Porcine circovirus infection

MA O'Dea

Animal Health Laboratories
Department of Agriculture and Food WA
3 Baron-Hay Court, South Perth, WA 6151
mark.odea@agric.wa.gov.au

Part 1. Diagnostic Overview

Summary

There are two porcine circoviruses. Both are members of the family Circoviridae, which are small (17 nm in diameter), non-enveloped DNA viruses with a 1767-1768 nucleotide circular viral genome. Porcine circovirus type 1 (PCV1) has not been associated with disease in pigs and was first reported as a persistent contaminant in laboratory PK15 cell lines. Porcine circovirus type 2 (PCV2) is considered to be a key agent in the development of a number of diseases, which has led to the adoption of the term PCV2-associated disease (PCVAD), combining all syndromes under a single heading. Specific diseases included under the term 'PCVAD' include; enteric form, respiratory form, systemic form, postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS). Due to a herd seroprevalence of almost 100%, serological assays are generally not used in the diagnosis of PCVAD. The demonstration of PCV2 antigen by immunostaining, in-situ hybridisation or PCR methods, in association with characteristic histological lesions, is specific for the diagnosis of PCVAD. PCV2 is ubiquitous in the global pig herd, including Australia and New Zealand, and some forms of PCVAD have been diagnosed in Australia

Aetiology

PCV2 is present in most pig producing countries and by definition is the key infectious agent involved in PCVAD.

Australian strains of PCV2 show very high nucleotide sequence homology to overseas strains (in the order of 94-99%) and in general show no consistent differences in amino acid sequences.[6-8] It has been demonstrated that PCV2 can be split into two major strains on the basis of genetic sequence. These are designated PCV2 group 1 (new-type) and PCV2 group 2 (old type) based on the North American nomenclature[9], or PCV2 group a (old-type) or group b (new type) based on the European nomenclature. There is no clear indication that group 1 or group 2 strains differ in pathogenicity. Both strains are present in Australia[10] and in New Zealand (Ross H. and Tham KM., manuscript in preparation).

The PK15 cell line-associated PCV1 shows only 80% nucleotide homology to PCV2[11]. It has been widely accepted that PCV1 is non-pathogenic.

Clinical Signs

Clinical signs of PCV2 infection vary greatly depending on the disease manifestation encountered.[12]

In outbreaks of systemic PCVAD,[9] the predominant clinical signs are weight loss/emaciation, tachypnoea, dyspnoea, icterus and pallor in pigs of weaning and post-weaning age. Lymphadenomegaly may also be present. Due to the immunosuppressive nature of PCV2 infection, less severe cases of PCVAD may have clinical signs related to co-infection with secondary agents (bacterial or viral) rather than those directly associated with PCV2 infection.

The clinical signs and gross pathology of PDNS can be similar to classical swine fever and African swine fever,[13] both of which are exotic to Australia and New Zealand, and these present important differential diagnoses. PDNS can present as a particularly striking cutaneous purpura, seen as petechiae and ecchymoses over the hindlimbs and perineum in particular. These lesions may coalesce to form large plaques which may darken and become necrotic. The flanks, ventral abdomen, forelimbs, head and ears may also be affected. Skin lesions in mildly affected pigs may resolve within 2-3 weeks. However, severely affected pigs can become acutely ill with pyrexia, depression, ventral oedema and, ultimately, death.[14]

Reproductive disorders associated with PCV2 are somewhat non-specific; clinical presentations include mummifications, stillbirths,[15] late term abortions and pre-weaning mortalities.[16]

Epidemiology

PCV2 is ubiquitous in the global domestic pig population. Despite this, not all herds develop severe PCVAD, and in many cases not all pigs within an affected herd develop PCVAD, indicating that PCV2 is not the only factor required for disease expression. There have been numerous studies on coinfections with PCV2 and other agents including porcine parvovirus (PPV),[17] porcine reproductive and respiratory syndrome virus (PRRSV)[18] and mycoplasma.[19] Although some researchers favour the theory of a single as yet undiscovered agent (Agent X) as being the cause of disease, it is likely that a number of factors including management and secondary disease agents act in concert with PCV2 to produce disease. Due to the presence of serum antibodies in most pig herds, serological assays are of limited use in diagnosing PCVAD, but do provide an historical perspective on virus circulation within the herd. They may also be used to determine the immune status of dams prior to mating as maternal antibodies may play a key role in development of PCVAD in young pigs. Studies have shown that decline of maternal antibodies correlates with detectable serum viraemia, and that sows with high levels of colostral PCV2 antibodies produce litters that are less susceptible to PCVAD.[20]

PMWS generally occurs in weaner pigs in the 5-12 week age range.[21] It has a morbidity of 4-25% and, at its most severe, can have a case fatality rate approaching 90%.[22] There are no clear indications of differences in breed susceptibility to development of disease. In contrast, PDNS tends to occur in older pigs in the 40-70 kg weight range, and usually has a low morbidity (<1%), although mortalities can rise to 80%.[13]

PCV2 can be transmitted both horizontally through faecal-oral[23] or respiratory routes,[24] and vertically[15]. Virus has also been demonstrated in the semen of infected boars,[25] and in urine. Horizontal transmission is generally via direct contact, although the virus can be transmitted via aerosols through sheds. Despite the virus persisting in cells of the macrophage/monocyte lineage, which is hypothesised to be a method for dissemination around the body,[26] there is no indication that a persistently infected

carrier state develops. Once a herd is infected, environmental contamination results in continual exposure of naïve animals to PCV2, with infection of weaners coinciding with waning of maternal antibodies. For this reason infected herds tend to remain seropositive once seroconversion has been demonstrated. Derivation of PCV2 negative pigs from positive herds has been demonstrated in an experimental setting,[27] although this is impractical for production herds.

