1 **Development and use of a multiplex real-time quantitative polymerase chain reaction assay**
2 **for detection and differentiation of *Porcine circovirus-2* genotypes 2a and 2b in an**
3 **epidemiological survey**

4

5 Carl A. Gagnon,[1] Jérome R. E. del Castillo, Nedzad Music, Guy Fontaine, Josée Harel, Donald
6 Tremblay

7

8 From the Groupe de recherche sur les maladies infectieuses du porc (Gagnon, del
9 Castillo, Music, Harel), the Service de diagnostic (Gagnon, Fontaine, Harel, Tremblay), and the
10 Groupe de recherche en pharmacologie animale du Québec (del Castillo), Faculté de médecine
11 vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada.

12

13 [1]Corresponding Author: Carl A. Gagnon, Faculté de médecine vétérinaire, Université de
14 Montréal, 3200 rue Sicotte, St-Hyacinthe, Quebec, Canada, J2S 2M2.
15 carl.a.gagnon@umontreal.ca

16

17 Short running title: PCV-2 genotypes differentiation using a new real-time PCR assay
18

Abstract. By the end of 2004, the Canadian swine population had experienced a severe increase in the incidence of Porcine circovirus-associated disease (PCVAD), a problem that was associated with the emergence of a new *Porcine circovirus-2* genotype (PCV-2b), previously unrecovered in North America. Thus it became important to develop a diagnostic tool that could differentiate between the old and new circulating genotypes (PCV-2a and -2b, respectively). Consequently, a multiplex real-time quantitative polymerase chain reaction (mrtqPCR) assay that could sensitively and specifically identify and differentiate PCV-2 genotypes was developed. A retrospective epidemiological survey that used the mrtqPCR assay was performed to determine if cofactors could affect the risk of PCVAD. From 121 PCV-2–positive cases gathered for this study, 4.13%, 92.56% and 3.31% were positive for PCV-2a, PCV-2b, and both genotypes, respectively. In a data analysis using univariate logistic regressions, PCVAD compatible (PCVAD/c) score was significantly associated with the presence of *Porcine reproductive and respiratory syndrome virus* (PRRSV), PRRSV viral load, PCV-2 viral load, and PCV-2 immunohistochemistry (IHC) results. Polytomous logistic regression analysis revealed that PCVAD/c score was affected by PCV-2 viral load ($P = 0.0161$) and IHC ($P = 0.0128$), but not by the PRRSV variables ($P > 0.9$); suggesting that mrtqPCR in tissue is a reliable alternative to IHC. Logistic regression analyses revealed that PCV-2 increased the odds ratio of isolating 2 major swine pathogens of the respiratory tract, *Actinobacillus pleuropneumoniae* and *Streptococcus suis* serotypes 1/2, 1, 2, 3, 4, and 7, which are serotypes commonly associated with clinical diseases.

Key words: Epidemiological survey; genotyping; molecular diagnostic test; *Porcine circovirus-2*.

Introduction

Postweaning multisystemic wasting syndrome (PMWS) is a disease of swine initially identified in Canada in 1991.[13] Now, it is known as a worldwide disease with outbreaks reported in swine herds of North and South America, Europe, and Asia.[7] The disease affects 5- to 12-week-old piglets and is characterized in part by weight loss, dyspnea, jaundice, and enlarged lymph nodes, as well as by degeneration and necrosis of hepatocytes, multifocal lymphohistiocytic pneumonia, lymphocytic depletion, and multinucleated giant cell formation.[26] The etiological agent responsible for PMWS has been identified as a circovirus particle named *Porcine circovirus-2* (PCV-2; family *Circoviridae*, genus *Circovirus*).[1,19,25] PCV-2 is a small nonenveloped virus that possesses a single-stranded ambisense circular DNA genome of about 1.76 kb in length.[20,33,41,54] Viral DNA possesses at least 3 functional open reading frames (ORFs): ORF1 encodes the Rep proteins involved in virus replication,[9,10,38] ORF2 encodes the nucleocapsid (Cap) protein,[42] and ORF3 encodes a protein that induces apoptosis and is involved in viral pathogenesis in vivo.[35,36] Today, the clinical expression of PCV-2 infection in swine is acknowledged to be more complex than initially established because it can play a pivotal role in several syndromes, such as porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failure, granulomatous enteritis, necrotizing lymphadenitis, exudative epidermitis, and congenital tremor.[8,27] Consequently, the term "Porcine circovirus-associated disease (PCVAD)" is now accepted to describe the syndromes in which PCV-2 plays a role.

At the end of 2004, the swine industry in the province of Québec in Canada started to experience a significant increase in death rate related to PCVAD.[14] It was hypothesized that this emerging problem was caused by the presence of a new type of circulating PCV-2 strain. This

1 was confirmed by the sequencing of the entire genome of several PCV-2 strains originating from
2 PMWS-affected herds.[23] Interestingly, this newly recovered PCV-2 genotype (named PCV-2b in
3 comparison with the circulating genotype named PCV-2a) had already been reported in Asia and
4 Europe[15] and had also been recently reported in the United States.[11,30]
5 The current international consensus is that there are at least 2 major PCV-2 genotypes
6 that are presently circulating worldwide.[11,17,23,24,43] It is noteworthy that different nomenclatures
7 have been used to identify the PCV-2 genotypes: for instance, the PCV-2b and -2a genotypes[23]
8 respectively correspond to the restriction fragment length polymorphism (RFLP) classification
9 321 and 422,[6], and to the genotypes 1 and 2.[24] Based on RFLP and gene sequence comparison
10 results, PCV-2b was suggested to be associated with the mortality rate increase observed in
11 several Canadian swine herds.[6] Consequently, even if no experiment confirms yet if the PCV-2b
12 strains found in Canada are more virulent than previously circulating PCV-2a strains, it became
13 important to develop a low cost (compared to entire viral genome sequencing) diagnostic test
14 that could rapidly and efficiently differentiate both genotypes. Previous results indicated that the
15 protein that best distinguishes PCV-2a from PCV-2b was the Cap protein, with an amino acids
16 (aa) sequence identity varying from 88–94% between both genotypes.[23] Therefore, a new
17 multiplex real-time quantitative polymerase chain reaction (mrtqPCR) assay was developed to
18 detect with high sensitivity PCV-2 strains present in tissue samples of pigs. Furthermore, this
19 new PCR assay was able to identify PCV-2 genotypes by targeting the ORF2 gene. Afterwards, a
20 retrospective epidemiological survey was conducted using PCV-2 mrtqPCR positive cases that
21 were submitted at the molecular diagnostic laboratory of the Faculté de médecine vétérinaire
22 (FMV) of the University of Montreal to determine the prevalence of PCV-2 genotypes; and to
23 establish if other viral or bacterial pathogens were associated with the clinical disease.

