelling is caused by an extension of the endoplasmatic reticulum, Golgi apparatus and mitochondria while the swelling of the nucleus is the result of production of viral particles.

In the nucleus a prominent oval, strongly basophilic, granular intranuclear inclusion (8-12 mm), connected with the nuclear membrane by fine strands, can bee seen (Done, 1955; Rac, 1961; Duncan et al., 1965). Eventually, the inclusion body fills up almost the whole nucleus, displacing the nucleoli towards the periphery, giving infected cells a so called "bird's eye" appearance (Done, 1955; Rac, 1961; Shirai et al., 1985).

These inclusion bodies are thought to consist of dense granular chromatin and membrane bound particles (Done, 1955; Rac, 1961; Duncan et al., 1965). Acidophilic inclusion bodies, markedly smaller than the basophilic, can be seen in the cytoplasm and, occasionally, in the nucleus (Shirai et al., 1985).

Specific features of replication

With electron microscopy, the nucleocapsids can be seen, solitary clustered or arranged as crystalloid arrays in the nucleus and the cytoplasm. Mature virions, carrying external projections on the membrane, are located inside the rough endoplasmatic reticulum or free in the cytoplasm.

The crystalloid arrangements as well as the solitary and clustered nucleocapsid appear in the cytoplasm at a late stage of replication and appear to pass through the nuclear membrane after it has been damaged, this however not completely accepted. The question of wether structures can be seen in cell cultures infected with PCMV, is disputed.

Non-enveloped nucleocapsids that are seen free in the cytoplasm may become enveloped by budding into vacuolar or tubular membranes or via the Golgi apparatus. Acquisition of envelope may also result from budding into intranuclear vesicular structures that probably represents reduplications of the nuclear membrane.

The existence of dense bodies, viroids not carrying DNA, as described for HCMV, is disputed (Duncan et al., 1965; Valicek et al., 1973; Yoshikawa et al., 1981; Valicek and Smid, 1979; Shirai et al., 1985).

Transmission, clinical signs and laboratory diagnosis

Virus transmission and clinical signs

The virus is transmitted horizontally and vertically. Horizontal transmission is likely to occur during weaning in herds were PCMV is endemic or at the introduction of new stock in free herds (Edington, 1989).

The nasal cavity is the principal port of entry and site of primary viral replication. The virus is excreted with nasal discharge, saliva, urine and cervical discharge, even in the presence of antibodies (Edington, 1992).

In piglets infected at the age of three weeks, or more, virus excretion lasts for a around two days. Virus can be isolated from the epithelium of the nasal mucosa and the kidneys, the predominant sites of replication in pigs over three weeks.

In such pigs, the infection is more likely to be subclinical, but poor weight gain and lengthy respiratory problems may develop, sometimes complicated by secondary bacterial infections. In herds were the virus has been endemic for some time, clinical signs tend to occur sporadically because maternal antibodies may confer partial protection of piglets and reduce the risk for vertical transmission. The occurrence of clinical signs in seropositive herds appears to be partly dependent on management practices (Edington, 1992).

Vertical transmission is most likely due to virus introduced in PCMV free herds. In non-immune sows and gilts, transplacental infection of foetuses may occur at any stage but is more frequent in mid or late gestation. Infection of foetuses takes at random and mummified, stillborn, congenitally infected, as well as uninfected piglets can be seen at the time of farrowing. Abortions are rare.

In non-immune herds, the introduction of PCMV also leads to early horizontal transmission with infection of young piglets. Congenitally and neonatally infected piglets frequently die soon after birth.

In piglets, clinical signs are most frequently seen around the age of two weeks. These pigs show dyspnoea, sneezing and often also anorexia. A serous nasal discharge may be observed, becoming mucopurulent, and sometimes blocking the nostrils (Murphy, 1999).

This condition is named *inclusion body rhinitis* because inclusion bodies appear in the histopathological sections from the mucus glands in the turbinate epithelium. Piglets with blocked nostrils have difficulties with suckling, they rapidly lose weight and die within a few days (Murphy, 1999).

Infection, caused by early horizontal or vertical transmission, may also result in generalised spread of virus frequently leading to death especially in neonates. Secondary bacterial infection is a common feature.

Introduction of PCMV in seronegative herds may cause serious losses due to foetal death, high piglet mortality and unthriftiness in young pigs due to lengthy respiratory infections.