Occurrence and Distribution

PCV2 is widely distributed throughout the pig populations of Australia and New Zealand. Serological surveys based on the 2001/2002 Australian National Pig Serum Bank have demonstrated seroprevalence levels ranging from 75.8% to 86.9%,[28] indicating the presence of PCV2 in most, if not all pig herds. PMWS was first diagnosed in New Zealand on pig farms on the North Island in 2003, and on pig farms on the South Island in 2006.[29] Despite PMWS not being diagnosed in Australia, less severe manifestations of PCVAD have been diagnosed in New Zealand and most states of Australia. These include the enteric and respiratory forms, and those with generalised lymphoid lesions. The occurrence of PDNS has also been reported in Australia. PMWS has not been diagnosed in Australia, with a case definition for its initial diagnosis agreed by Australian authorities in 2005 (Appendix 1).

Gross Pathology

The gross pathology of PCVAD can vary greatly depending on the severity of the disease, ranging from no obvious lesions through the spectrum of lesions seen in severe PCVAD. The lesions can also concentrate in one or more organ systems, depending on whether respiratory, enteric or systemic PCVAD is encountered.

Pigs with low-grade PCVAD may not have grossly visible lesions, and the disease may be limited to the lymph nodes. For this reason, if PCVAD is suspected as part of a disease investigation, lymphoid tissues should be collected for histopathological examination.

In PMWS and severe cases of systemic PCVAD there is obvious lymphadenomegaly, with the inguinal lymph nodes being easily visible when the pig is suspended by the hind limbs. Affected lymph nodes are usually homogenously pale on the cut surface. The lungs fail to collapse after the thorax is opened and are heavy and palpably firm. The lung surfaces show mild to severe mottling due to randomly scattered grey to tan coloured lobules. In severe cases, alveolar haemorrhage results in the appearance of dark red to brown lobules. Consolidated areas are often present in the cranial and middle lung lobes. The mediastinal and bronchial lymph nodes are enlarged and firm. There is usually moderate splenomegaly. Liver pathology occurs in approximately 50% of cases, and consists of varying degrees of mottling and mild to moderate, diffuse atrophy. The cut surface of the liver shows multifocal to diffuse pallor. Gross renal pathology is detected in 50% of cases. The renal capsule is easily removed and the surface of the kidneys has numerous white foci which, on the cut surface, are seen to involve the cortex and medulla. In some cases renomegaly occurs and the kidneys may be up to five times normal size and have a waxy or translucent appearance. The fundic mucosa of the stomach may be mottled with patchy opaque, white areas. The mucosal surfaces of the caecum and proximal spiral colon are sometimes mildly hyperaemic and petechiated. In a few cases, the wall of the caecum is severely thickened by oedema.[30]

In PDNS, postmortem examination reveals extensive dermal and subcutaneous haemorrhage and oedema. In all cases there is renomegaly and pallor of the kidneys with petechiation on the capsular surface. The lymph nodes are enlarged and may be haemorrhagic. There may be ventral subcutaneous oedema. Some pigs have mucosal ulceration of various regions of the stomach or ileocaecocolic valve.[14,31]

The gross lesions associated with reproductive disease are non-specific, and aside from the foetal abnormalities outlined above, there are no lesions of the dam or foetal tissues specific to PCV2 infection.[15,16]

Diagnostic Tests

Diagnostic tests for PCVAD can be grouped into 3 main categories; histopathology, identification of the agent and identification of an antibody response to the agent.

For a diagnosis of PMWS to be made in Australia, the disease must meet 3 criteria as outlined by the Consultative Committee on Emergency Animal Diseases (CCEAD) (Appendix 1).

The first criterion is clinically based on current or historical evidence of a herd syndrome characterised by elevated morbidity and mortality, associated with a non-responsive, otherwise unexplained wasting, in pigs between weaning and 12 weeks of age. Where the syndrome is newly recognised, a mortality and morbidity rate of twice the baseline for the herd is considered significant. Other signs may be present including, dyspnoea or tachypnoea, enlarged lymph nodes, and less frequently, diarrhoea, pallor or jaundice.

The second criterion is based on histopathology. To satisfy the histopathological criteria, lymphoid tissues (lymph nodes and spleen) obtained from affected pigs must have evidence of depletion of lymphoid cells associated with lymphohistiocytic to granulomatous inflammatory infiltration. Multinucleated giant cells may be observed and basophilic intracytoplasmic (botryoid) inclusion bodies which are highly characteristic for the syndrome may also be present. Similar inflammatory lesions may be found in a variety of organs including lung, liver, kidney and intestine and parenchymal cell necrosis may also be seen.

The third criterion is demonstration of abundant PCV2 antigen within the characteristic histological lesions described above.

The entire CCEAD definition can be seen in Appendix 1.

The diagnostic criteria are similar for New Zealand, although the diagnosis of PMWS does not require that viral antigen detection be performed (by in-situ hybridisation (ISH) or Immunohistochemistry (IHC)), but it may be used as an adjunct to clinical parameters and histopathology.[32]

The lesions seen on histological examination of PCVAD cases are characteristic and must be present for the diagnosis of PMWS. Histological lesions are quite specific for PCVAD, although the sensitivity of histopathology as a diagnostic test, particularly for the diagnosis of herd-level PCVAD, is dependent on the number of cases examined. The most characteristic lesions are found in the lymphoid tissues and consist of lymphoid depletion, infiltration of histiocytes, formation of syncytia (giant cells) and presence of botryoid cytoplasmic inclusions in histiocytic cells.

The usual lung lesion is a subacute diffuse interstitial pneumonia, with histiocytic and multinucleate giant cells present in thickened alveolar walls.