1 Furthermore, since PCV-2 antigen detection in microscopic lesions by immunohistochemistry
2 (IHC) is one of the three criteria used for the diagnostic of PCVAD cases,[51] the survey was
3 conducted to determine the relationship between the PCV-2 IHC results and the PCV-2 viral load
4 with PCVAD.
5
6

Materials and methods

7

Organisms and culture methods

8 All bacteria and viruses used as controls were isolated and characterized at the FMV. The
9 PCV-2 viruses, IAF 2897 (genotype PCV-2a) and FMV-06-1717 (genotype PCV-2b), used in the
10 mrtqPCR assay were previously characterized reference strains.[23] Those viruses were propagated
11 into PK15A cells, a subclone of PCV noninfected PK15 cells,[49] and the viruses were purified
12 following a 4-hr period of ultracentrifugation on a 30% sucrose cushion using the SW28
13 Beckman Coulter rotor[a] at 83,000 relative centrifugal force (rcf). The virus pellets were
14 resuspended in 2 mL of a phosphate buffered saline (PBS) containing 2% fetal bovine serum,
15 and aliquots of the virus stocks were then conserved at –70°C for future use. The infectious dose
16 of the virus stock was calculated from a 96-well microplate of PK15A-infected cells following
17 fluorescent antibody test[49] by the Kärber method.[47] Virus titers were expressed in tissue culture
18 infectious dose 50 per mL ($TCID_{50}$/mL).
19

Porcine circovirus-2 recombinant plasmids

20 Extraction of PCV-2 IAF2897 and FMV-06-1717 isolates of DNA was performed using
21 the Qiagen QIAamp DNA Mini Kit[b] according to the manufacturer's instructions. The PCR
22 products encompassing the entire viral genome of both strains were produced using the
23 following primers: Fw-SacII-PCV2 (GAACCGCGGGCTGGCTGAACTTTTGAAAGT) and

Rv-SacII-PCV2 (GCACCGCGGAAATTTCTGACAAACGTTACA). The PCR products were subsequently cloned in the plasmidic vector pCR2.1 using the TA (Taq-amplified) Cloning Kit[c] according to the manufacturer's instructions. Finally, the recombinant plasmids (pCR2.1/PCV-2a and pCR2.1/PCV-2b) were sequenced to verify the accuracy of the nucleotide sequences and were used as DNA templates to validate the new mrtqPCR diagnostic assay.

DNA/RNA extraction

Extraction of DNA and RNA from tissue samples (lungs and/or lymph nodes) and virus isolates was performed using the Qiagen QIAamp DNA Mini Kit[b] and the Qiagen QIAamp Viral RNA Mini Kit,[b] respectively, according to the manufacturer's instructions. For DNA/RNA tissue extraction, 100 mg of tissues was put in a 1.5-mL homogenization tube with 0.5-mm glass beads and 1 mL of PBS. The mix was homogenized at high speed for 3 min in a BeadBeater[d] apparatus and centrifuged for 1 min at 16,100 rcf. DNA was extracted using 200 µl of diluted and homogenized viral solutions and recombinant plasmid solutions. A volume of 140 µl of diluted and homogenized viral solution was used for RNA extraction.

Multiplex real-time quantitative polymerase chain reaction assay

Primers and probes were selected within 2 regions of the PCV-2 genome based on nucleotide alignments generated by the BioEdit Sequence Alignment Editor (version 7.0.5.2)[e] of available sequences in public domain databases (over 76 sequences of PCV-2a and -2b strains were obtained from GenBank and compared) and sequences obtained from viral specimens in the authors' molecular diagnostic laboratory (over 49 sequences of PCV-2a and -2b strains).[23] For general detection of PCV-2, a conserved region of PCV-2 genomes was targeted by the mrtqPCR using the following primers and probe: Circo-Gen-F (GGCCACCTGGGTGTGGTAAA), Circo-Gen-R (CCCACCACTTGTTTCTAGGTGGTT), and Circo-Gen-Probe (6-FAM-

1 TTTGCAGACCCGGAAACCACATACTGGA-BHQ-1). For genotyping, a hypervariable region
2 localized in the ORF2 of the PCV-2 was targeted by the mrtqPCR using the following primers
3 and probes: Circo-DF (GGGCCAGAATTCAACCTTAA), Circo-DR
4 (CGCACCTTCGGATATACTATCA), Circo-2a-Probe (Cy5-
5 GGGGACCAACAAAATCTCTATACCCTTT-BHQ-2), and Circo-2b-Probe (Cy3-
6 GGGCTCAAACCCCCGCTCTGTGCCCTTT-BHQ-2).
7 In the multiplex reaction, 5 µl of sample containing the DNA template was added to a 20-
8 µl reaction mixture (4.8 µl of LightCycler FastStart DNA MasterPLUS HybProbes,[f] 400 nM of
9 each primer and genotyping probes, and 120 nM of Circo-Gen-Probe). Thermocycling and
10 detection were performed in a SmartCycler 6400 System.[g] The PCR reaction started with an
11 initial denaturation and polymerase-activating step of 95°C for 10 min, followed by 45
12 amplification cycles of a 3-step PCR (95°C for 30 sec; 60°C for 60 sec; and 72°C for 60 sec).
13 To assess the sensitivity and specificity of the mrtqPCR assay and to determine its ability
14 to differentiate the 2 PCV-2 genotypes, 10-fold serial dilutions of PCV-2a and -2b reference
15 viruses and PCV-2 recombinant plasmids (pCR2.1/PCV-2a and pCR2.1/PCV-2b) described
16 above were assayed. The viruses and recombinant plasmids were then quantified by mrtqPCR.
17 Furthermore, validation of the mrtqPCR PCV-2 genotype classification from clinical submitted
18 samples was confirmed following sequencing of 49 PCV-2–positive cases (10 PCV-2a and 39
19 PCV-2b cases). A conventional multiplex PCR amplification (mPCR) for the detection of type 1
20 and 2 circoviruses was also performed as previously described.[46] The specificities of the primer-
21 probe sets were determined by testing several specimens from different animals, bacterial DNA,
22 and nucleic acids of swine viruses. The samples consisted of PCV-2–negative porcine tissues,
23 semen, and sera, as well as bovine tissues, and various pathogens isolated by the FMV diagnostic