Vertical transfer of virus across the placenta sometimes occurs in seropositive sows (Edington et al., 1988). Virus excretion can be detected for two weeks to one month p.i. in piglets infected congenitally or during their first day of life (Edington et al., 1976; 1977).

In infected foetuses virus can be isolated most frequently from lungs and liver but also from spleen, kidney, brain, nasal mucosa, peritoneal macrophages and periosteal cells (Edington et al., 1977; Hornedo and Edington, 1987).

In the adult pig, including pregnant sows, infection is silent, apart for a few days of lethargy and anorexia.

PCMV has a predilection for epithelial cells in pigs over three weeks, whilst in the foetus or neonate the viruses tend to infect reticuloendhotelial cells, like the endothelium of capillaries and the sinusoids of lymphoid tissues (Edington et al., 1976).

The high prevalence of PCMV in the pig population with a low incidence of clinical disease indicates that this virus is well adapted to its host.

Laboratory diagnosis by conventional means

Histopathology can be used to diagnose inclusion body rhinitis and tentatively for detection of systemic infections in young pigs. Electron microscopy is a practical tool to detect virus, but the method lacks sensitivity and specificity.

Virus isolation also a provides means of diagnosis, but requires a source of PCMV free PAM and is very time consuming due to the slow replication of the virus and the need for repeated passages. Other important limitations of this system are: 1) considerable batch variation in cell culture quality and sensitivity for virus isolation, 2) sensitivity to toxic substances in the inoculum and, 3) long replication cycle for PCMV *in vitro* which in combination with the restricted life span of PAM (approx. twelve days) The continuous detachment of cells leads to decreased sensitivity of the culture and hinders the development of higher virus titres (Plowright et al., 1976).

Antibodies to PCMV can be detected by indirect immunofluorescence, using infected PAM cultures (Watt et al., 1973; Rondhuis et al., 1980). This technique also requires a source of PAM free from PCMV.

Since the establishment of PCMV free herds is cumbersome, difficult and expensive, the availability is strictly limited and cannot be relied on for more extensive testing purposes.

The detection of antibodies by ELISA using plates coated with whole virus, has been attempted in the past (Assaf et al., 1982; Tajima et al., 1993). For this

technique, a prerequisite is an efficient system for production of virus in large quantities. As mentioned above, this is a serious constraint.

The above listed problems, underlines the need to develop novel diagnostic methods for PCMV.

PCMV and Xenotransplantation

The shortage of human tissues and organs suitable for transplantation has provided an incentive for research into alternative sources.

At present two different approaches are being investigated. These are: 1) Cloning of human stem cells and 2) Transplantation of tissues or organs from a non-human donor (xenotransplantation). Research into these two areas is currently very active. However, we have to admit that although both are promising, it will take long time until any of the two approaches will be introduced in practice.

The greatest barrier to the application of xenotransplantation is the immunological rejection of transplanted tissues. The recent development of so called knockout transgenic pigs has reduced some of the problems of graft rejection (Lai et al., 2002). However, the possibility of transferring infectious agents from the donor to the recipient also constitutes a major issue.

Transplant recipients are treated with immunosuppressive drugs, to reduce the risk of graft rejection, and therefore have impaired immunological defences. Furthermore, an infectious agent contained in the transplanted tissue will bypass important barriers to infection like the skin, and the mucosal linings of the airways and the gastrointestinal tract.

Because of these circumstances, the risk is a significant that an infectious agent of non-human origin, transferred together with the xenograft may cause damage to the transplanted tissue, infects the recipient and possibly spreads to other human beings. It is also possible that an infectious agent of human origin could inflict damage on the transplanted tissue.

The ability of HCMV to infect porcine cells has recently been proven (Degre et al., 2001). Furthermore, human and non-human infectious agents may enhance each other. Moreover, if a recombination between human and non-human virus occurs, the cross species transfer may be facilitated. The prolonged and very close contact between the transplanted organ and the recipient's tissues and body fluids increases the likelihood for such an event to take place.

The risk for xenogeneic infections is considered to be greatest with the use of non-human primates as donors because of their close evolutionary relationships. This risk in combination with ethical considerations and the scarcity of primates with an appropriate size for successful organ transplantation (chimpanzees) make it highly unlikely that non-human primates will be used for this purpose.

At present, the pig is considered as the most suitable donor animal. This is based on similarity in size, cardiovascular system and renal function amongst others. (Tumbleson, 1986; Cooper et al., 1991; Sachs D.H, 1994). However, consideration of possible infectious agents present in the donor animal still has to be taken.