Demonstration of viral antigen by IHC and ISH allows visualisation of the viral antigen in association with lesions and is an integral component of the diagnosis of PCVAD. Antibodies used in IHC, must be directed against the capsid protein of PCV2, in order to avoid cross-reactivity with the non-pathogenic, but antigenically similar PCV1. Similarly, nucleic acid probes developed for use in ISH must be directed against regions of the PCV2 genome that are not homologous with PCV1, and again this is most commonly the capsid gene. A PCV2-specific monoclonal antibody is available in Australia. A commercial antiserum is also available, although this may not be as suitable for IHC.

Published methods for ISH involve hybridisation with specific PCV2 digoxigenin or biotinylated labelled DNA probes.[33-35] An anti-digoxigenin antibody conjugated to alkaline phosphatase is used to detect hybridisation. For biotinylated probes, streptavidin conjugated with alkaline phosphatase or peroxidase is used, followed by a suitable substrate.

Although it is generally accepted that ISH is a more sensitive technique than IHC, the relative simplicity of IHC makes it the preferred method for antigen detection in some laboratories. IHC has been shown to be sensitive enough to detect approximately 10^8 PCV2 genomes per 500 ng of DNA[36] and is highly specific when monoclonal antibodies are used. Given that cases of moderate to severe PCVAD display extensive amounts of antigen in affected tissues, the difference in sensitivity between the methods is unlikely to be significant.

PCV2 isolation is a highly sensitive method which may be undertaken to demonstrate presence of the agent. The specificity of the method is generally high, although this relies on the technique and reagents used for detection of the agent following passage. PCV2 can be isolated from most affected tissues, although lymphoid tissue generally contains the highest viral titre. The most commonly used cell line is a PK15 clone which is free of PCV1 contamination. PCV2 does not cause cytopathic effect in cell culture, and following blind passage the presence of PCV2 must be detected by ISH, IHC or PCR-based techniques.

PCR-based techniques are highly sensitive and have been widely used for the detection of PCV2 DNA in both tissues and blood. However, the use of these techniques is considered by some to be too sensitive for diagnostic applications, as many clinically normal animals will test positive for PCV2 DNA. It is therefore important that the detection of PCV2 DNA from tissue is interpreted in combination with histological examination of that tissue. The development of multiplex PCR primer sets has enabled the detection and differentiation of PCV1 and PCV2 in tissue samples,[37] and multiple protocols have been published for the detection of PCV2 DNA in samples including tissue, blood, faeces and semen. A protocol for the detection of PCV2 mRNA has also been published,[38] but it holds little value as a diagnostic tool and is more suited to research.

Quantitative PCR (qPCR) or real-time PCR allows for visualisation of results in real-time, without the need to run post-amplification products in agarose gels. This method is also more sensitive than standard gel-based PCRs, and allows the determination of a copy number of virus per unit of serum or tissue. It has been suggested that a value of 10^7 PCV2 genomes/mL of serum corresponds to a pig developing PCVAD.[36] The determination of copy numbers requires the inclusion of a standard curve in each assay, and this is not routinely performed in Australian laboratories. However, the qPCR assay is a screening tool which may be used in

preference to gel-based PCR. Recently, a multiplex real-time PCR was published for diagnosis of and differentiation between group 1 and 2 PCV2 viruses in tissue samples.[39]

Sequencing of the PCV2 genome from isolates may be undertaken in diagnostic laboratories, but this is not a routine requirement for PCVAD diagnosis. Most laboratories have an 'in-house' system for sequencing, and primer sets for amplifying the entire genome for sequencing purposes have been published elsewhere.[6]

Although a number of serological assays have been developed to measure antibodies to PCV1 and PCV2, such as ELISAs[40,41] and the immunoperoxidase monolayer assay (IPMA),[42] they are mainly used for research purposes. This is because the viruses are ubiquitous with an almost 100% herd seroprevalence and seroconversion does not differ to a significant extent between PCVAD-affected and non-affected farms. Also, there are often cross-reactions between PCV1 and PCV2, particularly if antigens are composed of whole virus rather than the capsid subunit. Negative results to serological assays may be of benefit in excluding PCV2 involvement from syndromes such as foetal infections.

Guidance on Safety and Containment Requirements

PCV2 is not a zoonotic agent and poses no risk to human health. Although the virus is endemic in Australia and New Zealand, samples containing, or suspected of containing, the virus should be handled in an appropriate PC2 laboratory.

Part 2. Test Methods

Histopathology

Test procedure

For investigations of suspected PCVAD, a range of tissues should be sampled. These include lymphoid tissues (mesenteric, inguinal and bronchial lymph nodes, tonsil and spleen), liver, lung, heart, kidney, small and large intestine and brain. A simplified sampling protocol has been suggested whereby either two lymphoid tissues are sampled (from lymph node, tonsil and spleen), or a lymphoid tissue and another organ system (lung, liver, kidneys or intestine).[43] A system for scoring PCVAD lesions from HE sections, modified from previously published methods[43,44] is outlined in Table 1.

Interpretation of Results

Table 1: Criteria for scoring histopathological lesions of PCVAD

Lesion	Negative	Mild	Moderate	Severe
Score*	0	1	2	3
Lymphocyte depletion in germinal centres of lymphoid follicles	Absent	Focal	Multifocal	Diffuse
Granulomatous inflammation including formation of syncytial giant cells and granulomas	Absent	Focal	Multifocal	Diffuse
Intracytoplasmic botryoid inclusion bodies in	Absent	Focal	Multifocal	Abundant

histiocytes and macrophages				
Immunohistochemical PCV2 antigen staining in histiocytes and fibrocyte matrix	No staining demonstrated	Focal	Multifocal	Diffuse

*Score definition

0. No lesions or antigen demonstrated
1. 1 – 3 foci per section of body organ examined, or less than 10% of lymphoid follicles with lesions, inclusion bodies or antigen.
2. Multifocal to confluent areas of lymphoid depletion, granuloma or giant cell syncytia formation or granulomatous inflammation, multifocal inclusion bodies present (4 – 20 per section) and multifocal to confluent areas of antigen deposition in a body organ or involving 10 – 50% of lymphoid follicles in lymphoid tissue.
3. Multifocal confluent to diffuse lymphoid depletion, granulomatous inflammation or antigen deposition in body organs or involving more than 50% of lymphoid follicles. Abundant inclusions bodies present (more than 20 per section).