1 laboratory such as *Brachyspira hyodysenteriae*, *Campylobacter* spp., *Clostridium perfringens*,
2 *Enterococcus* spp., *Escherichia coli*, *Haemophilus parasuis*, *Lawsonia intracellularis*,
3 *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Porcine circovirus-1* (PCV-1), *Porcine*
4 *reproductive and respiratory syndrome virus* (PRRSV), *Salmonella* spp., *Staphylococcus hyicus*,
5 *Streptococcus suis*, swine influenza viruses (SIV) H1N1 and H3N2, and *Transmissible*
6 *gastroenteritis virus* (TGEV).

7 **Retrospective epidemiological survey**

8 Sick or dead pigs that were submitted to and processed by the Institut National de Santé
9 Animale (INSA), a subsidiary of the Ministère de l'Agriculture, des Pêcheries et de
10 l'Alimentation du Québec (MAPAQ), were subsequently submitted to the diagnostic services of
11 FMV. All live animals or carcasses submitted to INSA originated from the province of Quebec
12 (Québec, Canada). Following necropsy of all submitted animals and evaluation of the gross and
13 microscopic lesions, several diagnostic tests were realized on tissue samples (such as lung and
14 lymph nodes) including standard bacteriological identification, PCR identification of viruses and
15 bacteria, and PCV-2 antigen assessment by IHC. All test results and observed clinical signs
16 reported by the veterinarians were gathered in a databank at the INSA. The following pathogens
17 were tested: *Actinobacillus pleuropneumoniae*, *Bordetella bronchiseptica*, *B. hyodysenteriae*, *E.*
18 *coli*, *H. parasuis*, *M. hyopneumoniae*, *M. hyorhinis*, *P. multocida*, PCV-2, PRRSV, *Salmonella*
19 ssp., *Staphylococcus aureus*, *S. suis* with serotype identification, SIV, and a few others in very
20 rare occasions (e.g., Rotaviruses, *Klebsiella* ssp., *Lactobacillus* ssp., and *Pasteurella* ssp.). A
21 total of 121 cases submitted between October 2006 and January 2007 were selected based on the
22 results of the PCV-2 mrtqPCR. In these cases, a positive mrtqPCR result was recorded for at
23 least one of the 2–3 submitted live animals or carcasses, all of which were examined and tested

1 individually. All pigs submitted for a case originated from the same farm and had the same age,
2 which ranged between 3 and 15 weeks. In the selected population of PCVAD compatible
3 (PCVAD/c) cases, 40.5% had the highest PCVAD/c rank and showed moderate to severe signs
4 of wasting or weight loss, dyspnea, coughing, pallor, and jaundice, with characteristic
5 histopathological lesions such as multifocal lymphohistiocytic pneumonia, lymphocytic
6 depletion, and multinucleated giant cell formation. An additional 40.5% of cases had the lowest
7 PCVAD/c rank and showed neither clinical signs nor histopathological lesion (Table 1). Among
8 the 121 PCVAD/c cases, 42 had their PCVAD status confirmed by IHC testing and 79 were IHC
9 untested. In addition, the PRRSV and PCV-2 PCR quantification results were included in the
10 databank generated for statistical analysis. The quantification of PCV-2 was determined with the
11 mrtqPCR by comparing the sample results with a standard curve based on the amount of serially
12 diluted PCV-2 recombinant plasmids (pCR2.1/PCV-2a and pCR2.1/PCV-2b). Moreover, the
13 probe that could identify all PCV-2 genotypes (Circo-Gen-Probe) was also used to quantify
14 PCV-2 in submitted samples. The PCV-2 quantification results were reported as the number of
15 DNA copies/gram of tissue. A PRRSV real-time PCR diagnostic kit[h] specific for the North
16 American genotype of PRRSV was used for PRRSV identification as recommended by the
17 manufacturer. The quantification of PRRSV was determined with the kit by comparing the
18 sample results with a standard curve based on the amount of serially diluted IAF-Klop PRRSV
19 reference strain[22] titrate following infection of MARC-145 cells and expressed as $TCID_{50}$/mL.
20 The PRRSV quantification results were reported as the number of $TCID_{50}$/gram of tissue. The
21 amount of PCV-2 and PRRSV in each case was established by calculating the mean value of
22 virus quantification of positive animals. The IHC assay was performed as previously described[53]
23 using an anti-PCV-2 rabbit polyclonal serum (kindly provided to the MAPAQ by Dr Dave

Cavanaugh, Iowa State University). A case was considered IHC positive if tissue (lung or lymph nodes) of at least one of its submitted animals gave a positive result.