There are several porcine viruses, which require a serious consideration when assuring the biosafety of xenotransplantation. Such agents are the endogenous retroviruses, which are invariably present in the pig genome and transplanted tissues are bound to harbour copies of the genome of this virus. Therefore, the risk posed by porcine retrovirus, to xenograft recipients, has to be assessed.

A number of other viruses present in the pig population, like for example Encephalomyocarditis virus, have to be taken into consideration.

Herpesviruses pose a special problem because of their ability to cause latent infections and to be transmitted vertically. This has to be taken into consideration when herds of donor pigs are established. The porcine herpesviruses known to date are: Aujeszky's disease virus (ADV, alias pseudorabies virus, PRV), PCMV and Porcine lymphotrophic herpesvirus (PLHV-1, PLHV-2, PLHV-3, Ehlers et al., 1999; Ehlers, 2002).

For ADV there are diagnostic assays and vaccines available. Eradication programmes have been successfully employed in many countries, including Sweden. For PLHV and PCMV there are neither practical diagnostic assays, nor vaccines available curently. Out of this collection of porcine viruses, raising a potential danger in biosafety, we selected PCMV as the subject of our studies. The very high prevalence of PCMV in the pig population and its ability to remain as a persistent or latent infection for life complicates the establishment of free herds and necessitates the use of very sensitive tools for monitoring of donor pigs.

A recent study has demonstrated a possible mutual enhancement between PCMV and Baboon cytomegalovirus (BCMV) in a Baboon xenotransplantation model (Mueller. et al., 2002). Cross-species transfer of virus was also demonstrated as well as viral infection with clinical disease in the tissues of the native species for each virus.

These findings clearly strengthen the view that PCMV may constitute risk factor in relation to xenotransplantation. The findings that HCMV replicates in porcine cells indicate a potential risk for viral recombination (Degre et al., 2001).

In view of the high prevalence of HHV-6 and the relatively close relationship to PCMV at the genetic level, the possibility of recombination between these two viruses also merits investigation.

These findings underline the need for effective diagnostic methods to ensure that donor pigs are free from PCMV and for monitoring of xenograft recipients for PCMV infections.

Aims of the study

1) To provide information on the genetic arrangements in the PCMV genome in order to clarify the evolutionary relationship to other members of the *Herpesviridae* family and to provide a basis for the development of novel diagnostic techniques.

2) To study the genetic stability of PCMV and identify conserved and variable regions in the viral genome.

3) To develop a sensitive, specific and rapid method for the direct detection of PCMV in tissues.

4) To study the possibility of producing recombinant PCMV antigen for use in serological assays, circumventing the need for tissue culture and use of live piglets.

Materials and methods, briefly summarised

Since the Materials and Methods are described in the attached articles, here only a shorter summary is given.

Samples

Viruses and tissue samples

The virus strain PCMV B6 was obtained from the Royal Veterinary College, London, U.K. The virus strain OF-1 was obtained from University of Osaka, Osaka, Japan.

The German pig spleen sample #489 was obtained from an abattoir in Eberswalde, Germany. The Spanish pig lung sample #55 was obtained from University of Barcelona, Barcelona, Spain. The British pig spleen sample P1 was obtained from the pathology department, VLA, Addlestone, UK. The Swedish pig spleen sample #1469 was obtained from the pathology department, National Veterinary Institute, Uppsala, Sweden. DNA extracted from these samples will be referred to as "DNA samples" in the text below.

Serum samples

The PCMV positive pig serum VLA-98 was obtained from Scientific Services, VLA, Addlestone, UK. The positive sera K199B and K199K were obtained from the Royal Veterinary College, London, UK. A PCMV negative pig serum was delivered by the Institute for Animal Science and Health, Lelystad, The Netherlands.

Swedish pig sera from the field used in paper IV were obtained from the Virology department (206 samples) and from the Department of ruminant and porcine diseases (5 samples), National Veterinary Institute, Uppsala, Sweden. The sera from the Virology department had been collected as part of a surveillance program for Aujeszky's disease (AD) in Sweden and from samples submitted for routine diagnostic testing. All these sera had yielded negative result for antibodies to ADV on ELISA. The sera from Department of ruminant and porcine diseases had been collected from a Specific Pathogen Free (SPF) pig farm.

Methods

Extraction and purification of DNA

Extraction and purification of DNA from tissue and blood samples

DNA was extracted from PAM collected from young pigs at the tissue culture department, VLA, U.K. The young pigs were originating from the Institute of

Animal Health, Compton, U.K. DNA was also extracted from tissue samples specified above.

