Australian Porcine Circoviruses

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By

Jill Muhling

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Declaration

I declare that this is my own account of my research and contains work that has not previously been submitted for a degree at any tertiary educational institution.

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Abbreviations

AA	amino acid
BBTV	banana bunchy top virus
BCV	bovine circovirus
BFDV	beak and feather disease virus
bp	base pairs
CaCV	canary circovirus
CAV	chicken anaemia virus
CFDV	coconut foliar decay virus
CoCV	pigeon (columbid) cicrcovirus
CMV	cytomegalovirus
CPE	cytopathic effect
CT	congenital tremor
DAB	diaminobenzidine
dH_2O	distilled water
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dNTP	dinucleotide triphosphate
DuCV	duck circovirus
DTT	dithiothreitol
EE	exudative epidermitis
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FCS	fading chick syndrome
GCV	goose circovirus
HPV	human papillomavirus
HRP	horseradish peroxidase
IFA	immunofluorescence assay
IL	interleukin
IPMA	immunoperoxidase monolayer assay
ISH	*in situ* hybridisation
LD	lymphoid depletion
Mab	monoclonal antibody
MCP	monocyte chemoattractant protein
NCBI	national centre for biotechnology information (USA)
nt	nucleotide
OCV	ostrich circovirus
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PCV	porcine circovirus
PDNS	porcine dermatitis and nephropathy syndrome
PMWS	postweaning multisystemic wasting syndrome
PNP	proliferative and necrotising pneumonia
PRDC	porcine respiratory disease complex
PRRSV	porcine reproductive and respiratory syndrome virus
Rep	replication protein
RF	replicative form
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SCSV	subterranean clover stunt virus
SPF	specific pathogen free
ssDNA	single stranded DNA

TLMV	TTV-like minivirus
TTV	TT virus
UTR	untranslated region
VP	viral proteins

List of Units

%	percent
°C	degrees celsius
cm^2	squared centimetre
g	gram
h	hours
kb	kilobase
kDa	kilodalton
L	litre
M	molar
mg	milligram
min	minutes
μL	microlitre
mL	millilitre
μM	micromolar
mM	millimolar
ng	nanograms
nm	nanometres
ρmoles	picomoles
s	seconds
V	volts
w/v	weight per volume
x g	gravity

Communications

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CHAPTER 1. GENERAL INTRODUCTION

Two types of porcine circovirus (PCV) exist, referred to as PCV1 and PCV2. PCV2 has been associated with disease syndromes in pigs, including that designated postweaning multisystemic wasting syndrome (PMWS), which has been identified in all regions of the world bar Australia (Hamel *et al.*, 1998; Allan *et al.*, 1999a; Onuki *et al.*, 1999; Martelli *et al*., 2000; Kyriakis *et al*., 2000; Wellenberg *et al.*, 2000; Done *et al.*, 2001; Trujano *et al*., 2001; Saradell *et al*., 2004; Castro *et al*., 2004; Jemersic *et al*., 2004; Maldonado *et al*., 2004; Wang *et al*., 2004; Motovski and Segales, 2004; Garkavenko *et al*., 2005). PMWS affects young weaner pigs and results in weight loss, tachypnea, dyspnea, enlarged lymph nodes and jaundice (Harding, 1998). PCV2 may also cause or contribute to other swine diseases such as congential tremors (CT) (Stevenson *et al*., 1999), porcine dermatitis and nephropathy syndrome (PDNS) (Rosell *et al*., 2000), reproductive failure (Meehan *et al.*, 2001) and several other emerging disease syndromes. PCV1 is currently considered to be non-pathogenic.

Although PMWS has not been reported in Australia, information on the distribution, variation and further characterisation of PCV in Australian pigs was necessary as it might provide insights into why there is no PCV-associated disease in this country.

The results reported in this thesis involved the detection and further study of porcine circovirus in Australia.

This chapter provides an outline of this thesis and the work undertaken, while Chapter 2 is a review of the relevant literature with particular reference to circoviral diseases. Chapter 3 describes the detection of both PCV1 and PCV2 in the Australian pig herd, using a multiplex PCR designed to differentiate between the two viral types. The association of Australian PCV with two disease outbreaks was also investigated. Following the detection of both viruses, it was important to genetically compare Australian PCV with overseas strains known to cause disease, and this was achieved with a sequencing and phylogenetic study as described in Chapter 4. Possible reasons for the genetic groupings and distribution of different PCV2 strains worldwide are also discussed in this chapter.

As PMWS is as yet unidentified in Australian pigs, the importation of pig meat into Australia from countries with the disease requires careful monitoring. Current protocols for the cooking of imported pig meat were designed to inactivate porcine reproductive and respiratory disease virus (PRRSV), and as such may not be effective against PCV. In this study (Chapter 5), Australian PCV2 was successfully infected into cell culture, and detected using a variety of techniques. Subsequently,

thermal stability experiments were performed using a newly-developed immunoperoxidase (IPMA) test. It was anticipated that this study would determine whether current importation protocols require revision, and the results would suggest that this is the case, with PCV2 unaffected by treatment comparable with current cooking protocols.

While no animal experiments were undertaken in this study, it may become necessary to infect pigs with Australian PCV to determine viral pathogenicity. Cell culture inoculums have been used in the past overseas, but problems with contamination and viral titre have been encountered (Fenaux *et al*., 2001). Viral infectious clones can be used to overcome these problems, so an infectious clone of Australian PCV2 was constructed, as described in Chapter 6. While time constraints prevented the clone from being infected into culture, it is anticipated that the construct would be infectious as it is based on a previously published method (Hattermann *et al*., 2004).

Chapter 7 is a general discussion of the results and conclusions from this study.

The detection and characterisation of Australian PCV as described in this study has provided further information on the status of PCV in the Australian pig herd, and also developed diagnostic tests to assist in future research. These tools will be important when assessing and managing the risk of Australia experiencing PCV-associated diseases.

CHAPTER 2. REVIEW OF THE LITERATURE

Circoviridae

The *Circoviridae* is a family of single-stranded DNA (ssDNA) viruses, with covalently closed, circular genomes (Todd *et al.*, 1991; Todd *et al*., 2005). There are three genera of animal circoviruses: *Circovirus*, *Gyrovirus* and *Anellovirus*. Porcine circovirus (PCV) is classified as belonging to the genus *Circovirus,* together with beak and feather disease virus (BFDV). Other animal circoviruses originating from geese, pigeons and canaries and cattle have been identified and tentatively placed in this genus. Chicken anaemia virus (CAV) has been assigned to the genus *Gyrovirus* (Todd *et al*., 2005) and the genus *Anellovirus* is proposed for the 2 human circoviruses, TT virus (TTV) and TTV-like minivirus (TLMV) (Phenix *et al*., 2001; Mankertz *et al*., 2000; Todd *et al*., 2001; Miyata *et al*., 1999; Takahashi *et al*., 2000; Hino 2002).

The plant circoviruses, such as coconut foliar decay virus (CFDV), banana bunchy top virus (BBTV) and subterranean clover stunt virus (SCSV) were previously classified as *Circoviridae*, however they are now classified separately as *Nanoviridae* (Todd *et al*., 2005). Although they are no longer in the same family, the plant and animal circoviruses do have conserved regions in their genomes associated with conserved functions such as replication (Bassami *et al.*, 1998).

Comparison of the animal circoviruses BFDV, PCV and CAV has revealed no common antigenic determinants and no DNA sequence identity (Todd *et al.*, 1991). The structure of PCV and BFDV is similar, but the structure of CAV is quite different, further supporting their separation into 2 genera (Crowther *et al*., 2003). The recent discovery and characterisation of a goose circovirus (GCV), a pigeon circovirus (CoCV), a canary circovirus (CaCV), and TTV and TLMV has provided more insight into possible evolution and relationships between members of the *Circoviridae* family.

Chicken anaemia virus (CAV)

CAV affects young chickens, causing transient severe anaemia due to the destruction of erythroblastoid cells, and immunodeficiency due to depletion of cortical thymocytes, which occurs via CAV-induced apoptosis, or programmed cell death (Noteborn *et al.*, 1994). The severity of the disease depends on the age of the affected chicks and the presence of secondary infections (Noteborn *et al.*, 1994). CAV has a virion diameter of 23-25 nm, and a genome approximately 2.3 kb in size. Three partially overlapping open reading frames (ORFs) encode potential viral proteins (VP) of molecular weight 52 kDa (VP1), 24 kDa (VP2) and

13 kDa (VP3) (Meehan *et al.*, 1992; Noteborn and Koch, 1995). The 52 kDa VP1 protein is the putative capsid protein and is probably encoded by ORF3, the only ORF large enough to produce it (Noteborn and Koch, 1995). VP2 is a putative non-structural virus protein required for assembly (Noteborn and Koch, 1995). Apoptin, the 13 kDa VP3, is considered to be responsible for depletion of chicken lymphoblastoid T-cells by inducing apoptosis. This is presumably achieved by regulating the appropriate genes involved in this process (Noteborn *et al.*, 1994). Apoptosis is important in CAV replication as it allows the release of new virus particles from the cell.

High genetic conservation of strains detected worldwide suggests there is only one CAV genotype (Kato *et al.*, 1995) although a putative strain of CAV designated CIAV-7, antigenically and serologically distinct from the reference strain, was recently identified (Spackman *et al*., 2002a). The pathogenesis of the 2 virus types is similar, although some of the lesions produced by CIAV-7 appear less severe than those caused by the reference strain of CAV. There is currently no sequence data available for CIAV-7, but Southern blot and PCR results suggested there was significant sequence variation between the 2 strains (Spackman *et al*., 2002b).

Beak and feather disease virus (BFDV)

Beak and feather disease affects both wild and captive psittacine birds (Pass and Perry, 1984; Bassami *et al.*, 1998), although recent evidence suggests that it may also affect some non-psittacine species (Rahaus and Wolff, 2003). In most affected birds, the disease is characterised by chronic progressive, symmetrical feather dystrophy and occasional beak deformity, and death usually occurs as a result of secondary infections (Pass and Perry, 1984; Bassami *et al*., 1998). The causative agent of beak and feather disease is beak and feather disease virus (BFDV), which has a virion diameter of 14-16 nm and a genome 1993 nt in size. The virus has 7 potential ORFs, but only 3 are present in all known isolates, suggesting there may be only 3 proteins encoded (Niagro *et al.*, 1998; Ritchie *et al*., 1989; Bassami *et al.*, 2001). Three BFDV proteins have been reported, with approximate molecular weights of 19.5, 23.7 and 26.3 kDa (Ritchie *et al.*, 1989). Based on similarity between BFDV, PCV and the plant circoviruses, the largest BFDV protein, possibly encoded by ORF1, is the putative Rep protein (Bassami *et al.*, 2001), while the protein encoded by ORF2 is the capsid protein (Niagro *et al*., 1998; Bassami *et al*., 2001). From the limited sequence data available, it appears that BFDV isolates are genetically conserved, with 84-97% nt homology (Bassami

et al., 2001).

Recently, isolates of BFDV have been found to cluster phylogenetically according to the avian host species in which they were detected, eg. parrots and cockatoos, suggesting the presence of BFDV genotypes (Ritchie *et al*., 2003; Raue *et al*., 2004).

Goose circovirus (GCV)

GCV was first identified in geese from a German commercial flock with a history of increased mortalities and runting (Soike *et al*., 1999). Circovirus-like particles were detected by electron microscopy, and after sequence analysis, the genome of the newly-identified GCV was found to be 1821 nt in length (Todd *et al*., 2001). The GCV genome had ambisense organisation, with one predicted major ORF on each of the viral and complementary strands (Todd *et al*., 2001). A potential circovirus-like stem-loop structure was also identified (Todd *et al*., 2001). The putative Rep protein, encoded from the largest ORF on the viral strand, had a predicted size of 293 amino acids, while the putative capsid protein, translated from the complementary strand, had a predicted size of 250 amino acids (Todd *et al*., 2001). Phylogenetic studies suggested that GCV was not as closely related to BFDV as was CoCV. In fact, based on capsid amino acid sequences, GCV may have a closer relationship to the PCVs than the other avian circoviruses (Todd *et al*., 2001). Based on sequence variation, it has also been suggested that several distinct groups of GCV are circulating in Europe and Asia (Chen *et al*., 2003).

Canary circovirus (CaCV)

CaCV was first detected in neonatal canaries with a condition known as 'black spot', characterised by abdominal enlargement, gall bladder congestion and failure to thrive (Goldsmith, 1995). The virus has since been identified by electron microscopy in adult canaries that died following a short illness characterised by dullness, anorexia, lethargy and feather disorder (Todd *et al.*, 2001). The CaCV genome is ambisense and 1952 nt in length, with 3 putative protein-encoding ORFs (Phenix *et al*., 2001). The largest ORF, on the viral strand, codes for the putative Rep protein, 290 amino acids in length and with a predicted size of 33.4 kDa. The complementary strand encodes a putative capsid protein 250 amino acids in length (30 kDa) and a protein 134 amino acids in length (14.9 kDa) (Phenix *et al*., 2001). A potential stem-loop structure was identified (Phenix *et al*., 2001). Phylogenetic analysis indicated CaCV was most closely related to CoCV, less closely related to BFDV and most distantly related to GCV and PCV (Phenix *et al*., 2001).

Columbid (pigeon) circovirus (CoCV)

A potential circovirus-like infection was first detected in a pigeon in 1993 (Woods *et al*., 1993). Further electron microscopy studies identified apparent circovirus particles in meat pigeons in association with lymphoid depletion (Coletti *et al*., 2000). Although subclinical infection with CoCV seems common, mortality in young birds with active infection may approach 10% (Pare *et al*., 1999). The pigeon (columbid) circovirus (CoCV) has a typical single-stranded circular DNA genome 2037 nt in size (Mankertz *et al*., 2000). Putative ORFs were identified on both the viral and complementary genome strands and a potential stem-loop structure was identified (Mankertz *et al*., 2000). The putative Rep protein, encoded from the largest ORF on the viral strand, had a predicted size of 316 amino acids (36.3 kDa), while the putative capsid protein translated from the complementary strand had a predicted size of 273 amino acids (31.9 kDa) (Mankertz *et al*., 2000). CoCV was related most closely to BFDV and more distantly to CAV, PCV1 and PCV2 (Mankertz et al., 2000).

Bovine circovirus (BCV)

Porcine circovirus will replicate in mononuclear cell cultures prepared from the peripheral blood of cattle, which has raised questions about the susceptibility of cattle to PCV (Allan *et al*., 1994). PCV antibody was also detected in approximately 35% of cattle sera tested, leading to speculation of a virus closely related to PCV infecting cattle (Tischer *et al*., 1995). When circovirus DNA was subsequently detected in aborted bovine foetuses and in cattle with respiratory disease, the agent was tentatively named bovine circovirus (BCV) (Nayar *et al*., 1999). This virus, however, was nearly identical to PCV2 (99%) therefore it is highly likely that the isolated virus was PCV2 replicating in cattle. It is unclear whether the isolated circovirus could have caused the clinical signs of respiratory illness and abortion, as experimentally, BCV failed to cause disease in cattle. Two subsequent studies have failed to find PCV antibody in cattle (Allan *et al*., 2000; Ellis *et al*., 2001) and the role, if any, of BCV or any other circovirus in cattle disease has never been established.

TT virus (TTV)

TTV was the first identified human circovirus, initially detected in a Japanese patient with post-transfusion hepatitis, which was not caused by any known hepatitis virus (Nishizawa *et al*., 1997). The genome of the unknown virus was determined to be DNA, but no homology to any other known nt or protein sequence could be found, and it could not be determined whether the infection was persistent or transient in nature (Nishizawa *et al*., 1997). Further research revealed that TTV was a single-stranded circular virus, extremely common in the human population (Mushahwar et

al., 1999) and capable of persisting in the plasma of infected individuals for many years (Miyata *et al*., 1999; Bendinelli *et al*., 2001; Pollicino *et al*., 2003).

The TTV genome is ssDNA approximately 3 kb in size, with 2 large overlapping ORFs (Bendinelli *et al*., 2001). The largest (ORF1) encodes a protein 770 amino acids in size, likely to be the capsid protein (Simmonds, 2002). It is possible that the second ORF, which is divided into ORF2a and ORF2b, may code for non-structural proteins. ORF2a is highly conserved between TTV genotypes, whereas ORF2b is highly variable (Kakkola et al., 2002).

Phylogenetic studies have shown that of the animal circoviruses, CAV appears to be most closely related to TTV (Bendinelli *et al*., 2001). Both viruses possess large genomes with potential coding and untranslated areas, and there is some amino acid motif conservation and identity in short areas of nt sequence (Bendinelli *et al*., 2001). However, unusual for circoviruses, there is significant size variation between TTV isolates, with genome lengths between 3808 and 3853 nt reported, and nt variation of up to 40% (Bendinelli *et al*., 2001). Several potential stem-loop structures have been identified in the untranslated areas of the TTV genome, but not all of them are conserved between all TTV isolates so their potential role in replication is uncertain (Bendinelli *et al*., 2001).

Several studies have shown that TTV is widespread in the global population, but more prevalent in post-transfusion hepatitis patients (Miyata *et al*., 1999; Bendinelli *et al*., 2001), and it is common for an individual to be infected with multiple strains of TTV (Mushahwar *et al*., 1999; Raffa *et al*., 2003; DeValle and Niel, 2004). The highest rates of infection detected were in South America and Africa, while the lowest rates were detected in the USA and Northern Europe (Bendinelli *et al*., 2001). TTV has also been identified in USA blood donors and patients suffering liver disease, and prior exposure to blood products is associated with an increased risk of TTV infection (Charlton *et al*., 1998). Although TTV is almost certainly transmitted laterally via blood products, it has also been theorised that TTV transmission may be vertical, based on the high rate of newborn infants negative for TTV that aquire the virus almost immediately after birth (Yokozaki *et al*., 1999). It has also been speculated that cross-species transmission of some genotypes of TTV can occur as TTV and TTV-like viruses are also common in non-human primates (Cong *et al*., 2000; Simmonds 2002; Thom *et al*., 2003).

The high incidence of viral infection in the absence of clinical disease has led to the proposal that TTV is a commensal human and primate virus, and despite some correlation with respiratory disease (Maggi et al., 2003) and liver disease (Miyata *et*

al., 1999), the clinical significance of TTV is yet to be determined. It has been suggested, however, that while TTV infection is extremely common, certain genotypes among the many (approximately 28) genotypes identified may be capable of causing disease (Takahashi *et al*., 2000). It is likely that the number of genotypes identified will increase as diagnostic tests are developed further. Recently, TTV-like viruses, highly divergent from those infecting primates, have been identified in pigs, cats and dogs (Okamoto *et al*., 2002) and it seems likely that more species-specific and cross-species infecting TTV-like viruses will be identified in non-primate mammals or other animals in the future.

TTV-like minivirus (TLMV)

TLMV was identified after the detection and characterisation of TTV (Takahashi *et al*., 2000). It is similar in genomic organisation to TTV and CAV, with a negative-sense genome approximately 2860 nt in size, including quite highly conserved untranslated areas accounting for approximately 25% of the viral genome. Similar to TTV, there are no predicted ORFs of significant size on the viral (positive) strand, and different isolates of TLMV differ markedly in genome size and sequence identity (Takahashi *et al*., 2000). The largest predicted ORF, ORF1, potentially encodes a protein about 660 amino acids in length, which is assumed to be a glycoprotein as there are several potential N-glycosylation sites, also found in CAV and TTV (Takahashi *et al*., 2000). A second potential ORF, ORF2, partially overlaps ORF1, and potentially encodes a protein approximately 90 amino acids in size (Takahashi *et al*., 2000) which may code for a novel dual-specificity phosphatase (Peters *et al*., 2002). A third potential ORF, ORF3, was originally predicted to encode a protein of about 130 amino acids (Takahashi *et al*., 2000), but this ORF is now suspected to be non-functional (Biagini *et al*., 2001). Sequence identity for both ORFs in 3 TLMV strains was low, but an amino acid motif $WX_7HX_3CXCX_5H$ common to CAV and TTV was identified (Takahashi *et al*., 2000). Based largely on its genetic organisation and some small areas of sequence identity, it was suggested that TLMV may be an intermediate between the rather remotely related CAV and TTV (Takahashi *et al*., 2000).

TLMV is estimated to infect approximately 75% of the human population (Biagini *et al.*, 2000) and it has been suggested that co-infection with TTV and TLMV is common (Thom *et al*., 2003). The low sequence identity found between TLMV strains would suggest there are a large number of genotypes infecting humans, similarly to TTV (Takahashi *et al*., 2000).

Ostrich circovirus (OCV)

A circovirus was first identified in the gut of an ostrich suffering from enteritis (Els and Josling, 1998). Since then, circovirus particles have also been found in cases of fading chick syndrome (FCS), a worldwide problem in the ostrich-farming industry, mostly affecting young chicks, causing depression, weight loss, anorexia and eventually death (Eisenberg *et al*., 2003). Mortalites of 30-90% have been reported (Terzich and Vanhooser, 1993). The cause of the syndrome is probably multifactorial, involving management issues, stress and bacterial and viral infections (Eisenberg *et al*., 2003). Circovirus DNA was identified by PCR in tissues of FCS cases, with partial sequence data indicating that is the virus detected was very similar to BFDV (Eisenberg *et al*., 2003). At present, however, there is insufficent information to determine whether the circovirus identified in ostriches is associated with disease, and more sequence data is needed to elucidate the relationship between this circovirus and other members of the *Circoviridae*.

Gull circovirus

A possible circovirus infection was identified in a terminally ill young black-backed gull in Manawatu, New Zealand (Twentyman *et al*., 1999). Suffering from chronic airsacculitis due to *Aspergillus* infection, the bird was one of many dead or dying gulls that had been seen in the same area. Histologically, there was moderately severe inflammation in the bursa of Fabricus with large basophilic, intracytoplasmic inclusions present that were similar to those detected in other circovirus infections. These pathological findings were considered to be consistent with the possibility that lymphocytic depletion and immunosuppression, caused by circovirus infection, allowed an overwhelming and ultimately lethal secondary *Aspergillus* infection (Twentyman *et al*., 1999). It was suggested that this circovirus may have been present in gulls for a long period, unnoticed previously as gulls are undomesticated and therefore not usually subject to pathological examination, and the mild clinical signs characteristic of most circoviral infections. Alternatively, the gull may have been an aberrant host for the virus (Twentyman *et al*., 1999). No sequence data has ever been published for gull circovirus, therefore it is not possible to determine whether this circovirus is a new virus specifically infecting gulls, or whether a known circovirus such as BFDV was able to infect a new host.

Duck circovirus (DuCV)

DuCV was first detected in a mulard duck with a feathering disorder and poor body condition (Hatterman *et al*., 2003). Electron microscopy revealed small non-enveloped spherical viral particles in bursal tissue, and it was determined by

sequence analysis that a new type of circovirus was present (Hatterman *et al*., 2003). The DuCV genome was 1996 nt in size, with a genomic organisation similar to the previously described avian circoviruses. The putative Rep ORF, located on the viral strand, was a predicted 292 amino acids in length (33.6 kDa), while the putative capsid protein was a predicted 257 amino acids in length (29.7 kDa) (Hatterman *et al*., 2003). Three potential minor ORFs were identified, 2 on the viral strand and one on the negative strand, but whether these ORFs encode proteins is uncertain (Hatterman *et al*., 2003). Similar to previously described circoviruses, DuCV possessed a small intergenic region thought to contain the origin of replication, but a second intergenic region was also present between the Rep and capsid ORFs, a feature unique to DuCV (Hatterman *et al*., 2003). Based on nt and amino acid sequences, DuCV is most closely related to GCV (Hatterman *et al*., 2003).

Raven circovirus (RaCV)

RaCV was identified in an Australian raven with feather lesions similar to those whch occur in beak and feather disease (Stewart *et al*., 2006). The virus was isolated and found to be 1898 nt in length, with two ORFs likely to encode a capsid and replication-associated protein. RaCV was found to be most closely related to CaCV and CoCV by phylogenetic analysis, however no further information on this virus is currently known.

Starling circovirus (StCV)

StCV was initially detected in Spanish wild starlings found dead during an outbreak of septicaemic salmonellosis (Johne *et al*., 2006). The virus was also identified in healthy birds from the same population, and no specific pathogenisis has been associated with StCV as yet. The virus was found to have the highest similiarity with CaCV, with ORFs potentially coding for capsid and replication-associated proteins also present (Johne *et al*., 2006).

Porcine circovirus (PCV)

PCV was first detected in 1974 as a persistent infection in the immortal PK-15 porcine kidney cell line (Tischer *et al.*, 1974). Eight years later, it was characterised as a small non-enveloped, icosahedral virus with a circular ssDNA genome (Tischer *et al.*, 1982). At 17 nm in diameter and with a DNA genome of 1759 nt (Tischer *et al.*, 1982), PCV was the smallest animal virus known. When first described, PCV was considered likely to be non-pathogenic, as it produced no cytopathic effect in the PK-15 cell line (ATCC CCL-33) (Tischer *et al.*, 1982). The virus was reported to replicate in monocyte/macrophage type cells of pigs and cattle, although not those of sheep or humans (Allan *et al.*, 1994b), but the

infection did not affect cell function (McNeilly *et al.*, 1996). However, more recent experiments with PCV suggest that it can in fact infect human mononuclear cells, and can even cause ultrastructural alterations (Arteaga-Troncoso *et al*., 2005). Later experimental infections of pigs with PCV did not result in any clinical signs of disease (Tischer *et al.*, 1986; Allan *et al*., 1995).

In 1997, an apparently new syndrome designated postweaning multisystemic wasting syndrome (PMWS) was described in Canadian pigs (Clark, 1997; Harding *et al.*, 1998). Retrospective data indicated that the disease may have been present in Canada as early as 1991 (Clark, 1997) and as early as 1986 in Spain (Rodriguez-Arrioja *et al*., 2003). Meehan *et al*. (1998) found that the PCV strains in PMWS-affected pigs in Canada and Europe had more than 96% nt identity when sequence homologies were compared, but they shared less than 80% identity at the nt level with the PCV infecting PK-15 cells (Hamel *et al.*, 1998). It was concluded that there were 2 types of PCV and Allan *et al*. (1998) proposed that the original PK-15 cell culture isolate should be referred to as PCV1 and the genetically different strains isolated from PMWS pigs should be referred to as PCV2. Both types of PCV have since been found to be common in pigs, and PCV antibodies and DNA have been detected in pigs in Asia, Europe and America (Dulac and Afshar, 1989; Tischer *et al.*, 1986; Allan *et al.*, 1994a; Edwards and Sands, 1994; Kiss *et al*., 2000; Kim and Lyoo, 2002; Toplak *et al*., 2004). PCV has also been detected in feral pigs (Schulze *et al*., 2003; Ellis *et al*., 2003).

Both types of PCV have multiple potential ORFs, only 3 of which probably encode proteins, and are referred to as ORF1, ORF2 and ORF3. ORF1 encodes a protein of approximately 36 kDa, thought to be the Rep protein as it contains conserved motifs previously found in proteins involved in rolling circle DNA replication (Niagro *et al.*, 1998). The 27.8 kDa protein encoded by the PCV1 ORF2 is the putative capsid protein (Mahe *et al.*, 2000). A slightly larger capsid protein (30 kDa) is encoded by PCV2 ORF2 (Liu *et al.*, 2001). The function of the ORF3 protein, if any, is still to be determined, although it may have a role in apoptosis (Liu *et al*., 2005). However, the ability of PCV2 to cause apoptosis is still uncertain.

The ORF1 proteins of PCV1 and 2 were antigenically related, with cross-reactivity shown in serological assays (Mahe *et al.*, 2000). This correlated with the high sequence conservation of ORF1 between PCV1 and PCV2 and suggested conservation in the antigenicity and probably functions of the Rep protein (Mahe *et al.*, 2000). Other similarities of PCV1 and PCV2 have also been detected: both

have very similar stem-loop structures, essential for viral replication, and the amino acid sequence of the ORF1-encoded proteins of PCV1 and PCV2 are 85% similar (Hamel *et al.*, 1998). However, the capsid proteins from PCV1 and PCV2 are antigenically distinct, with no serological cross-reactivity, consistent with their lower sequence identity (around 56%) (Mahe *et al.*, 2000). PCV1 and PCV2 have been successfully differentiated using antisense ORF2 riboprobes, although ORF1 probes failed to differentiate between the 2 types (Nawagitgul *et al.*, 2000). When the sequences of PCV1 and PCV2 were compared, Hamel *et al.* (1998) found 65% similarity and Morozov *et al.* (1998) found 80% similarity. It was suggested that the differences in amino acid sequences between these 2 strains may account for variation in pathogenicity (Hamel *et al*, 1998), and that point mutations may have occurred in PCV1, generating a pathogenic variant (PCV2) from the non-pathogenic PCV1 (Ellis *et al.*, 1998).

Comparative genetics

Genome organisation

Genome organisation is variable between the genera of animal circoviruses. Viruses classified in the genus *Circovirus* (PCV, BFDV, GCV, CoCV, CaCV and DuCV) all have ambisense genomes, that is, proteins are encoded by both the viral and complementary DNA strands (Niagro *et al.*, 1998; Mankertz *et al*., 1998; Todd *et al*., 2001). Conversely, CAV, TTV and TLMV have negative-stranded (antisense) genomes, and only one of the 2 strands of the double-stranded replicative form is transcribed to produce mRNA (Thom *et al*., 2003; Phenix *et al*., 1994). The replication strategy of OCV has not been studied, however it seems likely that it would possess an ambisense genome. PCV, GCV, CaCV, CoCV and BFDV all have a compact genomic arrangement, with a small intergenic region flanked by 2 ORFs in opposing orientation (Mankertz *et al*., 2004). In contrast, the 3 viruses currently placed in the genera *Gyrovirus* and *Anellovirus* (CAV, TTV and TLMV) all possess highly conserved untranslated regions (UTR) in their genomes (Miyata *et al*., 1999). Interestingly, while DuCV is similar in genome organisation to the other members of the *Circovirus* genus, a 231 bp UTR was identified between the 3` ends of the Rep and capsid ORFs (Hatterman *et al*., 2003). This region contains several direct repeat sequences, however the function and sequence conservation of this UTR is unknown (Hatterman *et al*., 2003).

Transcription

While CAV produces one large unspliced transcript approximately 2 kb in size (Phenix *et al*., 1994), both PCV1 and PCV2 produce multiple transcripts, many

from the same ORF (Mankertz *et al*., 1998;Cheung and Bolin, 2002; Cheung, 2003b). TTV produces 3 mRNAs of 3 kb, 1.2 kb and 1 kb from the complementary DNA strand only, all of which are subsequently spliced (Kamahora *et al*., 2000). The transcription strategies employed by TLMV, BFDV, GCV, CoCV, CaCV, DuCV and OCV have not been studied in detail, although it was reported that BFDV and CoCV can both use alternative start codons in different virus strains (Bassami *et al*., 1998; Todd *et al*., 2001).

Regulatory elements

Potential regulatory motifs and direct repeat sequences have been identified in the GC-rich UTRs which account for 25% of the TLMV genome, 20% of the CAV genome and 30% of the TTV genome (Miyata *et al*., 1999; Takahashi *et al*., 2000; Phenix *et al*., 1994). CAV promoter activity is associated with its UTR, while a tandem array of four 19 bp repeats in this region is thought to be an enhancer element (Phenix *et al*., 1994; Noteborn *et al*., 1994). Similar tandem repeat sequences have also been identified in CaCV, DuCV, PCV and BFDV in their much smaller intergenic regions (Mankertz *et al*., 1997; Bassami *et al*., 2001; Phenix *et a*l., 2001; Hatterman *et al*., 2003). While there is very little nt or amino acid sequence identity between TTV, TLMV and CAV, the presence and organisation of regulatory elements such as ATF/CREB binding sites and TATA boxes is similar (Phenix et al., 1994; Miyata *et al*., 1999; Thom et al., 2003). No TATA box has been identified in the genome of PCV, GCV or CoCV, although putative CAAT boxes have been identified in the sequence of all 3 viruses (Mankertz *et al*., 1998; Mankertz *et al*., 2000). A putative TATA box has been identified in the genome of DuCV but no CAAT box was present (Hatterman *et al*., 2003). Rolling circle replication-associated motifs and P-loop motifs are present in the genomes of GCV, DuCV and CoCV (Mankertz *et al*., 2000; Todd *et al*., 2001; Hatterman *et al*., 2003).

***Circovirus* Rep protein sequences**

The sequence of the ORF encoding the replication-associated (Rep) protein, is highly conserved between PCV, BFDV, GCV, CoCV, CaCV, the plant circoviruses and some geminiviruses (Bassami *et al*., 1998; Phenix *et al*., 2001;Todd *et al*., 2001). The highest similarity was found between BFDV and PCV, where there was 65% amino-acid similarity (Bassami *et al.*, 1998).

The Rep proteins of viruses in the genus *Circovirus*, the plant nanoviruses and the geminiviruses all contain common dNTP-binding motifs, characteristic of proteins involved in rolling circle replication (Mankertz *et al.*, 1998a; Niagro *et al.*, 1998). A

nt-binding site, often referred to as a P-loop motif, is also conserved between the Rep proteins of these viruses (Mankertz *et al.*, 1998a; Niagro *et al.*, 1998). There is little variation in these motifs between PCV, BFDV, CFDV, BBTV and subterranean clover stunt virus (SCSV), and their Rep proteins exhibit a high degree of amino acid similarity (Mankertz *et al.*, 1997). Based on this similarity, it has been suggested that the Rep proteins of these viruses evolved from a common ancestor (Bassami *et al*., 1998).

Stem-loops

A 9 nt motif, highly conserved in plant geminiviruses, plant nanoviruses and bacterial replicons such as ϕx174, all of which also have circular, ssDNA genomes, is thought to be the initiation site of rolling circle replication (Meehan *et al*., 1997; Mankertz *et al.*, 1998; Mankertz *et al*., 2000). Usually located in the putative intergenic region in the form of a stem-loop structure (Phenix *et al*., 2001), this nonamer element is conserved in circoviruses, with the exceptions of CAV in which only the nonamer is present, and TTV in which several putative stem-loop structures have been reported in the UTR but no nonamer appears to be present (Miyata *et al*., 1999). There have been no potential stem-loop structures or nonamer sequences reported for TLMV, possibly the result of insufficient sequence data. The nonamer sequence may vary slightly between the circoviruses, and the number of bases comprising the stem-loop itself may also vary (Meehan *et al.*, 1997; Bassami *et al.*, 1998; Todd *et al.*, 2001). Mutation of this nonanucleotide sequence has been shown to prevent replication of PCV (Mankertz *et al.*, 1997). The sequence of the nonanucleotide motifs for the animal circoviruses and some plant circoviruses are shown in Table 2.1.

Table 2.1. Stem-loop sequences of the animal and some plant circoviruses. From Harding *et al*. (1993), Boevink *et al*. (1995), Miyata *et al*. (1999), Todd *et al*. (2001b), Phenix *et al*. (2001), Rohde *et al*. (1995) and Hattermann *et al*. (2003).

Virus	9 nt sequence	Stem-loop(s)	Stem-loop size (bp)
PCV1	TAGTATTAC	Yes	12
PCV2	AAGTATTAC	Yes	10
BFDV	TAGTATTAC	Yes	10
CAV	TACTATTCC	No	-
GCV	TATTATTAC	Yes	11
CoCV	TAGTATTAC	Yes	20
CaCV	CAGTATTAC	Yes	8
DuCV	TATTATTAC	Yes	10
TTV	No motif	Yes (?)	-
TLMV	No motif identified?	??	-
OCV	Unknown	Unknown	Unknown
SCSV	TAGTATTAC	Yes	11
BBTV	TA(G/T)TATTAC	Yes	11
CFDV	TAGTATTAC	Yes	10

? indicates a degree of uncertainty.

PCV transcription and replication

The full transcription pattern of both PCV1 and PCV2 have recently been elucidated, and shown to be much more complex than previously thought.