The scores for each tissue and each criterion are summed and the total divided by the number of tissues examined. In the context of a simplified sampling protocol, the maximum score per animal is 12 based on assessment, for example, of mesenteric lymph node and tonsil. Depending on the final calculated score, a pig is categorised as having no PCVAD lesions (score = 0), mild PCVAD lesions (score = 1-3), moderate PCVAD lesions (score = 4-6) or severe PCVAD lesions (scores greater than 7).

In cases of PDNS, for which the presence of specific clinical signs coupled with histopathology is diagnostic, the skin lesions display acute to sub-acute haemorrhagic epidermitis and necrotic dermatitis associated with severe necrotising vasculitis. The renal lesions present as subacute glomerulonephritis with plasma protein leakage into Bowman's capsules, often accompanied by severe fibrinoid necrosis of arcuate arteries. Frequently, there is necrotic lymphadenitis, particularly in the superficial inguinal, mesenteric and mediastinal lymph nodes.[13,31] Diagnosis of PDNS is not reliant on demonstration of PCV2 antigen in the tissues, and in most cases antigen is not demonstrated in direct association with lesions in the skin or kidneys. However, PCV2 antigen is usually present in lymphoid tissues.

The presentation of PCVAD may correlate more specifically with an affected organ system, leading to a diagnosis of PCV2-associated respiratory disease, enteritis or reproductive disease. The common findings in these presentations are outlined in Table 2.

Table 2: Key microscopic lesions associated with other forms of PCVAD

PCVAD category	Key lesions	PCV antigen staining by IHC	Systemic lesions present also
Enteric	Lymphoid depletion in Peyers patch of the ileum with villous atrophy and bridging	Multifocal, confluent to diffuse, within lamina propria	No

	of the mucosal epithelium. Many PCV2 inclusions may be present within submucosal tissue.	and submucosal tissue	
Respiratory	Granulomatous interstitial pneumonia with lymphoid depletion of BALT	Multifocal, confluent to diffuse, within submucosa of bronchial epithelium and within alveolar septa.	Yes
Reproductive	Non-suppurative, necrotizing and fibrosing myocarditis	Multifocal, confluent to diffuse, within foetal myocardium	No

Immunohistochemistry

The IHC technique is used for the detection of PCV2 antigen in either tissue sections or cell culture material. It is an integral component of making a diagnosis of PCVAD, as it allows demonstration of viral antigen in concert with lesions. The following method is suitable for formalin-fixed, paraffin-embedded (FFPE) tissues, although it can be adapted for use in fixed cell culture material by starting the process at the peroxidase treatment step.

Reagents and Materials

- Suitable monoclonal or polyclonal antibody directed specifically against PCV2. A monoclonal antibody is available from the Animal Health Laboratories, Department of Agriculture & Food, Western Australia.
- Suitable secondary antibody and detection system. In this case the DakoCytomation EnVision™ kit.
- Xylene solutions
- Graded ethanol solutions
- 3% hydrogen peroxide
- Tris-buffered saline (TBS)
- Rabbit serum
- Antibody dilution solution (DakoCytomation)
- AEC (3-amino-9-ethylcarbazole)
- Haematoxylin

Test Procedure

- Take FFPE tissue sections on glass slides, and de-paraffinise in xylene.
- Rehydrate tissue sections in graded ethanol solutions, before rinsing in tap water.
- Treat sections for the presence of endogenous peroxidases by application of a 3% hydrogen peroxide solution. Incubate for 10 minutes at room temperature.
- Gently wash slides 3 times in TBS.

- Add blocking solution (25% rabbit serum in antibody diluting solution) to the slide and incubate for 10 minutes at room temperature.
- Remove blocking solution and add anti-PCV2 antibody (at appropriate dilution in blocking solution) to slide. Place slides in a humidified chamber and incubate overnight at 4°C.
- Gently wash slides 3 times in TBS.
- Add secondary antibody (HRP conjugated anti-mouse) and incubate for 30 minutes at room temperature.
- Gently wash slide 3 times in TBS.
- Add AEC substrate to slide and allow to develop for 5-30 minutes at room temperature.
- Rinse slides 3 times in distilled water, counterstain in haematoxylin and mount.

Quality Control Aspects

All batches of slides for IHC must have a negative and positive control included. Control slides are usually developed in-house from material that has previously been shown to be positive for the presence of PCV2 antigen. Ideally, 4 controls should be used in each test, with a negative tissue, a 1+ tissue, 2+ tissue and 3+ tissue section. This may not be feasible for some laboratories and the inclusion of a single positive control is sufficient to ensure the assay is operating correctly. In order to run a negative control slide, the positive control material is used, however the primary antibody is replaced by blocking solution in the corresponding step. A test run can only be reported as valid if there is strong brown/red staining on the positive control slide, and no staining on the negative control slide.

Interpretation of Results

A system for scoring IHC-based staining is outlined in Table 3, although it should be noted that the scoring of IHC and ISH staining is subjective, and scores may differ between examiners.

Table 3. Scoring system for the presence of PCV2 IHC staining

Score	0	1+ (mild)	2+ (moderate)	3+ (marked)
Staining	None present	Less than 10% of tissue section	Between 10 and 50% of tissue section	Greater than 50% of tissue section

Virus Isolation

Virus isolation may be used as an adjunct to sequencing, or to verify that viable virus is present in tissues. The method described below is suitable for any post-mortem tissue including serum and whole blood, although lymphoid organs generally yield the highest titre of virus. Propagation of PCV2 may require an ongoing infection technique to ensure the virus doesn't die out during ongoing blind passage. This essentially involves re-infecting a culture with itself every time it is passaged, and this is outlined below. The use of glucosamine is also essential for maximising PCV2 titre in cell culture. Volumes outlined below may be scaled for use in different culture vessels accordingly.