Statistical analyses were performed with the SAS system, version 9.1.[i] The relevance of the PCVAD/c score system was evaluated by establishing its statistical relationship with the PCV-2 IHC test using the Mantel-Haenszel χ^2 test. Afterwards, the association between the PCVAD/c score system and the presence of PRRSV was established with a likelihood ratio χ^2 test; separate univariate logistic regressions were performed to test its relationship with the logarithms of the PCV-2 and PRRSV viral loads (PCV-2/vl and PRRSV/vl, respectively), which were expressed in DNA copies/gram of tissue and $TCID_{50}$/gram of tissue, respectively. In addition, Kruskal-Wallis non-parametric tests were performed to detect differences in PCV-2/vl as a function of IHC result (untested, negative and positive) in each of the 4 PCVAD/c score levels. This series of tests were performed at the $\alpha = 0.0125$ level (i.e., Bonferroni adjustment) to set the overall type-1 error risk at 0.05. Then, a multivariate polytomous logistic regression[2,31] was performed to examine the concurrent effects on the PCVAD/c score of the following variables: 1) the PCV-2/vl, 2) the presence of PRRSV, 3) the PRRSV/vl, 4) IHC testing, 5) IHC result, and 6) all possible interactions of these predictor variables. Inclusion of two IHC-related variables allowed comparing the differences in PCVAD/c score of the two subgroups, and assessing the potential redundancy of the mrtqPCR with respect to IHC, which is a key diagnostic criterion.[51] The reference outcome value of the PCVAD/c score was set to 0 to focus on variation from clinical normality, and the cumulative logit function was used as a link between the predictors and response variables. The statistical model was built by stepwise-forward selection of the predictors, where marginal P-value thresholds of 0.15 and 0.20 were used, respectively, for introducing a predictor in the model or deleting it from the model.[31] The

1 likelihood ratio test was performed at each step to evaluate the significance of the newly added

2 predictor. The score test for the proportional odds assumption[i] was used to verify that the

3 proportional odds model[39] was valid for analyzing the ordinal response variable. In addition, the

4 authors dichotomized among the levels of the PCVAD/c score system (i.e., between 0 and ≥1,

5 between ≤1 and ≥2, etc.) and performed separate binary logistic regressions to further investigate

6 the validity of the proportional odds assumption.[3] Once the best logistic regression model was

7 obtained, odds ratio (OR) and 95% confidence intervals (95% CI) were calculated for each

8 significant predictor or interaction of predictors.[31] Afterwards, a series of binary logistic

9 regressions was performed to determine the odds of recovering the above listed viral and

10 bacterial pathogens as a function of the PCVAD/c score, the PCV-2/vl, the presence of PRRSV,

11 and the PRRSV/vl. Relevant predictors and interactions were introduced in the model using the

12 stepwise-forward strategy, with P = 0.15 and P = 0.20 thresholds for inclusion and deletion of

13 predictors.

14

1 **Results**

2 **Identification of the ORF2 hypervariable region by sequence analysis**

3 Based on sequence analysis of 125 PCV-2 strains (46 PCV-2a strains and 79 PCV-2b
4 strains), the ORF2 gene was identified to be the most variable gene between both PCV-2
5 genotypes, as previously described by the authors and others.[23,24] Consequently, the
6 hypervariable region of ORF2 was targeted to differentiate both PCV-2 genotypes. The
7 hypervariable region of ORF2 was localized between nucleotides (nt) position 222 and 279 from
8 the gene start codon (data not shown). In the hypervariable region, 20 nt over 57 are different
9 between both genotypes, which represents overall 35% nt variation in this region (data not
10 shown). Because the 3'end hypervariable region is more conserved than the 5'end nt sequence
11 within PCV-2a strains, the 3'end region was selected to design DNA probes that could
12 differentiate genotypes and quantify PCV-2 viruses (data not shown).

13 **Sensitivity of *Porcine circovirus-2* primers and probes**

14 To compare the sensitivity of the mrtqPCR with that of a conventional mPCR (an assay
15 designed to differentiate PCV-1 from PCV-2 viruses but not between PCV-2 genotypes),[46] serial
16 10-fold dilutions of PCV-2b FMV-06-1717 and PCV-2a IAF2897 infectious isolates were tested
17 with both assays (Fig. 1). The mrtqPCR was determined to be at least 1000-fold more sensitive
18 than the mPCR (Fig. 1). The mrtqPCR standard curves were generated for both genotypes with
19 10-fold dilutions of quantified recombinant DNA (Fig. 2c). The coefficients of determination
20 (R^2) were over 0.99 for all the regression of the standard curves. By using the cycle threshold
21 (Ct) values and the regression equations of those standard curves, the DNA copy number
22 detected was calculated for each positive mrtqPCR reaction with a detection limit estimated to be
23 25 DNA viral genome copies/mrtqPCR reaction for all primers and probes sets (data not shown)

1 because a non-reproducible detection of PCV-2 recombinant DNA could be observed below this
2 value. The Ct value obtained at 25 DNA viral genome copies/mrtqPCR reaction was 37. All
3 results with a Ct value greater than 37 were considered to be negative for PCV-2. Moreover,
4 when the mrtqPCR was compared with a previously described PCV-2 real-time PCR assay that
5 could not differentiate PCV-2 genotypes,[23] the sensitivity of both real-time PCR assays was
6 similar for all mrtqPCR primers and probes sets (data not shown).

7 Two types of targets, recombinant DNA and purified viral DNA, were used to evaluate
8 the PCV-2 quantification efficiency of the mrtqPCR assay. From an initial concentration of
9 pCR2.1/PCV-2b recombinant plasmid corresponding to 1.0×10^{10} PCV-2 viral genome
10 copies/mL, 10-fold serial dilutions were made and tested by mrtqPCR. At the same time, the
11 FMV-06-1717 PCV-2 infectious virus (4.7×10^{3} $TCID_{50}$/mL) was serially diluted and DNA was
12 subsequently extracted from each dilution and tested with the mrtqPCR assay. Interestingly, the
13 mrtqPCR standard curves obtained with the 2 different templates (recombinant DNA and
14 purified viral DNA) were parallel with almost the same slope (Fig. 3). This indicates a very good
15 correlation between the mrtqPCR quantification using both templates. For each time point, a 10^{6}-
16 fold difference was consistently observed in the mrtqPCR using the 2 different templates.
17 Overall, similar results were obtained when the mrtqPCR was performed with the pCR2.1/PCV-
18 2a recombinant purified DNA compared to DNA purified from IAF 2897 infectious PCV-2a
19 isolate (data not shown). The mrtqPCR appears to be more sensitive than the culture of infectious
20 virus, as the limit of detection of the technique is under a single $TCID_{50}$ (Fig. 3).