The QIAmp blood kit, QIAmp tissue kit, the Qiagen DNeasy Tissue Kit (Qiagen GmBH, Hilden, Germany) and the Isoquick Nucleic Extraction Kit (Orca Research Inc., Bothell, WA, USA) were utilised. Briefly, for Qiagen kits, tissue and blood samples are treated with proteinase K, after which the DNA is adsorbed selectively to resin in the presence of high concentrations of chaotropic salts at neutral pH, washed and eluted under low salt conditions in distilled water or Tris-HCl buffer. For DNA extraction from PAM, the Isoquick system was used according to a modified protocol. Briefly, cells were lysed with a chaotropic buffer, mixed with extraction matrix, heated and centrifuged. The aqueous phase was collected and extracted once again. Transfer RNA from bakers yeast (tRNA, Sigma-Aldrich) was added prior to precipitation with sodium acetate and isopropanol. The addition of tRNA has been reported to improve the recovery of HCMV DNA from blood samples, particularly when low loads of target are anticipated (Amparo et al., 1996). The Isoquick method is similar to phenol extraction but less hazardous.

Purification of PCR products for sequencing
PCR products were purified by adsorption of the amplicon DNA to resin in the presence of high concentrations of chaotropic salts at neutral pH, washed and eluted under low salt conditions in distilled water or Tris-HCl buffer. The QIAquick PCR purification kit (Qiagen GmBH) was utilised.

Extraction and purification of plasmid DNA
E. coli containing the recombinant plasmid was grown in Luria-Bertoni broth under selective antibiotic pressure, overnight at 37° C in an orbital shaker. Harvested E. coli bacteria was lysed at alkaline conditions with SDS containing buffer and proteins, chromosomal DNA and SDS were precipitated, at neutral pH, with high salt buffer containing potassium acetate and chaotropic salts, while plasmid DNA remained in solution. Subsequently plasmid DNA in the supernatant was adsorbed to resin, washed and eluted under low salt conditions in distilled water or Tris-HCl buffer. The Qiagene plasmid purification kit (Qiagen) was utilised.

Amplification and cloning of DNA
The polymerase chain reaction (PCR)
When this study was initiated, no DNA sequence data for PCMV was available and therefore our first approach was to use degenerate primers based on alignments of the sequences from Mouse cytomegalovirus 1 (MCMV 1), HCMV and Guinea pig cytomegalovirus (CaHV 2). However, this approach was not successful. We therefore tested a set of primers first used to amplify a short segment in the DPOL gene of HCMV and subsequently also for MCMV 1 and MCMV 2 (Rozenberg and Lebon, 1991; Beuken et al., 1996). PCMV specific primers were designed and used subsequently.

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PCR was performed as described by (Ref). For paper I, PCR was performed using Amplitaq *Taq* polymerase (Perkin-Elmer) while for papers II, III and IV, PCR we used Amplitaq Gold (Perkin-Elmer) and 5% dimethyl sulfoxide (DMSO).

Cloning of DNA

For paper I, the PCR product was cloned directly in to the plasmid vector pCR 2.1(Invitrogen Corporation, Carlsbad, CA, USA). In paper III, the amplicon from DNA sample P1 was cloned in to the plasmid pCR-XL-TOPO (Invitrogen). For paper IV, see "Protein expression" below.

Genome walking

The genome walking procedures described in the literature are basically of three types: inverse PCR (Ochman. et al, 1990), randomly primed PCR (Weber et al. 1998) and adaptor ligated PCR (Lagerström et al., 1991). The method used in this study was based on adaptor ligated restriction fragment libraries, using a nested PCR approach (Siebert. et al., 1995, Universal Gene Walker Kit, Clontech, Palo Alto, CA, USA and Genexpress GmBH, Berlin). Briefly, Genomic DNA is digested with *EcoRV, ScaI, DraI, PvuII* and *SspI* separately and phenol extracted, followed by ethanol precipitation. The digestion products are ligated to an adaptor possessing one blunt end. The fragments are ligated to an adaptor and used as templates in a long distance PCR with one gene specific primer and one adaptor specific primer. The product from the first PCR is used as template in a second PCR assay with a nested pair of gene specific and adaptor specific primers.

The long distance PCR method employs a mixture of DNA polymerases some with proof reading activity and some without.