Reagents and Materials

- Appropriate porcine cell lines free of PCV1 and PCV2. A PCV-free PK15 clone is generally used.
- Cell culture media (DMEM supplemented with 5% foetal calf serum and antibiotics).
- Phosphate buffered saline (PBS)
- Trypsin
- 300 mM D-glucosamine (prepared in Hank's balanced salt solution)
- 25cm^2 tissue culture flasks (T25 flasks)

Test Procedure

- Prepare PK15 cells in T25 flasks as per routine cell culture procedures. Grow cell cultures until approximately 50% confluent.
- Prepare a 10% tissue homogenate using cell culture media and clarify by centrifugation.
- Remove media from PK15 cells and wash monolayer in PBS. Inoculate PK15 cells by addition of 1 mL of clarified tissue homogenate. Incubate for at least 1 hour in a 37°C incubator.
- Remove inoculum, wash monolayer once with PBS, replace cell culture media and incubate cells at 37°C.
- At 24 hours post inoculation, cells may be treated with glucosamine. Remove media from infected cells and wash twice in PBS. Add 1ml of 300mM D-glucosamine per T25 flask and incubate at 37°C for 30 minutes. Wash cells twice in PBS, replace culture media and return to the 37°C incubator for 5-7 days.
- Blind passage of cultures is performed every 5-7 days. T25 flasks are trypsinised and split 1:2. Half of the material is re-seeded into the flask, and half is frozen. 24 hours post-trypsinisation, the frozen material is thawed and used to inoculate the re-seeded flask. The inoculation and glucosamine treatment is then the same as that outlined above. This procedure should be repeated at least 3 times before a sample is declared negative.
- Following at least 3 blind passages the detection of PCV2 antigen in inoculated cell cultures may be undertaken by either ISH/IHC or PCR.

Quality Control Aspects

It is important to ensure cross contamination doesn't occur during the preparation and passage of cell cultures. Good cell culture technique should be followed, with clean cultures kept in a separate area to inoculated cultures. Include a negative and positive control flask of cells with each isolation, treated in exactly the same manner as test flasks.

Interpretation of Results

Isolation of PCV2 from tissues does not necessarily imply a direct aetiological relationship with the disease under investigation, and should be interpreted in association with histological lesions. Conversely, failure to isolate the virus from an affected animal does not preclude a viral aetiology for the particular condition.

PCR Based Diagnostics

All PCR-based tests must undergo appropriate evaluation and laboratories should follow the guidelines developed by SCAHLS for the use of PCR technology. In particular the design and layout of the PCR testing facility must be such as to minimise contamination.

There are numerous published methods for both gel based and real-time PCR methods for the detection of PCV2 genomic material. While the primer sets and methods outlined below may be adopted and evaluated by Australian and New Zealand laboratories, there are a large number of alternatives available.

The methods outlined in this section utilise primers and probes directed against the capsid region of the PCV2 genome to avoid cross-reactivity with PCV1. The gel based PCR was initially developed as a tool for screening tissue samples, but has been superseded by the real-time PCR due to the latter's higher sensitivity, lower chance of cross-contamination and potential for copy number determination. It should be noted that determination of genomic copy numbers requires the inclusion of a standard curve, which is not covered in this document.

Reagents and Materials

- DNA extraction kit suitable for blood and/or tissues. Most laboratories have a preferred brand of kit which is commonly used.
- 2X PCR Mastermix (Promega), for gel based PCR
- Taqman Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems), for real-time PCR.
- Primers for gel based PCR (from preferred supplier)

Primer	Sequence	Position in genome
ScreenF	TACATACATGGTTACACGGATATT	1029-1052
ScreenR	CTATACCCTTTGAATACTACAGAA	1417-1394

- Primers and probe for real-time PCR (from preferred supplier)

Primer/probe	Sequence	Position in genome
PCV2quant61-76F	CAGATCCTCCGCCGCC	1625-1610
PCV2quant189168R	T(G/C/T)TGACTGTGGTA(C/G)(G/C)CTTGACA	1497-1518
PCV2probe147-159	*FAM*-CTCCCGCACCTTC-*MGBNFQ*	1527-1541

Test Procedure

For Gel based PCR;

- Prepare DNA extracts from tissues or blood according to kit manufacturer's instructions. PCR mastermixes are prepared as follows:

	1x (µL)	number of reactions____x
2x PCR buffer	10	

ScreenF (20µM)	0.25	
ScreenR (20µM)	0.25	
PCR grade water	7.5	
Template	2	-
Total	20	

- Cycling conditions for the gel based PCR are as follows:

Step	Temperature (°C)	Time (min:sec)	Number of cycles
1	95	3:00	1
2	95	00:40	35
	51	00:30	
	72	1:00	
3	72	10:00	1
4	14	Hold	1

- PCR reaction products are electrophoresed in a 2% agarose gel containing 10 µg/mL ethidium bromide at 80 V for 1 h, and viewed with an ultraviolet transilluminator. Samples are positive for the presence of PCV2 DNA when a band is visualised at the expected size of 391 base pairs.