PCV1

It was initially thought that 2 transcripts were synthesised from the Rep gene of PCV1, one comprising the full-length reading frame producing a protein of 312 amino acids (Rep), and the second transcript that is differentially spliced and encodes the Rep' protein, which was truncated to 168 amino acids (Mankertz and Hillenbrand, 2001). The splicing results in a frame-shifted C-terminal moiety of Rep', which deviates from the amino acid sequence of the Rep protein (Mankertz and Hillenbrand, 2001). Both the Rep and Rep` proteins were shown to be

essential for PCV1 replication (Mankertz and Hillenbrand, 2001), and it was suggested that the Rep protein may be a helicase while the Rep` protein is a nickase (Mankertz *et al*., 2003), although this has not been confirmed. The PCV1 origin of replication was mapped to an 111-bp fragment which included the stem-loop, and with 4 repeats of the sequence CGGCAG (H1, H2, H3 and H4) located adjacent to the stem-loop sequence (Mankertz *et al*., 1997), which are binding sites for the products of the Rep gene (Steinfeldt *et al*., 2001). The Rep and Rep` proteins both bind to the dsDNA containing the stem-loop and 4 repeat hexamer sequences in vitro, but their required binding sequences appear to be different (Steinfeldt *et al*., 2001). It is thought that the Rep-associated proteins then destabilize and unwind the replication origin DNA, nick the octanucleotide sequence (AGTATTAC) between the 6th T and the 7th A to generate a free 3′-OH end for initiation of plus-strand DNA replication (Cheung, 2005). Presumably for this reason, mutations of the 6^{th}, 7^{th} and 8^{th} nucleotides of this 8 nt sequence are lethal to the virus (Cheung, 2005). The PCV1 Rep gene promoter was mapped to an area immediately adjacent to the start of the Rep gene, where it overlapped the PCV1 origin of replication and also the intergenic region (Mankertz et al., 1997). This Rep promoter is repressed by the Rep protein, but not the spliced Rep` protein, the result of the Rep protein binding to 2 of the CGGCAG hexamer repeats (H1 and H2, not H3 or H4) (Mankertz and Hillenbrand, 2002). The Rep` protein has not been reported to influence the Rep promoter at all (Mankertz *et al*., 2004).

Based on Northern blotting analyses of PCV1 transcripts, a total of 3 mRNAs were initially thought to be transcribed from the PCV1 genome, of 1230 nt , 990 nt and 750 nt (Mankertz et al., 1998; Cheung and Bolin 2002). Mankertz *et al*. (1998) reported that 2 mRNAs were encoded by the viral negative strand and one by the positive strand. However, a subsequent study using RT-PCR detected 12 RNA transcripts associated with the PCV1 genome: a capsid RNA transcribed from the complementary DNA strand, 8 Rep-associated RNAs (Rep, Rep`, Rep3a, Rep 3b, Rep 3c-1, Rep 3c-2, Rep 3c-3 and Rep 3c-4), and 3 NS-associated RNAs (NS 462, NS 642 and NS0) all transcribed from the viral DNA strand (Cheung, 2003b). The 8 Rep-associated RNAs were found to share common 5` and 3` nt sequences with each other, and to share common 3` nt sequence with the 3 NS RNAs. It was determined that the Rep transcript is the primary transcript that gives rise to the other 7 Rep-associated transcripts by alternate splicing (Cheung, 2003b). The 3 NS transcripts were transcribed from 3 different promoters inside ORF1, all of

which were independent of the Rep promoter. The NS0 transcript appeared to be unspliced and coded for a potential protein 23 amino acids in length. The NS 462 and NS 642 transcripts do not appear to code for functional proteins as no AUG initiation codon was found (Cheung, 2003b). Commonly located in the cell nucleus, the PCV1 capsid protein is 234 amino acids in size, arginine-rich with a basic end-terminus (Mankertz *et al*., 2004). No regulation of the capsid gene by any PCV-encoded proteins has yet been demonstrated (Mankertz and Hillenbrand, 2002). The exact function of the Rep 3a, Rep 3b, Rep 3c-1, Rep 3c-2, Rep 3c-3, Rep 3c-4, NS 462, NS 642, and NS0 transcripts and any proteins transcribed from them is unknown (Cheung, 2003b). Mutational studies of PCV1 have revealed that only the Rep and Rep` transcripts are essential for PCV1 replication, and that directed mutation of all other transcripts does not affect Rep-antigen synthesis or DNA self-replication (Cheung, 2004).

PCV2

Nine RNA transcripts were detected from the genome of PCV2 using RT-PCR (Cheung, 2003), in contrast to the three transcripts found previously by Northern blot (Cheung and Bolin, 2002). The transcripts identified were the capsid RNA, 5 Rep-associated transcripts (Rep, Rep`, Rep 3a, Rep 3b and Rep 3c) and 3 non-structural protein transcripts (NS515, NS672 and NS0). The 5 Rep-associated RNAs all shared common 5` and 3` nt sequences, and they also shared common 3` nt sequence with the 3 NS transcripts. As in PCV1, it was suggested that the Rep transcript gave rise to the other 4 Rep-associated transcripts by alternate splicing and that the 3 NS transcripts were transcribed from 3 different promoters inside ORF1 (Cheung, 2003b).

Similarly to the PCV1 transcripts NS 462 and NS 642, NS 515 and NS 672 do not seem to code for functional proteins, and the function of the Rep 3c, Rep 3a, Rep 3b, NS 515 and NS 672 transcripts is not currently known (Cheung, 2003). The PCV2 capsid, Rep 3a, Rep 3b, Rep 3c, NS 515, NS 672 and NS0 transcripts have been demonstrated to be non-essential for PCV2 viral replication, but Rep and Rep`are essential for replication (Cheung, 2003c) as was previously reported for the equivalent PCV1 transcripts (Mankertz and Hillenbrand, 2001).

The requirement of both PCV types for 2 Rep-associated proteins is unusual as all other known examples of rolling-circle DNA replication require only one Rep protein (Handley-Bowdoin *et al.*, 2000). The Rep and Rep` proteins of PCV1 have been shown to be capable of initiating PCV2 replication (Mankertz and Hillenbrand, 2001) and the Rep 3a, Rep 3b and NS0 transcripts and potential

proteins are considered to have equivalent roles in PCV1 and PCV2 replication (Cheung, 2003b). PCV1 NS0 shares 61% nt homology with PCV2 NS0 (Cheung, 2003b), but there are differences in the expression levels of similar transcripts between PCV types. During PCV1 replication, the NS0 transcript is less abundant than the Rep` transcript, but during PCV2 replication the NS0 transcript is more abundant than the Rep` transcript (Cheung, 2003b). The remaining transcripts Rep 3c-1, Rep3c-2, Rep 3c-3, Rep 3c-4 and NS 462 and NS 642 (PCV1) and NS 515 and NS 672 (PCV2) appear unique to each PCV type (Cheung, 2003b).

The transcription patterns of both PCV types are complex. Both use similar initiation and termination signals at similar areas of the genome, but gene expression levels of some transcripts and splice junction selection result in some transcripts that are unique to each virus (Cheung, 2003b). Interpretation of the role of the transcripts in replication, and differences in the the transcripts of PCV1 and PCV2, could be important in determining the rates of replication of different strains and differences in pathogenicity between PCV1 and PCV2. Interestingly, the Rep and Rep` proteins of each PCV type are able to bind *in vitro* to the origin of replication of the other type, that is, the replication factors of PCV1 and PCV2 appear to be exchangeable (Mankertz *et al*., 2003). The implications of this are unclear.

Replication

Members of the *Circoviridae* all possess small genomes 1.76 kb (PCV) to 3.8 kb (TTV) in size (Todd *et al*., 2005). The absence of coding regions for DNA polymerases in the circoviruses would suggest that cellular enzymes are required for replication (Todd *et al*., 2001). Porcine circovirus is probably dependent on cellular proteins expressed during the S phase of cell growth (Tischer *et al*., 1987). The dependence of these viruses on cellular enzymes present during cell replication is supported by the finding that CAV antigen can be detected in only 30-40% of infected MSB1 cells in culture, the approximate proportion of cells that would have been undergoing cell division (Todd *et al*., 2001). The dependence of these viruses on dividing cells for replication is reflected in the type of cells that are infected *in vivo*, so circoviruses often replicate well in rapidly dividing tissues and cells, such as foetal tissue (Allan *et al*., 1995; Sanchez *et al*., 2003) and cells of monocyte/macrophage lineage (Rossell *et al*., 1999; Allan *et al*., 1994; Gilpin *et al*., 2003; Okamoto *et al*., 2002).

The replication of TTV and TMLV seems occur by a different mechanism. It has been suggested that TTV replication involves a dsDNA intermediate form

(Okamoto et al., 2000), and it is presumed that TTV and possibly TLMV replicate by rolling circle replication, however this has not been confirmed experimentally (Miyata *et al*., 1999). A possible intermediate replicative form of TTV was detected in the livers of monkeys experimentally-infected with the virus (Xiao *et al*., 2002). In TTV and TMLV, and perhaps relevant to their replication, the ORF3 possesses a degree of homology to the gene encoding DNA topoisomerase 1, a protein which catalyses ssDNA cleavage (Takahashi *et al*., 2000). It has been suggested that the protein encoded by the ORF3 of TTV and TLMV may play a role in replication (Takahashi *et al*., 2000). It is not certain, however, if the TLMV ORF3 is functional as this ORF is not present in all TLMV isolates (Biagini *et al*., 2001).

Circovirus replication probably occurs in the nucleus of the host cell, and OCV, PCV, CAV and CoCV have frequently been identified in the cell nucleus after infection (Studdert *et al.*, 1993; Tischer *et al.*, 1995; Coletti *et al*., 2000; Eisenberg *et al*., 2003). Liu *et al.* (2001) demonstrated that the nuclear localisation signal of the PCV2 capsid protein was conferred by the 41 N-terminal amino acids of ORF2.

A summary of the major steps thought to be involved in circovirus replication is shown in Figure 2.1.

Rolling circle replication

Viruses belonging to the family *Circoviridae* are thought to replicate genomic DNA by a mechanism known as rolling circle replication, a process of continuous elongation of the open positive-strand of DNA via the endless copying around a closed circular negative-strand template. This is a means of DNA replication common to the animal circoviruses, plant circoviruses and the geminiviruses (Dressler, 1970) (Figure 2.2).

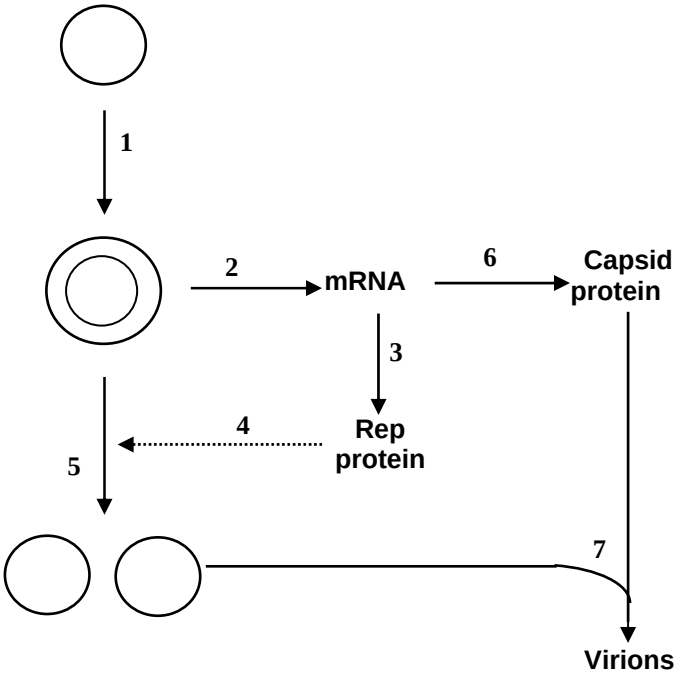

Figure 2.1. Summary of the major steps involved in circovirus replication. **1**. The ssDNA virus origin is converted into a dsDNA replicative form (RF), presumably by host DNA polymerase. **2**. Transcription of the RF DNA yields a number of mRNA transcripts. **3**. One of the mRNA transcripts produced is then translated into the Rep protein. **4**. The Rep protein introduces a nick into the RF DNA, initiating viral DNA replication. **5**. Rolling circle replication takes place, producing new progeny virus DNA. **6**. Another mRNA transcript is translated into the capsid protein. **7**. Assembly of the new progeny virus DNA and the capsid into new infective virions.

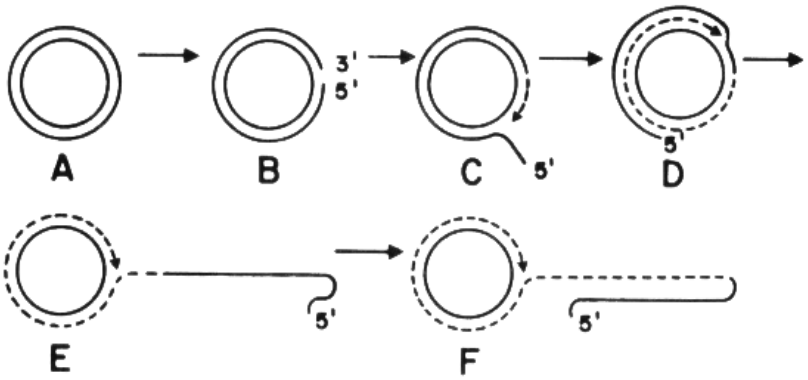

Figure 2.2. Diagrammatic representation of rolling circle replication. A circular dsDNA (an intermediate replicative form of DNA) molecule is shown in **A**. In **B** a nick opens one strand and the free 3'-OH end is extended by DNA polymerase. The newly synthesised strand displaces the original parent strand as it grows (**C, D**). By **E**, the polymerase has completed one revolution, and by **F**, 2 revolutions. The result is a molecule containing 3 unit genomes, one old and 2 new. The displaced strand can then serve as a template for a complementary strand. From King and Stansfield (1990).

Porcine circovirus replication

Within the nucleus, presumably using a polymerase of host cell origin, the naked single-stranded viral genome is converted to a double-stranded replicative form (Prescott *et al.*, 1993). Progeny virus is then produced via the circular dsDNA intermediate (Tischer *et al.*, 1995) which is assembled with the capsid protein to produce new virions. The new infective virions are then released when the infected cell goes through mitosis, thus infecting new daughter cells (Tischer *et al.*, 1995).

PCV has similarities with the *Microviridae* and *Geminiviridae*, in regions of the Rep proteins thought to be associated with DNA rolling-circle replication (Todd *et al.*, 2005), suggesting PCV replicates by a similar mechanism. As described above, a full-length and intact Rep protein, with a P-loop domain and rolling circle replication motifs, is required for replication of PCV1 (Mankertz and Hillenbrand, 2001) and PCV2 (Cheung, 2003c). The Rep and Rep` proteins appear to be co-localised in particular areas of the nucleus (Mankertz *et al*., 2004) and both PCV1 and PCV2 possess nuclear localisation signals (Cheung and Bolin, 2002). PCV2 can be detected in the nucleus of the host cell immediately after infection, but viral antigen becomes perinuclear and cytoplasmic as the infection progresses (Cheung and Bolin, 2002).

Virus evolution and genetic variation

Genetic change over time occurs in all viruses, due to mutation, recombination and reassortment. A high level of genetic diversity within a viral species may occur as a result of a widespread virus infecting a large number of hosts over a long period of time, and/or if the virus in question has a high mutation rate, either due to selective pressures or an inefficient replicative proofreading mechanism (Ritchie *et al*., 2003). The most common mutations involve single nt substitutions, deletions or insertions, collectively known as point mutations (White and Fenner, 1994).

The rate of mutation and therefore evolution is faster in RNA viruses than DNA viruses, and some RNA viruses evolve at higher rates than others (Ngui *et al.*, 1999). Mutations in the RNA viruses are largely caused by the absence of a 3` to 5` exonuclease proofreading activity in the RNA polymerase (Ngui *et al.*, 1999). Retroviral reverse transcription enzymes are particularly error-prone. Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase can introduce base-substitution errors translating to 5-10 errors per HIV genome per round of replication (Preston *et al.*, 1988). The low fidelity of HIV-1 reverse transcriptase is suggested to be responsible, at least in part, for the hypermutability of the virus (Preston *et al.*, 1988). Selection forces, such as the immune response and the

limited availability of appropriate target cells during transmission and persistence, can act on new viral variants and are likely to control the diversity and rate of evolution, and therefore sequence variation, of highly mutable viruses such as retroviruses (Overbaugh and Bangham, 2001).

The DNA viruses, such as the *Circoviridae*, are generally more stable and evolve more slowly, due to the proofreading ability of DNA polymerase (Ngui *et al.*, 1999). Replicative DNA polymerase proteins possess multiple proofreading mechanisms to prevent errors in replication (Perlow and Broyde, 2001) resulting in the lower mutability and therefore sequence variation of DNA viruses. The ssDNA viruses, however, despite this increased proofreading ability, have the potential for a large amount of intra-specific sequence variation, possibly because the DNA lacks the stability conferred by a double helix (Lindahl and Nyberg, 1974). For this reason ssDNA is susceptible to spontaneous deamination, which occurs when a cytosine or adenine residue loses an amine group through hydrolytic disassociation to form uracil or hypoxanthine, respectively (Duncan and Miller, 1980). This deamination is known to be a significant cause of spontaneous mutation (Coulondre *et al*., 1978). Most animal circoviruses, however, with the exception of TTV and TLMV, are highly conserved at the nt level.

Genus *Anellovirus*

Strains of CAV generally exhibit very limited genetic variability (Classens *et al*., 1991; Kato *et al*., 1995; Chowdhury *et al*., 2003). One study of 13 CAV isolates detected >96% identity at the nt level (Chowdhury *et al*., 2003), and this included the sequence of strains passaged multiple times in cell culture (Meehan *et al*., 1997b; Todd *et al*., 2002; Scott *et al*., 1999). The attenuation of CAV to a less pathogenic form in cell culture is well described, although cell culture attenuated strains will revert to virulence after inoculation into young chicks (Todd *et al*., 1995). Attenuation has been associated with minor nt and amino acid changes throughout the CAV genome (Scott *et al*., 1999; Meehan *et al*., 1997b). One specific change observed was a 21 bp insertion in the CAV UTR in attenuated strains, although this may be simply an adaption to replication in MDCC-MSB1 cells as the insertion was also found in pathogenic CAV strains (Meehan *et al*., 1997). Another possible explanation provided for attenuation is that capsid proteins with altered conformation caused by amino acid changes may be selected during multiple cell passages, and these altered protein may bind more efficiently to the receptor on host MDCC-MSB1 cells (Todd *et al*., 2002). Amino acid changes within the CAV VP1 (capsid) protein have been shown to affect the rate of CAV replication and the ability of CAV to replicate in

different types of MDCC-MSB1 cell lines, therefore it appears that minor amino acid changes may affect the cytopathogenicity and cell culture tropism (Renshaw *et al*., 1996). It has also been found that VP1 amino acid 394 is variable between high pathogenicity and low pathogenicity non-attenuated field strains (Yamaguchi *et al*., 2001).

TTV and TLMV are the only animal circoviruses that exhibit high genetic variability. This is unusual for DNA viruses, but possibly TTV is replicated by machinery with poor proofreading ability, leading to the high genome variation. The variation within the TTV genome is uneven: the UTR is conserved between isolates with segments more than 90% homologous at the amino acid level. However, the central region of the TTV ORF1 shares poor identity between isolates, with as little as 32% amino acid identity (Miyata *et al*., 1999). A possible reason for the high conservation of the UTR of TTV and TLMV is that they are situated close to TATA boxes and other regulatory elements (Thom *et al*., 2003).

CAV has been considered the closest known relative of TTV and TLMV (Miyata *et al*., 1999; Takahashi *et al*., 2000). While there are no significant nt or amino acid homologies between the 3 viruses, CAV and TTV share a similar genome organisation, including the presence and location of some putative regulatory sequences (Miyata *et al*., 1999). There are also some small areas of sequence which are conserved between CAV, TLMV and TTV (Thom *et al*., 2003) and an amino acid motif $WX_7HX_3CXCX_5H$ is common to all 3 viruses (Takahashi *et al*., 2000). CAV and TTV share a 36 nt region with approximately 80% homology, but whether this area is involved in any function conserved in TTV and CAV is unknown. The ORF1 proteins of all 3 viruses (TTV, CAV and TLMV) are arginine-rich at the amino terminus. The amino terminus of TLMV is also tryptophan and phenylalanine rich suggesting a common relationship between CAV and TLMV. The ORF2-encoded protein of TLMV, approximately 90 amino acids, is much shorter than the corresponding protein of CAV or TTV (Takahashi *et al*., 2000).

Additional evidence of a distant relationship between CAV and TLMV is the observation that the regions of CAV encoding VP2 and the TLMV ORF2 may both code for a novel dual-specificity phosphatase (Peters *et al*., 2002). Protein phosphatases are known to function in the regulation of mitogenesis, gene transcription, signal transduction, cell-cell interactions, and cellular differentiation and in cytokine responses of lymphocytes, and VP2 protein phosphatase activity during infection could be expected to induce regulatory changes in infected lymphocyte populations (Peters *et al*., 2002). Like all circoviruses, CAV is highly

dependent on the host cell for replication and it is possible that a capacity to regulate the cell cycle may be vital for this purpose (Peters *et al*., 2002). It is also possible that protein phosphatase activity may be required for replication, and effects on the host cell may be secondary. A similar situation may exist to that which operates during vaccinia virus infection, when the encoded VH1 phosphatase protein is able to block interferon signalling, which assists in the evasion of the host immune response (Najarro *et al*., 2001). This potential function of vaccinia VH1, the only viral phosphatase with a characterised *in vivo* function, suggests that virus-encoded phosphatases may be involved in immune evasion and virus persistence (Peters *et al*., 2002). The observation that CAV and TLMV both have ORFs capable of phosphatase activity would suggest a potential common strategy for infection and replication between CAV and the human circoviruses (Peters *et al*., 2002).

Due to the high prevalence of TTV infection in the general human population and its apparent inability to cause any overt disease, it has been proposed that TTV is a commensal human virus. Commensal viruses are usually persistent, highly transmissible and highly prevalent but fail to produce any lesion (Simmonds, 2002). TTV may be an 'old' circovirus, and indeed the fact that TTV and TLMV both commonly infect humans and non-human primates would suggest that the divergence of TTV and TLMV probably occurred before the human/chimp lineage split (Thom *et al*., 2003). Chimpanzees are infected by a range of TTV and TLMV-like viruses, some of which are also capable of infecting humans, and therefore it may be assumed that TTV and TLMV diverged earlier than chimps and humans did, an estimated 5 million years ago (Simmonds, 2002). Forms of TTV have been identified in apes, old world primates, new world primates and the tree shrew (the most divergent primate), supporting the theory of a long co-evolution of TTV and TLMV-like viruses and their primate hosts over tens of millions of years (Thom *et al*., 2003; Simmonds, 2002). The TTV and TLMV-related viruses may have split from their non-human primate variants, the forms found in African monkeys and other apes, around 90 million years ago (Thom *et al*., 2003). The long evolution of TTV and TMLV is similar to that which seems to have occurred with the herpesviruses, which have also shared a long co-evolution with mammals (Simmonds, 2002).

Some suggested reasons for the high genetic diversity evident for TTV and TLMV are genetic drift over very long time periods (probably millions of years) and cross-species transmission between human and non-human primates (Simmonds,

2002). Mixed infection with multiple TTV genotypes is a frequent occurrence (Simmonds, 2002) that would increase the chance of recombination between TTV isolates (Worobey, 2000). Long lasting viral infections such as in TTV and TMLV may also allow the host immune system to exert genetic and therefore evolutionary pressure. Other low pathogenicity viruses that may have co-existed with primates for millions of years, for example the papillomaviruses, also show high levels of genetic variation (Bendinelli *et al*., 2001).

It has been estimated that CAV could represent a divergence from the TTV/TLMV virus lineage that occurred approximately 300 million years ago (Simmonds, 2002). Other animals, including non-primate mammals, reptiles, amphibians and marsupials could also have CAV or TTV-like viruses (Simmonds, 2002) that, given the high genetic variability of the known TTV-like viruses, may be difficult to detect using present PCR methods (Thom *et al*., 2003).

Genus *Circovirus*

PCV1 and PCV2 have been shown to be genetically stable, with higher genetic conservation than BFDV, a close relative (Choi *et al*., 2002). All PCV2 strains examined so far have shared greater than 90% nt identity (Kim and Lyoo 2002; Choi *et al*., 2002; Larochelle *et al*., 2002), and a 40 year old PCV1 isolate had 99% identity to modern strains (Choi *et al*., 2002).

Although not as highly conserved as PCV, other animal circoviruses are still genetically stable. In one study of 8 Australian BFDV isolates, the sequence variation between isolates was 84-97% (Bassami *et al*., 2001). Twelve GCV strains varied by only 7.7% (Chen *et al*., 2003), although 11 of these strains were from Taiwan and more data is needed to confirm the genetic variation in GCV between countries and over time. Two strains of CoCV exhibited approximately 96% nt identity (Todd *et al*., 2001). Only one strain of CaCV and one strain of DuCV have been sequenced (Phenix *et al*., 2001; Hatterman *et al*., 2003) and only partial sequence data has been reported for OCV, so the sequence variation and genetic variability of these viruses is unknown.

While inter-specific phylogenetic analysis of the viruses in the genus *Circovirus* has revealed that they all appear to be genetically closely related, PCV1 and PCV2 are the circoviruses that share the greatest similarity to each other (Phenix *et al*., 2001; Hatterman *et al*., 2003). Based on the amino acid sequences of the Rep and capsid proteins of these 2 viruses, the closest relative to PCV is GCV, and the 2 viruses sharing the closest relationship with them are GCV and DuCV (Hatterman *et al*., 2003).

In contrast to the *Anelloviruses*, there has been little research on the theoretical evolution of viruses placed in the *Circovirus* genus. PCV and BFDV are thought to have evolved from plant nanoviruses (Bassami *et al*., 1998; Gibbs and Weiller, 1999). This theory is based on the observation that part of the circovirus nt sequence encoding the Rep protein is similar to that of plant nanoviruses, while the remainder closely resembles the Rep protein of a single-stranded RNA virus. Gibbs and Weiller (1999) considered that plant nanovirus DNA was probably transferred from a plant to a vertebrate, possibly by an animal being exposed to sap from an infected plant, and that as the transferred DNA included the origin of replication the virus was still able to replicate. They hypothesised that once this host switch had taken place, the plant viral DNA recombined with a vertebrate-infecting RNA virus, most likely a calicivirus, to produce circoviruses capable of infecting vertebrates. This theory was proposed before the characterisation of GCV, CaCV, CoCV, DuCV and OCV, and given the nt and amino acid identity and the preservation of replication-associated motifs between PCV, BFDV, CaCV, CoCV, GCV and OCV, it would be assumed that if such a recombination event did occur that it would have involved the ancestor of all these viruses.

The reason for the very high sequence conservation of the PCV types is unclear. Presumably PCV2 evolved from PCV1, or vice versa, by a series of point mutations (Ellis *et al*., 1998). No intermediate forms have been found to exist, and both types of PCV are genetically very stable over time (Choi *et al*., 2002, Larochelle *et al*., 2002), suggesting that PCV1 and PCV2 have been divergent and relatively unchanged for a long period of time.

Comparative pathology and pathogenicity of circoviruses

Target cells and sites of replication of circoviruses

The target cells for replication of CAV, PCV and TTV *in vivo* have been well studied, with less information available for the other circoviruses.

The main cellular hosts of CAV are considered to be haemocytoblasts in the bone marrow and precursor lymphocytes in the thymus (McNulty, 1991; Todd, 2004), although CAV antigen has been detected in the thymus, spleen, bone marrow, proventriculus and the duodenum of experimentally infected birds (Smyth *et al*., 1993). It was suggested that CAV may be lymphotrophic during the early stages of infection, but later infects other cell types (Cardona *et al*., 2000). CAV infection in chickens seems age-dependent, and CAV-associated disease has only been reported in chicks infected before 3-weeks of age, or in older immunosuppressed chickens (Cardona *et al*., 2000). Adult chickens do not seem to be as susceptible to

CAV as young chicks, which could be due to a lack of suceptible thymocytes in older birds (Jeurissen *et al*., 1992).

BFDV appears to be epitheliotropic, targeting the actively dividing cells of the basal epithelial layer of the feather and feather follicles (Pass and Perry, 1984). Virus particles have also been detected by electron microscopy in the liver and bursa of diseased birds (Raidal *et al*., 1993; Schoemaker *et al*., 2000), and intracytoplasmic inclusions have been demonstrated within macrophage cells in the feather epithelium and spleen (Latimer *et al*., 1991; Sanada *et al*., 1999).

It appears that both types of PCV may infect several porcine tissues but the tissues infected may vary, possibly depending on the amount of virus circulating in the body and the health status and age of the host. In experimental PCV1 infections, PCV1 antigen has been detected in the thymus, spleen, mesenteric lymph node, kidney, small intestine and lung (Allan *et al*., 1994 b; Allan *et al*., 1995). Allan *et al*. (1994a) reported that PCV1 could infect monocyte/macrophage cell types but not T or B cells *in vitro*. In experimental PCV2 infections, antigen has been demonstrated in the liver, lung, kidney, pancreas, myocardium, intestines, testis, brain and salivary, thyroid and adrenal glands (Kennedy *et al*., 2000), and also tonsil, spleen, lymph nodes and ileum (Pogranichnyy *et al*., 2000). In naturally infected pigs with PMWS, PCV2 has been detected in the lungs, lymph nodes, spleens and tonsils (Hamel *et al*., 1998). It is thought that PCV2 initially infects the tonsils and lymph nodes (Rosell *et al*., 1999) or the thymus (Darwich *et al*., 2003) and is disseminated to other tissues by viraemia (Krakowka *et al*., 2002). It is not certain whether PCV2 can infect and replicate in lymphocytes, with some reports supporting infection of B-lymphocytes (Shibahara *et al*., 2000) and others claiming that it does not replicate in any type of lymphocyte (Kim and Chae, 2003). A study using flow cytometry suggested that PCV2 probably does not infect resting lymphocytes *in vitro,* and there was evidence of viral infection in both monocyte and macrophage cells (Gilpin *et al*., 2003).

Both PCV1 (Choi *et al*., 2002) and PCV2 (Stevenson *et al*., 2001) have been identified in the neural tissues of pigs suffering from congenital tremors (CT), although no causal relationship has been proven. PCV2 can also infect and persist in dendritic cells, although active replication does not seem to occur in these cells (Vincent *et al*., 2003).

PCV2 has also been shown to replicate in porcine embryos (Mateusen *et al*., 2004) and the demonstration of PCV2 antigen in the tissues of foetuses would suggest that prenatal infection with PCV2 does occur (West *et al*., 1999). Differences in the distribution of PCV2-infected cells in tissues between foetal and postnatal animals

would suggest that PCV2 may target different cells in foetal and newborn animals (Sanchez *et al*., 2003). Possibly, the differentiating tissues in foetuses may provide the rapidly replicating cells suitable for PCV2 replication (Sanchez *et al*., 2003).

TTV seems to be widely distributed in the body tissues of patients with liver disease, but no causal association between the virus and liver disease has been established (Pollicino *et al*., 2003). The major TTV replication site is probably the liver (Okamoto *et al*., 2002) although the virus does circulate in the blood and has been found to replicate in bone marrow cells and stimulated PBMC (Bendinelli *et al*., 2001; Mariscal *et al*., 2002). TTV has also been identified in the brain and spinal fluid of patients with liver disease, suggesting that TTV can invade the human central nervous system (Pollicino *et al*., 2003). It has also been detected in the respiratory tract of children with acute respiratory disease (Maggi *et al*., 2003). When inoculated into monkeys, TTV viral DNA was detected in the liver, bone marrow and small intestine, suggesting that TTV can replicate in all these tissues (Xiao *et al*., 2002). It is not certain whether the distribution of TTV in tissues is the same in healthy and diseased individuals.

TLMV DNA has been detected in human plasma and PBMC (Takahashi *et al*., 2000; Biagini *et al*., 2001) but no data was found on the distribution of TLMV in human tissues, presumably due to its recent discovery and the invasiveness required for tissue testing.

The target cells for the replication of CoCV, GCV, TLMV, OCV, DuCV and CaCV are not known. CoCV virus has been detected in the bursa, spleen, thymus, liver, kidney, brain, crop and intestine of infected pigeons (Smyth *et al*., 2001; Duchatel *et al*., 2005), and GCV and DuCV DNA have been amplified from the bursa, where virus particles had previously been identified by electron microscopy (Todd *et al*., 2001; Ball *et al*., 2004; Hatterman *et al*., 2003) with GCV also identified by ISH in the liver, small intestine, lung, spleen, thymus, kidney, heart and bone marrow of infected birds, with particularly large amounts of virus in the liver (Smyth *et al*., 2005). OCV has been identified in the gut content of an ostrich (Els and Josling, 1998) and also detected in ostrich embryos, suggesting vertical transmission is possible, and in liver tissue of young chicks (Eisenberg *et al*., 2003).

The target cells used for replication is a key issue needed to elucidate the pathogenesis of virus infections, and while macrophages have been suggested to play a key role, there is some uncertainty as to whether circoviruses commonly replicate in these type of cells. PCV1 has been detected in

monocyte/macrophage cell lines (Allan *et al*., 1994; McNeilly *et al*., 1996). BFDV antigen has been identified within macrophage cells in the feather epithelium and spleen of diseased birds (Latimer *et al*., 1991; Sanada *et al*., 1999). PCV2 has been detected in macrophage cells of PMWS-affected pigs (Kiupel *et al*., 1998; Rosell *et al*., 1999; Kim *et al*., 2004). CAV has not been shown to replicate in macrophages, although macrophages from CAV-infected birds have reduced IL-1 production, Fc receptor expression, phagocytosis, and bactericidal activity (McConnell *et al*., 1993). This may be due to the withdrawal of cytokines essential for the maintenance of these functions, which could be caused by viral destruction of T-cells and precursor T-cells (McConnell *et al*., 1993). The decrease in IL-1 production can affect T-cell maturation in the thymus, therefore more precursor T-cells are likely to be available for CAV replication (McConnell *et al*., 1993b).

A hypothesis was proposed that the replication of PCV2 in macrophages contributed to the PMWS disease process, as experimentally or naturally induced immunoproliferation could lead to increased macrophage production and therefore increased PCV2 replication, precipitating disease (Krakowka *et al.*, 2001). However, while in both naturally occurring and experimentally induced cases of PMWS, cells of histiocyte, monocyte and macrophage lineage are often positive for PCV2 antigen, the viral DNA is commonly contained in the cell cytoplasm rather than the nucleus (Allan *et al*., 1999; Balasch *et al*., 1999; Ellis *et al*., 1999; Krakowka *et al*., 2000; Rosell *et al*., 2000; Harms *et al*., 2001; Fenaux *et al*., 2002; Kim *et al*., 2004) which would suggest that PCV2 replication within these cells is very limited or non-existent. Consequently, it has been suggested that the viral material of PCV2 and other circoviruses accumulates in macrophages as a result of phagocytosis, and that the presence of circovirus-containing inclusions in the cytoplasm of macrophages may result from the phagocytosing activity of these cells and is not the result of endogenous replication (Krakowka *et al*., 2002; Shibahara *et al*., 2000; Todd, 2004). Chang *et al*. (2005) demonstrated with *in vitro* studies, that PCV2 can passively enter and accumulate in the cytoplasm of alveolar macrophages (AMs), which may assist the virus to evade detection by the host immune system.

Phagocytosis as an explanation for the presence of circovirus antigen in macrophages is not universally accepted. Phagocytosis of CAV by macrophages *in vitro* is rare (Gilpin *et al*., 2003). While circoviruses commonly replicate well in rapidly dividing cells (Rossell *et al*., 1999; Allan *et al*., 1994; Okamoto *et al*., 2002; Gilpin *et al*., 2003), presumably due to their reliance on host-cell polymerases,

macrophages are considered to be in the terminal stages of monocytic cell differentiation. Their replicative machinery is largely inactive, therefore they were considered an unlikely target for PCV replication (Gilpin *et al*., 2003). An alternative reason for the non-productive circovirus infection of macrophages was proposed. This was that macrophages can express high levels of DNA polymerase activity in response to damage to their DNA (Williams et al., 2002), and this expression could facilitate PCV replication.