The first short stretch of sequence from the DPOL gene, published in paper I, served as a base for attaining more sequence data. Short fragments of new sequence were obtained.

PCR primers were designed to amplify the sequence between the new DNA fragment obtained through the genomic walking procedure and the old fragment. A combination of long distance PCR and conventional PCR was used to extend the known sequence in the DPOL and the gB gene. Conventional PCR was performed as described for papers II and III above. Long distance PCR was performed using the Expand Long Template PCR system from Boehringer Mannheim. This system employs a combination of the *Taq* and *Pwo* polymerase enzymes. The *Pwo* polymerase has a proof reading exonuclease activity.

Expression of protein and utilisation for coating of ELISA plates

Protein expression

Polyacrylamid gel electrophoresis under denaturing conditions (SDS-PAGE), DNA transfer and Western Blot procedures were performed as described previously (Sambrook and Russel, 2001).

For expression of truncated gB protein in *E. coli* the pBAD/Thio-TOPO plasmid vector (Invitrogen) was utilised. Expression was induced by Arabinose. The cloned gene fragments were expressed as fusion proteins consisting of a Thioredoxin part that has been altered on two positions so it can form a His patch on the N-terminal side of the insert and carry a polyhistidine region on the C-terminal end, both having the ability to chelate divalent cations. This enables the purification of expressed protein on Nickel charged resin columns.

In order to analyse the expression of protein and to determine the optimal Arabinose concentration, the expression product was separated by polyacrylamid gel electrophoresis under denaturing conditions (SDS-PAGE).

This was followed by Western blot procedure using monoclonal antisera, against the fusion protein and polyclonal anti PCMV as primary antibody. As secondary antibodies, HRP conjugated rabbit anti swine HRP (Dakopatts) antibodies were used. Diamino benzidine was used as substrate to visualise antibody binding to protein.

Expressed protein was purified by adsorption to nickel charged resin (Probond, Invitrogen).

ELISA

The protein expressed in *E. coli* was used to coat Maxisorp 96 well ELISA plates (Nunc) overnight at +4° C. The assay was optimised by analysing the effect on the ELISA results of different buffers and other reagents. The known positive sera VLA-98 was used for the ELISA.. For coating, Tris-HCl buffer, pH 8.5, gave the best result and for blocking 1% Bovine serum albumin (BSA, Sigma) in phosphate buffered saline with Tween 20 (PBST), worked best. However, Casein in PBST gave almost identical results.

The optimal dilution of recombinant protein, horseradish Peroxidase (HRP) conjugated Protein G (BioRad) and primary antibody was determined by checkerboard titration. The combination that resulted in an optical density (O.D.) value around 1.0 at 450 nm with minimal background was chosen. This corresponds to a primary antibody dilution of 1:100, a conjugate dilution of 1:12000 and a protein dilution of 1:8000 for the U.K. strain B6 and a conjugate dilution of 1:6000 and a protein dilution of 1:4000 for the Swedish DNA sample 1469.

Known PCMV positive pig sera and known PCMV negative pig sera were used as controls in the assay. Positive and negative controls were included in duplicates on each ELISA plate. Each serum sample was tested in duplicate and both in wells coated with truncated gB protein (positive antigen) and with buffer only (control wells).

The O.D. values were read at 450 nm.

The O.D. value for the respective control well was subtracted from the value for the corresponding positive antigen well. The cut off point was calculated as twice the mean of the transformed O.D. value for the negative control. All O.D. values below 0.2 were considered negative regardless of the value for the negative control.

Results and Discussion

Sequence analysis of PCMV DNA

PCR based studies on the viral genome of PCMV

The PCR method is a versatile tool for the characterisation of genes, especially from slowly replicating or fastidious organisms. The application of conventional *Taq* polymerase, lacking exonuclease activity, may lead to erroneous results because mismatches introduced in the sequence are not corrected. The risk is accentuated by the sequencing of cloned amplicons. The application of conventional *Taq* polymerase mixed with a small amount of proof-reading DNA Polymerase, minimises the miss match rate. Repeated amplification and sequencing can also be used to overcome the problem with miss matches.

In the case of PCMV the *in vitro* host range is restricted to PAM and PFT cells. Since the utilisation of PFT cells was unsuccessful, PCMV free PAM needs to be replaced all the time and have strictly limited availability the employment of the PCR method was extremely constructive.