For real-time PCR;
(note this is based on an Applied Biosystems 7500 platform and primer/probe concentrations and cycling conditions may need to be adapted if alternative platforms are used)

- Real-time PCR mastermixes are prepared as follows:

	1x (µL)	number of reactions____x
Taqman PCR mix (2x)	10	
PCV2quant61-76F (10µM)	1.8	
PCV2quant189168R (10µM)	1.8	
Probe (5µM)	1	
PCR grade water	3.4	
Template	2	-
Total	20	

- Real-time PCR cycling conditions are as follows:

Step	Temperature (°C)	Time (min:sec)	Number of cycles
1	95	10:00	1
2	95	00:15	45
	60	1:00 (acquire fluorescence)	

- The baseline and threshold settings should be determined as appropriate for the platform used. For the ABI 7500 system the Delta Rn threshold is set to 0.1 and the baseline is set to auto. Alternatively, the automatic baseline and threshold settings may be chosen. A positive sample has a

characteristic amplification curve crossing the threshold at or before 35 cycles (Ct = 35). Samples that cross the threshold above 35 cycles and display a characteristic curve are classified as suspect. Samples that do not cross the threshold prior to 45 cycles are classified as negative for PCV2 DNA.

Quality control aspects

Quality control aspects for PCR diagnostics are highly important to prevent false positive results due to contamination. For gel-based PCR, a positive, negative and blank control should be included. For a test to be valid, the positive control must display a band at the expected size of 391 base pairs, while the negative control (preferably DNA from uninfected porcine tissue) and blank (consisting of PCR water in place of template) do not display any amplified bands.

For real-time PCR the controls should also include a positive, negative and blank control. The positive control should be a DNA sample that has been titrated to give a Ct value in the upper region of what is considered positive. It is suggested that the positive control is titrated to give a Ct of 25 to 30 cycles. This ensures that a drop in sensitivity of the reaction will be reflected in the positive control, a factor which may be missed if a strong positive control is used. Alternatively, laboratories may choose to use both a strong and weak positive control. For a test to be valid, the positive control must give a characteristic curve with a Ct </= 35, and no amplification curves should be seen in the negative and blank controls.

An 18S ribosomal RNA (18S rRNA) internal control (based on eukaryotic 18S rRNA) may be incorporated in the assay to validate the DNA extraction procedure, determine the integrity of the DNA sample and demonstrate the absence of significant levels of PCR inhibitors. The multiplex assay will then include a FAM labelled PCV2 probe, and a separately labelled (usually VIC dye) 18S rRNA probe. If such a control is included, all extracted samples should have a characteristic curve in the VIC dye layer regardless of whether or not they are positive for PCV2. PCV2 positive samples should have characteristic curves in both the FAM and VIC dye layers, and negative samples should only have a curve in the VIC dye layer. The blank sample should not have any 18S rRNA amplification, although due to the ubiquitous nature of the template, a curve may be seen in the late cycles.

Interpretation of results

Interpretation of gel-based (qualitative) PCR is simply as a positive or negative result. As stated previously these results do not imply direct relationship with the disease process, and should be interpreted in concert with histological lesions.

Real-time PCR as described in this document is also a positive/negative assay, and should be interpreted as for gel-based PCR. Methods for obtaining a quantitative result, such as running standard curves (absolute quantitation) or comparing viral load in healthy and diseased tissues (relative quantitation) may be adopted by laboratories, but currently these methods are used as research tools and are outside the scope of this document.

References

1. Tischer I, Rasch R, Tochtermann G. Characterization of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. *Zbl Bakt Hyg, I Abt Orig A* 1974;226:153-167.

2. Kim J, Chae C. Necrotising lymphadenitis associated with porcine circovirus type 2 in pigs. *Vet Rec* 2005;156:177-178.

3. Ladekjaer-Mikkelsen AS, Nielsen J, Storgaard T, et al. Transplacental infection with PCV-2 associated with reproductive failure in a gilt. *Vet Rec* 2001;148:759-760.

4. Kim J, Ha Y, Jung K, Choi C, Chae C. Enteritis associated with porcine circovirus 2 in pigs. *Can J Vet Res* 2004;68:218-221.

5. Rosell C, Segales J, Ramos-Vara JA, et al. Identification of porcine circovirus in tissues of pigs with porcine dermatitis and nephropathy syndrome. *Vet Rec* 2000;146:40-43.

6. Muhling J, Raye WS, Buddle JR, Wilcox GE. Genetic characterisation of Australian strains of porcine circovirus types 1 and 2. *Aust Vet J* 2006;84:421-425; discussion 416.

7. Muhling J. Australian porcine circoviruses. PhD Thesis, Murdoch University, Perth, 2006:149.

8. Raye WS. An investigation into the status of porcine circovirus in Australia. PhD Thesis, Murdoch University, Perth, 2004:163.

9. Cheung AK, Lager KM, Kohutyuk OI, et al. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. *Arch Virol* 2007;152:1035-1044

10. O'Dea MA. Pathogenesis and detection of porcine circovirus type 2 in the Australian pig herd. PhD Thesis, Murdoch University, Perth, 2008:185.

11. Meehan BM, McNeilly F, Todd D, et al. Characterization of novel circovirus DNAs associated with wasting syndromes in pigs. *J Gen Virol* 1998;79:2171-2179.

12. Harding JC. Post-Weaning Multisystemic Wasing Syndrome (PMWS): Preliminary Epidemiology and Clinical Presentation. *Proc Am Assoc Swine Pract 1997;28:503*

13. Done SH, Gresham A, Jackson G, et al. Porcine Dermatitis and Nephropathy Syndrome (PDNS) and Post-weaning Multisystemic Wasting disease (PMWS); Pathology, Virology and Circoviruses. *The Pig Journal* 2000;46:76-94.

14. Done S, Gresham A, Potter R, Chennells D. PMWS and PDNS - two recently recognised diseases of pigs in the UK. *In Practice* 2001:14-21.

15. West KH, Bystrom JM, Wojnarowicz C, et al. Myocarditis and abortion associated with intrauterine infection of sows with porcine circovirus 2. *J Vet Diagn Invest* 1999;11:530-532.