Persistent PCV2 infection of porcine macrophage and monocyte cells *in vitro* with no increase in infectious virus or production of replicative intermediates has been demonstrated (Gilpin *et al*., 2003). It has been thought that this type of PCV2 infection with minimal replication could well contribute to the disease process associated with PMWS: PCV2 could be disseminated throughout the body and via the persistent 'silent' infection of macrophage cells, remain undetected by the host immune system (Gilpin *et al*., 2003). Support for this hypothesis was a very low level of PCV2 replication detected in macrophages *in vitro,* and replication only in cells derived from certain individual pigs (Meerts *et al*., 2004). This is an unusual finding and the reasons for these differences between animals have not been identified. More investigation is needed to elucidate the possible role of macrophage cells in PCV2 infection and dissemination throughout the host animal.

Virus transmission and route of infection of circoviruses

BFDV is thought to be transmitted primarily by feather dust, faeces and crop secretions (Ritchie *et al*., 1991). However, research in this area is limited, and whether BFDV may be transmitted by any other means is largely unknown.

Vertical transmission of OCV was demonstrated by the detection of virus in embryos (Eisenberg *et al*., 2003), but no information is available on other forms of OCV transmission. There is no information available on the transmission of CaCV, GCV, DuCV or CoCV, although it would seem likely that shedding through faeces, crop dust and feather secretions would occur as it does with BFDV. The transmission of CAV has been studied more extensively, and it has been determined that CAV transmission may be both vertical, resulting in increased mortality of young chicks, and horizontal once maternal antibody has disappeared, resulting in a subclinical disease that can result in decreased productivity in adult chickens (Scott *et al*., 1999). CAV maternal antibody will protect chicks from clinical disease, but it does not protect from infection, and it does not prevent virus transmission or immunosuppression. As a consequence of vaccination programs, CAV-associated

clinical disease is rare today, although subclinical infection is ubiquitous (Sommer and Cardona, 2003). CAV can persist in the reproductive organs of specific-pathogen-free (SPF) chickens long after seroconversion and the virus can also infect embryos in the early stages of development (Cardona *et al*., 2000).

From the intensive study of TTV since its discovery in 1997, it would appear that the virus can be transmitted in a variety of ways, possibly accounting for the virus being so common in the human population. The high prevalence of TTV in donated blood would suggest that it could be transmitted by blood transfusion. The high prevalence of infection in very young (7-12 month old) children would also suggest that TTV is commonly aquired in infancy (Yokozaki *et al*., 1999; Peng *et al*., 2002) and TTV has been found in the breast milk of nursing mothers (Matsubara *et al*., 2001). TTV has also been identified in amniotic fluid (Matsubara *et al*., 2001) and cord blood (Matsubara *et al*., 2001) providing evidence for vertical transmission. Faecal-oral transmission has been suggested; it is thought that viral excretion in faeces may occur via secretion from the liver into the bile (Pollicino *et al*., 2003). It has also been suggested that TTV may be transmitted sexually (Krekulova *et al*., 2001).

Although there is only limited data on the transmission of TLMV, given that co-infection with TTV and TLMV is common (Matsubara *et al*., 2001; Thom *et al*., 2003) it could be assumed that TLMV is spread by similar routes to TTV. TLMV has been detected in plasma, PBMC, saliva, faeces and the cervix (Biagini, 2004). TLMV, like TTV, has also been identified in cord blood (Matsubara *et al*., 2001), suggesting that vertical transmission can occurr.

PCV2 has been identified in a wide variety of tissues, including oropharyngeal and nasal swabs, faeces, whole blood and serum after experimental infection, and the frequency of PCV2 in whole blood, nasal swabs and faeces from field samples has been reported to be 30.4%, 19.2%, and 20.4%, respectively (Shibata *et al*., 2003). Both types of PCV have been isolated from faecal samples and nasal swabs in experimentally infected pigs, and from naturally infected field pigs (Tischer *et al*., 1986; Shibata *et al*., 2003; Yang *et al*., 2003; Sibila *et al*., 2004; Segales *et al*., 2005). A study involving the experimental inoculation of piglets with PCV2 showed that uninoculated piglets in the same room as inoculated piglets could become infected with PCV2 through simple contact, viral infection becoming apparent in the in-contact pigs one day after the inoculated piglets (Okuda *et al*., 2003). This is supported by the finding of PCV2 in respiratory and nasal secretions (Segales *et al*., 2005). Sexual transmission of PCV has also been suggested, and both PCV1 and PCV2 DNA have both been identified in boar semen (Kim *et al*., 2001); PCV2 has been

shown to be shed in semen for up to 47 days after infection (Larochelle *et al*., 2000). Vertical transmission of PCV is probable: PCV2 appears to cross the placenta and to infect foetuses (West *et al*., 1999), and transplacental infection of foetuses causing reproductive failure has also been suggested (Ladekjaer-Mikkelsen *et al*., 2001).

Immunosuppression and concurrent infections as factors in circovirus infections

Common features of PMWS, BFD, and GCV and CoCV-associated diseases include intracytoplasmic inclusions within macrophages, depletion of T and B lymphocytes, ill-thrift and secondary infections, all of which are suggestive of immunosuppression (Todd, 2004). However the mechanisms of immunosuppression resulting from circovirus infection probably differ between circoviruses.

The mechanism of immunosuppression in birds suffering from BFD has not been fully elucidated. Damage to the thymus and bursa in diseased birds is thought to result in immunosuppression and secondary infections (Latimer *et al*., 1992). However, direct cell destruction of lymphocytes in these organs is probably not involved and maybe the immunosuppression is caused by a cytokine-mediated reduction in lymphoid tissue development (Todd, 2004). There may also be immunosuppression in association with CoCV infection: virus particles were found in association with lymphoid depletion (Coletti *et al*., 2000) suggesting that the virus might damage the immune system (Roy *et al*., 2003).

Immune function and immunosuppression in CAV-infected birds has been studied more extensively. A decrease in the levels of CD3, CD4, CD8 and natural killer cells has been demonstrated in experimentally infected chicks, providing evidence for altered lymphocyte function (Bounous *et al*., 1995). The immunosuppression is thought to be the result of depressed macrophage function, which may in turn result from infection and destruction of activated T-cells and precursor T-cells (Adair *et al*., 1993; McConnell *et al*., 1993a). CAV-associated clinical disease occurs only in young chicks but adult birds may also be immunosuppressed as a consequence of CAV infection (McConnell et al., 1993b).

The effects of human circovirus infection appear to be clinically mild or inapparent, although both TTV and TLMV titres increase in immunosuppressed people, for example soldiers undergoing intense training and patients receiving immunosuppressive drugs prior to organ transplantation (Moen *et al*., 2003). This would suggest that these viruses can interact with and possibly affect the human immune system. As a majority of the human population appears to be infected

with TTV and/or TLMV, and sometimes several different isolates of TTV (DeValle and Niel, 2004; Raffa *et al*., 2003; Mushahwar *et al*., 1999), various theories have arisen for the possible consequences of this in inducing immunosuppression. One theory is that only high levels of viral replication may affect the immune system, and that in most people TTV infection is not associated with immunosuppression (Maggi *et al*., 2003). It was also hypothesised that infection with more than one genotype may lead to lymphocyte subpopulation imbalances, including low CD3 (total T lymphocytes), low CD4 (T helper lymphocytes) and high CD 19 (B lymphocytes) (Maggi *et al*., 2003). It was also suggested that different TTV isolates might interact with each other, or alternatively only some isolates are responsible for affecting lymphocyte subpopulations (Maggi *et al*., 2003). TTV infection may also favour the production of type 2 cytokines, thereby leading to immunosuppression (Maggi *et al*., 2003).

In piglets, artificially-induced immunosuppression seems to potentiate PCV2 replication and allow the virus to spread to hepatocytes and Kupffer cells as well as dendritic cells and macrophages (Krakowka *et al*., 2002); CD8+ cells are mainly responsible for the cytotoxic response, and DP cells are mature lymphocytes with properties of memory and effector cells. This had led to the development of a theory that there is a relationship between the immune status of the infected pig and PCV2-associated disease development (Krakowka *et al*., 2002). It was reported that PCV2 infection causes a shift in the animals relative counts of peripheral blood lymphocyte subsets, specifically a decrease in CD4+CD8+ double-positive (DP) and CD8+ cell counts (Darwich *et al*., 2002). Others have detected a decrease in CD4+ cells in PMWS-affected pigs (Segales *et al*., 2001; Sarli *et al*., 2001) although this may not be a direct affect of PCV2 infection as decreased CD4+ counts were also detected in wasted PCV2-negative pigs (Darwich *et al*., 2002). Others have found a decrease in both T and B cells in PCV2-infected pigs (Segales *et al*., 2001; Darwich *et al*., 2002; Darwich *et al*., 2003; Chianini *et al*., 2003; Kim and Chae, 2003), and depletion of T- and B-cell dependent areas in lymphoid tissues have been observed, the intensity of which correlated with the amount of virus present in those tissues (Darwich *et al*., 2002; Darwich *et al*., 2003; Chianini *et al*., 2003).

It was suggested that the destruction of B-lymphocytes leading to immunosuppression by PCV2 occurred by apoptosis, with phagocytosis of the cellular remains by macrophages, which serve as a source of infection as they travel throughout the body (Shibahara *et al*., 2000). However, there is conflicting

evidence regarding the ability of PCV2 to induce apoptosis – one study found that the incidence of apoptosis in lymphoid tissues of healthy and PMWS-affected pigs was inversely correlated with viral load and the severity of microscopic lesions (Resendes *et al*., 2004). In the lymphoid tissues of healthy pigs, the processes of apoptosis and proliferation are in balance to maintain a stable population of cells, and if PCV2-mediated apoptosis does not occur then decreased cellular proliferation in PMWS-affected pigs may be the primary cause of cell depletion in lymphoid tissues (Resendes *et al*., 2004; Mandrioli *et al*., 2004). An alternative theory proposed to explain the changes in lymphocyte populations is that virus overloading and subsequent neglect of cellular metabolism may contribute to cell death and phagocytosis (Krakowka *et al*., 2002).

However, a study of a novel PCV2 protein, designated ORF3, found that the ORF3 gene product was not essential for virus replication in cell culture, but was capable of inducing apoptosis involving the caspase-8 pathway (Liu *et al*., 2005). While the sequence of ORF3 was highly conserved between PCV2 isolates, the potential ORF3 sequence of PCV1 shared only moderate homology with that of PCV2, suggesting this novel protein and its apoptotic activity may be related to pathogenicity (Liu *et al*., 2005). *In vivo* studies using mice demonstrated that this ORF3 protein may be involved in viral pathogenesis by altering immune cell numbers (Liu *et al*., 2006b). Another study also found that PCV2 caused apoptosis in the spleens of infected mice, mediated through the activation of caspases 3 and 8 (Kiupel *et al*., 2005).

PCV2 is also thought to affect the host immune system by stimulating the production of pro-inflammatory cytokines, probably causing the typical PMWS granulomatous lesions. This could attract macrophage cells to the affected areas, increasing the chances of the virus infecting new target cells (Darwich *et al*., 2003b).

Further evidence for the ability of PCV2 to directly affect the host immune response was the finding that within the genome of PCV2, there are a number of CpG dinucleotide motifs (Hasslung *et al*., 2003). These motifs are thought to be responsible for the ability of plasmid DNA to increase and inhibit immunostimulatory activity in vertebrates, and as the PCV2 genome is similar in size and structure to a plasmid, it is possible that PCV2 is able to stimulate or repress the immune response in a similar fashion (Hasslung *et al*., 2003). Sixty five CpG sequences were identified in the PCV2 genome, 40% of these were stimulatory and 60% inhibitory (Hasslung *et al*., 2003). Immune modulation was

achieved by induction or repression of IFN-α production, an interferon molecule which contributes to the specific immune response to viral infections. PCV2 replication has also been shown to be increased by IFN-α *in vitro* (Meerts *et al*., 2005c). The ability of PCV2 to control IFN-α via CpG motifs may help PCV2 to avoid the host immune system (Hasslung *et al*., 2003).

Animals infected with circoviruses are commonly infected with other secondary pathogens, supporting the theory of immunosuppression. Birds suffering from BFD are commonly infected with secondary bacterial, chlamydial and fungal pathogens: cryptosporidiosis, a bacterial infection commonly associated with immunosuppression, has been identified in cockatoos with BFDV (Latimer *et al*., 1992); septicaemia, peritonitis, chlamydiosis and mycotic ventriculitis were also identified by Latimer *et al*. (1992) and Schoemaker *et al*. (2000) identified secondary bacterial or fungal infections in 9 of 14 African grey parrots infected with BFDV. A high prevalence of secondary infections in pigeons (*Escherichia coli*, *Salmonella, Pasteurella, Aspergillus, Mycoplasma*, candidiasis, nematodiasis, or capillariasis infections) and ostriches (*E. coli*, *Klebsiella pneumoniae*, *Streptococcus* and *Bacillus* infections) was detected in association with CoCV and OCV, respectively (Terzich and Vanhooser, 1993; Pare *et al*., 1999; Eisenberg *et al*., 2003; Roy *et al*., 2003). GCV is also considered to predispose infected birds to secondary infections by inducing immunosuppression, and a commercial goose farm affected by GCV suffered problems with runting, adverse production and increased mortality, partly due to infections with *Riemerella anatipestifer* and *Aspergillus fumigatus* (Todd, 2004). The only report thus far of circovirus infection in a gull concerned a young bird with an overwhelming *Aspergillus* infection, thought to be an opportunistic pathogen secondary to circovirus infection (Twentyman *et al*., 1999).

PCV2-infected pigs have been found to be co-infected with a number of different pathogens, including *Pasteurella multicoda*, *Mycoplasma hyopneumoniae, Streptococcus sp.,* swine influenza virus, porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhoea virus and porcine parvovirus (PPV) (Sato *et al*., 2000; Allan *et al*., 1999; Hirai *et al*., 2001; Pallares *et al*., 2002). The presence of the rare pathogen *Cryptosporidium parvum* in a pig co-infected with PCV2 was suggested to be an indication of immunosuppression (Nunez *et al*., 2003). Sato *et al*. (2000) found various mixed infections in tissue samples from diseased pigs suspected of having PMWS. Of those animals infected with PCV2, over 90% were also infected with another pathogen, including bacteria such as *P. multocida*, but the most common co-infection was with PRRSV, which was present in over 50%

of pigs with PCV2. A requirement of co-infections in the pathogenesis of PMWS is not essential; however, as Cook *et al.* (2001) could not establish any significant association between the presence of PMWS and any other infectious conditions in the United Kingdom. Interestingly, it has been reported consistently that despite the presence of secondary bacterial and fungal infections in cases of PMWS, antimicrobial treatments are ineffective in controlling the disease (Segales *et al*., 2004b).

Since no co-infecting pathogen seems to be present consistently in PMWS, PDNS and CT cases where PCV2 infection might be involved, it has been suggested that mixed infections with bacteria and other viruses are opportunistic, and that co-infection does not precipitate PCV2-associated disease but is the result of immunosuppression as a consequence of PCV2 infection (Sato *et al.*, 2000). This is supported by the finding that PCV2 infection may decrease the ability of alveolar macrophages to phagocytose bacterial and fungal pathogens (Chang *et al*., 2006). However, it is difficult to tell whether these co-pathogens are necessary for the induction of PMWS or if they are simply opportunistic infections as a result of immune suppression. Bacterial lipopolysaccharides are capable of increasing PCV2 replication *in vitro* (Chang *et al*., 2006b) and the co-infection of pigs with PCV2 and porcine parvovirus (PPV) or PRRSV does result in more severe disease than infection with PCV2 alone (Allan *et al*., 2000; Ellis *et al*., 2000c). In the case of PPV, this is possibly because PPV infection stimulates the immune system, providing more target cells for PCV2 replication (Krakowka *et al*., 2000). In CAV infections, it has been suggested that the destruction of B lymphocytes by other agents, for example a reovirus, may cause disease because the bird cannot mount an effective antibody response to CAV, therefore CAV becomes more pathogenic (Todd 2004). Synergistic effects of dual infection with CAV and *Cryptosporidium baileyi* have been reported (Hornok *et al*., 1998).

While most circoviruses cause severe disease in young animals, infection of older animals can still cause immunosuppression. As with CAV (see above) subclinical PCV2 infection in pigs has been shown to affect performance and immune system function (Markowski-Grimsrud *et al*., 2003).

Subclinical circovirus infections

PCV2 has been widely detected in healthy pigs (Done *et al.*, 2001) and antibodies have been detected on farms with no history of PMWS (Rodriguez-Arrioja *et al*., 2000; Larochelle *et al*., 1999) which indicates that PCV infections are commonly subclinical. Similarly, CoCV has been detected in clinically normal pigeons (Pare *et*

al., 1999) and BFDV DNA has also been detected in clinically normal birds (Rahaus and Wolff, 2003). Subclinical CAV infection is very common (Sommer and Cardona, 2003) and infection with TTV and TLMV in the absence of any specific identified disease syndrome suggests these viruses are also principally subclinical infections.

Subclinical circovirus infection may, however, still cause immunosuppression in animals in which there is no specific circovirus-associated disease syndrome. This is thought to occur in CAV and PCV2 infections. CAV infection in young chicks, after the loss of maternally-acquired antibody, may result in a subclinical disease that can result in decreased growth and productivity and affect T cell function in adult chickens, although anaemia is not observed (McNulty *et al*., 1991; Scott *et al*., 1999; Markowski-Grimsrud and Schat, 2003; Todd, 2004). PCV2 infection is common in the absence of specific PCV2-associated disease such as PMWS but this might result in mild lymphoid depletion (Allan *et al*., 1999; Ellis *et al*., 1999).

Circoviruses infection may be persistent, despite the production of antibody. CAV may persist in chickens long after seroconversion, possibly in reproductive tissues (Cardona *et al*., 2000). PCV2 infection, thought to occur mainly after the pig nursery stage of production, reaches a peak prevalence in 3 to 4-month-old pigs, suggesting it persists for a long period after the initial infection (Shibata *et al*., 2003). This persistence may be due to an inefficient immune response, as is thought to occur with TTV where infection is usually persistent, possibly lifelong in some cases (Bendinelli *et al*., 2001). As circoviruses seem capable of compromising the host immune system, it is possible that the weakened immune response would be unable to clear the virus.

It is assumed that the level of circovirus in subclinical infections is low, and that factors which lead to increased levels of virus replication may be critical for disease development. Any condition, such as co-infection with another pathogen or environmental factors, which promote PCV2 replication might therefore result in virus dissemination and disease (Krakowka *et al*., 2002). There has been little further development of this theory for other circoviruses, although it is thought that high levels of TTV replication may cause lymphocyte subpopulation imbalances (Maggie *et al*., 2003). It is possible that high levels of circovirus replication and the subsequent overpowering of the immune system may be a critical factor in determining whether an infected animal will develop disease or subclinical infection.

Severe circovirus-associated diseases commonly affect young animals. This is the case for PMWS, where affected pigs usually develop clinical signs at 6-8 weeks of age (Harding *et al.*, 1998), in CAV-associated disease that usually affects young

chicks (Noteborn *et al*., 1994; McNulty *et al*., 1991) and BFD, largely identified in birds under 3 years of age, although older psittacines may develop a chronic form (Pass and Perry, 1984; Schoemaker *et al*., 2000). From the information currently available, the recently described avian circoviruses also cause disease in young birds: CoCV commonly affects pigeons 1-12 months of age (Woods and Shivraprasad, 1997); GCV possibly causes ill-thrift in young geese (Soike *et al*., 1999); ostrich FCS was associated with death in birds less than 2 weeks of age, although in rare cases birds up to 4 years of age can be affected (Terzich and Vanhooser, 1993), and CaCV has been detected in diseased neonatal canaries (Goldsmith, 1995). It is possible that the reason for the susceptibility of younger animals to circovirus-associated disease is that their immune systems are not as developed as older animals. Todd (2004) suggested that the age resistance of older chickens to CAV-induced anaemia and severe immunosuppression was possibly due to greater immune competence and therefore a more rapid immune response of older birds.

Circovirus-associated diseases and their treatment

Beak and feather disease (BFD)

Psittacine birds affected by BFD suffer feather dystrophy, plumage loss and diarrhoea, with beak deformities a less frequent occurrence. The feather and beak abnormailities are caused by epidermal cell necrosis, epidermal hyperplasia and hyperkeratosis in the epidermis of the feathers and beak (Pass and Perry, 1984; Latimer *et al*., 1992). Liver necrosis, lymphoid necrosis and atrophy in the thymus and bursa of affected birds have also been observed (Pass and Perry, 1984; Schoemaker *et al*., 2000). The condition is progressive and death often occurs from secondary infections, so the treatment of affected birds is usually palliative. Such treatment involves antibiotics, a controlled environment and balanced nutrition (Jergens *et al*., 1988), although the use of antibiotics, antifungal agents and nutrient supplements may be unhelpful during acute disease (Jacobson *et al*., 1986).

CAV-associated disease

The acute clinical form of CAV-associated disease occurs when vertical transmission results in the virus infecting the egg, with subsequent infection of newborn chicks (McNulty, 1991). The disease becomes apparent at 7-14 days of age, when affected chicks are depressed and anaemic, with pale bone marrow, thymic, splenic and bursal atrophy, and haemorrhagic skin lesions (which are prone to secondary bacterial infections) (McNulty, 1991; Crowther *et al*., 2003). Histopathological changes include atrophy of the haematopoietic elements of the bone marrow and

severe depletion of lymphocytes in the thymus, followed by hyperplasia of reticular cells (Adair *et al*., 1993; Bounous *et al*., 1995). The mortality rate may be as high as 60% but unlike BFD and PMWS, surviving chicks usually make a full recovery, presumably because the CAV-associated disease differs histopathologically from BFD, PMWS and CoCV and GCV-associated disease. The observed lymphocyte depletion in CAV-infected chicks only affects T cells, whereas the other animal circoviruses affect both T and B cells (Todd, 2004). This destruction of B cells further impairs the host antibody response resulting in a longer term disease condition (Todd, 2004).

As CAV is very stable in the environment (Todd *et al*., 2001), virus eradication as a form of disease control is impractical, and disease control usually involves increased hygiene and the prevention of co-infection (Rosenberger and Cloud, 1998). Ensuring that flocks develop antibodies to CAV, usually by vaccination, before laying is thought to prevent vertical transmission of the virus (Todd *et al*., 2001). CAV vaccination is widespread and the severe clinical form of the disease is therefore rare (Sommer and Cardona, 2003).

Circovirus disease of non-psittacine birds

Circoviral infection of non-psittacine birds is usually accompanied by histological changes in the primary and secondary lymphoid tissues, commonly in the bursa (Woods and Latimer, 2000; Todd, 2004).

The macroscopic lesions of CoCV-infected birds include swelling and oedema in the bursa and spleen (Mankertz *et al*., 2000), pneumonia, liver necrosis and lymphoid necrosis (Pare *et al*., 1999).

FCS, which is associated with OCV, is characterised by depression, weight loss, anorexia, occasionally diarrhoea and death after a few days of illness. Gastric stasis, yolk sac infection and enteritis have also been observed (Eisenberg *et al*., 2003; Terzich and Vanhooser, 1993). Histopathological changes in FCS chicks include lymphocyte depletion in the bursa and lymphocytic infiltration of the lamina propria of the intestine (Terzich and Vanhooser, 1993).

CaCV has been identified in neonatal canaries with ‘black spot’, characterised by abdominal enlargement, gall bladder congestion and failure to thrive, and another illness in adult canaries which had died following a short illness characterised by dullness, anorexia, lethargy and feather disorders (Goldsmith, 1995; Todd *et al.*, 2001).

It should be noted that although GCV, DuCV, CoCV, CaCV and OCV have been identified in the tissues of diseased psittacine birds with lesions characteristic of

circoviral infection, such as feathering disorders, poor body condition and secondary bacterial or fungal infections, a causal relationship is yet to be established. Some control measures used for BFD may be applicable for these diseases, but quarantine is impractical for a disease of free-ranging birds such as pigeons, and thus far there has been little investigation into vaccination (Todd, 2004). Management conditions may also play a role in disease control, for example a study of 82 ostriches dead from FCS found that the co-infecting *E. coli* and *K. pneumoniae* bacteria present were antibiotic-resistant, possibly caused by the widespread use of antibiotics on the affected farms without veterinary supervision (Terzich and Vanhooser, 1993).

TTV and TLMV

No clinical condition has been definitely linked with TTV or TLMV, although TTV may be more common in patients with liver disease or those that have been exposed to blood products (Miyata *et al*., 1999; Bendinelli *et al*., 2001). It has been suggested that certain TTV genotypes may be capable of causing disease (Takahashi *et al*., 2000) but no such genotype has ever been identified. Treatment with interferon has been found to decrease TTV and TLMV titres, but only temporarily (Moen *et al*., 2003). Heavy TTV infection was reported to affect human lymphocyte subpopulations (Maggi *et al*., 2003).

PDNS

Porcine dermatitis and nephropathy syndrome (PDNS) was first recognised in the UK (Smith *et al*., 1993) and has subsequently been identified in several countries including Korea (Choi *et al*., 2002b), Northern Ireland (Allan *et al*., 2000), Hungary (Dan *et al*., 2003), Scotland (Thomson *et al*., 2000); England (Gresham *et al*., 2000) and Spain (Segales *et al*., 1998). PDNS-affected pigs, which are usually 12 to 14 weeks of age, develop dark skin lesions, especially on the hindquarters, and are pale, dull, depressed and reluctant to move (Done *et al.*, 2001). Lymphadenopathy and kidney dysfunction are common, and pneumonia has been observed in affected pigs (Done *et al.*, 2001; Thibault *et al*., 1998). In acutely affected pigs, mortality may approach 80% (Done *et al.*, 2001) and animals that do recover may suffer chronic kidney lesions (Segales *et al*., 1998). The severe glomerulonephritis lesions are consistent with an immune complex disorder (Thomson *et al*., 1998).

PCV2 nucleic acid has been identified in the tissues of pigs suffering from PDNS in Peyer's patches, tonsil, lung, spleen, kidney, liver, and skin. Viral nucleic acid was located mainly within the cytoplasm of monocyte/macrophage lineage cells, including follicular dendritic cells, macrophages, histiocytes and Kupffer cells

(Rosell *et al.*, 2000; Gresham *et al*., 2000; Saoulidis *et al*., 2002; Choi *et al*., 2002b). PCV2 antigen was found primarily in the lymphoid organs, especially the lymph nodes (Saoulidis *et al*., 2002).

No causative relationship between PCV2 and PDNS has yet been proven as the disease has not been reproduced experimentally, but PDNS development has been associated with very high levels of PCV2 antibody (Wellenberg *et al*., 2004b). The condition has also been associated with other pathogens such as PRRSV (Thibault *et al*., 1998; Segales *et al*., 1998; Choi and Chae, 2001) and *P. multocida* (Thomson *et al*., 1998). However, PRRSV is unlikely to be essential for PDNS to occur, as the disease was present in Ireland as early as 1990, before PRRSV was introduced in 1996 (Allan *et al*., 2000). As the pathological changes associated with PDNS are consistent with an immune-complex disorder, it is possible that viral infection may alter the immune response, allowing the clinical expression of the disease (Thomson *et al*., 2002). Since both PMWS and PDNS appear to be immune-mediated, and affected pigs display similar lesions such as lymphadenitis, it has been suggested that the 2 diseases are different manifestations of the same pathogenic process (Done *et al.*, 2000). PMWS and PDNS may occur separately, together or following each other, and a recent study using a multivariate model suggested that the 2 diseases were strongly associated (Cook *et al.*, 2001).

Sipos *et al*. (2005) suggested that PCV2 infection may be more recent in PDNS animals, as compared to PMWS animals, where the disease may be the end result of a longer period of infection. Also, the pro-inflammatory cytokines IL-1α and IL-6 were found to be much increased in PDNS pigs, with the chronic inflammation present in the disease possibly suggesting some kind of autoimmune condition.

Congenital tremor (CT)

Pigs affected by CT suffer from clonic contractions of their skeletal muscles, sometimes resulting in a fatal inability to suckle (Stevenson *et al.*, 2001) and while most piglets recover by the age of 3 weeks, some continue to shake throughout the growing and fattening periods (Lukert and Allan, 1999). The condition is associated with deficient and abnormal myelination of newborn pigs, and one form of the disease is possibly due to PCV2 infection of spinal cord motor neurons (Stevenson *et al.*, 2001).

Seemingly healthy neonatal pigs infected with PCV2 are commonly found in the same herd as those suffering from CT (Stevenson *et al.*, 2001). PCV1 has also

been associated with a CT outbreak in the 1960s (Choi *et al*., 2002). Despite the association between PCV and CT, a definitive link between PCV1 or PCV2 and CT has never been established, and one study of 40 CT-affected pigs from several different countries found no evidence of PCV infection (Kennedy *et al*., 2003).

Affected litters are usually from young breeding stock recently introduced to the affected farm, suggesting that exposure of seronegative stock to a virus at a critical time during gestation may precipitate disease (Lukert and Allan, 1999).

Reproductive failure

When PCV antibodies were first identified in pigs (at the time using serological tests that were not capable of distinguishing between PCV1 and PCV2), no correlation between PCV antibody and reproductive disorders was found (Tischer *et al*., 1995). However, PCV antibody was subsequently found associated with a few cases of abortion and stillbirth (Allan *et al*., 1995) and more definitive evidence was discovered with the association of PCV2 with a case of reproductive failure involving mummification and stillbirth in an SPF herd that became infected with the virus (Ladekjaer-Mikkelsen *et al*., 1999).

Reproductive problems associated with PCV2 infection, as with PMWS (see below) appears to be a fairly recent phenomenon (Meehan *et al*., 2001). Bogdan *et al*. (2001) failed to identify PCV2 in cases of reproductive failure between 1995 and 1998, suggesting that PCV2-associated reproductive failure was a relatively new clinical manifestation. Although PCV2 sequences isolated from cases of reproductive failure possess some amino acid differences to previously described strains, there is no evidence to suggest that PCV2-associated reproductive failure is caused by a particularly pathogenic isolate of PCV2 (Farnham *et al*., 2003; Meehan *et al*., 2001). Whether there is an association between PCV2 infection and co-infection with other agents has not been defined, but PCV2 was identified in stillborn piglets on a farm experiencing widespread reproductive problems where PPV and PRRSV were also present (O'Connor *et al*., 2001).

Sanchez *et al*. (2001) and Pensaert *et al*. (2004) suggested on the basis of field evidence that it was likely that PCV2 could cross the placenta and replicate in the foetus, although Mateusen *et al*. (2004) thought the chances of this event occurring in the field were low. However Park *et al*. (2005) demonstrated that sows infected with PCV2 3 weeks before farrowing experienced abortion and premature births, suggesting PCV2 crossed the placenta and replicated in the foetal piglets. PCV2 infection *in utero*, however, does not automatically mean

disease or death, as PCV2 virus and antibody have been detected in clinically normal newborn piglets (Sanchez *et al*., 2001). It has been proposed that the potential pathogenicity of PCV2 could depend on the age of the foetus at the time of infection (Farnham *et al*., 2003), with infection prior to foetal immunocompetence possibly causing foetal death and mummification (Pensaert *et al*., 2004). The results of experimental inoculation of foetuses 57, 72 and 95 days of age with PCV2 (Sanchez *et al*., 2001) supported this theory: foetuses inoculated at 57 days of age had high titres of virus in association with oedema, haemorrhages and congestion, and they did not develop antibody to PCV2, but foetuses inoculated at 72 and 95 days of age had low virus titres, no lesions, and developed antibodies to PCV2. Johnson *et al*. (2002) experimentally infected foetuses 86, 92 and 93 days of age with PCV2, and found that approximately 35% were mummified, stillborn or weak-born. Although the details are somewhat discrepant, both reports indicate that PCV2 can infect and cause reproductive abnormalities in late-term foetuses. Intra-uterine spread of PCV2 between foetuses is known to occur but progresses slowly (Pensaert *et al*., 2004), and the extent of *in utero* PCV2 spread may be affected by whether the sows are infected mid-gestation or late-gestation (Yoon *et al*., 2004).

PCV2 has been identified in heart, kidney, liver and lung tissue of aborted, mummified and stillborn foetuses (West *et al*., 1999; Lyoo *et al*., 2001; Sanchez *et al*., 2001; Park *et al*., 2005). Infection of the heart with associated myocarditis is a common finding in such cases, suggesting that the main target tissue for PCV2 replication in foetuses is the heart (West *et al*., 1999; O'Connor *et al*., 2001; Sanchez *et al*., 2001) although PCV2-associated myocarditis has also been identified in a weak-born 8-day old piglet (Mikami *et al*., 2005). Cardiovascular lesions such as granulomatous and necrotizing lymphadenitis have also been identified in the hearts of PCV2-infected animals aged 4-7 weeks (Oppreissnig *et al*., 2006).

While there is field evidence that infection of naïve gilts with PCV2 can cause foetal infections leading to mummification, it is unclear if this could occur in sows already exposed to PCV2 and with circulating antibody to the virus (Pensaert *et al*., 2004). Reports suggest that reproductive problems are most common in first-parity gilts: West *et al.* (1999) found field evidence of vertical transmission and associated reproductive failure in first-parity gilts. The introduction of naïve PCV2 seronegative gilts into a herd may be necessary for transplacental infection and resulting foetal pathogenesis (O'Connor *et al*., 2001; Mateusen *et al*., 2004).

Porcine respiratory disease complex (PRDC)

PRDC is a disease of growing and finishing pigs approximately 16-22 weeks of age. It is characterised by slow growth, lethargy, anorexia, fever, coughing and dyspnoea (Thacker, 2001). PRDC is considered to be the clinical manifestation of multiple disease agents working in concert with each other and with environmental and management factors (Halbur, 2001). Specifically, *M. hyopneumoniae*, combined with several opportunistic bacterial pathogens such as *P. multocida, Actinobacillus pleuropneumoniae* and *Streptococcus suis*, cause enzootic pneumonia which in turn seems to result in PRDC when combined with respiratory viruses such as various types of swine influenza virus and PRRSV (Halbur, 2001; Thacker, 2001; Holko *et al*., 2004). Although *M. hyopneumoniae* and PRRSV are considered to be the main pathogens responsible for PRDC, in recent years the prevalence of PCV2 in cases of PRDC has increased markedly. A study of pneumonia cases at the Iowa State University Veterinary Diagnostic Laboratory from 1993-2000 revealed that the incidence of PRRSV in such cases had increased 13-fold, while the incidence of PCV2 had increased 456-fold (Halbur, 2001). In another study of 105 PRDC cases, infections with PCV2 (81%), PRRSV (63%), PPV (57%) and swine influenza virus (13%) were identified, with PCV2-PRRSV co-infection identified in 55% of cases (Kim *et al*., 2003e). Interestingly, PCV2 antigen was mainly identified in the nucleus of macrophage-like cells in lung tissue (Kim *et al*., 2003e), whereas in previous studies of PCV2 distribution, the virus was found predominantly in the cytoplasm of lymphoid cells (Rosell *et al*., 2000).

Although it is unclear whether PCV2 is a primary, synergistic, secondary or opportunistic pathogen in cases of PRDC, the demonstration of lung lesions such as broncho-interstitial pneumonia in association with PCV2 DNA would suggest that PCV2 has an important role in PRDC (Kim *et al*., 2004) and that this and the sharp increase of PCV2 identified in cases of swine pneumonia would suggest that the clinical scope of PCV2 may be expanding, as suggested by Ellis *et al*. (2000).

Proliferative and necrotising pneumonia (PNP)

PNP was first described in Quebec in 1990 (Morin *et al*., 1990). First observed in the late 1980s, the condition was found to affect pigs between 15 days and 4 months of age, with a peak between the ages of 4 and 10 weeks (Morin *et al*., 1990). Adult pigs appeared clinically unaffected. The main clinical signs observed were fever, dyspnea and abdominal respiration, and surviving pigs displayed growth retardation and wasting (Morin *et al*., 1990).

Pneumonic lesions associated with PNP were found to have 3 distinctive features:

extensive lesions often involving the entire lung, fibrin deposits and necrotic cells in alveolar spaces, and severe and diffuse proliferation of bronchioalveolar epithelium (Morin *et al*., 1990). Lesions present in the lungs appeared to vary according to the stage of the disease: confluent consolidation of some or all lobes of one or both lungs was a common finding (Morin *et al*., 1990). Mediastinal and bronchial lymph nodes were enlarged and haemorrhagic, and in some cases evidence of heart failure was observed (Morin *et al*., 1990).

Histological lesions were characterised by alveoli filled with protein-rich oedema and large macrophages, with giant cells sometimes present. A proliferation of type II pneumocytes was also characteristic, particularly in the later stages of the disease (Morin *et al*., 1990).