Similarity search analysis of PCMV DNA

With the primer pair selected from the HCMV DPOL gene, we were able to amplify a DNA fragment of approximately 0.5 kbp. Based on this sequence, a nested set of specific primers was designed

Comparison of the generated sequence with the European Bioinformatics Institute (EBI), nucleotide databases using the FASTA algorithm demonstrated that the sequence had the highest similarity to Betaherpesvirus DPOL genes and especially to HHV-6 and HHV-7

Phylogenetic comparison indicated that PCMV is a member of the *Betaherpesvirinae* subfamily, groups together with HHV-6 in the genus *Roseolovirus* and is distinct from HCMV. However, since these results were based on a small fragment in an ORF that spans more than 3 kbp, it was necessary to confirm the results, from paper I, by sequencing the complete gene.

Genomic walking generated short stretches of sequence in the DNA Polymerase (DPOL) and Glycoprotein B (gB) genes. Gaps in the sequence were bridged by PCR. In this manner the sequencing of 7.2 kbp DNA was completed.

This sequence encompasses the complete ORF's from U37, U38 (DNA polymerase gene), U39 (glycoprotein B gene) and the partial ORF's U36 and U40 (both starting from the 3' end).

The results from the sequencing were analysed by FASTA and phylogenetic trees were based on the complete DPOL and the gB gene sequence respectively. In both phylogenetic trees the PCMV sequence group together with HHV-6 and HHV-7, however the identity is lower for the gB than for the DPOL gene.

The results in papers II and III also show that in PCMV a block of genes exists colinear with those in HHV-6 and HHV-7. These analyses confirm the results of article I. This firmly assigns PCMV to the subfamily *Betaherpesvirinae* and clearly indicates that it belongs to the genus *Roseolovirus*.

The present study has shown that there are considerable similarities in sequence between PCMV on the one hand and HHV-6, HHV-7 on the other, for the genes studied. Similar results for the Major capsid protein (MCP) have been published (Rupasinghe V. et al., 2001).

Sequence variation dependant on the geographical origin of PCMV

In paper II the DPOL gene sequences in strains and DNA samples from United Kingdom (U.K.), Germany, Spain and Japan were compared. These strains and DNA samples were found to differ from each other both in nucleotide sequences and in the deduced protein sequences. The strain B6 from U.K. was the most different of the sequences compared.

These results were confirmed in paper III by comparing the gB sequences from the same four strains and DNA samples as well as a Swedish DNA sample. Furthermore it was demonstrated in papers II and III that the marked difference seen for the U.K. strain was not due to temporal factors or repeated passaging in cell culture but represents a true difference. Moreover, it was shown that the gB gene as well as the deduced gB protein of PCMV is more variable than the DPOL gene. Characterisation and comparison of the DPOL gene from four geographically distinct strains and DNA samples also demonstrates the existence of conserved and variable regions within the gene.

Characterisation of the PCMV gB gene

In paper III we describe that the gB gene from five different strains and DNA samples had a length varying between 2580 and 2581 nt and it is coding for a protein of 860 and 861 amino acid residues respectively, depending on geographical origin. The characterisation demonstrated the conservation of certain motifs and of 10, out of 11, cysteine residues. The conservation of these 10 cysteine residues is found in all three subfamilies.

Furthermore, the studies indicated the presence of a hydrophobic stretch probably constituting a signal peptide motif and two putative transmembrane regions that may anchor the protein in the membrane. Moreover, a potential cleavage site was found indicating that this protein is cleaved into two subunits similar to many other herpesviruses, like for example HHV-6, Aujeszky's disease virus, Bovine herpesvirus 1 and HCMV but different from HSV 1. These data strengthen the assumption that this is really the gB gene of PCMV.

The existence of potentially immunogenic regions was also studied. In HCMV, two immunogenic domains, capable of binding neutralising antibodies, named AD-1, AD-II and a third antibody binding site, close to the carboxy terminal, have been described. The domain AD-I seems to be immunodominant (ref). In PCMV gB, the homologous region is 45.6% identical with the AD-1 region in HCMV. Compared with the identity between the complete gB sequences of PCMV and HCMV (35.1%) this is significantly higher.