16. O'Connor B, Gauvreau H, West K, et al. Multiple porcine circovirus 2-associated abortions and reproductive failure in a multisite swine production unit. *Can Vet J* 2001;42:551-553.

17. Krakowka S, Ellis JA, Meehan B, Kennedy S, McNeilly A, G. Viral wasting syndrome of swine: Experimental reproduction of Postweaning Multisystemic Wasting Syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and porcine parvovirus. *Vet Pathol* 2000;37:254-263.

18. Harms PA, Sorden SD, Halbur PG, et al. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. *Vet Pathol* 2001;38:528-539.

19. Opriessnig T, Thacker EL, Yu S, et al. Experimental reproduction of Postweaning Multisystemic Wasting Syndrome in Pigs by dual infection with Mycoplasma hyopneumoniae and porcine circovirus type 2. *Vet Pathol* 2004;41:624-640.

20. McKeown NE, Opriessnig T, Thomas P, et al. Effects of porcine circovirus type 2 (PCV2) maternal antibodies on experimental infection of piglets with PCV2. *Clin Diagn Lab Immunol* 2005;12:1347-1351.

21. Chae C. Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis and pathology. *Vet J* 2004;168:41-49.

22. Plana-Duran J, Balasch M, Segales J, et al. Post-weaning multisystemic wasting syndrome in Spain. *Vet Rec* 1999;145:87-88.

23. Sato K, Shibahara T, Ishikawa Y, et al. Evidence of porcine circovirus infection in pigs with wasting disease syndrome from 1985 to 1999 in Hokkaido, Japan. *J Vet Med Sci* 2000;62:627-633.

24. Magar R, Larochelle R, Thibault S, Lamontagne L. Experimental transmission of porcine circovirus type 2 (PCV2) in weaned pigs: a sequential study. *J Comp Pathol* 2000;123:258-269.

25. Hamel AL, Lin L, Sachvie C, Grudeski E, Nayar GPS. PCR detection and characterisation of type-2 porcine circovirus. *Can J Vet Res* 2000;64:44-52.

26. Gilpin DF, McCullough K, Meehan BM, et al. In vitro studies on the infection and replication of porcine circovirus type 2 in cells of the porcine immune system. *Vet Immunol Immunopathol* 2003;94:149-161.

27. Opriessnig T, Yu S, Thacker EL, Halbur PG. Derivation of porcine circovirus type 2 - negative pigs from positive breeding herds. *J Swine Health Prod* 2004;12:186-191.

28. Finlaison D, Kirkland P, Luong R, Ross A. Survey of porcine circovirus 2 and postweaning multisystemic wasting syndrome in New South Wales piggeries. *Aust Vet J* 2007;85:304-310.

29. Neumann EJ, Dobbinson SS, Welch EB, Morris RS. Descriptive summary of an outbreak of porcine post-weaning multisystemic wasting syndrome (PMWS) in New Zealand. *N Z Vet J* 2007;55:346-352.

30. Clark EG. Post-weaning multisystemic wasting syndrome. *Proc Am Assoc Swine Pract* 1997;28:499-501.

31. Smith WJ, Thomson JR, Done S. Dermatitis/nephropathy syndrome of pigs. *Vet Rec* 1993;132:47.

32. Stone M. Response to first diagnosis of post-weaning multisystemic wasting syndrome. *Surveillance* 2004;31:8-14.

33. Nawagitgul P, Morozov I, Sirinarumitr T, Sorden SD, Paul PS. Development of probes to differentiate porcine circovirus types 1 and 2 in vitro by in situ hybridization. *Vet Microbiol* 2000;75:83-89.

34. Sirinarumitr T, Morozov I, Nawagitgul P, et al. Utilization of a rate enhancement hybridization buffer system for rapid in situ hybridization for the detection of porcine circovirus in cell culture and in tissues of pigs with postweaning multisystemic wasting syndrome. *J Vet Diagn Invest* 2000;12:562-565.

35. Kim J, Chae C. Optimal enhancement of in situ hybridization for the detection of porcine circovirus 2 in formalin-fixed, paraffin-wax-embedded tissues using a combined pretreatment of thermocycler and proteinase K. *Res Vet Sci* 2003;74:235-240.

36. Brunborg IM, Moldal T, Jonassen CM. Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning multisystemic wasting syndrome using a TaqMan-based real-time PCR. *J Virol Methods* 2004;122:171-178.

37. Larochelle R, Antaya M, Morin M, Magar R. Typing of porcine circovirus in clinical specimens by multiplex PCR. *J Virol Methods* 1999;80:69-75.

38. Yu S, Carpenter S, Opriessnig T, Halbur PG, Thacker E. Development of a reverse transcription-PCR assay to detect porcine circovirus type 2 transcription as a measure of replication. *J Virol Methods* 2005;123:109-112.

39. Gagnon CA, del Castillo JR, Music N, et al. Development and use of a multiplex real-time quantitative polymerase chain reaction assay for detection and differentiation of Porcine circovirus-2 genotypes 2a and 2b in an epidemiological survey. *J Vet Diagn Invest* 2008;20:545-558.

40. Walker IW, Konoby CA, Jewhurst VA, et al. Development and application of a competitive enzyme-linked immunosorbent assay for the detection of serum antibodies to porcine circovirus type 2. *J Vet Diagn Invest* 2000;12:400-405.

41. Nawagitgul P, Harms PA, Morozov I, et al. Modified indirect porcine circovirus (PCV) type 2-based and recombinant capsid protein (ORF2)-based enzyme-linked immunosorbent assays for detection of antibodies to PCV. *Clin Diagn Lab Immunol* 2002;9:33-40.

42. Segales J, Domingo M. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. *Vet Q* 2002;24:109-124.

43. Opriessnig T, Meng XJ, Halbur PG. Porcine Circovirus Type 2 associated disease: Update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. *J Vet Diagn Invest* 2007;19:591-615.