21 **Specificity and differentiation capacity of the multiplex real-time quantitative polymerase**
22 **chain reaction**

1 From a panel of RNA and DNA extracted from bacterial and viral cultures, serum, and
2 tissues, only the samples containing PCV-2 were positive by mrtqPCR (data not shown). As
3 illustrated in Figures 2a and 2b, the mrtqPCR assay detected specifically each genotype. Also,
4 when recombinant DNA of both genotypes was mixed together, the mrtqPCR assay was able to
5 identify both genotypes simultaneously (Fig. 2c).

6 **Retrospective epidemiological survey**

7 Examination of the clinical samples (lungs and lymph nodes) from the authors' databank
8 of 121 cases originating from different farms revealed that 92.56%, 4.13%, and 3.31% cases
9 were positive for PCV-2b genotype alone, PCV-2a genotype alone, and both genotypes,
10 respectively (Table 2). This latter result indicates the existence of PCV-2 mixed infections in
11 Quebec swine herds, but the number of PCV-2a submitted cases recovered was too low to be
12 able to determine whether the PCVAD/c score is affected significantly by the genotype of PCV-2
13 that was involved as illustrated in Table 2 by the prevalence of PCV-2 genotypes in regards to
14 PCVAD/c status.

15 The PCVAD/c score was linearly associated with the result of the PCV-2 IHC assay
16 (Mantel-Haenszel χ^2; $P < 0.0001$), as an average of 93% (14/15) of cases with a score ≤ 1 came
17 out negative, whereas an average of 81% (22/27) of cases with a score ≥ 2 were positive (Fig. 4a).
18 This confirmed the validity of the PCVAD/c score system. In each PCVAD/c score grade, no
19 significant difference in PCV-2/vl was recorded for the IHC untested and IHC tested cases
20 (Kruskal-Wallis tests; $P > 0.25$). In the case of PCVAD/c score = 0, the median PCV-2/vl value
21 was 5.7735 for the IHC untested cases, and 6.8348 for the IHC negative cases. In the case of
22 PCVAD/c score = 3, the median PCV-2/vl value was 10.6821 for the IHC untested cases, and
23 10.3729 for the IHC positive cases. These results strongly suggest that the IHC untested cases

with a PCVAD/c score value of 0 are true negative PCVAD cases, and those with a score value of 3 are true positives PCVAD cases.

The univariate logistic regression analysis indicated that the value of the PCVAD/c score was significantly affected by the PCV-2/vl ($P < 0.0001$). To support this result, the mrtqPCR Ct mean values obtained were 30.31, 27.41, 19.74, and 15.54 for PCVAD/c grade score 0, 1, 2, and 3, respectively. Furthermore, this result is clearly illustrated in Figure 4a where a higher amount of PCV-2 correlated with a higher PCVAD/c grade score. In addition, the PCVAD/c score was significantly affected by the presence of PRRSV (likelihood ratio χ^2; $P = 0.0258$), as well as by the PRRSV/vl (univariate logistic regression; $P = 0.0249$), which granted further evaluation with the multivariate polytomous logistic regression.

This polytomous logistic regression for ordinal data, which simultaneously takes into account the above listed variables and their interactions, revealed that the level of PCVAD/c score was significantly affected by the PCV-2/vl ($P = 0.0161$) and by the result of the IHC assay ($P = 0.0128$). In addition, a significant interaction between the results of the IHC assay and the PCV-2/vl ($P = 0.0304$) was recorded. But the PCVAD/c score was significantly affected neither by IHC testing (untested vs. tested; $P=0.3502$), nor by the presence of PRRSV and PRRSV/vl ($P > 0.9$; Fig. 4b). The coefficient estimates of the final model (data not shown) revealed that IHC and PCV-2/vl considerably decreased the odds of a null score. The odds ratios of having a PCVAD/c score greater than 0 following PCV-2 PCR quantification and IHC testing are presented in Table 3. A positive IHC result increased the odds ratio of having a PCVAD/c score greater than 0 by 3.52×10^4 (Table 3). Furthermore, the PCV-2 quantification (PCV-2/vl) increased the odds ratio of having a PCVAD/c score greater than 0 by 4.08 with a PCV-2/vl log-scale unit increase (Table 3). Interestingly, both variables (IHC-positive result and PCV-2/vl)

1 increased the odds ratio of having a PCVAD/c score greater than 0 by 5.45×10^4 per PCV-2/vl
2 log-scale unit increase (Table 3).
3 Table 4 presents the effects of the PCVAD/c score, the PCV-2/vl, the presence of
4 PRRSV, and the PRRSV/vl on the odds of recovering a number of swine pathogens from
5 samples of a given case from the authors' databank. The prevalence of those pathogens in
6 regards to the PCVAD/c grade score 0 and 3 (which are considered to be non-PCVAD and
7 PCVAD cases, respectively, based on Kruskal-Wallis and multivariate polytomous logistic
8 regression analyses) are also given in Table 4. None of these variables (PCVAD/c score, the
9 PCV-2/vl, the presence of PRRSV, and the PRRSV/vl) had significant effect on the odds of
10 isolating *A. pyogenes*, *B. hyodysenteriae*, *S. hyicus*, or SIV ($P > 0.20$ in all cases). From the
11 predictors tested, the PCV-2 viral load (i.e., PCV-2/vl) was found a risk factor for 2 pathogens
12 and the PRRSV viral load (i.e., PRRSV/vl) was found a risk factor for 5 pathogens. The PCV-
13 2/vl significantly increased the risk of recovering *A. pleuropneumoniae* (OR = 1.887) and *S. suis*
14 serotypes 1/2, 1, 2, 3, 4 and 7, which are serotypes commonly associated with clinical diseases[29]
15 (*S. suis* pathogenic serotypes; OR = 1.3690). In contrast, PCV-2/vl had no significant effect on
16 the odds of isolating *S. suis* when all of its serotypes were included (i.e., generic *S. suis*). The
17 odds of isolating *M. hyopneumoniae* were significantly higher in pigs with a PCVAD/c score of
18 2, compared to the reference population with a PCVAD/c score of 0 (OR = 7.800). Nevertheless,
19 PCV-2 had null or significantly negative effect on the risk of recovering other pathogens,
20 especially *B. bronchiseptica*, *E. coli*, and *Salmonella* spp. On the contrary, PRRSV significantly
21 increased the risk of recovering *B. bronchiseptica*, *E. coli*, *H. parasuis*, *P. multocida*, and generic
22 *S. suis* (PRRSV/vl; OR = 1.305, 1.479, 1.443, 1.474, and 1.203, respectively). In 2 cases, the

PCV-2 viral load had opposite sign coefficients compared to PRRSV viral load, suggesting that their combined effect was not additive.