Several bacterial infections were identified in PNP-affected pigs: *P. multocida*, *Haemophilus parasuis*, *Pneumocystis carinii* and *S. suis* type 2. Pathogens such as PPV and *M. hyponeumoniae* were also occasionally identified (Morin *et al*., 1990). The presence of these apparently secondary infections in affected animals was suggestive of immunosuppression. There were also reports of stillbirths, mummifications and abortions either before or during the respiratory problems in some affected herds (Morin *et al*., 1990).

The most common pathogen, and postulated cause of PNP, identified from 5 separate outbreaks, was influenza A virus (Morin *et al*., 1990). However, a few years after this initial study a virus designated Lelystad virus was identified in the Netherlands, associated with a 'mystery swine disease' characterised by outbreaks of reproductive failure and respiratory disturbances (Wensvoort *et al*., 1991). 'Mystery swine disease' was subsequently named porcine reproductive and respiratory syndrome (PRRS) and Lelystad virus was redesignated PRRSV.

PRRSV is an enveloped, single-stranded RNA virus, classified in the in the genus *Arterivirus* of the *Arteriviridae* family (Rossow, 1998). The presence of PRRSV in association with PNP in the absence of influenza virus led to speculation that PRRSV was the causative agent of PNP, not influenza. A study of 38 PNP field cases in Quebec between 1988 and 1993 found PRRSV in 28 samples and influenza in only one (Larochelle *et al*., 1994), and a more recent retrospective study of 60 PNP cases demonstrated PRRSV in 92% of cases, influenza in 2% and PCV2 in 42% of cases (Drolet *et al*., 2003). A study of swine pneumonia cases for the year 1999 found PRRSV in 35% of cases, swine influenza virus in 21%, *M. hyopneumoniae* in 23% and PCV in 9% of cases (Harms and Sorden, 2000). This data would appear to confirm PRRSV as the causative agent of PNP, however the role, if any, of PCV2 in

this disease remains to be fully investigated. The fairly high prevalence of PCV2 in PNP cases may simply reflect the high infection rate of PCV2 in healthy pigs (Drolet *et al*., 2003). It was noted that in some cases the amount of PCV2 nucleic acid detected was so high that damage to the lungs would be likely (Drolet *et al*., 2003), but this abundance of PCV2 antigen may be a result of increased replication in diseased and possibly immunosuppressed pigs.

Exudative epidermitis (EE)

EE is a generalised skin infection characterised by a greasy cutaneous exudation, exfoliation, vesicle formation and death in suckling or newly weaned piglets (Sato *et al*., 2000; Wegener and Skov-Jensen, 1999). Mortality may be as high as 70%, and during an outbreak all litters on a farm may be affected (Wegener and Skov-Jensen, 1999).

Exfoliative toxin-producing strains of *Staphylococcus hyicus* are thought to be the cause of the disease, but predisposing factors seem necessary for it to develop (Andresen *et al*., 1993; Wegener and Skov-Jensen, 1999). Both virulent and avirulent strains of the bacterium have been reported, and both types may be present on the skin of diseased and healthy pigs (Andresen *et al*., 1993). While virulent strains of *S. hyicus* may be recovered from the skin of adult pigs, EE has not been reproduced experimentally in adult pigs (Wegener and Skov-Jensen, 1999). The disease has been reproduced experimentally in SPF piglets, and conventional pigs may be resistant to experimental infection, suggesting that immunological factors in the affected herd may be important for disease development (Wegener and Skov-Jensen, 1999). The disease may occur sporadically with low morbidity or it may affect all litters in an epidemic form, which commonly occurs following the introduction of new animals into a closed herd (Wegener and Skov-Jensen, 1999).

In 1993 a Swedish SPF pig herd suffered an outbreak of EE (Wattrang *et al*., 2002). This herd had been established by caesarean section and was closed to all outside material except semen used for AI. A retrospective serological survey determined that the herd was seronegative to PCV2 until approximately 2 months before the EE outbreak, all animals had seroconverted by 4 months after the outbreak and all animals remained positive for PCV2 antibody for the next 7 years (Wattrang *et al*., 2002). *S. hyicus* was isolated from all piglets tested, irrespective of clinical signs, and PCV2 antigen was isolated from the lymph node of one affected piglet. An increase in the number of stillbirths and small litters was also observed at time of PCV2 seroconversion (Wattrang *et al*., 2002).

Exudative epidermitis has also been induced experimentally by the infection with

PCV2 of 3-week-old caesarean-derived colostrum-deprived (CD/CD) pigs, but the condition was noted in both PCV2-inoculated and sham-inoculated (PCV2-negative) piglets (Harms *et al*., 2001). Interestingly, the disease was more severe in the PCV2-inoculated group, and while the PCV2-negative piglets responded to antibiotic therapy, the PCV2-inoculated pigs did not (Harms *et al*., 2001). This suggests that PCV2 could play a role in the development of the disease, possibly as a predisposing factor rather than the main cause of the condition (Wattrang *et al*., 2002).

A retrospective study conducted on 142 cases of EE between 1997 and 2002 found that PCV2 and porcine parvovirus (PPV) were highly prevalent in these cases, however both viruses were absent in noninflammed skin from pigs with *S. hyicus* infection and from normal healthy pigs (Kim and Chae, 2004). This suggests that PCV2 and PPV may be involved in lesion development and/or progression during EE (Kim and Chae, 2004).

Various combinations of antibiotics, plus skin disinfectants, vaccination and improved herd management and hygiene are recommended for the treatment of EE (Wegener and Skov-Jensen, 1999).

Enteritis

Enteritis may occur in pigs for a number of reasons including bacterial infections with agents such as *Yersinia pseudotuberculosis* (Slee and Button, 1990), *Salmonella enterica* and *Lawsonia intracellularis* (Moxley and Duhamel, 1999). While pigs affected by PMWS may suffer from diarrhoea (Harding et al., 1998), a recent study found 6 cases of PCV2-associated enteritis in the absence of any signs of PMWS (Kim *et al*., 2004). The most unique lesions in these cases were granulomatous inflammation affecting Peyer's patches, characterised by infiltration of epitheliod macrophages and multinucleated giant cells. No microscopic lesions were observed in the lymphoid tissues as is found in cases of PMWS, and PCV2 was detected in abundance by *in situ* hybridisation in association with the lesions observed in Peyer's patches (Kim *et al*., 2004). Furthermore, no pathogenic bacteria or other viruses were detected in the intestines of the affected pigs, suggesting that the enteritis was associated with and possibly caused by PCV2. Other cases of PCV2-associated enteritis have not been reported, but as the clinical scope of PCV2 appears to be expanding, it would appear likely that more cases will be identified.

Post-weaning multisystemic wasting syndrome (PMWS)

Diagnosis

The major disease syndrome that has been associated with PCV2 is PMWS. The link was first established in 1997 (Clark, 1997), and 3 criteria must be met for a

diagnosis of PMWS to be made: the presence of clinical signs (usually wasting), the presence of characteristic histopathological lesions in lymphoid tissue, and the presence of PCV2 within those lesions (Sorden, 2000; Quintana *et al*., 2001). A diagnosis of PMWS is usually confirmed by the presence of microscopic lesions such as lymphocyte depletion, multinucleate giant cell infiltration and cytoplasmic inclusions (Segales *et al*., 2004). However these criteria are not absolute or infallible, and it appears that the best samples to use for PMWS diagnosis are lymphoid tissues from pigs in the first week of clinical disease (Segales *et al*., 2004). In addition, most of the clinical signs of PMWS, such as wasting and respiratory distress, are non-specific and may be present in pigs affected by a variety of other swine diseases (Segales *et al*., 2004).

Clinical signs and lesions of PMWS

Pigs suffering from PMWS usually develop clinical signs between 6 and 8 weeks of age (Harding *et al.*, 1998). Major clinical signs are weight loss, tachypnea, dyspnea, enlarged lymph nodes and jaundice. Less common signs are diarrhoea, productive coughing and central nervous system disturbances (Harding *et al.*, 1998). Anaemia is a common finding in PMWS-affected pigs, and is probably caused by gastric ulceration (Segales *et al*., 2000). Morbidity is usually low, but case fatality may be high. Death is usually due to respiratory, gastrointestinal and/or urinary system failure in the field, although deaths from liver failure have been observed in gnotobiotic pigs experimentally infected with PCV2 (Krakowka *et al*., 2002).

Organ failure is caused by the virus disseminating out of lymph tissues into other target organs, accompanied by granulomatous inflammatory cell infiltrates (Krakowka *et al*., 2002). It is thought that PCV2 may induce monocyte chemo-attractant protein-1 (MCP-1) expression by macrophages and other inflammatory cells in infected lymph nodes, suggesting that MCP-1 could be involved in lesion development or progression during PCV2 infection and associated disease (Kim and Chae, 2003b). MCP-1 is known to be produced during granulomatous inflammation and to participate in it, and in the case of PMWS, macrophages, epithelioid cells and multinucleated giant cells may ‘recruit’ mononuclear cells to a site of granulomatous inflammation by producing MCP-1 (Kim and Chae, 2003b). This progressive angiocentric granulomatous inflammation compromises organ function, leading to death (Krakowka *et al*., 2002).

Post-mortem examination of PMWS-affected pigs usually reveals skin pallor and muscle wasting, with lymph nodes 3-4 times normal size (Clark, 1997). The lungs

are rubbery and non-collapsed, the spleen is moderately enlarged, and the liver may be mottled or atrophic (Clark, 1997). Generally, the most consistent abnormalities occur in the lymph nodes and lungs (Clark, 1997). Interstitial pneumonia is often present, and apoptosis of liver cells may occur (Meehan *et al*., 1998; Clark, 1997). Gross and histopathologic lesions are found in virtually all tissues, but are most common in the lungs, liver, kidneys and lymph nodes (Harding *et al.*, 1998).

The main histopathological findings of PMWS are lymphocyte depletion, interstitial pneumonia and hepatitis (Allan *et al*., 1998; Rosell *et al*., 1999). Not all histological lymphoid lesions are found together in PMWS-affected pigs, suggesting that these lesions may change throughout the course of the disease. Quintana *et al*. (2001) found that histiocytic infiltration and lymphocyte depletion were much more common and severe in the early stages of PMWS, and were not always found in the later stages of the disease. Atrophy of the thymus, causing severe lymphocyte depletion in peripheral blood and tissues in a similar fashion to cases of BFD and CAV-associated disease, has also been observed in PMWS cases (Darwich *et al*., 2003; Todd *et al*., 2000). The inflammatory lesions seen in PMWS are thought to be immune-mediated, probably antiviral humoral and cellular responses to viral antigenic epitopes (Krakowka *et al*., 2002).

A major retrospective Japanese study of tissues from affected pigs, collected from 1985 to 1999, found lesions suggestive of PMWS from 1990 onwards (Sato *et al.*, 2000). In this study, histopathological lesions were found mainly in the lymphoid organs, and included lymphocellular depletion and apoptosis, with replacement by multinucleate giant cells. In lymph nodes, granulomatous inflammation was present in some cases, and spherical inclusion bodies were present in macrophages and also occasionally in giant cells (Sato *et al.*, 2000). Similar changes were noted in the spleen, tonsils and Peyer's patches, but the changes in these organs were less severe (Sato *et al.*, 2000). Cytoplasmic inclusions were also found in the lungs, liver and kidneys. Macrophages and lymphocytes usually accumulated in the kidneys and liver, and hepatocytic necrosis was also present in a large percentage of cases (Sato *et al.*, 2000). Interstitial pneumonia was commonly found in the lungs. PCV2 antigen was found mainly in cytoplasmic inclusions of macrophages, but also in lymphatic organs, small intestine, lungs, kidney and liver. Generally, disease was more severe in those animals where various tissues were affected, rather than just the tonsils and lymph nodes (Sato *et al.*, 2000).

PCV2 has been found in pigs with wasting disease worldwide (Hamel *et al.*, 1998;

Allan *et al.*, 1999a; Onuki *et al.*, 1999; Martelli *et al*., 2000; Kyriakis *et al*., 2000; Wellenberg *et al.*, 2000; Done *et al.*, 2001; Trujano *et al*., 2001; Saradell *et al*., 2004; Castro *et al*., 2004; Jemersic *et al*., 2004; Maldonado *et al*., 2004; Wang *et al*., 2004; Motovski and Segales, 2004; Garkavenko *et al*., 2005; Vigre *et al*., 2005). Several techniques have been used to demonstrate the presence of PCV2. Ouardani *et al*. (1999) used a multiplex PCR to detect PCV2 in 94.2% of suspected PMWS-affected pigs tested. Immunohistochemistry has also been used to demonstrate virus antigen in tissues (Sorden *et al.*, 1999).

PCV1 does not seem to be associated with the condition. PMWS-affected pigs infected with PCV1 are usually co-infected with PCV2 (Ouardani *et al.*, 1999). However, LeCann *et al*. (1997) detected PCV1 only in piglets in Brittany affected by a wasting disease, and the lesions found, such as enlarged lymph nodes and respiratory distress, were similar to PMWS. This is, however, the only report of an association between PCV1 and any type of wasting disease.

Experimental reproduction of PMWS

Determination of the pathogenesis of PMWS has been frustrated by the inconsistency of clinical signs between and within infected herds, and the difficulty of inducing PMWS experimentally. Attempts to reproduce PMWS by experimental inoculation of PCV1 or PCV2 into piglets have been largely unsuccessful, with no clinical signs of disease recorded in most cases (Tischer *et al*., 1986; Pogranichnyy *et al*., 2000) although mild to moderate lesions typical of PMWS have been induced. Lesions induced with PCV2 have included lymphoid depletion (Allan *et al.*, 1999b), mild pneumonia, lymphoid inflammation (Ellis *et al*., 1999; Magar *et al*., 2000), multinucleated giant cells (Magar *et al.*, 2000) and mild nephritis and hepatitis (Ellis *et al.*, 1999). Pigs inoculated with tissue homogenates from PMWS-affected pigs also have not developed major clinical signs of the disease, only slight lymphocyte depletion (Balasch *et al.*, 1998), and nephritis, hepatitis, pneumonia and myocarditis (Ellis *et al.*, 1999). Adding to the confusion regarding the role of PCV2 in the disease is that PCV2 has also been widely detected in clinically normal pigs (Done *et al.*, 2001) and antibodies have been detected on farms with no history of PMWS (Rodriguez-Arrioja *et al*., 2000; Larochelle *et al*., 1999; Sorden, 2000). It has been suggested by some that another factor must be involved in disease development, such as concurrent infections with other organisms and immune activation.

A frequent suggestion is that other co-pathogens may be required for the development of PMWS, accounting for the difficulty of reproducing the disease

using PCV2 alone. Several studies have provided evidence for this theory, with co-infection of pigs with PCV2 and other viral and bacterial pathogens such as PPV (Krakowka *et al.*, 2000; Allan *et al*., 1999b; Ellis *et al.*, 2000; Kim and Chae, 2004; Cao *et al*., 2005), PRRSV (Allan *et al*., 2000c; Krakowka *et al*., 2000; Wellenberg *et al*., 2004), porcine pseudorabies virus (PRV) (Cao *et al*., 2005), candidiasis (Zlotowski *et al*., 2006) and *M. hyopneumoniae* (Opriessnig *et al*., 2004) resulting in more severe clinical signs of PMWS than infection with PCV2 alone.

Co-infection of pigs with PCV2 and PPV produced more severe clinical signs of PMWS, such as depression, anorexia, icterus and liver disease, than infection with PCV2 alone (Krakowka *et al.*, 2000). PPV by itself is not considered a major pathogen, so it has been suggested that either temporary PPV infection of macrophages could promote higher levels of PCV2 replication, or that PPV is immunosuppressive, allowing the spread of PCV2 throughout the body (Krakowka *et al.*, 2000). It is also possible that a similar interaction of PCV2 and PPV could occur in cases of EE, with the increased levels of DNA replication and macrophage proliferation caused by PPV enhancing the replication of PCV2 (Kim and Chae, 2004). In support of the dual role of PPV and PCV2, experimental infection of pigs with PCV2 and PPV produced severe, sometimes fatal disease, with lesions in the liver, lymph nodes and kidneys (Allan *et al*. 1999b). However, only a small percentage of PMWS cases are naturally co-infected with PPV (Ellis *et al.*, 2000) and vaccination against PPV does not reduce the incidence of PMWS (Opreissnig *et al*., 2004b), suggesting that the role of PPV is indirect and that the effect it induces can be mimicked by other agents or factors.

Co-infections with PCV2 and PRRSV have also been suggested as a cause of PMWS. PRRS was recognised in the USA in 1987 as causing late-term reproductive failure and severe pneumonia in neonatal pigs (Rossow, 1998). Clinical signs include ill-thrift, dyspnea, tachypnea, anorexia, lethargy, pneumonia and rough hair coat, and gross lesions such as lung consolidation and enlarged lymph nodes (Rossow, 1998). PRRS can be difficult to differentiate clinically from PMWS and co-infections with PCV2 and PRRSV have been commonly detected in countries where PRRSV is endemic. Hamel *et al.* (2000) found PCV2 in 55% of pigs with respiratory signs and lesions similar to PRRS, and respiratory problems have been found to be more severe in herds co-infected with PCV2 and PRRSV (LeCann *et al.*, 1997; Quintana *et al.*, 2001). Experimental co-infection of pigs with PCV2 and PRRSV resulted in more severe disease than with either agent alone (Krakowka *et al*., 2000;

Allan *et al*., 2000c). PRRSV may enhance PCV2 replication possibly by macrophage activation, the enhanced proliferation of PCV2 target cells or a possible release of PCV2 from cell-cycle dependence (Krakowka *et al*., 2000). Macrophages are the only cell type known to support PRRSV replication (Rossow, 1998) and after infection, PRRSV replicates in the regional macrophages, followed by a prolonged viremia and systemic distribution of virus to other macrophage populations (Rossow, 1998). Pigs co-infected with PCV2 and PRRSV frequently suffer from interstitial pneumonia, and a recent study has demonstrated that this may be due to high levels of interferon alpha (IF-α) and tumor necrosis factor alpha (TNF-α) produced during PCV2 and PRRSV co-infection (Chang *et al*., 2005).

PCV2 may also facilitate secondary infections by reducing the ability of alveolar macrophages to phagocytose other fungal and bacterial pathogens (Chang *et al*., 2006).

The role of immune activation in the pathogenesis of PMWS

Krakowka *et al*. (2001) suggested that activation of the immune system during the early phase of PCV2 infection allows the rapid replication and distribution of PCV2, producing wasting disease. These authors injected piglets with keyhole limpet haemocyanin (KLH) in Incomplete Freund's adjuvant (ICFA), which stimulated the pigs immune system, inducing mitosis and proliferation of histiocytes and macrophages at the injection site, which seemed to provide the optimal conditions for PCV2 replication and dissemination *in vivo* (Krakowka *et al.*, 2001). It was theorised that PCV2 could replicate in macrophages, with the immunoproliferation induced by the adjuvant and KLH was leading to heightened PCV2 replication and therefore greater PCV2 virion production (Krakowka *et al.*, 2001). More recent studies have shown that PCV2 will infect macrophages, but does not replicate within them. Instead, it appears likely that macrophages act to distribute the virus in vivo to major organs, with the non-productive infection helping the virus to escape detection by the host immune system (Shibahara *et al*., 2000; Krakowka *et al*., 2002; Todd, 2004; Chang *et al*. 2005).

The most severe lesion produced in piglets inoculated with the adjuvant and PCV2 was liver damage. All piglets in this group suffered liver cell damage, with missing hepatocytes replaced by macrophages, while piglets inoculated with PCV2 only did not suffer any liver damage (Krakowka *et al.*, 2001). The hepatic macrophages contained PCV2 inclusion bodies, which were also found in the lymph nodes (Krakowka *et al.*, 2001). This lesion is similar to that detected in a more recent study, where there was massive liver damage, liver necrosis and

hepatocellular inclusion bodies in piglets experimentally infected with tissue homogenates from PMWS-affected pigs (Hirai *et al*., 2003).

It is unclear what secondary agent or factors could activate the immune system under field conditions. PRRSV and PPV have been suggested as possible candidates, but PMWS can affect herds free of both these pathogens (Segales *et al.*, 2001), In herds naturally infected with PMWS, the primary organ system affected and the mortality rate varies within and between infected herds (Krakowka *et al.*, 2001) and it has been suggested that this could be due to the effect of other infectious agents such as PRRSV and PPV, and how they combine with PCV2 to influence the major organ system affected (Krakowka *et al.*, 2001). For example, pigs co-infected with PCV2 and PRRSV may have more severe lung lesions (Krakowka *et al.*, 2001).

While the results of Krakowka *et al*. (2001) suggested an important role of immunostimulation in the pathogenesis of PMWS, further experiments cast doubt on this theory. Ladekjaer-Mikkelsen *et al*. (2002) also inoculated young SPF piglets with PCV2 and immunostimulated half of them using the same adjuvants as Krakowka *et al*. (2001). Unexpectedly, 2 of 5 non-stimulated pigs infected with PCV2 died and another in the same group had to be euthanased. These pigs developed a persistent fever 12 days after infection, and upon necropsy all had gross and histological lesions consistent with PMWS. In contrast, only one out of 5 immunostimulated piglets developed wasting disease, the remainder of the stimulated and non-stimulated piglets remained clinically normal throughout the experiment, although all ten PCV2-infected pigs seroconverted. Further investigation using quantitative PCR revealed that all 4 PMWS-affected piglets had high levels of PCV2 in serum, leading to the conclusion that the development of PMWS may be correlated with PCV2 titre in serum, rather than immunostimulation (Ladekjaer-Mikkelsen *et al*., 2002).

A subsequent study by Okuda *et al*. (2003) involving experimental inoculation of CD/CD piglets with a PCV2 isolate originating from a PMWS pig, provided support for the theory of Ladekjaer-Mikkelsen *et al*. (2002) that the development of PMWS is associated with PCV2 titre in serum. In their experiments, 6 of 16 inoculated piglets developed PMWS, with PCV2 detected in all tissues tested. The piglets that developed clinical signs of PMWS showed the highest virus titre in all tissues. Interestingly, serum antibody to PCV2 was detected in most of the PCV2-infected pigs at 14 and 21 days post-infection and antibody was not detected in pigs exhibiting clinical signs until 28 days post-infection. The results suggest the

development of PMWS may depend on quantitative aspects of PCV2 distribution in tissues and the antibody response (Okuda *et al*., 2003). Two other studies involving pigs naturally affected by PMWS also found that healthy pigs had lower titres of PCV2 than PMWS-affected pigs (Olvera *et al*., 2004; Brunborg *et al*., 2004) and that PCV2 viral load increased with the severity of microscopic lesions (Olvera *et al*., 2004) and the intensity of immunocytochemical staining (Brunborg *et al*., 2004) in lymphoid tissue. A more recent study directly correlated the PCV2 viral load in lymphoid tissues and liver with the severity of PMWS clinical signs (Krakowka *et al*., 2005), and a similar experiment using a PCV2 infectious clone as the inoculum also demonstrated that viral load was directly related to the severity of PMWS clinical signs. Interestingly, this study also used immunostimulation to enhance disease development.

Earlier studies on PCV2 infection also suggested that PCV2 viral load may affect disease development, although this was not necessarily recognised at the time. Experimental inoculation of conventional pigs with PCV2 and PRRSV produced increased febrile response in all dual-inoculated pigs and clinical signs of severe PMWS in some dual-inoculated pigs and one pig inoculated with PCV2 alone (Rovira *et al*., 2002). Pigs infected with PCV2 and PRRSV had significantly higher PCV2 viral titres than those infected with PCV2 only, and one dual-inoculated pig was found to have very high PCV2 viral load at every sampling point and subsequently died before the end of the experiment (Rovira *et al*., 2002). Another study that examined healthy PCV2-positive pigs taken from a farm experiencing PMWS (Quintana *et al*., 2001) found they also had microscopic lymphoid lesions typical of the disease, albeit much less severe than affected pigs, and much less PCV2 nucleic acid than diseased pigs (Quintana *et al*., 2001), again suggesting that PCV2 viral load may be crucial for disease development.

Interestingly, it was also found that PDNS-affected pigs had fairly low viral loads when compared to PMWS pigs at any stage of the disease, suggesting that PCV2 viral load probably does not affect PDNS disease development (Olvera *et al*., 2004).

The emergence of PCV2-associated diseases

Shortly after it was first described, PMWS was detected in several countries, and the sudden emergence of the disease has not been explained. It was not due to the emergence of PCV2, as antibodies to this virus were present in sera from Japan dating back to 1985 (Sato *et al*., 2000), in North America from 1987 (Nawagitgul *et al*., 2002) and Northern Ireland from 1973 (Walker *et al*., 2000).

After the reproduction of PMWS by immunostimulation it was proposed that pathogenic PCV2 infection and therefore PMWS may have resulted from a combination of changed management practises such as early weaning and an overwhelming challenge to a pig's immune system as a result of the growing use of a variety of vaccines administered to young pigs (Allan *et al.*, 2001). To test this theory, these authors withdrew a *M. hyopneumoniae* vaccination program from about half the piglets born on a farm over a 4 month period. In the non-vaccinated animals, losses due to PMWS were significantly reduced in 4 out of the 5 groups studied (Allan *et al.*, 2001). In another study involving vaccination with bacterins in PCV2-infected pigs, it was found that vaccination along with PCV2 infection caused mild transient respiratory disease and diarrhoea (Opriessnig *et al*., 2003). It was also found that vaccinated pigs were viraemic for longer, had a higher copy number of the PCV2 genome in their sera, a wider tissue distribution of PCV2 antigen and increased severity of lymphoid depletion compared to unvaccinated pigs (Opriessnig *et al*., 2003). The age of vaccination was also suggested to be important as pigs vaccinated later than 5-weeks-of-age did not develop clinical signs of PMWS (Opreissnig *et al*., 2003). It was also suggested that the vaccination of gilts for PPV and erysipelas separately during their 'acclimatisation period' might increase the risk of PMWS (Rose *et al*., 2003) but a subsequent study of 7 French herds with and without PMWS found no evidence linking PMWS to any vaccine (Larochelle *et al*., 2003).

While the appearance of PMWS in some countries such as Denmark has resembled an infectious disease outbreak (Vigre *et al*., 2005), suggesting the introduction of a new pathogen, the emergence of new genetic variants of PCV2 is not currently considered to be a satisfactory explanation for the apparent differences in pig and herd susceptibility to PMWS and other PCV2-related diseases. While it has been suggested that PCV2 is a pathogenic variant generated from the non-pathogenic PCV1 by point mutation (Ellis *et al.*, 1998), no genetic differences have been detected in PCV2 strains from healthy and diseased pigs (Larochelle *et al*., 2002; Pogranichniy *et al*., 2002; Choi *et al*., 2002; de Boisseson *et al*., 2004; Grierson *et al*., 2004). Although one study of CAV identified a single amino acid change as a potential determinant of viral pathogenicity (Yamaguchi *et al.*, 2001), no motif or amino acid changes have been identified as a basis for explaining the pathogenicity of PCV2 (Choi *et al*., 2002; Larochelle *et al.*, 2002). While different PCV2 sequences isolated from the same country may be quite variable, as is the case with Hungary (Dan *et al*.,

2003), Korea (Kim and Lyoo, 2002) and France (de Boisseson *et al*., 2004), isolates from different continents may be quite similar and not cluster on any geographic basis (Choi *et al*., 2002; Larochelle *et al*., 2002). This suggests that the existence in some countries of widespread PCV2 infection but no PCV-associated disease is not due to the presence of an apathogenic PCV2. For example, no PMWS outbreak was confirmed in Sweden until 2003, although the virus had been present for 10 years (Wattrang *et al*., 2002; Wallgren *et al*., 2004).

Recently it has been shown that the replication factors of PCV1 and PCV2 are exchangeable, and that both types of PCV replicate at a similar rate *in vitro*. This would suggest that viral replication strategy may not be the main factor determining the difference in pathogenicity between PCV1 and PCV2 (Mankertz *et al.*, 2003).

Whether co-infecting pathogens are associated with PCV2-associated disease is uncertain. Certainly, some pathogens such as PPV and PRRSV have been shown to act synergistically with PCV2 to produce more severe disease than with PCV2 infection alone (Ellis *et al*., 1998; Allan *et al*., 2000), and it has been found that farms which are positive for PPV and PRRSV have an increased chance of experiencing PMWS (Rose *et al*., 2003). However, no other infectious agent or agents have been consistently identified in cases of PMWS, suggesting either that most co-infections are opportunistic in nature, or if they are involved that their effect is indirect. It has been suggested that co-infecting agents which can cause cell death and therefore lead to tissue regeneration may provide the rapidly dividing cells optimal for PCV2 replication, therefore increasing viral load and possibly causing disease (Ellis *et al*., 2004). Similarly, an increase in the production of cytokines and other growth factors affecting cell division may also cause an increase in PCV2 replication by increasing the number of target cells (Ellis *et al*., 2004). Immune activation, which may be caused by management practices such as vaccination, has been demonstrated experimentally to increase cytokine secretions in experimentally infected animals which subsequently developed PMWS (Krakowka *et al*., 2001). Meerts *et al*. (2005b) demonstrated that even gnotobiotic pigs raised and inoculated with PCV2 in identical fashions may differ in their responses to the virus, some mounting an efficient immune response while others were unable to control viral replication. The amount of maternal antibody can also influence the degree of protection from PCV2 infection; high titres of maternal antibody can protect against infection, but low titres cannot (McKeown *et al*., 2005).

It seems therefore, despite the fact that the PMWS disease syndrome has emerged recently and quite suddenly all over the world, that PCV2 is not a 'new' pathogen. It is more likely that changed management and environmental conditions are responsible for the emergence of the disease, and that the health and immunological status of herds or individual pigs possibly relating to early vaccination, high virus prevalence in the environment and low maternal antibody status may affect disease development.

Control of PCV-associated disease

There is no cure for the various PCV-associated disease syndromes although the use of antibiotics to control secondary infections may be of use (Ohlinger *et al*., 2000). As circoviruses are resistant to environmental degradation such as changes in temperature and pH (Todd, 2000) these viruses persist in the environment and the eradication of PCV is not a practical consideration. Control is focused therefore on altering management conditions on affected farms (Chen, 2000). Management practises such as batch mixing, adoptions, litter mixing and large pens are not recommended (Guilmoto and Wessel-Robert, 2000). On-farm semen collection, versus the use of insemination centres, may also increase the risk of PMWS (Rose *et al*., 2003). Management practises such as improving hygiene and regular herd treatment against external parasites have been recommended (Rose *et al*., 2003).

It has been suggested that PCV2 may be carried from farm to farm by some kind of secondary vector such as birds (MacKinnon, 2000). However as most circoviruses are host-specific, birds or other small animals as a vector for PMWS-associated virus spread seems unlikely (Todd, 2004).

PCV2 vaccination

As it has been theorised that PCV2-associated disease may occur when seronegative animals are exposed to the virus, either *in utero* or postnatally (O'Connor *et al*., 2001; Lukert and Allan, 1999), treatment of PMWS and PDNS by 'serum therapy' has been tried in the UK. Serum from older pigs in the herd has been administered to young piglets at weaning, to confer passive immunity to PCV2 (Dean, 2002). No data is available on the success rate of this treatment, although there is some anecdotal evidence that mortality is reduced. However, the risk of transferring other pathogens by movement of serum from an abbatoir back to the farm of origin may be dangerous and somewhat irresponsible (Potter, 2002).

PCV2 vaccines have been trialled in several forms. A DNA vaccine expressing

the capsid protein of PCV2 was found to be effective at raising PCV2 antibodies in a mouse model (Kamstrup *et al.*, 2004). The possibility of vaccination against PCV2 using chimeric infectious clones has been considered (Fenaux *et al*., 2003). To create a chimeric clone, PCV2 capsid DNA was cloned into the backbone of PCV1, and when inoculated into young pigs, PCV2 antibodies were produced. A subunit vaccine to enhance the immune response of piglets was reported to provide protection against severe PMWS but this method remains to be validated (Blanchard *et al*., 2003). Recently, recombinant viral vaccines have been developed as potential treatments for PCV2; a recombinant pseudorabies virus expressing the fused ORF1-ORF2 of PCV2 was shown to induce significant anti-PCV2 immunity in mice and pigs, although the vaccine has not yet been tested in response to experimental PCV2 challenge (Ju *et al*., 2005). In addition, a recombinant adenovirus expressing the capsid protein of PCV2 was shown to elicit immune responses against PCV2 in mice (Wang *et al*., 2006).

RNA interference (RNAi)

RNA interference is a process by which double-stranded RNA directs sequence-specific post-transcriptional gene silencing, which may be used to reduce viral RNA production, and therefore DNA and protein production. This technique reduced PCV2 transcription and translation in PK-15 cells, and also in a mouse model (Liu *et al*., 2006). Therefore this strategy could be useful to decrease PCV2 viral load, shown to be directly correlated with disease severity (Okuda *et al*., 2003; Olvera *et al*., 2004; Brunborg *et al*., 2004; Krakowka *et al*., 2005). However, experimental data using pigs is required to validate this technique as a potential therapy for PMWS and PCV2-associated diseases.

Management practises as a factor in PCV-associated diseases

As the incidence of PMWS and other PCV2-related diseases cannot be explained by simple infection with PCV2 (Done *et al*., 2001) or genotypic variation of PCV2 (Choi *et al*., 2002; Larochelle *et al*., 2002), management conditions may well play a role in disease development. Draughts, overcrowding, poor air quality, mingling of different age groups and infection with other agents may contribute to the occurrence of PMWS and PDNS (Choi *et al*., 2002b). Both PMWS and PDNS are common in eastern Great Britain, and the high prevalence of disease is possibly caused by high levels of pig movement, and pigs from up to 15 different sources may be together on one farm (MacKinnon, 2000). A high proportion of PDNS and PMWS cases are associated with corporate production, a fairly recent occurrence in the UK (MacKinnon, 2000). Such operations usually have large numbers of pigs from many

different sources on the one farm, and this may contribute to the development of PMWS and/or PDNS (MacKinnon, 2000).

A variety of management-related treatments for PMWS have been proposed but there is no clear indication of the efficacy of any of these procedures. Multivitamin supplements and reduced handling and stress were proposed by Baird *et al*. (2000). The use of anti-inflammatory drugs may be beneficial (Yoon *et a*l., 2004). The control of secondary bacterial infections is important, and intramuscular corticosteroids may also help, although the improvement is only temporary (Baird *et al*., 2000). Improved hygiene and segregation of sick animals may be important to reduce viral challenge and the spread of infection (Harding, 1997). Changes in diet could be beneficial, and one report suggested that conjugated linoleic acid (CLA), when compared to a control diet of soybean oil, appeared to ameliorate PCV2 viral disease and reduce the severity of pneumonic lesions (Bassaganya-Riera *et al*., 2003).

While it seems likely that changed pig farm management conditions have contributed to the recent increase in PCV2-associated disease, PCV2 has also been identified in cases of multisystemic disease in free-range wild pigs (Ellis *et al*., 2003; Schulze *et al*., 2004). However, wild pigs are still subject to environmental stresses (Ellis *et al*., 2003) which may help to precipitate PCV2-associated disease.

PCV in Australia

Analysis of a relatively small number of samples from pigs submitted to this laboratory has identified PCV1 and PCV2 in the tissues of pigs originating from several Australian states (Raye, personal communication). Initial sequence analysis has shown these strains have very high nt sequence identity, and are very similar to strains that have been detected in association with PMWS overseas. There have been no outbreaks of PWMS in Australia, and while PDNS has been detected (Cameron, 1995) it has been observed infrequently. It seems likely that the Australian situation is similar to Sweden, where PCV2 infection is widespread and the strains present are able to produce PMWS under experimental conditions, but no naturally-occurring cases of PMWS were identified until 2003 (Allan *et al*., 2002; Wattrang *et al*., 2002; Wallgren *et al*., 2004). A prevailing hypothesis is therefore that there are other, as yet unidentified factors, required for the development of PMWS which are not present in Australia. PRRSV and PPV have been suggested as possible co-factors combining with PCV2 to produce disease in other countries (Krakowka *et al*., 2000; Sato *et al*., 2000) and while PPV was, before the onset of vaccination programs, widespread in Australia (Buddle, personal communication),

PRRSV is not present in Australia. Herd management variables such as early weaning, early and extensive vaccination programs and large herd sizes have recently been suggested to affect the occurrence of PMWS and PDNS (Allan *et al*., 2001, Cook *et al*., 2001) and perhaps the management practises used in Australian pig herds are sufficiently different to explain why PMWS is not present.