Development of new methods for laboratory diagnosis of PCMV

Comments on conventional methods

Laboratory diagnosis of PCMV infection has previously been based on virus replication in PAM, either for detection of virus by direct or indirect immunofluorescence (IF) or for serological assay by indirect immunofluorescent antibody test (IFAT). As mentioned previously, the limited access to PCMV free PAM and the low titres for PCMV replicating in PAM limit the usefulness of these techniques. An IFAT, utilising a virus stock with a titre of 2.0-2.5 TCID/50, may suffer seriously from poor repeatability and low sensitivity. The poor replication of PCMV in PAM should also have a negative effect on virus isolation in this system. This is accentuated by a limited life span of PAM. Therefore it is reasonable to believe that the use of PAM for isolation of PCMV is inefficient.. PFT cells have also been used for replication of PCMV (Kawamura et al., 1992). All PFT in use can be traced back to the same original clone. One group reported that the use of TPA, a mitogen substance, in the cell culture media accelerated the replication and caused marked enlargement of infected cells (Kawamura and Matsuzaki, 1995). In our hands, the addition of TPA to the media caused enlargement of infected as well as uninfected cells. It can be concluded that the direct and indirect detection of PCMV can be significantly improved by the introduction of techniques that circumvent the need for tissue culture.

Development of PCR assays for PCMV

Based on the conserved regions in the DPOL gene, two PCR assays, that presumably detect any PCMV strain irrespective of geographical origin, were developed. These assays have a detection limit of 1-10 PCMV DNA copies.

The PCR assays developed in this study provides a quick, sensitive, robust, repeatable and fairly simple assay for PCMV detection.

Expression of protein and analysis of its immunoreactivity

In paper IV, the existence of potentially immunogenic regions was studied further. This was done by calculation of antigenic index by the Jameson-Wolf algorithm in the computer program DNA Star (DNA Star, DNA Star Inc., Madison, Wisconsin, U.S.A.). Regions with high scores for antigenicity were chosen as candidates for expression experiments. Several short fragments were cloned in the pBAD/ Thio-TOPO plasmid vector and tested for protein expression. The analysis for antigenicity indicated that the region at the carboxy terminal end of gB downstream of the transmembranous anchor region contains long continuous stretches of protein sequence with high antigenic index. A 336 nt long fragment from this region was amplified by PCR and subsequently cloned in frame with the Thioredoxin gene in the expression vector pBAD-Thio/TOPO, as described above. A 112 residues long truncated protein from the carboxy terminal part of the gB was successfully expressed as a fusion protein. This truncated protein will be referred to as "gB3' protein". The region in PCMV gB gene that is homologous to the AD-1 region of HCMV gB was also cloned in same vector but expression of the corresponding truncated protein was unsuccessful.

When the expressed gB3' protein was analysed on Western blot, a single prominent band of the expected size was seen. After purification, this protein was used for coating of ELISA plates. Negative and positive control sera gave the expected results when tested at the predetermined dilution. Uncoated wells (buffer only) and wells coated with expression control protein gave similar O.D. values.

This demonstrates that background O.D. values are almost entirely due to unspecific binding of pig antibodies to the plastic surface. The negative control as well as tested field sera's had generally slightly higher background than the positive control.

Analysis of selected sera

This ELISA was used to analyse 198 Swedish pig sera the ratio of positives was 54%, the ratio of negatives was 37% while 7% of the samples gave inconclusive results.

Cellular location of the C-terminal part of glycoprotein B

The truncated protein used for coating of ELISA plates was selected because of a high antigenic index. This segment is part of a region described as an antibody-binding site in HCMV (Kniess, J. Virol 1991).

One can speculate that the part of the glycoprotein B where this segment is situated is actually the intracytoplasmatic domain (Whitbeck J.C. et al., 1988). The computer analysis of the gB protein sequence using the programme TMHMM 2.0 (Sonhammer et al., 1998) indicates that this is actually the case.
Nevertheless, the results from the use of topology prediction algorithms have to be interpreted with caution. Short transmembrane domains may be overlooked and the algorithms are based on the limited information about membrane proteins that is currently available. Thus, the latest generation of computer programmes, based on a Hidden Markov Model, are trained on a small number of samples with an over representation of proteins that are easier to analyse structurally. These two facts give a bias in the topology prediction (Ott et al., 2002).

The results from the application of the truncated protein in ELISA and Western blot demonstrate that it contains immunogenic domains that are indeed capable of inducing a strong immune response. A possible explanation for this is that the carboxy terminal part of gB is exposed to the immune system after cell lysis.

Another possibility is that the C-terminal part of the protein is located extracellulary on the infected cells' membrane. Our characterisation of the deduced PCMV glycoprotein B, described in paper III, indicates the presence of two transmembrane anchor sequences. As a consequence, the C-terminal part of the protein should be located on the extracellular side of the cell membrane thereby being exposed to the immune system.