Discussion

One of the main objectives of the swine industry and health scientists is to establish if there is virulence variation between PCV-2a and PCV-2b viruses. In fact, a recent retrospective study[5] showed a statistically significant increase in the frequency of PCVAD characteristic histological lesions observed in lymph node, spleen, lung, small intestine, colon, and kidney, in pigs infected with PCV-2b strains, compared to pigs infected with PCV-2a strains. Viral burden, as estimated with IHC staining in lymph node, was also significantly increased in pigs infected with the PCV-2b strains, compared to the PCV-2a strains.[5] Because these results were derived from retrospective analysis of cases submitted to diagnostic laboratories, the conclusion may be biased. Nonetheless, it strongly suggests that PCV-2b virus could be more virulent than PCV-2a virus. Moreover, others have reported that genetic variations may have a great impact on the virulence of PCV-2.[21] Consequently, it became critical for swine producers and veterinarians to establish which PCV-2 genotypes are present in their herds. In that context, an mrtqPCR assay was developed to sensitively identify and differentiate PCV-2 viruses in clinical samples (Figs. 1, 2; Table 2). With the exception of the conventional PCR assay developed previously,[30] all other PCR assays reported until now did not differentiate between PCV-2 genotypes.[4,12,34,55] Moreover, the conventional PCR diagnostic test reported previously[30] required 2 PCR reactions to identify both genotypes whereas only 1 reaction is required in the authors' mrtqPCR. Furthermore, real-time PCR assays are usually more sensitive than conventional PCR assays as illustrated in Figure 1.

1 Interestingly, a good correlation was established with the mrtqPCR assay between 2
2 quantification methods, the DNA copies/mL versus the $TCID_{50}$/mL (Fig. 3). The relationship
3 between the 2 PCV-2 quantification methods (DNA copies vs. infectious virus) was determined
4 to be: DNA copies/mL value = $(1.76\times10^{6}) \times TCID_{50}$/mL value (Fig. 3). It is unknown whether
5 this equation could be generalized to all PCV-2 isolates, since new recombination or mutations
6 of the viral genome within the mrtqPCR targeted regions could interfere with both quantification
7 and genotyping results. Furthermore, PCR inhibitors in clinical specimens could adversely affect
8 the PCV-2 quantification. Nonetheless, it will help veterinarians in their interpretation of the
9 diagnostic results obtained with the mrtqPCR assay.

10 The standard PCVAD definition consists in three criteria: 1) presence of characteristic
11 clinical signs, 2) presence of characteristic histopathological lesions in lymphoid tissues, and 3)
12 detection of moderate to high amount of PCV-2 within the lesions in lymphoid and other tissues
13 by different techniques such as IHC. In order to statistically test the respective roles of PCV-2
14 viral load and the result of IHC testing in regards to the PCVAD status of the clinical cases, it
15 was purposely decided not to apply the above definition.[51] Specifically, the third criterion was
16 omitted in the PCVAD case definition as detailed in Table 1. Noteworthy, both the Kruskal-
17 Wallis and multivariate polytomous logistic regression analyses revealed that at identical PCV-
18 2/vl values, the IHC untested cases had similar PCVAD/c score grades compared to IHC
19 negatives and positives cases. Consequently, these results indicated that the PCVAD/c grade 0
20 and 3 cases are not significantly different from their IHC-tested counterparts (standard negative
21 and positive PCVAD cases), and strongly suggests that both groups are identical.

22 A very strong logistic (sigmoid) relationship was recorded between the PCVAD/c grade
23 score and the result of IHC assay, as well as the logarithm of the amount of PCV-2 virus

1 recovered from the tissue samples using the mrtqPCR assay and the interaction between these 2
2 independent variables (Fig. 4a). Interestingly, the stepwise-forward logistic regression analysis
3 suggests that the PCV-2 viral load may be a reliable alternative to the IHC assay as the third
4 criterion of the PCVAD definition since at PCVAD/c grade 3 score, the lowest amount of PCV-2
5 detected was $10^{7.5}$ DNA copies/gram of tissue, at which the OR of having PCVAD/c was
6 established to be $1/e^{(-1.4057\times7.5)} = 3.79\times10^4$ (95% C.I. = $1/e^{(-1.4057\times7.5 \pm 1.96\times7.5\times0.2180)} = [1.53\times10^3 -$
7 $9.34\times10^5]$), which favorably compares to the OR and 95% C.I. of a positive IHC result (Table 3,
8 scenario #2). Moreover, with the median PCV-2/vl value of the grade-3 score, the resulting OR
9 and 95% C.I. are 3.11×10^6 and $[3.31\times10^4 - 2.93\times10^8]$, respectively. These results are not
10 surprising because PCR has been shown to be more sensitive than IHC. Indeed, other
11 investigators have reported that from 20 PCV-2 experimentally infected pigs, 0% were PCR-
12 negative but 5% were IHC-negative when testing lymph nodes, and 10% were PCR-negative but
13 44% were IHC-negative when testing the lung tissue.[32] Furthermore, from 88 pigs tested in a
14 commercial herd with severe PMWS, only 3% were PCR-negative but 23% were IHC-
15 negative.[40] The latter result is similar to our findings, as 4 of the 19 (21%) cases with PCVAD/c
16 score = 3 were IHC negative despite showing high PCV-2 viral load at the mrtqPCR testing.
17 This relationship between PCV-2 viral load and clinical expression of PCVAD confirms
18 results previously reported.[4,44] Consequently, the higher the PCV-2 viral load in tissue samples,
19 the higher the probability to have PCVAD (Fig. 4a). This remains a probability since a high
20 amount of PCV-2 viral load has been observed in some cases unrelated to PCVAD and vice
21 versa (Fig. 4a). Surprisingly, the stepwise-forward logistic regression analysis revealed that a
22 positive IHC result significantly increased the odds of clinical disease, even if PCV-2 viral load
23 is already present in the model (Table 3). These results suggest that PCV-2 viral load and IHC

testing may represent 2 separate components of the overall disease process, but their statistically independent biological meaning could not be determined within the retrospective study.