CHAPTER 3. THE DETECTION OF PORCINE CIRCOVIRUS IN THE AUSTRALIAN PIG HERD

Summary

The objective of this study was to determine if porcine circovirus (PCV) was present in the Australian pig herd. A multiplex polymerase chain reaction (PCR) was utilised to detect PCV in tissues from weanling pigs found dead and from pigs originating from herds experiencing outbreaks of disease such as congenital tremors (CT). Tissues from approximately 350 pigs from Western Australia (WA), New South Wales (NSW), South Australia (SA) and Queensland were tested for the presence of PCV type 1 (PCV1) and PCV type 2 (PCV2) DNA. PCV1 DNA was detected in pig tissue samples from WA, SA and NSW and PCV2 DNA was detected in pig tissue samples from WA, NSW and Queensland. Sequence analysis of the PCR products was conducted to confirm the PCR results. It was concluded from these results that both PCV types (PCV1 and PCV2) were present in Australia, although no causal relationship with any disease outbreak was proven.

Introduction

Porcine circovirus was first identified in 1974 as a persistent infection of the immortal PK-15 pig kidney cell line (Tischer *et al.*, 1974). As it was non-cytopathic it was assumed to be non-pathogenic (Tischer *et al.*, 1982) and it was not possible to associate any disease with it, either experimentally or in the field (Tischer *et al*., 1986). The virus infecting the PK-15 cell line was subsequently designated PCV1 (Meehan *et al*. 1998). A second type of PCV, designated PCV2 was identified and characterised (Meehan *et al*., 1998) and has since been detected in pigs suffering from a number of conditions, including PMWS (Hamel *et al.*, 1998; Meehan *et al*., 1998; Allan *et al.*, 1999a; Onuki *et al.*, 1999; Kyriakis *et al*., 2000; Martelli *et al*., 2000; Wellenberg *et al.*, 2000; Done *et al.*, 2001; Trujano *et al*., 2001), porcine dermatitis and nephropathy syndrome (PDNS) (Gresham *et al*., 2000; Rosell *et al.*, 2000; Choi *et al*., 2002b; Saoulidis *et al*., 2002) and congenital tremor (CT) (Stevenson *et al.*, 2001; Choi *et al*., 2002). The clinical scope of PCV2 may be expanding, with recent increases in the prevalence of PCV2 found in cases of reproductive problems (West *et al*., 1999; Lyoo *et al*., 2001; Sanchez *et al*., 2001), proliferative and necrotising pneumonia (Drolet *et al*., 2003) and porcine respiratory disease complex (Halbur, 2001; Kim *et al*., 2003).

Although PMWS was not formally described until 1997, the disease was found to have been present in Canada since 1991 (Clark, 1997). Furthermore, retrospective studies of archival pig sera have demonstrated that PCV2 has been present in pigs

from Japan, dating from 1985 (Sato *et al*., 2000), America from 1987 (Nawagitgul *et al*., 2002) and Northern Ireland from 1973 (Walker *et al*., 2000), suggesting that the virus has been circulating in pigs for some time.

One of the difficulties associated with the study of PCV-associated disease is that both PCV1 and PCV2 are common in clinically normal pigs (Tischer *et al*., 1995b; Done *et al.*, 2001). It is common for pig herds to be seropositive to PCV2 without experiencing disease and individual pigs within a diseased herd may be infected with PCV2 yet remain healthy (Larochelle *et al*., 1999; Rodriguez-Arrioja *et al*., 2000; Sorden, 2000). Studies of the dynamics of PCV2 infection within and between pig herds have not provided conclusive answers to how it is associated with disease, but recent research suggests that the presence or absence of PMWS and its development may depend on the response of individual pigs to infection. Factors such as the age of the pig at the time of PCV2 infection, the immune status of the pig and PCV2 viral load are thought to influence PMWS development (Ladekjaer-Mikkelsen *et al*., 2002; Okuda *et al*., 2003; Olvera *et al*., 2004).

It seems that PCV2 is not a new virus and that unknown factors or conditions are causing this previously non-pathogenic or mildly pathogenic virus to produce disease. For example, for a long time after PCV2 was detected, Sweden remained free of PMWS but clinical disease was reproduced experimentally in Irish and Swedish pigs using a Swedish PCV2 isolate taken from a healthy pig (Allan *et al*., 2002; Hasslung *et al*., 2005) before the disease appeared in 2003 (Wallgren *et al*., 2004). The available evidence seems to suggest that PCV2 occurs in all pig populations as a subclinical viral infection, and that there are other factors which operate in conjunction with PCV2 to produce PMWS (see Chapter 2). There is no evidence that there are variations in pathogenicity of different strains of PCV2.

Very little is known of the status of PCV infection in the Australian pig herd. Initial investigations by this laboratory have identified both PCV1 and PCV2 in pigs in Australia, but there have been no reported outbreaks of PMWS in Australia. Both CT and PDNS have been reported in Australia and there is some evidence from other countries that both these diseases might be associated with PCV (see Chapter 2).

Assuming that PCV2 is present in Australia, the reasons for the absence of PMWS are unknown. In the absence of PMWS, a unique opportunity existed to examine strains of PCV in Australia and to compare them with strains detected elsewhere where PMWS has occurred. Tissues from Australian pigs were therefore examined to provide information on the presence and prevalence of PCV infection, especially in weanling pigs but also in tissues of any other young pigs that could be obtained. The

prevalence of PCV in one farm with a low prevalence of ill-thrift, and another 2 farms with CT cases, was also investigated.

Materials and methods

Tissue samples

Pig tissues were sent by consulting veterinarians when weaned pigs on farms were found dead or presenting with signs of ill-thrift. The most common tissue sent for testing was spleen, but liver, lymph node, brain, spinal cord and lung tissue were also received on some occasions. Pig tissue samples were transported to Murdoch University under refrigeration and were received from WA, NSW, SA and Queensland.

DNA extraction and PCR

Viral DNA was extracted from approximately 5 mg of tissue using a Qiagen DNeasy tissue kit (QIAGEN) as recommended by the manufacturer, or by using the phenol-chloroform method described by Sambrook *et al*. (1989). Pigs infected with PCV were identified using multiplex PCR based on a previously published method, which employed primers able to differentially amplify fragments of PCV1 or PCV2 (Larochelle *et al*., 1999a). PCR assays were performed in a 25 μL reaction mix consisting of 3 μL extracted sample DNA, 1X PCR reaction buffer, 2 mM $MgCl_2$, 1 mM each dNTPs, 6.4 pmol each primer and 0.5 U AmpliTaq® DNA polymerase (PerkinElmer). Amplification was performed with an initial denaturation step of 5 min, followed by 35 cycles of amplification at 95^{o}C for 1 min, 65^{o}C for 1 min, and 72^{o}C for 1 min, with a final extension step of 72^{o}C for 10 min. PCR products were electrophoresed in a 2% agarose gel at 80 V for 1 h and visualised using an ultra-violet transilluminator (UVP).

Cloning and transformation

To confirm that positive PCR results were PCV, amplified PCR products were cloned into the pCR®2.1 TOPO® vector (Invitrogen) and sequenced.

The various PCR products were cloned into the pCR®2.1 TOPO® vector using a TA cloning kit, Version M (Invitrogen) as recommended by the manufacturer. Constructed plasmids were used to transform competent Top 10 F' *E. coli* (Invitrogen). Plasmids containing the desired PCV1 or PCV2 inserts were identified by α-complementation blue/white screening, and purified recombinant plasmids were digested with *Eco R1* restriction endonuclease to confirm the presence of the insert.

Sequence analysis of PCR products

Plasmids containing the PCV1 and PCV2 fragments were sequenced using the M13 forward (5`-GTAAAACGACGGCCAG-3`) and reverse (5`-CAGGAAACAGCTATGAC-

3`) primers. Sequencing was performed using rhodamine dye terminator (PerkinElmer), and an ABI PRISM™ fluorescent cycle sequencing system (PerkinElmer). Data was analysed using the SeqEd program (v 1.0.3). The sequences obtained were then compared using the BlastN program (version 2.0) to PCV sequences available from the National Centre for Biotechnology Information (NCBI), USA.

Case studies

Case study 1. Unthrifty weaner pigs, September-November 2002

This case study involved unthrifty weaner pigs from a single WA herd, with clinical signs of ill-thrift that could have been consistent with PMWS. Twelve affected pigs 5-13 weeks of age were submitted for examination over a I month period, and each pig was subjected to a thorough haematological, gross pathological and histopathological examination. In addition, tissue samples were collected from the 12 pigs and tested for PCV DNA by PCR as described above.

Case study 2. Congenital tremor (CT) cases, October 2002 – February 2003

An outbreak of CT occurred in a WA pig herd (Farm A) in mid 2002. Although CT has occurred in WA herds before, this outbreak was considered to be unusually severe, with the epidemiology of a classical infectious disease outbreak. As the Farm A outbreak appeared to be resolving, another WA farm (Farm B) was also affected by an outbreak of CT. Two CT-affected pigs from Farm A and 15 affected pigs from Farm B were necropsied at Murdoch University and tissue samples were collected. Brain and spinal cord samples were selected for analysis as these tissues have been shown to contain the most PCV2 infected cells in CT-affected animals (Stevenson *et al*., 2001). DNA from these tissues was extracted and tested for PCV DNA by PCR as described above. Semen samples were also collected from Farm B on the 18th of November 2002, and DNA was extracted using a DNeasy kit (QIAGEN) using methods recommended by the manufacturer for cultured animal cells.

Results

Multiplex PCR

A multiplex PCR was used to identify PCV1 and PCV2 from tissue samples received from 4 Australian states. An example of the multiplex PCR is shown in Figure 3.1.

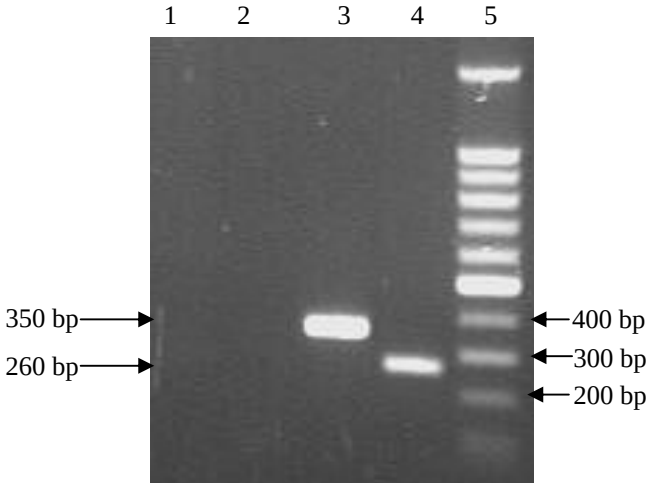

Figure 3.1. Amplification of a 350 bp fragment of PCV1 and a 260 bp fragment of PCV2 by multiplex PCR. Lane 1, negative tissue control; lane 2, negative PCR control; lane 3, PCV1 positive reaction; lane 4, PCV2 positive reaction, lane 5, 100 bp DNA ladder (Lifetech). The size of the bands in the DNA ladder is indicated on the right, and the expected size of the PCR product on the left.

PCV1 was detected in tissues received from WA, NSW and SA. PCV2 was detected in tissues received from WA, NSW and Queensland. The multiplex PCR results are summarised in Table 3.1.

Table 3.1. Multiplex PCR results on porcine tissues from farms in 4 Australian States.

State	Number of farms sampled	PCV-positive farms	Total number of samples received	Percent PCV1 positive	Percent PCV2 positive	Percent dual infection PCV1 and PCV2
WA	14	12	190	30.5	22	2.1
NSW	7	5	112	10	10.5	2.7
Queensland	4	1	29	0	31	0
SA	2	1	17	6	0	0
Total	27	19	348	20	18	2

Sequence analysis of PCR products

The multiplex PCR used in this study produced PCV1 DNA fragments of 350 bp, and PCV2 fragments of 260 bp. These DNA fragments were sequenced to confirm that PCR positive results were PCV. Two samples originating from WA and NSW that

were PCV1 positive, and a further 2 samples also originating from WA and NSW that were PCV2 positive were cloned, sequenced and compared to previously sequenced isolates of PCV available on the NCBI and Genbank databases (Table 2).

Table 3.2. Similarity of Australian strains of PCV to overseas PCV isolates.

Isolate	Nucleotide identity (%) to overseas PCV isolates	
	PCV1	PCV2
PCV1 NSW	98.9 - 99.7	89.6 - 90.2
PCV1 WA	98.6 - 99.4	89.5 - 90.2
PCV2 Queensland	60.6 - 63.2	94.4 - 97.7
PCV2 WA	60.9 - 63.2	94.0 - 98.1

Case studies

Case study 1. Unthrifty weaner pigs

Of the 12 pigs submitted, 2 were considered to be possible PMWS cases based on their haematological, gross pathological and histopathological examinations. Of the remaining ten pigs, seven were not considered to be PMWS-affected while the diagnosis of the remaining three was uncertain. Although there were limited histopathological lesions in some of these pigs that could have been considered consistent with PMWS, the small number of such lesions and their very mild nature resulted in an uncertain diagnosis. Several pigs had respiratory signs with pneumonia present in 8 of the 12 pigs. Lymph enlargement was a common finding and two pigs had multinucleated giant cells in lymph nodes. However the lesions were very inconsistent between pigs, and several were infected with various bacteria which could have accounted for their low body weight. IPMA (see Chapter 5) was performed on several sections from the unthrifty pigs, but no PCV2 antigen was demonstrated anywhere in any of the sections. PCV2 nucleic acid was, however, identified by PCR in all 12 pigs, while PCV1 was detected in 9 of the 12 pigs (Table 3.3). The most common PCV2-positive tissues were the mesenteric lymph node and the lung, followed by the spleen and the tonsil (Table 3.3). The most common PCV1-positive tissue was the spleen, followed by the lung, mesenteric lymph node and the tonsil (Table 3.3).

Table 3.3. Multiplex PCR results from the 12 unthrifty weaner pigs.

Pig	Age (wks)	Mesenteric lymph node		Lung		Spleen		Tonsil	
		PCV-1	PCV-2	PCV-1	PCV-2	PCV-1	PCV-2	PCV-1	PCV-2
02/635	5	-	-	-	+	+	+	-	-
02/622	6	-	+	-	+	-	-	-	-
02/728	7	-	+	-	+	+	+	-	-
02/728	7	-	+	-	+	-	-	-	+
02/714	7-8	+	+	-	+	+	+	-	+
02/715	7-8	+	+	-	+	+	+	-	+
02/716	7-8	-	+	-	-	+	-	-	+
02/634	8	-	+	+	+	-	+	-	-
02/726	9	-	+	+	+	-	-	+	-
02/623	10	-	+	-	+	-	+	-	+
02/633	12	-	+	+	+	-	-	-	-
02/624	13	+	+	+	+	+	+	+	-

Case study 2. CT outbreaks

No histopathological lesions were detected in the brain or spinal cord of any of the affected pigs, and PCV2 could not be detected by IPMA in any tissue sections.

The brains and spinal cords of 2 pigs from Farm A and 15 pigs from Farm B, plus 4 semen samples from boars originating from Farm B, were tested for the presence of PCV1 and PCV2 DNA using the multiplex PCR. The results are given in Table 3.4.

PCV2 was detected in brain and spinal cord tissues of both CT-affected pigs from Farm A, while PVC1 was detected in brain or spinal cord tissues of 8 of 15 pigs from Farm B. One semen sample from boars used to inseminate the gilts on Farm B, sample 400, was positive for PCV1.

Table 3.4. Multiplex PCR results from CT-affected pigs received from 2 farms in Western Australia.

Pigs	Brain		Spinal cord		Semen	
	PCV1	PCV2	PCV1	PCV2	PCV1	PCV2
Farm A						
02/016	-	+	-	+	NT	NT
02/017	-	+	-	+	NT	NT
Farm B						
02/992	-	-	-	-	NT	NT
02/993	-	-	-	-	NT	NT
02/994	+	-	+	-	NT	NT
02/995	+	-	+	-	NT	NT
02/996	+	-	+	-	NT	NT
02/997	+	-	+	-	NT	NT
02/998	+	-	+	-	NT	NT
02/999	-	-	+	-	NT	NT
02/1000	+	-	+	-	NT	NT
02/1001	+	-	+	-	NT	NT
02/1002	-	-	-	-	NT	NT
02/1003	-	-	-	-	NT	NT
02/1004	-	-	-	-	NT	NT
02/1005	-	-	-	-	NT	NT
02/1006	-	-	-	-	NT	NT
331 Semen	NT	NT	NT	NT	-	-
400 Semen	NT	NT	NT	NT	+/-	-
1050 Semen	NT	NT	NT	NT	-	-
1070 Semen	NT	NT	NT	NT	-	-

NT denotes not tested.

Discussion

In this study of PCV in Australian pigs, a multiplex PCR that was developed elsewhere (Larochelle *et al*., 1999a) was used to detect PCV infection. At the time of the study, this was the only available assay. No suitable type-specific serological tests were available, and no PCV-specific monoclonal antibodies were available for the development of immunocytochemical tests. The overall prevalence of PCV1 and PCV2 detected in pigs by the multiplex PCR was 20% and 18%, respectively.

The prevalence of PCV2 in the Australian pig herd (18%) was lower in comparison to some other studies but prevalence rates seem to reflect the presence or absence of PCV2-associated disease, and the 18% prevalence seems approximately equivalent to what has been detected elsewhere in regions where PMWS has not been detected. This is illustrated by the results of a study by Calsamiglia *et al*. (2002) who found that in pigs without PMWS lesions there was a 29% prevalence of PCV2 infection, much less than the 96% prevalence detected in pigs with PMWS lesions. Similarly, PCV2 was identified in 26.8% of pigs in Germany that were not affected by PMWS (Mankertz *et al*., 2000b). In a retrospective study of Japanese archived tissue blocks, PCV2 prevalence rates were 21% in 1991, 27% in 1994, rising to 50% in 1999 (Sato *et al*., 2000) when PMWS had begun to affect Japanese pig herds (Onuki *et al*., 1999).

The 20% prevalence of PCV1 in Australia detected by PCR appears higher than the approximately 5–10% PCV1 prevalence rates reported globally (Larochelle *et al*., 1999b; Ouardani *et al*., 1999; Quintana *et al*., 2001; Calsamiglia *et al*., 2002). Although PCR detection methods have generally indicated prevalence rates of less than 10%, serological studies have indicated that infection rates with PCV1 are higher than that. A Spanish serological study based on the use of an immunoperoxidase assay (IPMA) and sera from 90 pigs from 37 farms detected PCV1 antibody in 52.2% of samples and PCV2 antibody in 97.8% of samples (Rodriguez-Arrioja *et al*., 2000). While PCV2 was shown to be more prevalent than PCV1, the rate of detection of PCV1 was still high. Quintana *et al*. (2001) also tested for both types of PCV antibody using IPMA, and found that although PCV1 and PCV2 nucleic acid were present in 9% and 67% of samples respectively, and all pigs had seroconverted to PCV2, PCV1 antibodies were also present in all but one pig (94% seroprevalence rate). A retrospective serological survey using sera from 1985, 1989 and 1997 also found a high rate of PCV1 antibodies; 8%, 41.4% and 38.1% respectively (Magar *et al*., 2000b). The extent to which these high rates of PCV1

antibody in these reports might represent cross-reaction to PCV2 antigen is unknown but it is a factor that does need consideration.

The interpretation of prevalence rates of PCV1 and PCV2 based on serological results is difficult due to problems of interpreting the specificity of the assays used. Due to problems of serological cross-reactivity, not realised at the time but since well documented (Dulac and Afshar, 1989; Allan *et al*., 1998; Rodriguez-Arrioja *et al*., 2000), tests such as immunofluorescence assays in these early studies probably detected antibody to both PCV1 and PCV2 and did not differentiate one from the other. In addition, after 1997 when it became clear that there were two types of PCV and that only PCV2 appeared to be pathogenic, PCV1 was considered of little interest and it became common practice to survey pig herds for the presence of PCV2 only. A retrospective serological survey of Canadian pigs from the years 1985, 1989 and 1997, using indirect immunofluorescence which does not discriminate between PCV1 and PCV2 antibodies, found a higher prevalence of PCV2 in all 3 years, suggesting that PCV2 may have been more common than PCV1 in the Canadian pig herd even before PMWS became a recognised problem (Magar *et al*., 2000b).

Prevalence rates detected using serological tests are difficult to compare with detection of virus based on PCR: serological tests detect past infections and not necessarily current infections, and PCR detects current infection only. In a Canadian study using multiplex PCR, 94.2% of 35 pigs were infected with PCV2, compared with only 5.7% infected with PCV1, and both PCV1-positive pigs were also infected with PCV2 (Ouardani *et al*., 1999). Similar results were reported in a study of a PMWS-affected farm, with PCV1 DNA found in 9% of 33 pigs, while PCV2 was found in 67% of the same 33 pigs (Quintana *et al*., 2001). This high rate of PCV2 infection is supported by another study using multiplex PCR where 97.6% of pigs were PCV2-positive, while only 4.8% of samples were positive for PCV1 (Larochelle *et al*., 1999b). It should be noted however that all 3 of these studies used tissue samples from pigs with various clinical signs and conditions, including respiratory signs, weight loss (Ouardani *et al*., 1999), pneumonia, tremors and PDNS (Larochelle *et al*., 1999b) and PMWS (Quintana *et al*., 2001). All these signs have been observed in cases of PCV2-associated disease, so a high rate of PCV2 would perhaps be expected.

The reason for the apparently lower prevalence of PCV1 than PCV2 globally is unknown. The non-pathogenic PCV1 is probably capable of faecal-oral transmission (Tischer *et al*., 1986; Shibata *et al*., 2003; Sibila *et al*., 2004) and widespread

subclinical replication might be expected, much like TTV in humans (Mushahwar *et al*., 1999). A possible explanation is the low efficiency of PCV1 replication, resulting in virus titres too low for efficient infection of pigs, quite different to the widespread TTV which produces at least $3{\cdot}8x10^{10}$ virus particles per day in chronically infected patients (Maggi *et al*., 2003). It is also possible that there is competition between PCV1 and PCV2, evidence for which is that PCV1 has not been detected by PCR in any samples from PMWS affected and non-affected farms in the French region of Brittany since PMWS became a problem in the area, possibly due to the massive multiplication of PCV2 (De Boisseson *et al*., 2004). Another possible explanation is that PCV1 infection in pigs may be cleared by the immune system more rapidly than PCV2, with less persistence of infection.

The question of whether the Australian strains of PCV2 are different from those reported elsewhere in association with PMWS will be addressed in a subsequent Chapter (Chapter 4). The sequence analysis that was conducted during the investigation reported in this current Chapter was conducted only to validate the identity of the PCR amplification products that were obtained with the multiplex PCR. This limited analysis does demonstrate that the Australian PCV1 and PCV2 strains were genetically similar to PCV strains examined elsewhere. The initial data obtained also indicated that Australian PCV1 had a higher similarity to overseas isolates than the Australian PCV2 strains, but the PCR primers utilised to amplify PCV1 were situated in the highly conserved viral ORF1, while the PCV2 primers were situated in the more variable ORF2 (Mahe *et al*., 2000), and direct comparison of the sequencing results would not be valid.

It is evident that in Australia there is no widespread PMWS disease, but whether there is a low prevalence of PMWS-like lesions in individual weanling pigs in Australia is unknown. One property in WA was investigated over a 1-month period, and 12 unthrifty weaner piglets were examined. The criteria for a diagnosis of PMWS were those suggested by Sorden (2000) and Quintana *et al*. (2001) and were three-fold: the presence of clinical signs (usually wasting), the presence of characteristic histopathological lesions in lymphoid tissue, and the presence of PCV2 within those lesions. While some pigs had signs typical of PMWS such as pneumonia, poor body condition and coughing, a diagnosis of PMWS was eliminated in 10 of the 12 pigs due to the absence of PMWS histopathological lesions in the lymph nodes. Two of the 12 pigs exhibited wasting, pneumonia, bronchitis, multinucleated giant cells in the lung, liver inflammation, secondary bacterial infection in the colon, rhinitis and anaemia; however no PCV2 antigen could be detected by

the newly-developed IPMA in any tissues from these two pigs.

In all 12 unthrifty pigs, PCV2 DNA was detected in tissues by PCR with the most common positive tissues being the mesenteric lymph node and the lung. The next most common PCV2-positive tissue was the spleen, followed by the tonsil. PCV1 was also identified in 9 of the 12 pigs, with the most common infected tissue being the spleen, followed by the lung, mesenteric lymph node and the tonsil (Table 3.3). Comparison of these results with those reported in a study of 50 PMWS-affected pigs by Segales *et al*. (2004) showed there was a similar frequency of PCV2 infection in the mesenteric lymph node, tonsil and lung; however infection in the spleen was less common at around 70% (Table 3.5).

Table 3.5. Distribution and frequency of detection of PCV2 nucleic acid by *in situ* hybridisation in different tissues from 50 PMWS-affected pigs. From Segales *et al*. (2004).

Tissue	Frequency[a]	%
Tonsil	33/40	82.5
Mesenteric lymph node	37/45	82.2
Inguinal lymph node	33/41	80.5
Peyer's patches	31/39	79.5
Lung	33/45	73.3
Mediastinal lymph node	19/26	73.0
Submandibular lymph node	21/29	72.4
Spleen	30/43	69.8
Intestinal mucosa	25/39	64.1
Liver	25/45	55.6
Kidney	17/31	54.8
Pancreas	5/11	45.5
Gastric mucosa	2/5	40.0
Bone marrow	4/10	40.0
Perirenal lymph node	6/17	35.3
Nasal turbinates	1/3	33.3
Adrenal gland	3/13	23.1

While the diagnosis of PMWS in these unthrifty pigs was ruled out due to the absence of PCV2 in association with microscopic lesions, retrospective studies in some countries have shown that sporadic and isolated cases of PMWS in association with PCV2 may occur many years or even decades before severe PMWS outbreaks are reported. For example, PCV2 has been detected in association with typical PMWS microscopic lesions and clinical signs by ISH and immunoperoxidase in archived tissue blocks and sera from 1986 (Rosell *et al*., 2000c) and 1985 (Rodriguez-Arrioja *et al*., 2003). As PMWS was not recognised at these times, these lesions in these pigs were initially thought to be due to stress and management

conditions, Glasser's disease, gastric ulcers and colitis, with the presence of secondary agents also confusing the diagnosis (Rosell *et al*., 2000c; Rodriguez-Arrioja *et al*., 2003). PCV2 was also detected in isolated cases of PMWS from Thailand in 1993 (Kiatipattanasakul-Banlunara *et al*., 2002) and Japan in 1990 (Sato *et al*., 2000) and in sporadic cases of PCV2-associated PDNS in the United Kingdom dating back to the 1980s (Allan *et al*., 2000e; Grierson *et al*., 2004b). It was thought that the low prevalence of PMWS, along with the presence of secondary agents and the non-specificity of clinical signs, may have helped PMWS to remain undiagnosed for many years (Rodriguez-Arrioja *et al*., 2003). This has also been demonstrated in Denmark, where PMWS was officially identified in 2001, but two suspicious cases had occurred the previous year, and histopathological changes suggesting PMWS were seen in archived tissue from 1989. Similiarly to the present case in Australia, these cases were initially suspected to be PMWS, but the histopathological criteria required to diagnose the disease were not completely fulfilled (Vigre *et al*., 2005).

It has been established that the worldwide outbreaks of PMWS, beginning in the late 1990s, were not due to the emergence of a new pathogen, as PCV2 has been identified in archival tissues and sera decades before PMWS became a recognised disease. PCV2 antibodies have been identified in sera from Japan in 1985 (Sato *et al*., 2000), America in 1987 (Nawagitgul *et al*., 2002), Northern Ireland in 1973 (Walker *et al*., 2000) and the United Kingdom in the 1970s (Grierson *et al*., 2004b) and both PCV1 and PCV2-positive sera have been present in Belgium since at least 1985, 14 years before PMWS was recognised in that country (Vyt *et al*., 2000). Immunohistochemistry on archival lymph tissues also demonstrated that PCV2 was present in Swiss pigs since at least 1986, long before the first description of PMWS in Switzerland in 2001 (Staebler *et al*., 2005). It appears that PCV2 was present in many countries, causing isolated cases of PMWS in some situations, for several decades at least before PMWS was first officially described. Presumably, as yet unidentified factors or agents caused an increase in numbers of the previously isolated and sporadic cases of PMWS to severe epidemic proportions. Presumably the secondary factors or agents that are required to produce epidemic PMWS are not yet present in Australia; the situation in Australia seems to be similar to Switzerland, where PCV2 is widespread in pigs but has only been associated with a handful of PMWS cases to date (Staebler *et al*., 2004).

The opportunity to investigate cases of CT on WA farms occurred during 2002 and 2003, and although CT had been identified in WA herds before, these outbreaks were considered to be unusually severe, with the epidemiology of a classical

infectious disease outbreak. Previous reports have identified PCV1 (Choi *et al*., 2002) and PCV2 (Stevenson *et al*., 2001) in the neural tissues of CT-affected piglets, but there is debate as to whether one or either type of PCV is the cause of CT. A definitive link between PCV1 or PCV2 and CT has never been established and recent studies of CT-affected pigs from several different countries found no evidence of PCV infection at all (Kennedy *et al*., 2003; Ha *et al*., 2005). CT-affected litters are usually from young breeding stock recently introduced to the affected farm (Lukert and Allan, 1999) or, as may have happened in the WA outbreak, seronegative first-parity gilts moved during pregnancy to a farrowing area where they may be exposed to whichever agent causes CT, suggesting that exposure of seronegative stock to a virus at a critical time during gestation may precipitate disease (Lukert and Allan, 1999).

The first outbreak of CT in WA was detected in September 2002 on Farm A, a nucleus breeding herd that sold breeding stock and semen to other WA herds. Farm A was considered a 'closed herd' except for semen and hysterectomy-derived piglets from high-health herds in the eastern states that were owned by the same breeding company. The CT-affected litters initially came from fourth to sixth parity sows, with first-parity sows not affected until the fourth week of the outbreak. By the eighth week, affected litters were mostly from younger sows, suggesting a cycling of the virus through the herd via pregnant sows. As the outbreak on Farm A appeared to be resolving, cases were detected on Farm B, where up to 60% of litters affected and up to 30% pre-weaning mortality due to CT occurred at the peak of the outbreak. Although the boars on Farm B were sourced from Farm A, the last delivery to Farm B was some 5 weeks prior to clinical signs emerging in Farm A, therefore a delay of 3-4 months was seen between the introduction of boars to Farm B and the start of the CT outbreak. After the initial outbreaks at Farms A and B, several other farms were subsequently affected, although not as severely as Farm B. These pig herds were all owned by the same breeding company, they had all received boars and/or semen from Farm A and all farms had been established within the previous 8 years.

Tissues from CT-affected piglets from Farms A and B and semen from 4 boars that may have been associated with the transmission of CT from Farm A to Farm B were all tested by multiplex PCR for PCV (Table 3.2). Strangely, pigs from Farm A were positive for PCV2, while pigs from Farm B were positive for PCV1. One semen sample was PCV1-positive, suggesting that PCV could have been transmitted in semen as was reported by Kim *et al*. (2001) and Larochelle *et al*. (2000). There are at least 2 possible conclusions that may be drawn from this data: either the presence

of either PCV type in the neural tissues of the affected pigs was unrelated to the occurrence of CT, or that PCV1 and PCV2 acted alone or in combination to cause the CT outbreak.

The prevalence of PCV in the brain and spinal cord of the Australian CT-affected pigs was high, which does suggest that the viruses were involved in the condition, as the neural tissues are not commonly infected with either type of PCV. Lymphoid tissues, lungs, spleen, thymus and the liver are the most common sites for the recovery of virus in infected pigs (Allan *et al*., 1995; Hamel *et al*., 1998; Rosell *et al*., 1999; Pogranichnyy *et al*., 2000). PCV2 antigen, however, has been found in the brain of pigs experimentally infected with a PCV2 infectious clone (Fenaux *et al*., 2002) and with a cultured isolate of the virus (Kennedy *et al*., 2000). The presence of virus in brain tissue does not therefore necessarily indicate an association between PCV and CT. There is little information available on the incidence of PCV1 infection in the brain or spinal cord of healthy or diseased pigs, so it is not possible to say whether the presence of PCV1 in neural tissues is commonplace or not. If the presence of PCV in the neural tissues of these CT-affected pigs is indeed purely coincidental, then it is possible that an unknown agent was responsible for the Australian CT outbreak. If this is so, the unknown agent may have acted in concert with PCV1 or PCV2, perhaps in a similar fashion to the synergistic effect observed when pigs are co-infected with PCV2 and PPV (Krakowka *et al*., 2000; Kim and Chae, 2004), where the increased levels of DNA replication and macrophage proliferation caused by PPV might enhance the replication of PCV2. Therefore the presence of PCV in the brains and spines of CT-affected pigs could have been a result of increased PCV replication and possibly dissemination throughout the body, facilitated by an unknown organism.

If this CT outbreak in WA was caused by a virus or other microorganism, either PCV or another agent, presumably this agent must already be present in Australia and cause a subclinical infection most of the time. Therefore the WA outbreak may have been due to the increased use of genes from a large breeding company, and the start of several new pig herds, possibly naïve to whichever agent was causing the CT cases.

CHAPTER 4. GENETIC CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF AUSTRALIAN PORCINE CIRCOVIRUS STRAINS

Summary

Genetic characterisation of Australian porcine circovirus (PCV) strains, including 3 strains of PCV1 from WA and NSW and 7 strains of PCV2 from WA, NSW and Qld, was conducted and phylogenetic analysis of the results was performed to determine the similarity or otherwise of Australian PCV to overseas strains. Based on the results, several conclusions were made regarding the status and possible history of PCV in Australia.

Australian PCV1 strains were very similar to PCV1 strains detected in other countries. Six of the 7 Australian PCV2 strains were very similar to each other, and were most closely related to a Japanese PCV2 strain associated with an outbreak of PMWS. The seventh PCV2 strain was genetically distinct, and was most closely related to a Chinese strain and a Japanese strain associated with an unspecified clinical syndrome and PMWS, respectively. No unique amino acid motifs could be identified in the genomes of Australian PCV2, and there was no apparent genetic reason to believe that Australian PCV2 strains would not be capable of producing diseases such as PMWS given suitable conditions, which are as yet undefined.

The high degree of genomic conservation in PCV2 around the world and including Australia indicate a very low error rate in the transcription of the PCV genome for replication and the long term evolutionary history of the porcine host, in particular possible genetic bottleneck events resulting in the very limited diversity of modern pigs.

The reason for why 6 of the 7 Australian PCV2 strains were very similar and the seventh was genetically distinct was not determined but possible reasons were discussed. The reasons included the possibility of a long coevolution of PCV and pigs, and multiple introductions of PCV2 into the country.

Introduction

Australia is one of the only countries in the world where PCV is present in pig herds, yet confirmed PCV-associated disease has not been detected. It was assumed that either Australian PCV2 strains were non-pathogenic due to unique genetic properties, or that the viruses were the same as detected elsewhere and that co-factors required for PCV-associated disease development are not present or not operating under Australian conditions. There is no evidence of any marked variation in the genotype of PCV2 strains or any evidence of correlation between genotype and pathogenicity (Choi *et al*., 2002; Larochelle *et al*., 2002; Pogranichniy *et al*.,

2002; de Boisseson *et al*., 2004; Grierson *et al*., 2004) but in the absence of any sequence data on PCV strains in Australia, strain variation was a possibility that needed to be considered.

In Chapter 3, DNA of PCV1 and PCV2 was identified in a large number of pigs from herds within Australia. Preliminary analysis indicated this DNA was genetically similar to that of PCV strains detected in other countries, and to virus strains associated with PMWS. To confirm whether Australian PCV was genetically similar to strains associated with PMWS overseas, the complete genomes of viruses detected in pigs in Australia were sequenced and subjected to phylogenetic analysis. The objective of this analysis was to provide information on the genetic characteristics of Australian strains of PCV and to therefore determine if the strains present in Australia were likely to have the potential to cause PMWS, and if the reason for the absence of PMWS was due to genomic differences or to a probable lack of accessory factors required for PCV2 to cause disease. It was anticipated that this data would provide further information on the risk of Australia experiencing PMWS and other PCV-associated diseases in the future.