Comparison of the truncated protein sequences from the UK strain B6 and the Swedish DNA sample #1469 revealed differences at two positions. These changes involve residues at positions 792 and 836. The substitution at position, 792 serine (1469) for phenylalanine (B6), has a marked effect on the antigenic index for this position. Thus, the antigenic index at position 792 is lower for the U.K. B6 strain than for the Swedish DNA sample 1469. It was demonstrated that the amino acid substitutions, mentioned above, have no significant effect when the truncated proteins are used for ELISA.

Validation of the serological assay

Validation of serological tests involves five stages of rigorous assessment (Anonymous, 2000). The ELISA developed in this study has been taken through the feasibility study (stage 1) and assay development and partly through standardisation (stage 2). The feasibility study demonstrated that the assay clearly discriminates between positive and negative control samples. The raw O.D. values were normalised by dividing them with the O.D. value for the positive control on respective plate. This was done to enable comparison of results from different

plates and from different occasions. During the assay development and standardisation stage optimal incubation temperatures and durations, coating buffer and blocking buffer were determined. Furthermore, optimal dilution of recombinant protein, serum samples and conjugate were determined by checkerboard titration. A limited test of repeatability was performed. The results from this test was acceptable since the coefficient of variation for the positive and negative control were below 20%. The analytical sensitivity was determined by end point dilution. The positive control had an end point of 1:800 while several serum samples had an end point of 1:1600. For further validation of the assay it is important to compare it with another assay. In this case the ELISA should be compare with the IFAT performed on PAM since this is the only serological assay that has been used extensively. So far this has not been possible because of the very limited supply of PAM and the poor replication of PCMV in these cells.

However, because of the limited validation performed, the test results from this ELISA have to be interpreted with caution. Although the results from the work presented in paper IV is indeed very promising it has to be emphasised that the ELISA is not ready for application in routine testing of pig serum samples. Further work is needed to study the diagnostic specificity and sensitivity of the test.

Concluding remarks

1. When these studies were initiated, no molecular characterisation of PCMV had been published. The initial study provided the first molecular characterisation and the first development of a PCR assay for PCMV.

2. The studies presented here provide information regarding the evolutionary relationship of PCMV to other members of the *Herpesviridae*, firmly assigning it to the subfamily *Betaherpesvirinae* and indicating that it belongs to the genus *Roselovirus* by demonstrating a relative high degree of relatedness of the DNA sequence to homologous genes in HHV-6 and HHV-7. The studies also provide information regarding conserved and variable regions in the DPOL gene and the variability of the DNA sequence related to interstrain differences and geographical origin of the virus DNA sample.

3. The molecular characterisation of the DPOL gene enabled the development of a novel, robust, sensitive, fast, effective and user friendly diagnostic techniques for the direct detection of PCMV irrespective of geographic origin.

4. The molecular characterisation of the gB gene and subsequent analysis of the deduced gB protein demonstrated that it contains a number of features that are conserved amongst many gB proteins of herpesviruses and indicates that it undergoes post translational cleavage and is anchored in the plasma membrane of infected cells by one or two hydrophobic trans membrane anchor regions.

5. The studies shows that there are considerable variation in the DPOL gene and gB gene sequences, as well as in the respective deduced protein sequences, related to strain or geographical origin and that the British strain B6 differs most and that this difference cannot be attributed to temporal factors.

6. Furthermore, the molecular characterisation of the gB gene made it possible to express a highly immunogenic truncated gB protein and to develop a serological assay, in an early stage of validation, that has given promising results when applied to a collection of field sera from pigs. This assay provides a novel, efficient, fast and user friendly technique for detection of antibodies to PCMV.

7. The direct and indirect diagnostic techniques developed in this study will facilitate future studies on PCMV as a pig pathogen and will be of use in the establishment of PCMV free pig herds. Furthermore, these techniques will be useful for routine diagnostic investigations of pig health problems by veterinary diagnostic laboratories.

8. In view of the plans to use pigs for xenotransplantation purposes, the molecular characterisation of PCMV and the diagnostic techniques presented in this study are useful for the establishment of PCMV free breeding herds free. The diagnostic techniques can possibly also be applied to the screening of human xenotransplant recipients. The molecular characterisation and the diagnostic techniques will also

be useful for research into the potential risks for xenogeneic PCMV infections and interactions between PCMV and human viruses. However in order to certify that donor pigs are free even from latent PCMV it is necessary to perform further research.

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