In the present study, the prevalence of PCV-2b virus was very high as previously reported.[23] In fact, 95.9% of the submitted cases were infected with PCV-2b virus (Table 2), even though 40.5% of those submitted cases had no clinical sign and microscopic lesion related to PCVAD. This suggests that not all animals infected with PCV-2b virus may develop PCVAD. However, the difference observed between the presence of PCVAD and the number of PCV-2b cases may be due to the stage and time course of infection when the tissue samples were collected. As reported previously,[30] PCV-2a and -2b mixed infections were also observed but at a lower prevalence in Quebec province (3.31%) compared to that in a Kansas study (62.5%). The difference between prevalence values could be due to specimen origin because samples were obtained from pigs experiencing severe PCVAD in the Kansas study,[30] whereas in the present study almost half of samples were obtained from animals that had no PCVAD clinical signs (Table 1). Nonetheless, even if the disease evolution following PCV-2 mixed infections is unknown, it may have an effect since recombination between PCV-2 genomes has been reported.[28,37,43]

The course of PCV-2 infection that will lead to the occurrence of the disease has been associated with the presence of others pathogens such as *M. hyopneumoniae*, *Porcine parvovirus* (PPV), PRRSV, and SIV.[16,18,45,48] Thus, it was not surprising to find at the preliminary stage of data analysis, that a significant association existed between the presence of PRRSV and the appearance of PCVAD, as previously reported. Similar to the correlation between PCV-2 viral load and PCVAD/c (Fig. 4a), there was a significant association between PRRSV viral load and the appearance of PCVAD. In contrast, the multivariate polytomous logistic regression analysis

1 suggested that the presence and viral load of PRRSV had no significant effect on the PCVAD/c
2 score, as shown in Figure 4b, when both PCV-2 viral load and IHC result parameters are
3 included in the regression model. This suggests that PRRSV may be less involved as a cofactor
4 responsible for the induction of PCVAD than expected.[18,48] Indeed, animals with PCVAD may
5 be PRRSV negative or have PRRSV low titers (Fig. 4b). In contrast, animals that have no
6 PCVAD may possess a high viral load of PRRSV (Fig. 4b). Because of bias inherent to a
7 retrospective epidemiological survey, caution must be taken in the interpretation of these results.
8 In contrast to what has been reported previously,[16] epidemiological assessment of the OR
9 of PCV-2 coinfection with other swine pathogens in the present study did not involve SIV as an
10 important cofactor (Table 4). This could be explained by the fact that the number of SIV-positive
11 cases was very low in this studied population. Interestingly, statistical analyses revealed a high
12 odds ratio (OR = 7.800) of recovering *M. hyopneumoniae* in PCVAD/c grade 2 score animals,
13 which represents animals with PCVAD-specific microscopic lesions without having PCVAD
14 clinical signs (Tables 1, 4). In a previous report,[16] a 3.77 OR of recovering *M. hyopneumoniae*
15 was found in PCV-2–positive animals, irrespective of their PCVAD status. Surprisingly, no
16 association was found in the present study between *M. hyopneumoniae* and PCVAD/c grade 3
17 score, which corresponds to pigs that show PCVAD clinical signs with the presence of PCVAD-
18 specific histopathological lesions. Consequently, even if there is a good chance of identifying *M.*
19 *hyopneumoniae* in PCV-2–positive animals, the present study did not involve *M. hyopneumoniae*
20 in animals that have clinical signs related to PCVAD (Table 4). The most interesting finding was
21 the significantly increased odds ratios of isolating 2 major porcine respiratory pathogenic
22 bacteria, *A. pleuropneumoniae* and *S. suis* pathogenic serotypes, as PCV-2 viral load increases
23 (Table 4). As opposed to previous studies,[16] the present study did distinguish between serotypes

of *S. suis*. Herein, association with PCV-2/vl was significant only for the pathogenic serotypes of *S. suis* (Table 4). Yet, despite a good statistical relationship between *A. pleuropneumoniae*, *S. suis* pathogenic serotypes, and PCV-2 viral load, it is impossible to determine if those bacterial pathogens increased the risk of PCVAD development or if they colonized the host respiratory tract following PCV-2 infection.

In conclusion, the new mrtqPCR diagnostic assay is suitable for the sensitive identification and differentiation of PCV-2 and is a more convenient approach than RFLP, partial or entire viral genome sequencing, and conventional PCR techniques.[6,23,30] Furthermore, it could efficiently quantify PCV-2 in submitted samples and provide a risk assessment in regards to the odds of developing PCVAD (Fig. 4a). Until now, it was believed that PRRSV played a major role in PCVAD development, and experimental infections with both pathogens (PRRSV and PCV-2) may confirm that.[50] Nevertheless, if there is apparent statistical relationship with PCVAD and PRRSV in the present report, the impact of PRRSV in regards to PCVAD seems negligible, as previously reported,[52] when statistical analysis takes into account other relevant variables like the PCV-2 viral load. Since the odds of isolating *A. pleuropneumoniae* and pathogenic *S. suis* is significantly higher when the PCV-2 viral load increases, new experiments are now being conducted to evaluate the impact of PCV-2 and the mechanisms involved on the bacterial colonization of the swine respiratory tract.

Acknowledgements

The authors are grateful to Cynthia M. Guilbert for critically reviewing the manuscript. This work was supported by the Fédération des Producteurs de Porcs du Québec (FPPQ), the Centre d’Insémination Porcine du Québec Inc. (CIPQ), and the Conseil pour le Développement de l’Agriculture du Québec (CDAQ). The authors are also extremely grateful to the Ministère de

l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ) and his subsidiary, the Institut National de Santé Animale (INSA), for their permission to access complete diagnostic result files to compile the databank that was used in the retrospective epidemiological survey.