Materials and methods

Tissue samples and the PCV1-infected PK-15 cell line

The pig tissue samples used as a source of PCV1 and PCV2 DNA were those previously described in Chapter 3. Four PCV1 and 7 PCV2 strains were sequenced in this study, with their origins shown in Table 4.1 and 4.2 respectively. One of the PCV1 strains was derived from DNA extracted from the PCV1-infected PK-15 cells (ATCC-CCL31), a pig kidney cell line persistently infected with PCV1, purchased from the American Type Culture Collection and cultured by conventional methods.

DNA extraction and PCR

Viral DNA was extracted from approximately 5 mg of tissue or cultured PK-15 cells using a DNeasy tissue kit (QIAGEN) using the methods described by the manufacturer, or by a phenol-chloroform method as described by Sambrook *et al*. (1989). Pigs infected with PCV were identified using a multiplex PCR as described in Chapter 3, which employed primers able to differentially amplify fragments of PCV1 or PCV2. Additional primers were then used to amplify the whole genome of PCV1 (JM4, JM5, JM4Comp and JM5Comp) and PCV2 (PCV2SeqA, PCV2SeqB, PCV2SeqAComp, PCV2SeqBComp) from the DNA samples as shown in Table 4.3.

Table 4.1. Source of PCV1 DNA sequenced.

Virus strain	Date Isolated	Origin of virus	Tissue type
PCV1 Farm A NSW	2000	Pig found dead	Spleen
PCV1 Farm C WA	2002	Unthrifty weaner pig	Lymph node
PCV1 Farm D WA	2002	Pig suffering CT	Spinal cord
PCV1 PK-15A	2003	PK-15 cell line	Porcine kidney cells

Table 4.2. Source of strains of PCV2 subjected to sequence analysis.

Virus strain	Date Isolated	Origin of virus	Tissue type
PCV2 Farm B WA	1999	Pig found dead	Spleen
PCV2 Farm A NSW	2000	Pig found dead	Spleen
PCV2 Farm A WA	2000	Pig found dead	Lymph node
PCV2 Farm A (1) Qld	2000	Pig found dead	Lymph node
PCV2 Farm A (2) Qld	2000	Pig found dead	Spleen
PCV2 Farm B NSW	2000	Pig found dead	Spleen
PCV2 Farm C WA	2002	Unthrifty weaner pig	Lymph node

Table 4.3. Primers used to amplify whole genomes of Australian PCV.

Primer	Sequence	Position in genome	PCV type
JM4	5`-GGGATGATCTACTGAGACTGTG-3`	705 - 727	PCV1
JM5	5`-CCTTCAGAAACCGTTACAGATG-3`	1627 - 1605	PCV1
JM4Comp	5`-CACAGTCTCAGTAGATCATCCC-3`	727 - 705	PCV1
JM5Comp	5`-CATCTGTAACGGTTTCTGAAGG-3`	1605 - 1627	PCV1
PCV2SeqA	5`-AATGGTAC(T/C)CCTC(A/G)(A/G)CTGC-3`	832 - 850	PCV2
PCV2SeqB	5`-G(A/C)TATGACGT(A/T)TCCAAGGAG-3`	1719 - 1738	PCV2
PCV2SeqAComp	5`-GCAG(T/C)(T/C)GAGG(A/G)GTACCATTC-3`	850 - 832	PCV2
PCV2SeqBComp	5`-CTCCTTGGA(T/A)ACGTCATA(G/T)C-3`	1738 - 1719	PCV2

PCR assays were performed in a 25 μL reaction mixture consisting of 3 μL extracted sample DNA, 1X PCR reaction buffer, 2 mM $MgCl_2$, 1 mM each dNTPs, 6.4 ρmol each primer and 0.5 U AmpliTaq® DNA polymerase (PerkinElmer). Amplification was performed with an initial denaturation step of 5 min, followed by 35 cycles of amplification (95°C for 1 min, 60°C for 1 min, 72°C for 2 min), with a final extension step of 72°C for 10 min for all the primers, except PCV2SeqAComp and PCV2SeqBComp, where the annealing temperature step was 62°C. PCR products were electrophoresed in a 2% agarose gel at 80 V for 1 h and visualised using an ultra-violet transilluminator.

Cloning and sequence analysis of viral DNA

The various PCV1 and PCV2 PCR products were cloned into the pCR®2.1 TOPO® vector using a TA cloning kit, Version M (Invitrogen) as recommended by the manufacturer. Constructed plasmids were used to transform competent Top 10 F' *E. coli* cells (Invitrogen). Plasmids containing the desired inserts were identified by α-complementation blue/white screening, and purified recombinant plasmids were digested with *Eco R1* restriction enzyme to confirm the presence of the insert.

Plasmids containing the correct fragments were sequenced using the M13 forward (5`-GTAAAACGACGGCCAG- 3`) and reverse (5`-CAGGAAACAGCTATGAC-3`) primers. Sequencing was performed using rhodamine dye terminator or Big Dye terminator Version 3.1 (Applied Biosystems), with the ABI PRISM™ fluorescent cycle sequencing system (Applied Biosystems). Sequence data was analysed using the SeqEd program (v 1.0.3). The sequences obtained were then compared, using the BlastN program (version 2.0), to existing sequences available from the National Centre for Biotechnology Information (NCBI).

The virus sequence produced was compared to the nucleotide sequences of 8 PCV1 strains and 28 PCV2 strains available from GenBank and NCBI databases as shown in Table 4.5.

Table 4.5. Overseas PCV strains used for phylogenetic analysis.

Accession number	Country of origin	Disease association	Year isolated
PCV1 strains			
AF071879	Canada	From PK-15 cells	1998
AY193712	China	From PK-15 cells	2003
PCCOMGEN	Germany PK-15	From PK-15 cells	1996
PCU49186	Ireland	From PK-15 cells	1997
AY184287	USA	From PK-15 cells	2003
AF012107	France	PMWS-like disease	1997
AY219836	Taiwan	Unknown	2003
AY099501	USA	CT	1960s
PCV2 strains			
AY424401	Austria (1)	PMWS	2003
AF117753	Canada (1)	Unknown	1999
AF109398	Canada (2)	Unspecified clinical syndrome	1998
AF055392	Canada (3)	PMWS	1998
AF118097	Canada (4)	PMWS	2000
AF408635	Canada (5)	CPE in cell culture	2001
AF381177	China (1)	Unspecified clinical syndrome	2001
AF381175	China (2)	Unspecified clinical syndrome	2001
AY181946	China (3)	PMWS	2002
AY181947	China (4)	PMWS	2002
AY181945	China (5)	PDNS	2002
AF055394	France (1)	PMWS	1998
AF201311	France (2)	PMWS	1999
AY321993	France (3)	Unknown	2003
AF201307	Germany (1)	PMWS	1999
AY256459	Hungary (1)	PMWS	2003
AY256455	Hungary (2)	PDNS	2003
AB072302	Japan (1)	PMWS	2001
AB072303	Japan (2)	PMWS	2001
AF520783	Korea (1)	PDNS	2002
AF544024	Korea (2)	Unknown	2002
AF201897	Netherlands (1)	PMWS	1999
AF201309	Spain (1)	PMWS	2000
AY325495	South Africa (1)	PMWS	2003
AF166528	Taiwan (1)	Unspecified clinical syndrome	1999
AY146991	Taiwan (2)	Unspecified clinical syndrome	2002
AY180397	Taiwan (3)	Unspecified clinical syndrome	2002
AF364094	Taiwan (4)	Unknown	2001
AJ293869	UK (1)	PDNS, abortion	2000
AF055391	USA (1)	PMWS	1998
AF147751	USA (2)	Unknown	1999
AF264039	USA (3)	PMWS	2000
AY099500	USA (4)	CT	2002
AY099497	USA (5)	PMWS	2002

Sequence alignments were obtained using the ClustalW program. The Seqboot program (1000 datasets) was used for bootstrap resampling, before the DNAdist, Fitch, and Consense programs (Felsenstein, 1989) were used to obtain a consensus tree diagram. This diagram was visualised and edited using Treeview (Page, 1996).

Results

Sequence analysis of Australian PCV1 strains

The full genomes of 3 Australian strains of PCV1were successfully sequenced, as was the PCV1 strain persistently infecting the PK-15 cell line.

The 3 PCV1 strains were either 1759 nt (Farm D WA) or 1760 nt (Farm C WA, Farm A NSW) in length. The 3 strains shared 98-99% nucleotide identity to each other and 98-99% nucleotide identity to the other overseas PCV1 sequences used for comparison. A potential stem-loop structure was identified in all 3 sequences, with the sequence TAGTATTAC. Four potential ORFs with the ability to encode proteins longer than 80 amino acids were consistently identified in each strain, including the putative replication-associated (Rep) protein (nt 46–985) and capsid protein (nt 1724–1023). Two other minor ORFs were also identified in each strain, both on the complementary DNA strand (nt 658–46 and nt 552–205 (the nt locations were calculated based on the PCV sequence beginning at the stem-loop sequence ACCA).

An unrooted phylogenetic tree was constructed based on the nucleotide sequences of the 4 Australian strains (including the Australian PK-15 sequence) plus 8 other PCV1 sequences available on GenBank and NCBI (Figure 4.1). The PCV1 strains examined appeared to be divided into 3 clusters, the first containing PK-15 cell culture strains from Germany, Australia and the USA, the second containing field strains from Australia, the USA and France and the third containing the remaining PK-15 strains and the Taiwanese field strain. The 3 Australian field strains did not cluster together in a distinct branch.

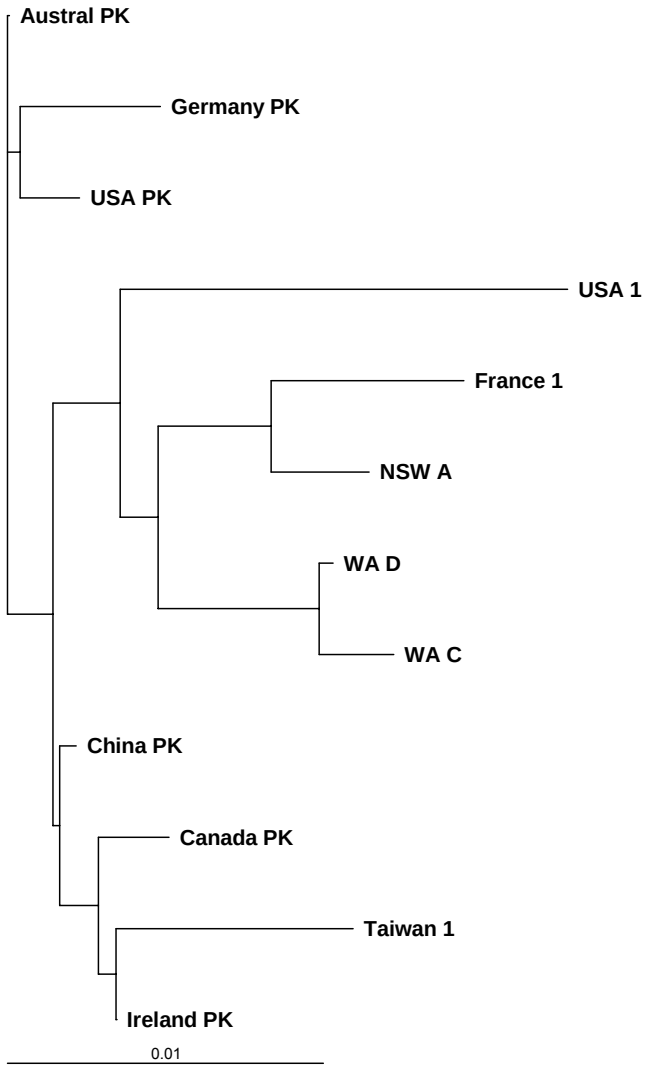

Figure 4.1. Unrooted phylogenetic tree based on the complete nt sequence of PCV1 strains. Sequence accession numbers are given in Table 4.4. The bar represents a 1% difference in nucleotide homology between 2 sequences.

Sequence analysis of Australian PCV2 strains

The complete genomes of 7 Australian strains of PCV2 were sequenced. All 7 were 1768 nt in length. The 7 strains shared 95-99% nucleotide identity to each other and 94-99% identity to overseas PCV2 strains. The total nucleotide sequence variation between all 41 PCV2 sequences (from Australia and overseas) was 93-99%. A potential stem-loop structure was identified in all 7 Australian sequences, with the sequence AAGTATTAC. Three potential ORFs with proteins longer than 80 amino acids were consistently identified in each strain, including the putative replication-associated (Rep) protein (nt 51–995) and capsid protein (nt 1735–1034). One other minor ORF was also identified for each strain, located on the complementary DNA

strand (nt 671–357). The strain from Farm C WA had 4 potential ORFs, the 3 described above plus one other potential ORF on the positive DNA strand (nt 1016–1363).

An unrooted phylogenetic tree was constructed based on the nucleotide sequences of the 7 Australian strains plus 34 other PCV2 sequences available on GenBank and NCBI (Figure 4.2). Phylogenetic analysis of the 41 PCV2 strains showed most of the PCV2 strains clustered into 5 genetic groups. The first group contained all the Australian strains except Farm C WA, plus Japan (1), Canada (1) and Canada (3). The second group contained all 3 French strains, plus China (3), China (4) and China (5), Netherlands (1) and UK (1). The third group contained all 4 Taiwanese strains. The fourth group contained the 2 Hungarian strains plus Spain (1), and the fifth and largest group contained all the USA strains, both the Korean strains, Canada (2), Canada (4) and Canada (5), Farm C WA, Japan (2), China (1) and (2), and South Africa (1). The Germany (1) and Austria (1) strains did not cluster with any particular group.

Amino acid variation and motifs in PCV1 and PCV2

Variable and conserved areas were identified in the aligned ORF2 amino acid sequences of the Australian and overseas PCV1 and PCV2 strains. In PCV2, amino acids 137-177 were highly conserved in all 41 strains (95-100% similarity, or a maximum of 2 amino acid changes) while amino acids 72-92 were much more variable (57-100% similarity, or a maximum of 9 amino acid changes). In all strains of PCV1, amino acids 137-177 were also conserved (95-100% similarity or a maximum of 2 amino acid changes) as were all the PCV1 ORF2 amino acid sequences with the exception of the region between amino acids 51-74 (73-100% similarity, or a maximum of 6 amino acid changes).

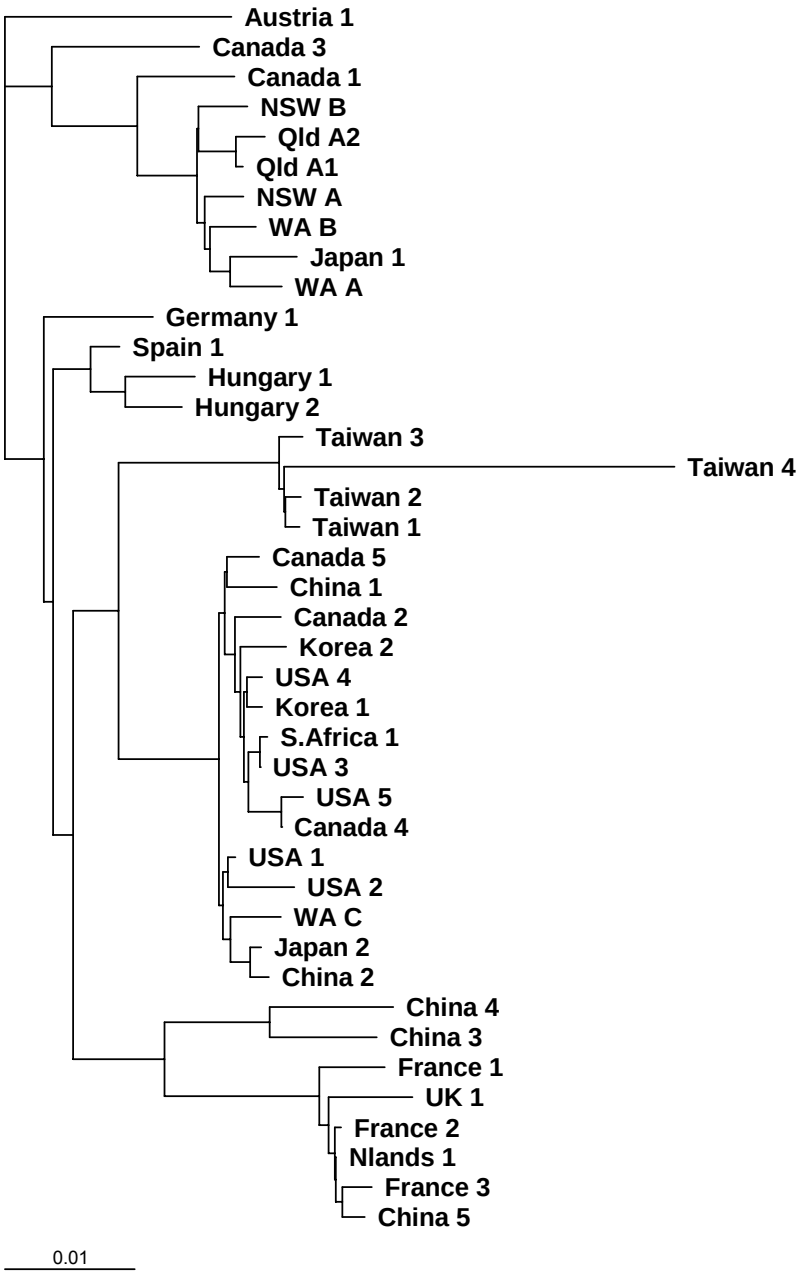

Figure 4.2. Unrooted phylogenetic tree based on complete nt sequences of PCV2 strains. Sequence accession numbers are given in Table 4.4. The bar represents a 1% difference in nucleotide homology between 2 sequences.

Discussion

The Australian PCV1 strains were very similar to each other and to the reported sequence of other strains of PCV1. The 3 Australian strains were 1759 nt (Farm D WA) or 1760 nt (strains Farm C WA and Farm A NSW) in length, and shared 98-99% nucleotide identity with each other and 98-99% nucleotide identity with overseas strains of PCV1. Likewise, the 7 Australian PCV2 strains were all 1768 nt in length, and very similar to each other. They shared 95-99% nucleotide identity with each other and 94-99% identity to overseas PCV2 strains. The total nucleotide sequence variation between all 41 PCV2 sequences from Australia and overseas was 93-99%. All PCV sequences had higher nt variability in the ORF2 than ORF1 as has been previously reported (Fenaux *et al*., 2000; Larochelle *et al*., 2002).

The intergenic region between ORF1 and ORF2 was between 38 (PCV1) and 39 (PCV2) nt in length. This region was 100% conserved in all PCV1 sequences examined except for a 1 nt insertion at nt 949 in strains WA C and NSW A. The 41 PCV2 strains were found to have 97-100% nt conservation in this area, with several strains having a maximum of 1 nt change only. This high conservation may indicate a possible regulatory function of this area, and it has been speculated that disruption of this intergenic region may affect the stability and viability of PCV2 (Cheung, 2004).

When subjected to phylogenetic analysis, the 12 PCV1 strains for which sequence data was available separated into 3 clusters, the first containing the same PK-15 cell culture strain but sequenced in Germany, Australia and the USA, the second containing field strains from Australia, the USA and France and the third containing the remaining sequences of the PK-15 strain and a Taiwanese field strain (Figure 4.1). As all 12 PCV1 strains analysed shared 98-99 % nt identity, the phylogenetic clusters were based on a very small number of nucleotide changes and therefore this division into clusters could be artificial.

When the nucleotide and amino acid sequences from all the PCV1 sequences were aligned (data not shown), there were only 4 nucleotide positions where bases from the 3 clusters were consistently different: at nt positions 1028 (T → C), 1044 (T → C), 1197 (G → C) and 1538 (A → G). All these changes are located in ORF2. When ORF2 was translated, 3 of these base changes were translated to amino acid changes, at amino acid positions 63 (Y or F → H, from nt change at 1538), 176 (H → Q, from nt change at 1197), and 233 (K → E, from nt change at 1028). The change at amino acid 176 is located on an immunorelevant epitope identified by Mahe *et al*. (2000).

While both PCV1 and PCV2 have been identified in the tissues of pigs with CT type

A2 (Stevenson *et al*., 2001; Choi *et al*., 2002), PCV has not been detected in other CT pigs (Kennedy *et al*., 2003; Ha *et al*., 2005). The PCV1 detected in the neural tissue of 2 pigs in one outbreak of CT (Chapter 3) did not differ from the other PCV1 strains, which suggest that these outbreaks of CT were not associated with a unique genotype of PCV1. The CT-associated PCV1 strains differed in only 9 nt positions (resulting in 3 amino acid differences) from the strain identified in a CT outbreak in the 1960s (USA (1)).

The 6 different analyses by different groups of the sequence of the PCV1 present in the PK-15 cell line were all very similar and the differences could represent sequencing errors, although minor nt changes with time in culture may have occurred. Although the data is limited to only a few strains of PCV1, it is apparent that PCV1 appears to have changed very little in culture and in the pig population over a 40 year period.

Seven Australian PCV2 strains were sequenced, 3 from WA and 2 each from NSW and Qld. When subjected to phylogenetic analysis, these strains and the other PCV2 strains used for the phylogenetic analysis separated into 5 major clusters, with all but one of the Australian strains appearing closely related (Figure 4.2). None of the PCV2 strains, Australian or international, clustered according to time of detection (from 1998 to 2003) or disease condition (PMWS, PDNS or CT). This conclusion is in agreement with previous phylogenetic studies of PCV2 (Choi *et al*., 2002; Fenaux *et al*., 2000; Larochelle *et al*., 2002) and indicates that the same or genetically similar forms of PCV2 are associated with the variety of diseases identified as possibly caused by PCV2. While viral genetic variation may be responsible for differences in pathogenicity and virulence of other viruses, such as chicken anaemia virus (Yamaguchi *et al.*, 2001), herpes simplex virus (Griffiths and Coen, 2003) and white spot bacilliform virus (Lan *et al*., 2002), this does not appear to be the case with PCV.

Some PCV2 strains appeared to cluster according to geographic origin, particularly the Taiwanese and French strains, but other PCV2 strains, from geographically distinct areas such as Japan, China, the USA and Australia were closely related. One of the Australian strains (Farm C WA) which differed from the other Australian strains appeared to be most closely related to a Japanese strain (Japan 2) and a Chinese strain (China 2), while the remaining Australian strains were most closely related to another Japanese strain (Japan 1) and a Canadian strain (Canada 1). It is possible that PCV2 may have travelled from Asia and North America to Australia or vice versa, or this result may just reflect the genetic diversity of PCV2 strains found worldwide, with the country of origin being incidental.

Nucleotide variations are significant in terms of their potential to cause amino acid changes which can affect function and antigenicity of individual virus proteins. Larochelle *et al*. (2002) identified 3 main regions of amino acid heterogeneity in PCV2 strains on ORF2, between amino acids 59-80, 121-136 and 180-191, the first 2 of these regions also being dominant immuno-reactive areas (Mahe *et al*., 2000). Variation at these sites could result in variability of regions at the external surface of the capsid protein, possibly affecting immune recognition (de Boisseson *et al*., 2004). These areas could be involved in the development of PCV2 variants and potential changes in pathogenicity (Larochelle *et al*., 2000). In the Australian and overseas PCV2 ORF2 strains these 3 amino acid regions displayed some variation but were not as variable as the region between amino acids 72-92. The region from amino acids 137-77 is well conserved between PCV1 and PCV2 strains, suggesting that it may be a possible key region for virus–host interactions or virus assembly, such as receptor binding or interactions between capsid monomers (de Boisseson *et al*., 2004), and it is therefore not surprising that very little variation was found in this region.

Amino acid motifs previously identified as being important for nuclear localisation and potential posttranslational modification sites (Liu *et al*., 2001) were present and generally well conserved in the Australian and overseas strains, and 2 amino acid mutations which contribute to the possible enhancement of PCV2 replication *in vitro* and the attenuation of the virus *in vivo* (Fenaux *et al*., 2004b) were unchanged in the Australian PCV2 sequences.

Although 4 of the Australian PCV2 strains (Farm C WA, Farm A WA, Farm B NSW and Farm B WA) possessed unique amino acid point mutations, the 7 Australian strains did not consistently contain any unique amino acid changes in either ORF1 or ORF2 when compared to the 35 overseas strains. The Farm C WA strain was genetically different to the other 6 other Australian PCV2 strains, which appeared closely related to a Japanese strain associated with PMWS (Figure 4.2), but the significance of this is unknown although no particular unique motifs were identified in this strains. Viral virulence determinants may be multigenic (Larochelle *et al*., 2002), so it is possible that other gene regions may be involved in PCV pathogenicity. However, as a genetic basis for PCV2 pathogenicity has not been identified (Choi *et al*., 2002; Larochelle *et al*., 2002). It appears likely that non-genetic factors are more likely responsible for absence of any confirmed PCV-associated disease.

Despite Australia being an isolated country, the Australian strains of PCV seem to be essentially indistinguishable to overseas strains. Clearly, both PCV1 and PCV2 are

highly conserved all over the world. This is in agreement with previous observations that the degree of conservation in PCV2 is very high, considering the large range of their geographical origins and the general prevalence of the virus in pig herds worldwide (de Boisseson *et al*., 2004). The similarity of all strains sequenced so far would suggest that viral mutations are rare and that PCV has evolved and continues to evolve very slowly.

PCV infections are often persistent and the persistent infection of animals by PCV and many other small DNA viruses may help to confer genome stability by maintaining viral genotypes in host populations (Shadan and Villarreal, 1993). Like other readily transmitted and environmentally resistant viruses such as the influenza viruses (Ina and Gojobori, 1994) and picornaviruses (Saiz *et al*., 1993), PCV is probably able to spread rapidly across and between geographic regions, resulting in similar strains of the virus being found worldwide.

The genetic stability of PCV may also be partly due to the low error rate in the transcription process. The replication of small DNA viruses is thought to linked to the host cell metabolic machinery, and that this is associated with low-error and species-specific replication (Eki *et al*., 1991). DNA viruses such as circoviruses which replicate using a DNA polymerase of the host cell (Todd *et al*., 2001), are assumed to have mutation rates similar to those of the host DNA, usually somewhere in the vicinity of 10^{-8} to 10^{-9} nucleotide changes per replication (Shadan and Villarreal, 1993). The newly-identified starling circovirus (StCV) has undergone co-evolution with its avian host, demonstrated by phylogenetic analysis of the virus and the starling cytochrome b gene (Johne *et al*., 2006).

The reliance of small DNA viruses on the host cell for replication has probably allowed host-pathogen co-evolution and co-speciation (Shadan and Villarreal, 1993). If PCV and pigs have shared a long co-evolutionary history, this may explain why strains of PCV do not appear to group phylogenetically according to the country of origin, as the infection of modern pigs with PCV may predate the current isolation of pig herds according to country and breed. If this is the case, the high conservation of the PCV genome could also be connected with the long term evolutionary history of the pig host. Genetic bottlenecks could therefore have limited the number of individual hosts that were available to maintain the strain, and could lead to the extinction of rare and/or divergent forms of a virus (Stewart *et al*., 1996). The relationship between humans and human papillomavirus (HPV) is an example of this effect, with the pattern of HPV variation indicative of a severe host bottleneck, then subsequent expansion, resulting in widespread extinction of most ancestral forms of

HPV, and the modern-day separation of HPV into discrete viral types with some variation within each type (Stewart *et al*., 1996).

As suggested by de Boisseson *et al*. (2004), PCV might also have been subjected to one or more genetic bottlenecks, possibly linked to the history of the modern pig. Many modern pig breeds are 'hybrids' of Asian and European ancestors, and subspecies of European wild pigs and South-East Asian pigs are thought to be the main contributors to the genetics of modern pigs (Clutton-Brock, 1987; Giuffra *et al*., 2000). Currently recognised pig breeds group into one of 2 clusters, an Asian cluster or a European cluster (Giuffra *et al*., 2000; Kim *et al*., 2002). Molecular evidence suggests that the domestication of the pig occurred approximately 9000 years ago, and there was a massive introduction of Asian pigs into European breeds during the 18th and 19th centuries (Giuffra *et al*., 2000). There is very little genetic variation between individual pig breeds, in contrast to other domestic animals such as horses where much larger variations between breeds and between individual animals are found (Kim *et al*., 1999). There is also evidence of genetic bottlenecks within the Asian and European groups, with some groups of breeds possibly descended from a single ancestor. This limited genetic diversity and the possibility of common ancestry both between and within the Asian and European pig lineages could partially explain why PCV is so homogenous. Maybe current strains of PCV are descended from a very few viral forms circulating in a small number of ancestors to the modern pig, assuming that the virus has been infecting pigs before the Asian and European pig groups diverged (Kim *et al*., 2002). A phylogenetic study of French PCV2 suggested that the very close relationship shared by most French PCV2 strains could be indicative of their derivation from a very restricted genetic pool of PCV2 disseminated throughout the country, with variant strains possibly associated with multiple introductions of PCV2 into France (de Boisseson *et al*., 2004). The close similarity of 6 of the 7 Australian strains could also be due divergence from a restricted genetic pool, and the genetically different seventh strain (Farm C WA) may have been due to a more recent introduction of PCV2 into Australia.

Genetic bottleneck events may also explain why there are no PCV types intermediate between PCV1 and PCV2. PCV1 and PCV2 are likely to have evolved from a common ancestor (Fenaux *et al*., 2000) but recent divergence from the 2 types has not occurred (Choi *et al*., 2002). The occurrence of the 2 discrete PCV types may indicate a genetic bottleneck in the host with subsequent expansion, and the loss of ancestral intermediate viral forms, as is suggested for HPV (Stewart *et al*., 1996). Alternatively, it is possible that each PCV type may have evolved separately in either

European or Asian-origin breeds for many thousands of years after divergence of the two pig genetic groups.

The theory of PCV diversity being constrained by the genetic history of pigs relies on PCV only infecting pigs, with no other animal species acting as a reservoir. Indeed, cross-species transmission of PCV1 or PCV2 has never been demonstrated to occur under natural conditions. Although there was one report of PCV2 in cattle (Nayar *et al*., 1999) this has not been confirmed by other investigators. There is no evidence that recombination between PCV isolates is a common event, indeed it is very unusual to find more than one isolate of PCV2 infecting one pig at the same time (de Boisseson *et al*., 2004). This is in contrast to the highly variable TTV that has cross-infected other primates such as apes, old world primates, new world primates and the tree shrew (the most divergent primate) (Simmonds, 2002; Thom *et al*., 2003). Non-human primates seem to be infected by a range of TTV and TTV-like viruses, some of which are also capable of infecting humans (Simmonds, 2002) and mixed infections with multiple TTV genotypes is a frequent occurrence (Simmonds 2002) and the chance of recombination between TTV strains is high.

Due to poor and incomplete records, it is not certain how pigs were first introduced to Australia. There are records of pig introductions with the first European settlement of Sydney in 1788 (Tisdell, 1982), as well as smaller introductions of pigs from Timor in 1827 to the Northern Territory, from New Guinea into North Queensland before 1900, from the Indonesian Island of Kisar to the Northern Territory in 1838 (Letts, 1962). It is also possible that small numbers of pigs were brought to Australia by the Chinese during the gold rush (Pullar, 1950). Genetic evidence has also been found suggesting a relatively recent introduction of Asian pigs into the Northern Australian feral pig herd, possibly from the release of Asian pigs during early settlement, or alternatively Asian pigs may have been traded by early Asian traders with indigenous Australians (Gongora *et al*., 2004). PCV is known to infect wild boar populations (Schulze *et al*., 2004; Ellis *et al*., 2003; Toplak *et al*., 2004), and PCV2 found in wild boars is very similar in sequence to other isolates found in domestic pigs (Toplak *et al*., 2004). Therefore multiple introductions of pigs into Australia from Asia and Europe may be responsible for variety of PCV2 isolates found in this study, in particular the divergence of the Australian PCV2 isolate Farm C from the other six isolates sequenced (Figure 2). It was not determined in this study whether Australian wild boars are infected with PCV, however it seems likely. Interestingly, a Slovenian study of PCV2 infecting wild boars found very high sequence diversity between PCV2 isolates within a small region, suggesting that PCV2 may have been infecting

these feral populations over a long period of time, or that the virus may have been introduced into Slovenian wild pigs several times (Toplak *et al*., 2004). A large-scale sequencing survey conducted on feral and domestic pigs would be required to test the theory that there are many different types of PCV2 circulating in Australia, as appears to be the case in Canada, the USA and China (Figure 2).

CHAPTER 5. THERMAL STABILITY OF PCV2 IN CELL CULTURE

Summary

To enable an investigation of the thermal stability of porcine circovirus type 2 (PCV2), an Australian strain of PCV2 was successfully adapted to replicate in the PK-15 pig kidney cell line. To quantitate the virus in cell culture, an immunoperoxidase monolayer assay (IPMA) was developed using a monoclonal developed in this laboratory against the recombinant PCV2 capsid protein, and compared to detection using a previously published RT-PCR procedure to detect viral RNA transcripts. The IPMA was used to investigate the thermal stability of Australian PCV2, where it was found that the virus was able to tolerate heating at 56°C, 65°C and 70°C for 15 minutes with no apparent loss of infectivity, with partial loss of infectivity at 85°C for 15 min, but was completely inactivated when heated at 95°C for 15 min.

Introduction

PCV1 and PCV2 are able to be cultured in variety of cell lines, both porcine and other, usually with no cytopathic effect observed (Tischer *et al.*, 1982; Allan *et al.*, 1994b; McNeilly *et al.*, 1996; Allan *et al*., 1994c; Harms *et al.*, 2001; Cheung and Bolin, 2002; Meerts *et al*., 2005). One study found evidence of PCV2-mediated apoptosis in PK-15 cells (Liu *et al*., 2005), and although some CPE was also observed after the infection of PCV2 into human Rd cells (Hattermann *et al*., 2004) this has not been confirmed. PCV1 has also been reported to cause some ultrastructural alterations in human mononuclear cells, although cell death was not observed (Arteaga-Troncoso *et al*., 2005). Due to the general absence of a cytopathic effect in cell culture, other methods are required to detect PCV infection and replication *in vitro*. PCR detects viral genetic material, but will not discriminate between live and inactivated virus. Immunofluorescence assays (IFA) with antisera to whole virus may also be used, but this technique is time-consuming and prone to cross-reaction between PCV types (Dulac and Afshar, 1989; Allan *et al*., 1998). Immunoperoxidase assays based on the use of monoclonal antibody against the capsid protein (IPMA) are commonly used for the detection of PCV in cell culture (Dulac and Afshar 1989; Harms *et al*., 2001; Gilpin *et al*., 2003; Meerts *et al*., 2005; Emmoth *et al*., 2004) and allow visualisation of antigen within infected cells and are specific for each PCV type. Reverse transcription PCR (RT-PCR) assays are also useful as they detect viral RNA transcripts and can therefore differentiate between replicating and inactivated virus. The assays used to detect replicating virus and to quantitate infectious virus and reported in this Chapter were a PCV2-specific IPMA based on a monoclonal antibody produced at Murdoch University against a

recombinant capsid protein of PCV2 and a previously published RT-PCR (Yu *et al*., 2005).

While sequence analysis of Australian strains of PCV2 (reported in Chapter 4) failed to detect differences in these strains from those associated elsewhere with postweaning multisystemic wasting disease (PMWS), the possibility that there may be unrecognised biotypes that could be imported into Australia and cause PMWS exists and has not been refuted scientifically. Amid considerable controversy, Australia has maintained restrictions on the importation of pig meat from other countries, requiring that all pig meat products be cooked at a temperature of 70°C for 11 minutes before distribution and sale in Australia, conditions based on inactivation data for PRRSV (Australian IRA for pig meat, 2004).

The aim of the studies reported in this Chapter were to examine the thermal stability of PCV2 to determine if the virus would indeed survive at the temperatures required for the heat treatment (cooking) of pork meat prior to distribution within Australia. For this, it was required to adapt an Australian strain of PCV2 to replicate in cell culture, to develop a method of titrating viable virus in the culture, and to determine the effect of a variety of heat treatments on the viability of the virus.