44. Krakowka S, Ellis J, McNeilly F, Waldner C, Allan G. Features of porcine circovirus-2 disease: correlations between lesions, amount and distribution of virus, and clinical outcome. *J Vet Diagn Invest* 2005;17:213-222.

Part 3. Reagents and kits

PCV free PK15 cell line

The cell line is available from the CSIRO Australian Animal Health Laboratories, Geelong, Victoria. Telephone 03 5227 5000

Anti-PCV2 monoclonal antibody

A monoclonal antibody directed against the capsid protein of PCV2 of mouse origin. It was originally produced at Murdoch University and works very well in IHC applications. It is available from the Animal Health Laboratories, Department of Agriculture and Food Western Australia. Telephone 03 9368 3351

Porcine circovirus antiserum

An anti-PCV2 polyclonal antiserum of porcine origin was made against a field isolate of PCV2; it does not stain PCV1. It is useful for IFA assays. It has not been successfully applied to IHC on formalin-fixed, paraffin-embedded tissues. A monoclonal antibody is preferred for IHC applications. It is produced by VMRD.

Porcine circovirus type 2 FITC conjugate

An anti-PCV2 polyclonal antiserum of porcine origin, conjugated to fluorescein isothiocyanate, was made against a field isolate of PCV2; it does not stain PCV1. It is suitable for staining PCV2 in cell cultures. It does not stain PPV, TGE or PRRS. It is produced by VMRD.

SERELISA® PCV2 Ab Mono Blocking ELISA

The SERELISA PCV2 Ab Mono Blocking detection kit uses a single well blocking technique for the detection of anti-PCV2 antibodies in swine faeces or serum. This test can be used to provide a quantitative measure of serum antibodies, although this decreases the test capacity of the kit by 1/3. The kit is most useful when seeking a quantitative measure of antibodies in serum or faeces; however, it is expensive. The kit is produced by Synbiotics.

Commercial suppliers

Australia: VMRD and Synbiotics products
Laboratory Diagnostics Pty Ltd
Serenity Cove Corp. Park
Unit 7, 260 Captain Cook Dr.
Kurnell
Sydney, NSW 2231
Australia
Phone: 61296680600
FAX: 61296688533

New Zealand: VMRD products
Diagnostic Bioserve, Ltd.
1029 Papamoa Beach Road
Papamoa Beach, Bay of Plenty 3118
New Zealand
Phone: +64 (7) 5422325
FAX: +64 (7) 5422326

New Zealand: Synbiotics products
Gribble-Alpha Scientific
57 Sunshine Avenue
Hamilton, New Zealand
Phone: +64 (7) 8500777
FAX: +64 (7) 8500770
www.alpha-scientific.co.nz

Appendix 1

CCEAD Case definition for the initial diagnosis of PMWS in Australia

Any initial diagnosis of PMWS in Australia will be made by the Consultative Committee on Emergency Animal Diseases (CCEAD). This case definition has been developed by an expert panel to assist CCEAD in making this diagnosis.

Each of the following three elements must be met.

I. Clinical criteria:

Current or historical evidence of a herd syndrome characterised by elevated morbidity and mortality[1], associated with a non responsive[2], otherwise unexplained[3] wasting[4], in pigs between weaning and 12 weeks of age[5]. Where the syndrome is newly recognised, a mortality and morbidity rate of twice the baseline for the herd is

---

[1] Mortality.

- Assessment is to be based on meaningful and accurate data including historical and/or current data. In the absence of adequate data, arrangements should be made to collect data prospectively to allow for meaningful analysis.
- Data should include number of pigs weaned per week and number of pig deaths per week from weaning to 12 weeks of age.
- Mortality figures should be assessed for pigs from weaning, and include all dead and destroyed/culled animals. The New Zealand C8MR statistic [Stone M 2004 *Response to first diagnosis of post-weaning multisystemic wasting syndrome*, Surveillance 31(4) p9] may be useful for this purpose.
- Where possible a baseline mortality rate for the herd should be established.

[2] Non responsive:

- Means an ongoing lack of response to interventions, even though there may be partial or temporary response to some treatments.
- Interventions include management changes, dietary changes or chemical/therapeutic treatments.
- Initiated interventions fail to achieve changes or responses that would be expected for the herd in question under normal circumstances.

[3] Otherwise unexplained.

- May be indicated by signs of unusual or atypical disease for the herd in question.
- Diagnostic intervention, including necropsy examinations, is required to exclude endemic diseases or other explanations for the clinical picture.

[4] Wasting means loss of body condition, typically over a 7-10 day period.

[5] Older pigs, to about 18 weeks, may be involved. Wasting in pigs due to PMWS can occur at any time during the growing phase, but should be distinguished from poor growth during the immediate post-weaning period.

considered significant. Other signs may be present including, dyspnoea or tachypnoea, enlarged lymph nodes, and less frequently, diarrhoea, pallor or jaundice.

II. Histopathological criteria:

To satisfy the histopathological criteria, lymphoid tissues (lymph nodes and spleen) obtained from affected pigs[6] must have evidence of depletion of lymphoid cells associated with lymphohistiocytic to granulomatous inflammatory infiltration. Multinucleated giant cells may be observed and basophilic intracytoplasmic (botryoid) inclusion bodies which are highly characteristic for the syndrome may also be present. Similar inflammatory lesions may be found in a variety of organs including lung, liver, kidney and intestine and parenchymal cell necrosis may also be seen.

III. Presence of the agent

Demonstration of abundant PCV2 antigen within the characteristic histological lesions described above.

[6] A representative group of affected pigs must have been subject to post mortem examination with submission of appropriate tissue samples. As a guide, submission of appropriate samples from at least six affected pigs would be expected. Characteristic histological lesions should be observed in a substantial proportion of samples submitted.