Materials and Methods

Cell cultures

PCV-free PK-15 cells (PK-15A) were obtained from Mr Chris Morrissey (Australian Animal Health Laboratory, Geelong). This cell line was cultured *in vitro* in 25 cm^2 tissue culture flasks (Nunc) at 37°C in a 7% CO_2-in-air atmosphere. When confluent monolayers were formed, the medium was removed from the culture flask and the cells washed in 2 mL phosphate buffered saline (PBS). The PBS was removed and discarded and cells were then treated with 0.5 mL trypsin solution (NaCl 80 g L^{-1}, KCl 4 g L^{-1}, glucose 10 g L^{-1}, $NaHCO_3$ 6 g L^{-1}, trypsin (Difco 1:250) 5 g L^{-1}, ethylenediaminetetra-acetic acid (EDTA) 2 g L^{-1}, phenol red [1%] 4 mL, dH_2O to 1L) for 10-15 min at 37°C. Cells were then suspended in 14 mL of Dulbecco's modified Eagle's medium (DMEM; Thermo) supplemented with amphotericin B (2.5 μg mL^{-1}), penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}), gentamycin (4 μg mL^{-1}) and 4% (v/v) newborn calf serum. Aliquots of 7 mL were dispensed into 25 cm^2 sterile tissue culture flasks and incubated at 37°C in 7% CO_2-in-air atmosphere.

PCV2 infection of cell cultures

Approximately 0.2 g of liver or lung tissue from a pig from Farm C in Western Australia infected with PCV2 and detected by multiplex PCR as previously described (Chapter 3) was frozen, then ground in 1 mL of semi-frozen media using a cold

mortar and pestle. The resulting mixture was frozen and thawed, before being mixed with 1 mL of trypsinised PCV-free cells (containing approximately 3.1 x 10^6 cells). The cells were incubated at 37°C on a rocker for 2 h, washed 3 times with PBS, divided into 2 aliquots and each suspended in 7 mL of DMEM supplemented with 4% newborn calf sera and incubated overnight at 37°C in 7% CO_2-in-air atmosphere. When the cells were 80% confluent they were treated with D-glucosamine HCl (Sigma) prepared as a 300 mM solution in Hank's salt solution (ICN Biomedicals) and stored at –20°C until required. For treatment with glucosamine, infected and control cells were washed, trypsinised and pelleted at 300 rpm, before being resuspended in 500 μL 300 mM glucosamine solution at 37°C for 45 min. Following incubation, the cells were pelleted at 300 rpm, resuspended in DMEM growth medium, split 1:2, dispensed into 25 cm^2 flasks and left for at least 4 days at 37°C in 7% CO_2-in-air atmosphere.

Infected cells were passaged either in the conventional manner, as outlined above, or by freeze-thawing. To passage by freeze-thawing, cells were washed, trypsinised and resuspended in 0.5 mL of DMEM growth medium. The resulting mixture was freeze-thawed 5 times and used to infect fresh PK-15 cells using the same method outlined above for the initial PCV infection.

To enhance PCV2 infection and replication in culture, a technique described by Allan *et al*. (1998) designated "superinfection" was used. Briefly, infected cells were split into 2 flasks, one flask was freeze-thawed and used to re-infect the other. These super-infected cells were then incubated at 37°C in 7% CO_2-in-air atmosphere and treated with glucosamine when 80% confluent as described earlier.

Thermal stability of virus

An Australian PCV2 isolate (Farm C WA) inoculated into cells using the methods described above, was detected in the cells by PCR and IPMA. After the virus was passaged in culture 3 times, the infected cells were freeze-thawed and the resulting virus-cell suspension divided into 1 mL aliquots for use in PCV2 thermal stability assays.

Each 1 mL of virus suspension in thin-walled glass tubes was heated at 56°C, 65°C, 70°C, 75°C, 85°C or 95°C in a heated water bath. Positive and negative controls were kept at room temperature (about 23°C). After treatment, the virus suspensions were immediately chilled on ice and used to infect PCV-free PK-15 cells in 25 cm^2 culture flasks, one flask for each temperature. After infection, the cells were left for at least 24 h then treated with 500 μl glucosamine by removing the medium from each flask and washing the cells in 2 mL PBS. The PBS was then removed and 500 μL

mL 300mM D-glucosamine was added to each flask, before incubation for 30 min at 37°C in 7% CO_2-in-air atmosphere. The glucosamine was then removed and the cells were washed twice with 2 mL PBS for 5 min each time. Following the addition of 5 mL of growth media to each flask, cells were incubated for at least 7 days. The cultures were tested for the presence of PCV2 by IPMA.

DNA and RNA extraction

Viral DNA was extracted from approximately 3.1 x 10^6 cultured PK-15 cells using a DNeasy tissue kit (QIAGEN) using the methods described by the manufacturer, with the exception that DNA was eluted in 100 μL of elution buffer instead of the recommended 200 μL.

Viral RNA was extracted from approximately 3.1 x 10^6 cultured PK-15 cells using a RNeasy mini kit (QIAGEN) according to the manufacturer's instructions.

PCR

PCR primers for detecting PCV2 *in vitro* (PCV2 5`F and PCV2 5`R) were based on the sequence of the isolate (FC-WA) that was used to infect the cell cultures and are shown in Table 5.1.

Table 5.1. PCR primers used to detect Australian PCV2 in vitro

Primer	Sequence	Position in genome
PCV2 5`F	5`-TGGAAAGCTTGAGATTTAATATTGACGACT-3`	1451 – 1480
PCV2 5`R	5`-GTGAGGTACCGGGAGTGTTAGGA-3`	1201 – 1223

PCR assays were performed in a 25 μL reaction mixture consisting of 3 μL extracted sample DNA, 1X PCR reaction buffer with 2 mM $MgCl_2$ (Roche), 1 mM each dNTPs, 6.4 pmol each primer and 1.16 U Expand high-fidelity DNA polymerase (Roche). Amplification was performed with an initial denaturation step of 5 min, followed by 35 cycles of amplification (95°C for 1 min, 57°C for 1 min, 72°C for 2 min), with a final extension step of 72°C for 7 min. PCR products were electrophoresed in a 2% agarose gel at 80 V for 1 h and visualised using an ultra-violet transilluminator (UVP).

RT-PCR

The RT-PCR used to detect PCV2 in cultured cells was based on the test developed by Yu *et al*. (2005) with the primers modified slightly (Table 2) to detect the Australian strain (Farm C WA) that had been adapted to replicate in cell

culture.

Table 5.2. RT-PCR primers used to detect Australian FC-WA strain of PCV2 in cell culture.

Primer	Sequence	Position in genome
CapF014	5`- GGAGTCAAGAACAGGTTTGGGTG -3`	1229 - 1251
CapR014	5`- AGACTCCCGCTCTCCAACAAG -3`	425 - 445

RT-PCR assays were performed using a Superscript II kit (Invitrogen). Extracted RNA was diluted 1:10 with DEPC water and 1 μL added to a reaction mixture with 1 μL oligo $DT_{(12-18)}$ (Invitrogen), 10 mM each dNTP (Invitrogen) and 7 μL DEPC water. Following incubation at 65°C for 5 min, samples were place on ice for 1 min. The 10 μL reaction was added to a prepared mixture with 1X RT buffer (Invitrogen), 25mM $MgCl_2$ (Applied Biosystems), 0.1 M DTT, 1 μL 40 U/μL RNase OUT (Invitrogen), 1 μL 50U/mL Superscript II (Invitrogen) except for the –RT control sample, and DEPC water to a total of 20 μL. Following incubation at 50°C for 50 min, the reaction was terminated by incubation at 85°C for 5 min and then chilled on ice. After the addition of 1 μL RNase H (Invitrogen) to each sample, tubes were incubated at 37°C for 20 min. Two μL of the resulting cDNA was added to a PCR mix with 10X PCR buffer (Applied Biosystems), 5 mM $MgCl_2$ (Applied Biosystems), 10 mM each dNTPs (Invitrogen), 3.2 pmol of each primer (CapF014 and CapR014), 0.1 μL Taq polymerase (Applied Biosystems) and DEPC water to 50 μL. Amplification was performed with an initial denaturation step of 5 min, followed by 35 cycles of amplification (95°C for 1 min, 64°C for 1 min, 72°C for 2 min), with a final extension step of 72°C for 7 min. PCR products were electrophoresed in a 2% agarose gel at 80 V for 1 h and visualised using an ultra-violet transilluminator (UVP).

IPMA

PCV2-infected and uninfected PK-15A control cells were grown in 8-chambered culture slides (BD Biosciences). When the monolayers were confluent, the medium was removed from the slides and the cells washed with PBS. The cells were fixed using cold acetone for 10 min, washed 3 times in PBS and blocked using NBCS in PBS (1:5) at room temperature. Excess blocking solution was removed and then

reacted with a PCV2 monoclonal antibody prepared against a recombinant PCV2 capsid protein (mAb) diluted 1:1,000 in 10% NBCS in PBS for 1h at room temperature. Slides were washed 3 times in PBS and a biotinylated secondary anti-mouse (DakoCytomation LSAB 2 System-HRP) was applied for 10 min at room temperature. Slides were washed 3 times in PBS and a streptavidin-HRP conjugate (DakoCytomation) was applied for 10 min at room temperature. Slides were washed a further 3 times in PBS and cells were stained with DAB (DakoCytomation) for 5 min at room temperature. Slides were finally washed 3 times with distilled water and cells were counterstained with haematoxylin. After dehydration with 100% ethanol, cells were mounted in permanent mounting medium (DPX) and examined by light microscopy.

As a control, the IPMA was performed on both fixed cultured PK-15 cells and tissue sections originating from New Zealand and French pigs with PMWS. When using the IPMA to detect PCV2 in fixed tissues, sections were deparaffinised and rehydrated as described by Lillie (1965), washed with PBS then processed in the same way as the fixed cultured cell monolayers.

Titration of PCV2 in cell culture

Ten-fold dilutions of freeze-thawed cell cultures were serially diluted up to 10^{-5} and used to infect PCV-free PK-15 cells as described above. When the infected cells were confluent, IPMA was used to detect PCV2 infection and an approximate viral titre. An approximate titre was determined as the highest dilution producing a positive IPMA result in cell culture.

Results

Inoculation of PCV2 into cell culture

PCV2 was successfully inoculated into cell culture and was detected using PCR (Figure 5.1) and subsequently RT-PCR (Figure 5.2) and IPMA (Figure 5.3). The IPMA developed with the mAb against the capsid protein of PCV2 detected PCV2 in both cultured PK-15 cells (Figure 5.3) and in tissues originating from PMWS-affected pigs from New Zealand (Figure 5.4) and France (Figure 5.5). PCV2-specific staining was mostly observed in the nucleus, with occasional cytoplasmic staining. To ensure no cross-reaction of the IPMA with PCV1, the PK-15 persistently PCV1-infected cell line was also tested and no staining was observed.

Viral infection was monitored by PCR for 26 passages, and the cell cultures remained PCR positive throughout. Using titration based on IPMA, the titre was found to be approximately $1x10^3$ virus particles per mL of infectious material after superinfection was adopted. Superinfection, or re-infecting PCV2-positive cells using

previously freeze-thawed cultures, was very successful, increasing the number of positive cells observed by IPMA from below 10 per slide chamber to approximately 5-10% of cells (Figure 5.3).

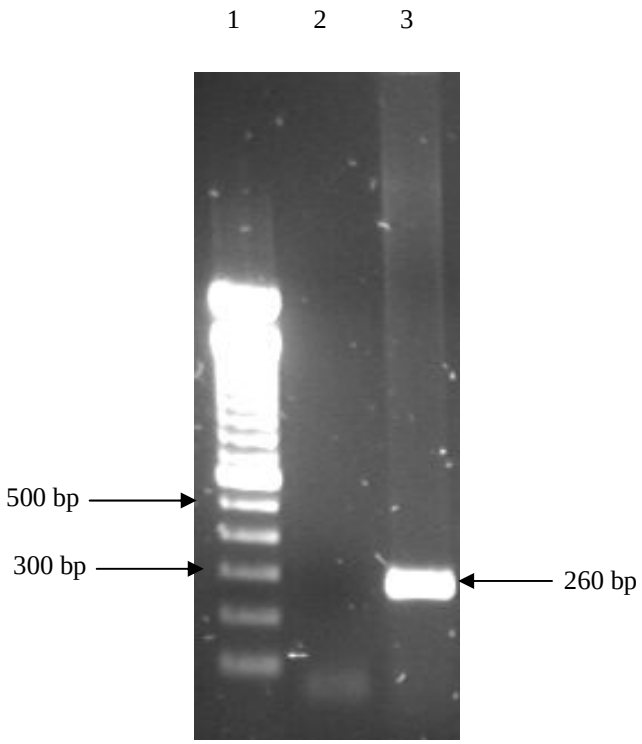

Figure 5.1. Detection of PCV2 DNA in PK-15 cell culture by PCR. Lane 1, 100 bp molecular weight marker (Lifetech); lane 2, negative PCR control; lane 3, FCWA PCV2 positive cell cultrue sample. The size of the bands in the DNA ladder is indicated on the left, and the expected size of the PCR product on the right.

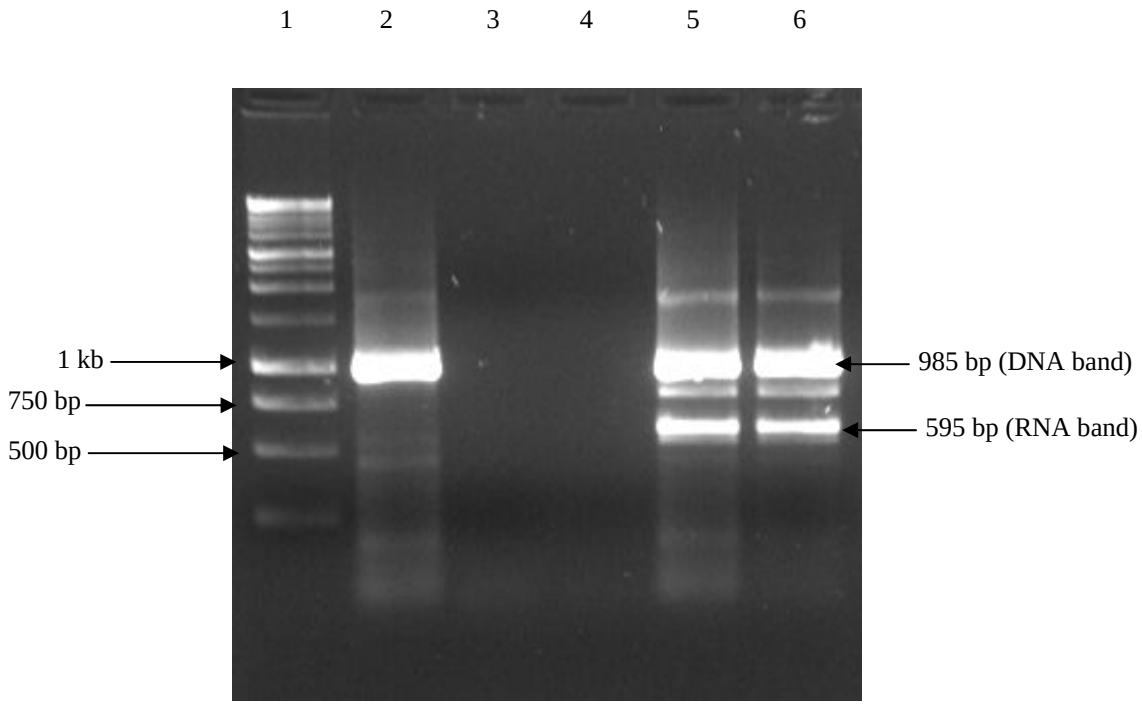

Figure 5.2. Detection of PCV2 replication in PK-15 cell culture by RT-PCR. Lane 1, 1 kb plus molecular weight marker (Promega); lane 2, FCWA DNA extraction from tissue; lane 3, negative PCR control; lane 4, negative cell culture control; lane 5, FCWA PCV2 positive cell culture sample, lane 6, FCWA PCV2 positive cell culture sample diluted 1:10. The size of the bands in the DNA ladder is indicated on the left, and the expected size of the PCR product on the right.

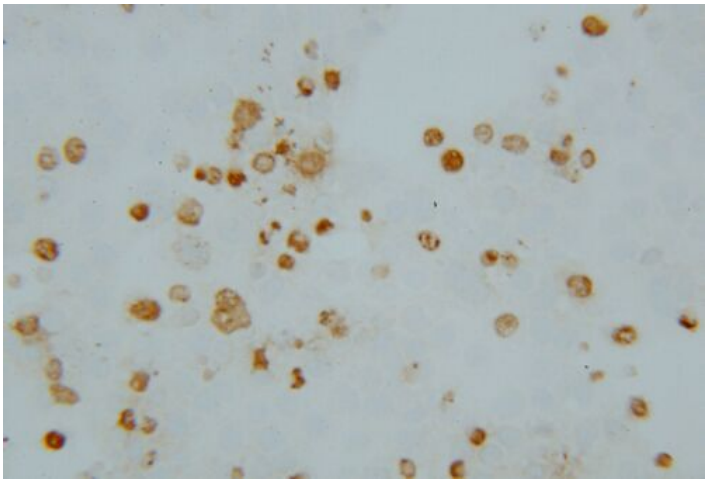

Figure 5.3. Detection of PCV2 antigen in PK-15 cell culture by IPMA. Brown staining indicates an infected cell.

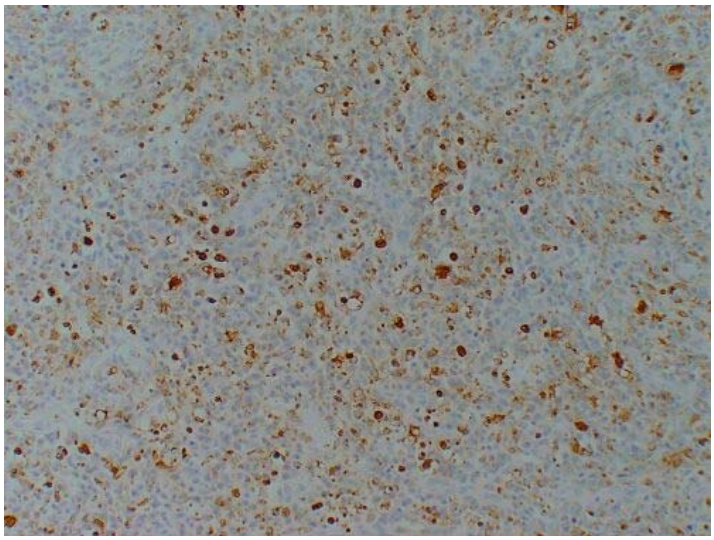

Figure 5.4. Detection of PCV2 antigen by IPMA in the lymph node of a PMWS-affected pig originating from New Zealand.

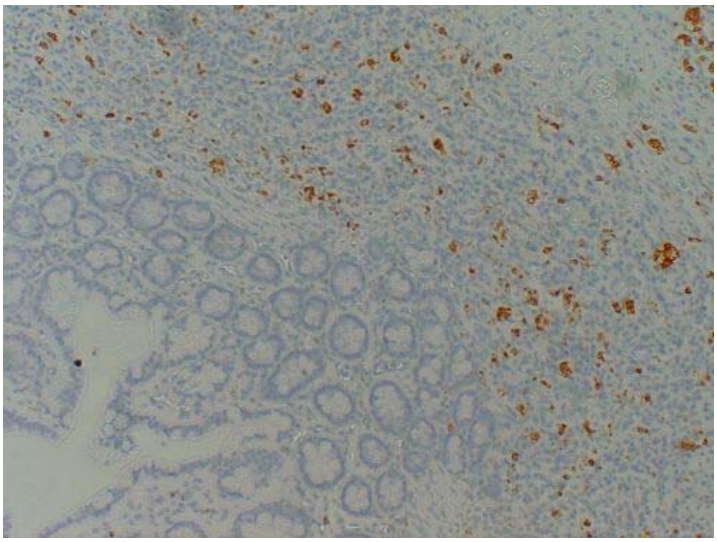

Figure 5.5. Detection of PCV2 antigen by IPMA in the gut of a PMWS-affected pig originating from France.

Thermal stability of PCV2

PCV2 positive cells were observed in cell cultures after viral inocula had been heated at 56°C, 65°C, 70°C, 75°C and 85°C for 15 min, while no positive cells were seen at 95°C or in the negative control slide (Table 5.1, Figures 5.6 to 5.13). Higher numbers of positive cells were observed in the cells infected with the inocula heated at 65°C and 70°C (Table 5.1, Figures 5.8 and 5.9).

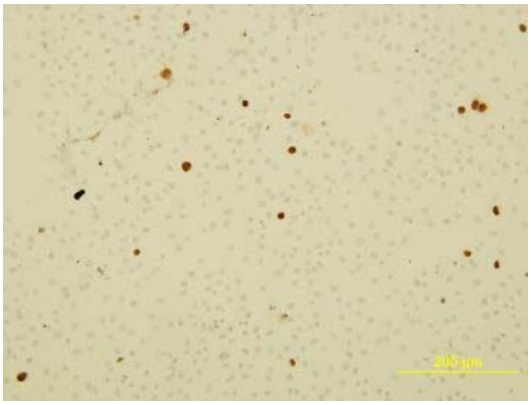

Figure 5.6. Positive control, PCV2 thermal stability experiment

Figure 5.7. Detection of PCV2 antigen following heat treatment at 56 degrees (^{o}C).

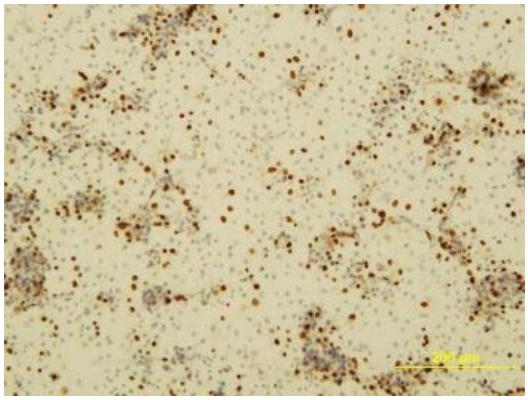

Figure 5.8. Detection of PCV2 antigen following heat treatment at 65 degrees (^{o}C).

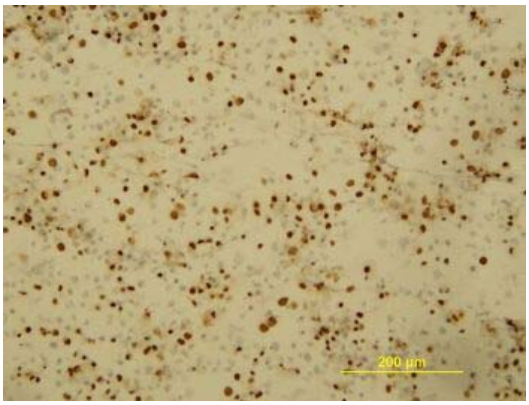

Figure 5.9. Detection of PCV2 antigen following heat treatment at 70 degrees (^{o}C).

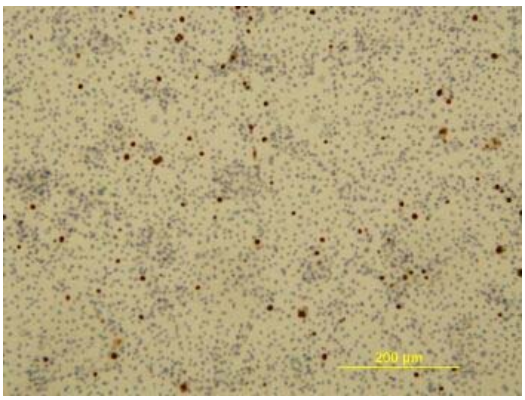

Figure 5.10. Detection of PCV2 antigen following heat treatment at 75 degrees

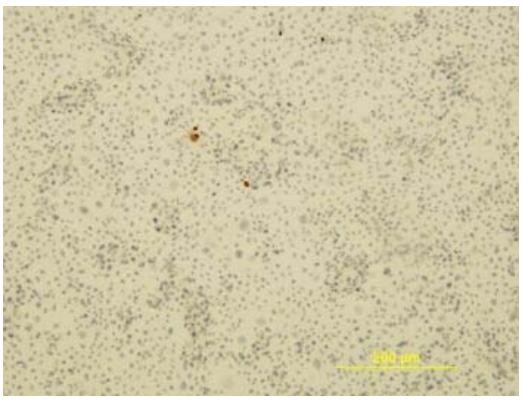

Figure 5.11. Detection of PCV2 antigen following heat treatment at 85 degrees (^{o}C).

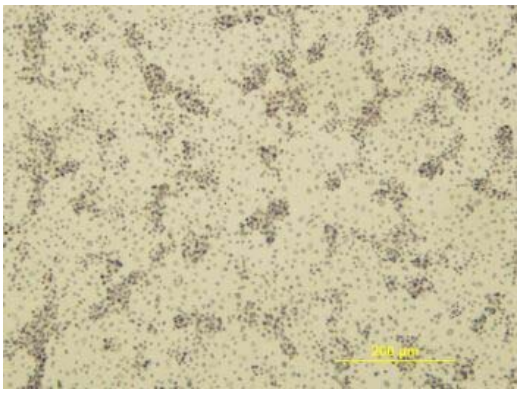

Figure 5.12. No PCV2 antigen detected following heat treatment at 95 degrees (^{o}C).

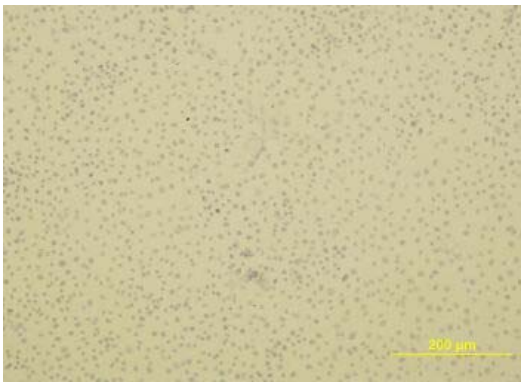

Figure 5.13. Negative control, PCV2 thermal stability experiment.

Table 5.3. Average number of PCV2-positive cells observed per well by IPMA following heat treatment for 15 minutes at temperatures from 56°C to 95°C.

Temperature (°C)	Average number positive cells/well
56	~4 500
65	~17 000
70	~18 000
75	~3 000
85	160
95	0
+ve control	800
-ve control	0

Discussion

The primary aims of this study were to determine the thermal stability of PCV2. Prerequisites for this study were the adaptation of an Australian strain of PCV2 to replicate in the PK-15 continuous cell line, and as the virus failed to induce a cytopathic effect, an efficient method of detecting virus in cell cultures.

The IPMA was a suitable method, relatively easy to perform, and because it utilised a capsid-specific mAb, a specific method of detecting viral antigen in the cell cultures. PK-15 cells persistently infected with PCV1 were also tested as controls, and no positive cells were observed. Although the number of PCV2-infected cells initially positive by IPMA was low, as has been previously reported (Fenaux *et al*., 2004b) when the technique was applied to tissue sections from French and New Zealand PMWS-affected pigs, abundant PCV2 antigen was detected (Figures 5.4 and 5.5). Therefore the small number of positive cells originally detected by IPMA in cell culture is most likely a reflection of a low infection rate rather than a lack of sensitivity of the IPMA. The IPMA allowed visualisation of PCV2 antigen within infected cells, and staining was observed mostly in the nucleus, with cytoplasmic staining rare.

IPMA has been utilised by several research groups for the detection of PCV antigen (Dulac and Afshar 1989; Harms *et al*., 2001; Gilpin *et al*., 2003; Meerts *et al*., 2005; Emmoth *et al*., 2004) but it is only one of several methods have been used to detect PCV in cell culture. Other techniques have included various immunofluorescence assays (Tischer *et al*., 1982; Tischer *et al*., 1987; Dulac and Afshar 1989; Allan *et al*., 1998; Allan *et al*., 1994a; Gilpin *et al*., 2003; Fenaux *et al*., 2002; Allan *et al.*, 1994b; Allan *et al.*, 1994c; Tischer *et al*., 1995a; McNeilly *et al*., 1996; Liu *et al*., 2001; McNeilly *et al*., 2001; Vincent *et al*., 2003; Hattermann *et al*., 2004), electron microscopy (Stevenson *et al*., 1999), *in-situ* hybridisation (ISH) (Allan *et al*., 1998),

PCR (Hattermann *et al*., 2004; Fenaux *et al*., 2004b), RT-PCR (Cheung, 2003; Cheung, 2003b; Yu *et al*., 2005) and Northern blot (Mankertz *et al*., 1998; Cheung and Bolin, 2002). The techniques used are usually tailored to the specific requirements of the study, for example electron microscopy is useful for studying the structure of PCV (Stevenson *et al*., 1999), Northern blot and RT-PCR to detect PCV-specific transcripts, indicating viral replication (Yu *et al*., 2005; Cheung, 2003; Cheung, 2003b; Mankertz *et al*., 1998; Cheung and Bolin, 2002) and techniques such as immunofluorescence, ISH and IPMA are useful for the visualisation of PCV antigen within infected cells (Allan *et al*., 1994b; Allan *et al*., 1998; Gilpin *et al*., 2003). Detection of antigen is particularly important when attempting to infect cells other than the PK-15 cell line with PCV, as cytoplasmic staining of the PCV antigen in the absence of nuclear staining can indicate a non-productive infection (Gilpin *et al*., 2003; Vincent *et al.*, 2003; Allan *et al*., 1994c).

The successful replication of PCV2 in the PCV-free PK-15 cell line was confirmed not only by IPMA, but also by PCR and RT-PCR, and persisted for at least 26 passages as confirmed by PCR. PCR was also initially used to detect PCV2 from infected cell cultures but was of limited usefulness as the PCR used was not quantitative and given that PCV may replicate at a very low level in culture (Fenaux *et al.*, 2004b), it became clear that a test that allowed PCV2 antigen to be seen in infected cells would be advantageous, so the IPMA was preferred. This was particularly important for the thermal stability experiments where heat treatment at high temperatures was expected to inactivate a large percentage of the viral particles, meaning that a reasonable titre of PCV2 would be required initially.

Treatment of the infected cells with glucosamine was essential to maintain a reasonable level of PCV2 infection in the cultures, but even with glucosamine treatment the number of PCV2-infected cells in the cultures was very low. Superinfection, however, dramatically increased the number of infected cells, to a maximum of about 10% of the cells in the culture (Figure 5.3). IPMA indicated that the majority of the virus was nuclear. Titration of the virus in the freeze-thawed superinfected cells indicated that there were approximately $1x10^3$ infectious virus particles per mL present in the culture.

The initial problem of very low viral titre was not unexpected, as previous studies of PCV1 as a persistent infection in the PK-15 cell line have found only a small number of cells (less than 1%) contained antigen when tested by IFA (Tischer *et al*., 1982; Dulac and Afshar, 1989). Also, attempts to retrieve PCV1 DNA from persistently-infected culture have resulted in very small amounts (nanograms) of PCV1 DNA from

several litres of PK-15 culture (Dulac and Afshar, 1989).

It is well recognised that PCV replicates at a fairly low titre (usually below 10^5 $TCID_{50}$ per mL in PK-15 cells (Fenaux *et al*., 2004b), and that the number of infected cells is much higher in persistently infected or high-passage cultures than newly-infected cultures (Tischer *et al*., 1987). Titration of virus present in cultures has indicated that PCV2 after 120 passages in PK-15 cells, and PCV1 from persistently infected cells, both replicate much more efficiently than PCV2 after one passage (Fenaux *et al*., 2004b).

While the initial low titre of the Australian strain of PCV2 in cultures was expected, glucosamine treatment appeared to have a limited effect on virus replication and this was not expected. Glucosamine treatment at the time of initial infection did enable establishment of infection in the PK-15 cells whereas this was not achieved when glucosamine treatment was not used (data not shown) but despite a report by Tischer *et al*. (1987) that this treatment increased the number of infected cells in cultures by up to 50-fold, in the current attempts glucosamine resulted in doubling of the number of infected cells only.

The increase in the number of infected cells in culture following glucosamine treatment is due to enhanced PCV replication (Tischer *et al*., 1987). It was theorised that PCV replication is dependent on cellular enzymes expressed in the S phase of cell growth, and that glucosamine treatment allows the PCV genome to enter the cell nucleus, where viral replication occurs (Tischer *et al*., 1987). Under normal conditions, the size and charge of the PCV molecule prevents the virus from penetrating the nuclear envelope, and viral DNA only becomes included in the daughter nuclei at the end of mitosis, therefore viral replication can only begin when the host cells have passed mitosis. However, this requirement is removed if cells are treated with glucosamine, possibly because glucosamine can bind to the PCV molecule and neutralise it, therefore enabling the viral DNA to pass through the nuclear pores and into the cell nucleus (Tischer *et al*., 1987).

The results obtained clearly demonstrated that PCV2 was able to survive heat at 85°C but not 95°C for 15 min. These results indicate that PCV2 may be capable of surviving in meat cooked at the current importation standard of 70°C for 11 minutes (Australian IRA for pig meat, 2004), and therefore cooking of pig meat prior to importation into Australia would be most unlikely to inactivate PCV2.

Other interesting results were obtained in the thermal inactivation experiment. It was thought that there would be a linear relationship between the temperature used to treat the virus and the viral titre. However, the number of positive cells detected in

cultures infected with heat treated inocula actually increased from 56°C to 65°C, then from 65°C to 70°C, before decreasing at 75°C and 85°C. The reasons for this are not certain, however given that most of the IPMA staining was observed in the cell nucleus, the major location of virus replication, it is possible that heating up to 70°C assisted in releasing virus from the cell material after it was freeze-thawed. However this was only effective up to a certain point, and above 70°C it was likely that the virus was actually inactivated by the heat treatment.

The results demonstrating the thermal stability of PCV2 are similar to those reported previously for PCV by others. Emmoth *et al*. (2004) showed that PCV2 will tolerate heating at 70°C for 15 minutes with no loss of infectivity, 80°C and 90°C for 15 minutes with moderate loss of infectivity, and that PCV2 may resist heating at 95°C for 5 seconds, although with a very large loss in infectivity. This is in agreement with the results from this experiment, where PCV2 was unaffected or perhaps even assisted by treatment at 56°C, 65°C and 70°C, while treatment at 75°C and 85°C for 15 minutes resulted in a much reduced rate of infection and treatment at 95°C for 15 minutes inactivated the virus (Table 5.1). Further study is required to elucidate more information on the kinetics of PCV2 and also PCV1 when subjected to heat treatment.

PCV1 was also previously shown to be unaffected by treatment at 70°C for 15 min (Allan *et al*., 1994c) and studies on PPV have demonstrated that viral heat inactivation is caused by destruction of the viral capsid protein, and that differences in heat resistance between viruses could be due to differences in the properties or structures of their capsid proteins (Blumel *et al*., 2002).

It could be expected that PCV would resist heat treatment more successfully than many other viruses. PCV is known to be resistant to adverse chemical and physical conditions, e.g. PCV1 is resistant to treatment at pH 3 (Allan *et al*., 1994c) and also to chloroform (Allan *et al*., 1994c), and PCV2 has been shown to be resistant to a number of common disinfectants (Royer *et al*., 2001). Some other non-enveloped viruses are also very heat stable, e.g. the non-enveloped beak and feather disease circovirus, in the same genus as PCV, survives heating at 80°C for 30 min with only a minimal reduction in titre, and 85°C for 30 minutes with only some reduction in titre (Raidal, 1994), and the porcine parvovirus, a particularly heat-resistant virus is able to tolerate treatment at 60°C for 1 h (Blumel *et al*., 2002) and 70°C for 15 min (Emmoth *et al*., 2004) with no loss of infectivity. In contrast, other non-enveloped viruses are less resistant, e.g. poliovirus and human B19 viruses have are inactivated after 30 min at 50°C (Shiomi *et al*., 2004) and immediately at 60°C (Blumel *et al*.,

2002), respectively. The non-enveloped feline and canine caliciviruses are easier to inactivate than the circoviruses, and are inactivated at 71.3°C after 1 min (Duizer *et al*., 2004). The enveloped viruses have normally even less stability after heat treatment: West Nile virus is completely inactivated at 60°C for 30 min (Remington *et al*., 2004); the SARS coronavirus is largely inactivated after 5 min at 56°C (Kariwa *et al*., 2004); cytomegalovirus (CMV) is inactivated at 62.5°C for 30 min and 72°C for 5 seconds (Hamprecht *et al*., 2004).

CHAPTER 6. CONSTRUCTION OF A POTENTIALLY INFECTIOUS CLONE OF AN AUSTRALIAN STRAIN OF PCV2

Summary

Three methods for the construction of an infectious clone of an Australian strain of PCV2 were attempted. One of these methods, based on that described by Hattermann *et al*. (2004) resulted in the construction of a full-length clone of an Australian PCV2 strain.

Introduction

Viral infectious clones, commonly constructed by the ligation of all or part of a viral genome into a plasmid vector, have many applications. Infectious viral DNA or RNA may be recovered and manipulated, and segments may be exchanged between strains to produce chimaeras. This could enable examination of the function(s) of different regions of the viral genome, for example, regions controlling host range and pathogenicity determinants (Bloom *et al*., 1990). Viral clones can also be manipulated to determine how structural differences in different viral strains can translate to significant differences in biological behaviour.

Infectious clones are particularly useful when a virus cannot be cultured *in vitro*. For example, a tissue culture system supporting TTV replication is not yet available, so the construction of a full-length molecular clone provided important information about TTV replication and gene expression (Hino, 2002). The study of hepatitis C virus has also been severely hampered by the inability to culture the virus *in vitro* and the lack of a suitable animal model (Shimizu *et al*., 1993). However, the construction of HCV molecular clones has provided more information on the life cycle of this significant human pathogen (Ikeda *et al*., 2002; Zhong *et al*., 2005).

Although both PCV1 and PCV2 may be grown in culture, infectious PCV clones have other applications for the study of the virus. Several PCV2 infectious clones have been constructed previously, for investigation into viral gene function, kinetics and replication *in vitro* (Liu *et al*., 2001; Cheung and Bolin, 2002; Mankertz and Hillenbrand, 2002; Cheung, 2003c; Mankertz *et al*., 2003; Cheung, 2004b; Fenaux *et al*., 2004b) and the ability of PCV1 and PCV2 to infect different continuous cell lines (Hattermann et al., 2004). Also, chimaeric clones constructed using PCV1 and PCV2 DNA have provided information on epitope mapping (Lekcharoensuk *et al*., 2004) and potential PCV vaccines against PCV2-associated diseases such as PMWS (Fenaux *et al*., 2003; Fenaux *et al*., 2004).

The aim of this study was to construct a PCV2 infectious clone, based on an Australian PCV2 isolate, that could be used in cell culture experiments and in future

animal experimental work.

Materials and methods

DNA extraction and PCR

The method used for the construction of a PCV2 infectious clone was based on the method used by Hattermann *et al*. (2004), where single linear copies of the PCV2 genome are re-ligated to form infectious particles.

PCV2 DNA for virus amplification was from a previously extracted sample from pig tissue in Farm B NSW (Chapter 4). The primers used for amplification were based around the unique *Sac1* enzyme restriction site located in PCV2 ORF1 (Table 6.1).

Table 6.1. Primers based on the unique *Sac1* restriction site used to amplify PCV2 DNA from tissue.

Primer	Sequence	Position in genome	PCV type
*Sac1*bF(T)	5`-TGGAGCTCCTAGATCTCAAGGACA-3`	322-345	PCV2
*Sac1*bR(A)	5`-AGGAGCTCCACATTCCATCAGTAA-3`	305-328	PCV2

PCR was performed using the Expand High Fidelity kit (Roche) in a 50 μL reaction mixture consisting of 3 μL extracted sample DNA, 1X PCR reaction buffer with Mg (Roche), 2 mM $MgCl_2$ (Roche), 1 mM each dNTP (Applied Biosystems), 6.4 pmol each primer and 1.3 U high fidelity polymerase (Roche). Amplification was performed with an initial denaturation step of 5 min, followed by 35 cycles of amplification (95^{o}C for 1 min, 57^{o}C for 1 min, 72^{o}C for 2 min), with a final extension step of 72^{o}C for 10 min. PCR products were electrophoresed in a 2% agarose gel at 80 V for 1h and visualised using an ultra-violet transilluminator (UVP).

Cloning and ligation

Fresh PCR products were cloned into the pCR®2.1 TOPO® vector using a TA cloning kit, Version M (Invitrogen), according to the methods recommended by the manufacturer. Constructed plasmids were used to transform competent Top 10 F' *E. coli* cells (Invitrogen). Plasmids containing the desired inserts were identified by α-complementation blue/white screening, and purified recombinant plasmids were digested with *Eco R1* and *Sac1* restriction enzymes to confirm the presence of the insert.

Sequence analysis

Plasmids containing the correct fragments were sequenced using the M13 forward (5`-GTAAAACGACGGCCAG- 3`) and reverse (5`-CAGGAAACAGCTATGAC-3`) primers. Sequencing was performed using rhodamine dye terminator or Big Dye

terminator Version 3.1 (Applied Biosystems), with the ABI PRISM™ fluorescent cycle sequencing system (Applied Biosystems). Sequence data was analysed using the SeqEd program (v 1.0.3). The sequences obtained were then compared, using the BlastN program (version 2.0), with sequences available from the National Centre for Biotechnology Information (NCBI), USA.

Construction of an infectious clone

Top 10 F' *E. coli* cells containing a plasmid with the correct insert (as confirmed by restriction digestion and sequencing) were grown overnight in a volume of 250 mL 2YT media. Plasmid DNA was extracted using a Plasmid MidiPrep kit (BioRad) and the resulting elution reduced to 25 μL using ethanol precipitation. This solution was digested using 25 U *Sac1* endonuclease (NEB), 1X NEB buffer 1 (NEB), 100 μg/mL BSA (NEB) and 10 mL of Ultra-Pure water (Fisher Biotec) at 37°C overnight. Digested DNA was electrophoresed in a 2% agarose gel at 60 V for 2 h and visualised using an ultra-violet transilluminator (UVP). Bands of the correct size (1768 bp) were excised using a clean scalpel and DNA was extracted using a QIAquick Gel Extraction kit (QIAGEN) according to the manufacturer's methods.

The DNA elutions were pooled and diluted to a volume of 200 μL with Ultra-Pure water (Fisher Biotec) and DNA was ligated using 400 U T4 DNA ligase (NEB), 50 μL 10X T4 DNA ligase reaction buffer (NEB) and 249 μL Ultra-Pure water (Fisher Biotec) to give a total reaction volume of 500 μL. The ligation reaction was incubated at 16°C for 48 h then reduced to a volume of 70 μL using ethanol precipitation. Ligated fragments were electrophoresed in a 2% agarose gel at 60 V for 2.5 h and visualised using an ultra-violet transilluminator (UVP). Bands of the correct size (approximately 1000 bp) were excised using a clean scalpel and DNA was extracted using a QIAquick Gel Extraction kit (QIAGEN) according to the manufacturer's protocol.

To confirm that purified DNA was recircularised PCV2 genome, 2 different PCR assays were performed to amplify the region across the PCV2 unique *Sac1* site at nt 373 (Table 6.2). The PCR assays were performed using the Expand PCR system (Roche) as described above. Amplification was performed with an initial denaturation step of 5 min, followed by 35 cycles of amplification (95°C for 1 min, 62°C [PCV2Seq Comp primers] or 64°C [Cap primers] for 1 min, 72°C for 2 min), with a final extension step of 72°C for 10 min. PCR products were electrophoresed in a 2% agarose gel at 80 V for 1 h and visualised using an ultra-violet transilluminator (UVP).

Table 6.2. Primers used to amplify across Sac1 site to confirm PCV2 genome re-ligation.

Primer	Sequence	Position in genome	PCV type
PCV2SeqA Comp	5`-GCAG(T/C)(T/C)GAGG(A/G)GTACCATTC-3`	850 - 832	PCV2
PCV2SeqB Comp	5`-CTCCTTGGA(T/A)ACGTCATA(G/T)C-3`	1738 - 1719	PCV2
Cap F	5`- CCCATCAAGGACAGGTTTGGGGG -3`	1229 - 1251	PCV2
Cap R	5`- AGACTCCCGCTCTCCAACAAG -3`	425 - 445	PCV2

Results

The whole genome of the PCV2 isolate Farm B NSW was successfully amplified using primers based around the unique Sac1 site at nt 373 (Figure 6.1).

One pCR 2.1 plasmid containing the correct insert, as confirmed by restriction endonuclease digestion and sequencing, was digested using the *Sac1* enzyme, yielding bands 3.9 kb (plasmid) and 1.76 kb (insert) in size (Figure 6.2).

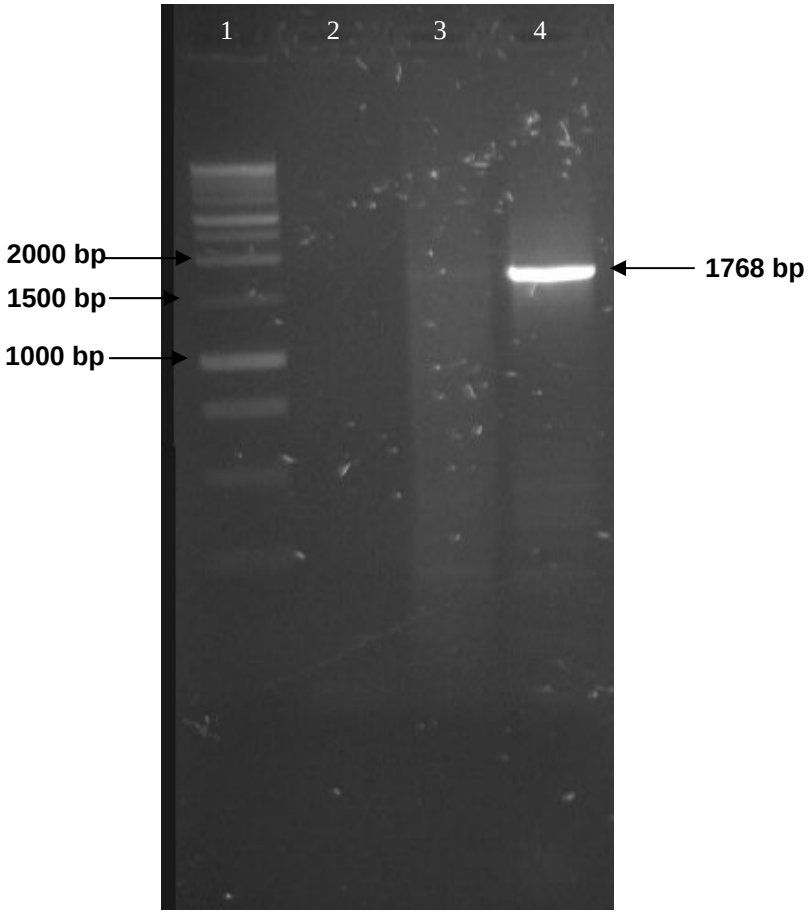

Figure 6.1. Amplification of the whole genome of PCV2 from tissues of a pig in Farm B NSW using primers PCV2 Sac1bF and PCV2 Sac1R. Lane 1, 1kb plus DNA ladder (Promega); lane 2, negative tissue control; lane , negative PCR control; lane 3, PCV2 isolate Farm C WA; lane 4, PCV2 isolate Farm B NSW. The size of the bands in the DNA ladder is indicated on the left, and the expected size of the PCR product on the right.

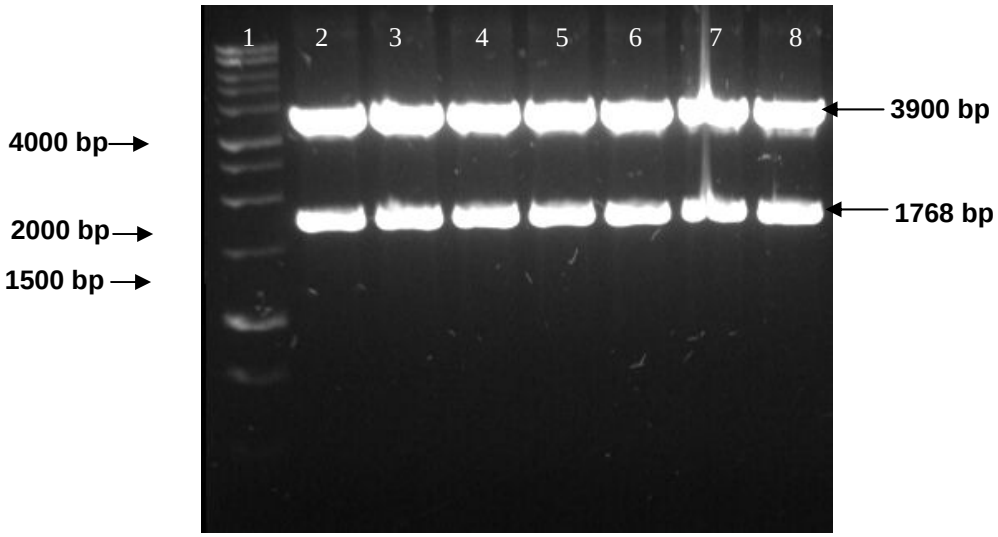

Figure 6.2. Endonuclease digestion of pCR 2.1 construct containing whole genome of PCV2 using Sac1 endonuclease. Lane 1, 1kb plus DNA ladder (Promega); lanes 2-8, digested plasmid construct. The size of the bands in the DNA ladder is indicated on the left, and the expected sizes of the digested plasmid and released linear genome on the right.

Linear genomes were successfully recircularised using T4 DNA ligase, with the circular genomes running at approximately 1000 bp on an agarose gel (Figure 6.3).

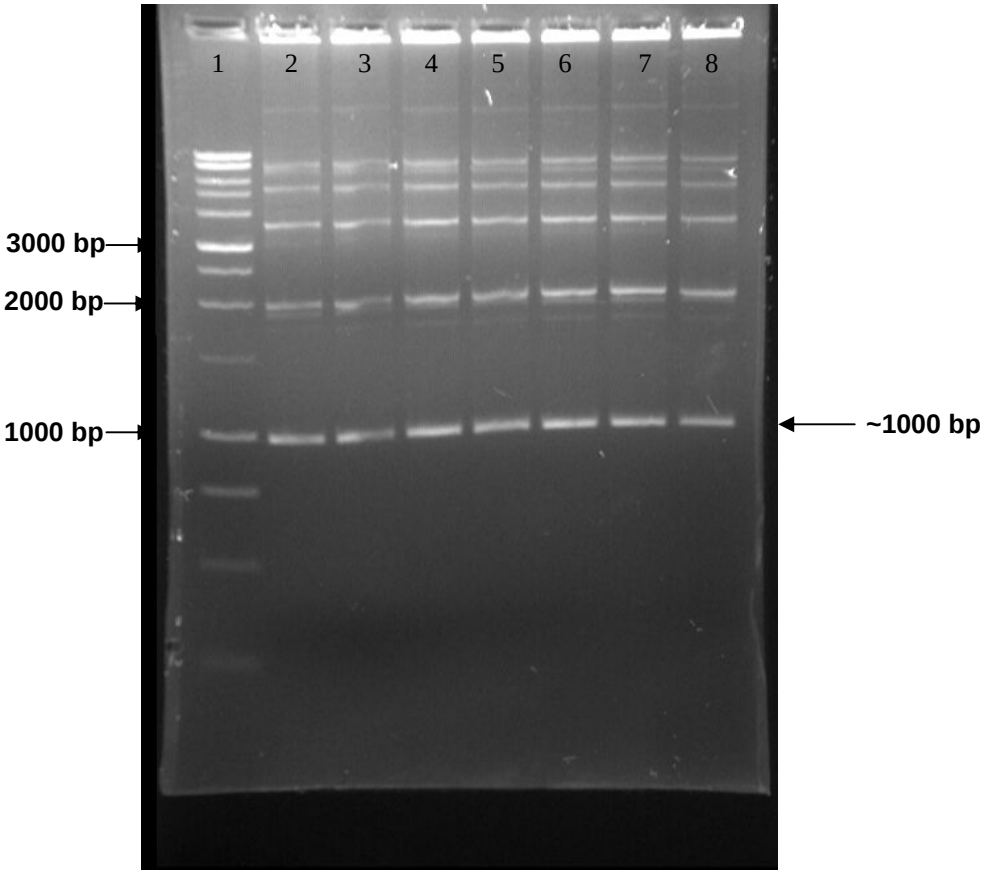

Figure 6.3. T4 ligation of the linear PCV2 genome, recircularised particles are at approximately 1000 bp. Lane 1, 1kb plus DNA ladder (Promega); Lanes 2-8, T4 DNA ligase reactions. The size of the bands in the DNA ladder is indicated on the left, and the size of the recircularised PCV2 genomes on the right.

The DNA was confirmed to be recircularised PCV2 genome by two PCRs, designed to amplify across the *Sac1* restriction site (Figure 6.4).

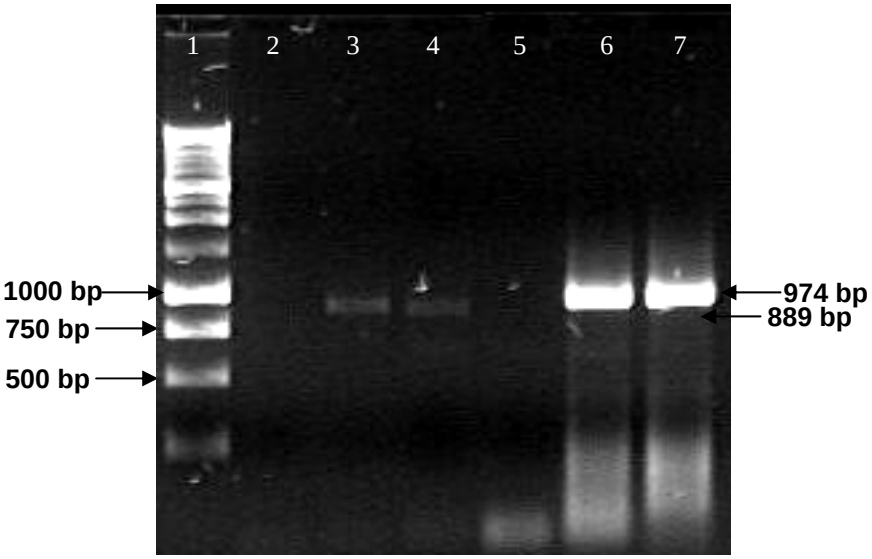

Figure 6.4. Amplification of the recircularised PCV2 genome using PCR assays designed to incorporate the *Sac1* endonuclease restriction site. Lane 1, 1kb plus DNA ladder (Promega); lane 2, PCR negative control; lane 3, recircularised PCV2 DNA (PCV2 Seq Comp primers); lane 4, recircularised PCV2 DNA 1:10 dilution (PCV2 Seq Comp primers); lane 5, negative PCR control; lane 6, recircularised PCV2 DNA (Cap primers); lane 7, recircularised PCV2 DNA 1:10 dilution (Cap primers). The size of the bands in the DNA ladder is indicated on the left, and the expected size of the PCR products on the right.

Discussion

The method used to construct an infectious clone of an Australian strain of PCV2 was closely modelled on the method of Hattermann *et al*. (2004), where the entire PCV2 genome was amplified, ligated into a holding vector, removed by restriction endonuclease digestion and then re-ligated before transfection into animal cells. Although the methods and results were not reported, 2 other methods of constructing a PCV2 infectious clone were also attempted before this method was tried. The other 2 methods involved the ligation of 2 copies of the PCV2 genome into an expression vector (Fenaux *et al*., 2002) and the ligation of several segments of the PCV2 genome into a holding vector (Roca *et al*., 2004). Infectious clones were not produced with either of these methods: while the initial amplification of the PCV2 genome was easily achieved, the ligation of different DNA fragments to each other and into various plasmid vectors was not achieved.

While the recircularised clone was confirmed by PCR, the infectious nature of the construct was not confirmed. Unfortunately there was not sufficient time to transfect the DNA into cell culture. Several attempts were made to purify sufficient DNA for transfection, however as this method does not involve the use of a plasmid backbone, obtaining large amounts of recircularised PCV2 DNA was very

difficult, as more DNA was lost with each purification step of the procedure. However, as the ligation process resulted in reasonable amounts of the desired recircularised PCV2 genome (Figure 6.3) it is anticipated that this method will be successful once sufficient infectious particles can be recovered from the final purification step.

In this study, construction of a PCV2 infectious clone was attempted with the intention of providing a biologically pure inoculum for future animal experiments. The reproduction of PCV-associated diseases (PMWS in particular) in gnotobiotic and conventional pigs using tissue homogenates or PCV2 isolates cultured *in vitro* has produced inconsistent results in the past (Fenaux *et al*., 2002). Pigs infected with PCV2 alone may develop mild to moderate lesions typical of PMWS, such as lymphoid depletion (Allan *et al.*, 1999b; Balasch *et al.*, 1998), mild pneumonia, lymphoid inflammation (Ellis *et al*., 1999; Magar *et al*., 2000), multinucleated giant cells (Magar *et al.*, 2000) and mild nephritis and hepatitis (Ellis *et al.*, 1999). However, despite the large number of studies performed after the initial identification of PCV2 in association with PMWS, in the majority of cases it has not been possible to produce clinical signs of the disease by infecting gnotobiotic or colostrum-deprived and caesarean-derived pigs (CD/CD) with PCV2 alone (Allan *et al*., 1999; Balasch *et al*., 1999; Allan *et al*., 2000c; Allan *et al*., 2000d; Kennedy *et al*., 2000; Krakowka *et al*., 2000; Pogranichnyy *et al*., 2000; Krakowka *et al*., 2001). It is possible that some of the clinical signs and lesions induced, especially using tissue homogenates, could have been produced by contaminants and were incorrectly attributed to PCV2. Therefore when conducting experimental infection studies using PCV2 it is highly desirable to use a homogeneous and biologically pure virus stock, and in recent times this has been accomplished by the use of PCV2 infectious clones. Infectious clones are also easier to quantify than virus grown in cell culture, and infectious clones overcome the problem of attenuation of virus or selection of variant viruses during serial passage in cell culture (Allan *et al*., 1994a).

PCV infectious clones have been used *in vivo* as inoculum for animal experiments (Fenaux *et al*., 2002; Oppreissnig *et al*., 2004; Roca *et al*., 2004; Grasland *et al*., 2005), although in 2 cases the infectious clone had to be administered intramuscularly or intraperitoneally or by injection directly into the liver and lymph nodes, as intranasal inoculation was not effective (Fenaux *et al*., 2002; Roca *et al*., 2004). The PCV2 infectious clones were shown to successfully infect young piglets, but none of the infected piglets showed any clinical signs of PMWS (Fenaux *et al*., 2002; Roca *et al*., 2004) unless the infectious clone was combined with another

agent (Oppreissnig *et al*., 2004) or if the pigs were artificially immunostimulated (Grasland *et al*., 2005). When compared with PCV2 originating from infected tissue and propagated in cell culture, it seems that pigs infected with infectious clones seroconvert later than those infected with the cell culture isolate (Roca *et al*., 2004).

Infectious clones are not only useful for providing pure inoculum for experimental work, but several of these constructs, whether in their original form or after manipulation, have also been used for studies on the kinetics and behaviour of PCV in culture and as possible vaccines. PCV1 and PCV2 full-length infectious clones and cloned partial fragments have been used for studying viral gene function, kinetics and replication *in vitro* (Liu *et al*., 2001; Cheung and Bolin, 2002; Mankertz and Hillenbrand, 2002; Cheung, 2003c; Mankertz *et al*., 2003; Cheung, 2004b; Fenaux *et al*., 2004b), the ability of PCV1 and PCV2 to infect different continuous cell lines (Hattermann *et al*., 2004) and chimaeric clones constructed using PCV1 and PCV2 DNA have provided information on potential PCV vaccines against PCV2-associated diseases such as PMWS (Fenaux *et al*., 2003; Fenaux *et al*., 2004). In particular, the immunogenic PCV2 ORF2 gene cloned into the PCV1 backbone induced a specific antibody response to the PCV2 capsid protein when infected into piglets, but caused the pigs no ill effects (Fenaux *et al*., 2003). While the chimaeric virus did not cause viraemia in all the infected pigs, 100% of infected animals developed antibodies, suggesting that the construct could be used as a potential vaccine against PCV2 and PMWS disease development (Fenaux *et al*., 2003). The same chimaeric infectious clone was then demonstrated to give protective immunity to PCV2-challenged pigs, whether the infectious clone itself or the progeny virus was used (Fenaux *et al*., 2004). Vaccinated pigs had reduced or no PCV2 viral load and were found to have significantly less virus in the lymph tissues and less lymphoid depletion (LD) than unvaccinated challenged pigs (Fenaux *et al*., 2004). This is significant because LD during initial PCV2 infection may be linked to the eventual occurrence of leukopenia prior to the development of PMWS (Segales *et al*., 2001; Nielsen *et al*., 2003). Therefore, the chimaeric vaccine may have the ability to stop the eventual progression to clinical PMWS by preventing the initial LD of lymphoid tissues.

CHAPTER 7. GENERAL DISCUSSION

The initial objective of this project was to determine whether PCV1 and PCV2 were present in the Australian pig herd, and this was achieved using PCR. PCR was the assay selected for detection as these viruses are not cytopathic in cell culture, and it was considered a sufficiently practical and rapid method for a large scale survey. An additional objective was to characterise the strains of virus present in Australia, with the DNA extracted from tissues and used as the PCR template also used for analysis of the entire genome of any viruses detected. A previously published multiplex PCR was used for the initial detection of PCV1 and PCV2 as this would detect and differentiate both types of PCV in a single reaction. Alternative serological tests were considered as a means of determining the prevalence of these viruses but at the time of initiating this study no type-specific serological tests were available in Australia, and even now the development of PCV1-specific serological tests has not been reported.

The pigs and tissues selected for examination by PCR were biased toward the possible association of viruses with pathological lesions and pig mortalities, as they were predominantly from pigs found dead within piggeries, and from pigs originating from herds experiencing disease outbreaks such as unthriftiness and congenital tremors (CT). While some of the tissues were from herds with illthrift problems, no evidence for the presence of PMWS had been reported in Australia during the period in which the survey was conducted. It was considered that the survey and the genetic analysis of the PCV strains present in Australia during this period provided background data against which strains detected in association with any future outbreak of PMWS or other PCV2-associated disease could be evaluated.

Tissues from approximately 350 pigs were tested for PCV genetic material in several Australian pig herds. PCV1 was detected in pig tissue samples from WA, SA and NSW and PCV2 in pig tissue samples from WA, NSW and Queensland. It was not possible to obtain tissues for examination from all States but there was no reason to believe that the results obtained in the States from which tissues were obtained and PCV was detected would not be similar to those detected in the States from which it was not possible to obtain tissues.

When compared to similar studies performed overseas, the prevalence of PCV1 in Australia (20%) appeared high while the prevalence of PCV2 (18%) appeared low. The reasons for the lower than expected prevalence of PCV2 infection are not known but could be explained by the lack of PCV2-associated disease in Australia, as several studies showing very high rates of PCV2 infection have been reported in

regions affected by PMWS (Onuki *et al*., 1999; Mankertz *et al*., 2000b; Calsamiglia *et al*. 2002). The onset of PMWS has also been associated with high levels of virus replication in pigs (Ladekjaer-Mikkelsen *et al*., 2002; Okuda *et al*. 2003). The reason for the higher than expected prevalence of PCV1 infection in Australian pigs is unknown: it could be due to the fairly small number of farms sampled, or the lack of competition with PCV2.

The presence of PCV in association with production problems was also investigated. In one, a farm experiencing problems with unthrifty weaner pigs, PCV2 was detected by PCR in the tissues of all 12 unthrifty pigs examined, and several of these pigs had lesions previously observed in cases of PMWS (Sorden, 2000; Quintana *et al*., 2001; Harding *et al.*, 1998), such as pneumonia, lymph node enlargement and the presence of multinucleated giant cells in tissues (Chapter 3). However, on this farm there was only a small number of affected pigs, there was no particular increase in mortality rates over the low level experienced in many farms, the lesions in the unthrifty pigs were mild, and a diagnosis of PMWS was excluded. Furthermore, no PCV2 antigen was detected by IPMA in association with lesions or indeed in any tissues from the affected pigs, even in those tissues positive by PCR, indicating that only low levels of virus were present in these pigs.

While the role of PCV2 in PMWS is generally accepted, the role of PCV in other conditions is less certain. PCV2 has been detected in neural tissue of newborn pigs with CT (Stevenson *et al*., 1999) but the association between PCV and CT has not been confirmed by others (Kennedy *et al*., 2003). During the course of the studies reported in this thesis outbreaks of CT occurred in several Western Australian farms, and the association of PCV with these pigs was also investigated. Both PCV1 and PCV2 were detected in neural tissues of newborn affected pigs examined from some but not all of the affected farms (Chapter 3). The detection of virus in the newborn pigs indicated that both PCV1 and PCV2 are able to cause *in utero* infections in pigs, and that at least in some cases this results in infection of the brain and spinal cord of foetal pigs. Attempts to detect virus antigen in the brain and spinal cords of the affected newborn pigs by IPMA were however, unsuccessful, indicating that the level of virus replication in the neural tissues was low. It was concluded that while the results certainly do not confirm a causal role of PCV in CT the high prevalence of virus in affected pigs in some of the affected herds does suggest that the role of PCV in CT should be investigated further. Factors such as the antibody status of the sow and the stage of gestation may be significant in determining whether there is persistence of the virus in the pig *in utero*.

The data obtained on the prevalence of PCV1 and PCV2 in the 350 pigs examined do not provide sufficient information to draw a reliable conclusion regarding the prevalence of PCV in pigs in Australia, it is only indicative. The detection of antibody by type-specific serological tests for both PCV1 and PCV2 are required for prevalence determinations. A commercial PCV2-specific serological test is available (Synbiotics) but is extremely expensive and was therefore not used, and an alternative PCV2-specific serological test based on a recombinant PCV2 capsid antigen has been developed in this laboratory and results obtained using this test will be reported by others. A similar PCV1-specific serological test based on a recombinant PCV1 capsid antigen is also being developed.

Although there is evidence that PCV2 can cause or is associated with PMWS and a variety of other disease conditions in pigs (reviewed in Chapter 2), with the exception of the occasional occurrence of PDNS (Cameron, 1995) these conditions have not been reported in Australia. The question is whether all strains of PCV2 are potentially able to cause disease, whether there may not be genetic variants that are capable of or more likely to be associated with disease , and particularly whether the strains present in Australia are potentially capable of causing disease. It is also evident that PCV2 is possibly not the only factor involved, and it is unknown whether the strains of PCV2 present in Australia may perhaps be non-pathogenic, or whether they are potentially pathogenic but the accessory factors required for the development of PCV-associated disease may be absent in Australia.

The genetic relationship between Australian strains of PCV and strains associated with PMWS and other disease conditions was determined (Chapter 4) as a basis for identifying any significant changes which might suggest differences in pathogenicity. Three Australian strains of PCV1 from WA and NSW were similar to the very small number of PCV1 field isolates sequenced overseas. One of the WA strains was from a newborn pig with CT, and there was therefore no evidence that this strain was different from strains not associated with CT. Of the 7 PCV2 strains sequenced, six were very similar to each other, and were most closely related to a Japanese PCV2 isolate associated with an outbreak of PMWS. The seventh PCV2 strain was also similar but distinct, and was most closely related to a Chinese isolate and a Japanese isolate, associated with an unspecified clinical syndrome and PMWS respectively. There were, however, only minimal differences between the Australian isolates and strains from other countries and the absence of unique amino acid motifs in the genomes of Australian PCV2 do not suggest that Australian PCV strains differ significantly from those found overseas and in association with diseases such

as PMWS. There is no genetic evidence that Australian PCV2 strains would not be capable of causing diseases such as PMWS given suitable conditions, which are as yet unknown.

One of the 7 Australian PCV2 isolates sequenced appeared genetically distinct from the other strains. This could be the result of multiple introductions of PCV2 into the country: multiple introductions of pigs into Australia in colonial and recent times have been either documented or suggested using molecular evidence, and these separate introductions may be responsible for the diversity of PCV2 found in the Australian pig herd. However, there was a very high conservation at the nucleotide and amino acid level with all the Australian strains and PCV2 strains from all locations throughout the world exhibit only minimal differences. This conservation may be due to a low error rate in transcription associated with the small size of the PCV genome, perhaps the reliance of PCV on the low error host cell machinery for replication and possibly associated with the long term co-evolution of the virus with the porcine host. Infection of modern pigs with PCV may predate the development of pig populations in separate countries, resulting in several slightly different strains of PCV2 but no marked difference between strains in different countries.

In earlier studies that have been conducted in this laboratory, an Australian strain of PCV2 was adapted to replicate in PK15 cells. Persistence and presumably replication of the virus over several passages was confirmed by PCR and a semi-quantitative PCR suggested the virus was present at a low titre. Unfortunately, attempts to infect weanling pigs with this cell culture adapted virus were unsuccessful in a majority of the pigs that were inoculated and it appeared that the titre obtained in cell culture was insufficient to provide an adequate infectious dose. A major contribution of the studies reported in this thesis that could overcome the problem of poor replication of virus in cell culture, was the construction of an infectious clone using an Australian PCV2 strain (Chapter 6). It was anticipated that the clone could be used in a variety of applications for examining replication of PCV2 and also for generating higher titre virus stocks for animal experiments. The use of infectious clones in animal experiments is common (Fenaux *et al*., 2002; Oppreissnig *et al*., 2004; Roca *et al*., 2004), due to their advantages over other methods of inoculation such as cell cultured virus. Infectious clones are biologically pure and particularly useful when the virus required cannot be cultured *in vitro*. In the case of PCV2, the inconsistency of results produced when infecting pigs with cell culture isolates (Allan *et al*., 1994a) has meant that PCV2 infectious clones are highly desirable for use in animal experiments (Fenaux *et al*., 2001).

Another major contribution arising from the results reported in this thesis which will assist in monitoring the Australian pig herd for PCV-associated diseases was the development of a PCV2-specific IPMA to enable PCV2 antigen to be seen in infected cells from tissue blocks and virus-infected cell cultures. This test was based on the use of a PCV2-specific mAb against the capsid protein of the virus that was produced in this laboratory. While similar assays have been developed elsewhere, these reagents have not been readily available commercially. At the commencement of this project, the only tools available for the detection of PCV were an IFA which detected both PCV1 and PCV2 without discriminating between the types of PCV, and PCR. The IFA was time-consuming and difficult to interpret, as well as cross-reacting with both PCV1 and PCV2, and while PCR does allow differentiation of the two viral types it did not allow the visualisation of PCV antigen within infected cells.

Another significant contribution of the results reported in this thesis was the determination of the thermal stability of PCV2, on which there exists very little data. This has particular significance in Australia where imported pig meat has to be cooked to an internal temperature of 70°C for 11 minutes (Australian IRA for pig meat, 2004) before release and sale and the industry has lobbied extensively to prevent the importation of pig meat on the basis that this treatment would perhaps be insufficient to inactivate PCV2 present in the meat, perhaps resulting in the introduction of PMWS (Australian IRA for pig meat, 2004). To test this, an Australian strain of PCV2 strain was adapted to replicate in the PCV-free PK-15 cell line, and using a technique known as superinfection, the viral titre in the cell cultures was increased to approximately $1x10^4$ virus particles per mL. The results of the IPMA showed that while PCV2 genetic material could be readily detected by PCR and RT-PCR from the PK-15 cells, the number of infected cells was extremely low, but 1% of cells infected increased to a maximum of 10% after superinfection.

The adaption of the Australian PCV2 strain to replicate in cell cultures to a reasonable titre, and its detection and quantitation by a combination of titration and IPMA (Chapter 5) enabled the thermal stability of PCV2 to be investigated. The stability of PCV2 after exposure to heat had not been investigated previously, and as a small, environmentally stable virus, data on virus inactivation was considered important in the context of trade and also decontamination of infected premises. Not unexpectedly, the Australian PCV2 strain resisted heat treatment at 56°C, 65°C and 70°C for 15 minutes with no loss of infectivity, and heat at 70°C for 15 min actually resulted in an apparent increase in titre of the virus from infected material possibly because it assisted release of the virus from the nucleus of infected cells. Heat

treatment at 75°C and 85°C decreased the titre of virus but did not totally inactivate the virus, which was only destroyed after heat treatment at 95°C for 15 mins (Chapter 5). These results could be important for trade, specifically the importation of pig meat in Australia from countries affected by PMWS, as the current heating conditions required for meat importation will not inactivate the virus present in pig meat.

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