Sources and manufacturers

a. Beckman Coulter Canada Inc., Mississauga, Ontario, Canada.

b. Qiagen, Valencia, CA

c. Invitrogen Canada Inc., Burlington, Ontario, Canada.

d. Biospec Products, Bartlesville, OK.

e. Ibis Therapeutics, Carlsbad, CA.

f. Roche Diagnostics, Laval, Quebec, Canada.

g. Cepheid, Sunnyvale, CA.

h. Tetracore Inc., Rockville, MD.

i. SAS Institute Inc., Cary, NC.

References

1. Allan GM, McNeilly F, Kennedy S, et al.: 1998, Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J Vet Diagn Invest 10:3–10.

2. Ananth CV, Kleinbaum DG: 1997, Regression models for ordinal responses: a review of methods and applications. Int J Epidemiol 26:1323–1333.

3. Bender R, Grouven U: 1998, Using binary logistic regression models for ordinal data with non-proportional odds. J Clin Epidemiol 51:809–816.

4. Brunborg IM, Moldal T, Jonassen CM: 2004, Quantitation of porcine circovirus type 2 isolated from serum/plasma and tissue samples of healthy pigs and pigs with postweaning

1 multisystemic wasting syndrome using a TaqMan-based real-time PCR. J Virol Methods
2 122:171–178.
3 5. Carman S, Cai H, DeLay J, et al.: 2008, The emergence of a new strain of porcine circovirus-2
4 in Ontario and Quebec swine and its association with severe porcine circovirus associated
5 disease—2004–2006. Can J Vet Res 72. In press.
6 6. Carman S, McEwen B, DeLay J, et al.: 2006, Porcine circovirus-2 associated disease in swine
7 in Ontario (2004 to 2005). Can Vet J 47:761–762.
8 7. Chae C: 2004, Postweaning multisystemic wasting syndrome: a review of aetiology, diagnosis
9 and pathology. Vet J 168:41–49.
10 8. Chae C: 2005, A review of porcine circovirus 2-associated syndromes and diseases. Vet J
11 169:326–336.
12 9. Cheung AK: 2003, Comparative analysis of the transcriptional patterns of pathogenic and
13 nonpathogenic porcine circoviruses. Virology 310:41–49.
14 10. Cheung AK: 2003, Transcriptional analysis of porcine circovirus type 2. Virology 305:168–
15 180.
16 11. Cheung AK, Lager KM, Kohutyuk OI, et al.: 2007, Detection of two porcine circovirus type
17 2 genotypic groups in United States swine herds. Arch Virol 152:1035–1044.
18 12. Chung WB, Chan WH, Chaung HC, et al.: 2005, Real-time PCR for quantitation of porcine
19 reproductive and respiratory syndrome virus and porcine circovirus type 2 in naturally-
20 infected and challenged pigs. J Virol Methods 124:11–19.
21 13. Clark EG: 1997, Post-weaning multisystemic wasting syndrome. *In*: 28th annual meeting of
22 the American Association of Swine Practitioners, pp. 499–501. AASP, Quebec City,
23 Canada.

1 14. D'Allaire S, Moore C, Cote G: 2007, A survey on finishing pig mortality associated with
2 porcine circovirus diseases in Quebec. Can Vet J 48:145–146.
3 15. de Boisseson C, Beven V, Bigarre L, et al.: 2004, Molecular characterization of Porcine
4 circovirus type 2 isolates from post-weaning multisystemic wasting syndrome-affected and
5 non-affected pigs. J Gen Virol 85:293–304.
6 16. Dorr PM, Baker RB, Almond GW, et al.: 2007, Epidemiologic assessment of porcine
7 circovirus type 2 coinfection with other pathogens in swine. J Am Vet Med Assoc 230:244–
8 250.
9 17. Dupont K, Nielsen EO, Baekbo P, Larsen LE: 2008, Genomic analysis of PCV2 isolates
10 from Danish archives and a current PMWS case-control study supports a shift in genotypes
11 with time. Vet Microbiol 128:56–64.
12 18. Ellis J, Clark E, Haines D, et al.: 2004, Porcine circovirus-2 and concurrent infections in the
13 field. Vet Microbiol 98:159–163.
14 19. Ellis J, Hassard L, Clark E, et al.: 1998, Isolation of circovirus from lesions of pigs with
15 postweaning multisystemic wasting syndrome. Can Vet J 39:44–51.
16 20. Fenaux M, Halbur PG, Gill M, et al.: 2000, Genetic characterization of type 2 porcine
17 circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in
18 different geographic regions of North America and development of a differential PCR-
19 restriction fragment length polymorphism assay to detect and differentiate between
20 infections with PCV-1 and PCV-2. J Clin Microbiol 38:2494–2503.
21 21. Fenaux M, Opriessnig T, Halbur PG, et al.: 2004, Two amino acid mutations in the capsid
22 protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and
23 attenuated the virus in vivo. J Virol 78:13440–13446.

22. Gagnon CA, Lachapelle G, Langelier Y, et al.: 2003, Adenoviral-expressed GP5 of porcine respiratory and reproductive syndrome virus differs in its cellular maturation from the authentic viral protein but maintains known biological functions. Arch Virol 148:951–972.

23. Gagnon CA, Tremblay D, Tijssen P, et al.: 2007, The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. Can Vet J 48:811–819.

24. Grau-Roma L, Crisci E, Sibila M, et al.: 2008, A proposal on porcine circovirus type 2 (PCV2) genotype definition and their relation with postweaning multisystemic wasting syndrome (PMWS) occurrence. Vet Microbiol 128:23–35.

25. Hamel AL, Lin LL, Nayar GP: 1998, Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J Virol 72:5262–5267.

26. Harding JC: 1997, Post-weaning multisystemic wasting syndrome (PMWS): preliminary epidemiology and clinical presentation. *In*: 28th annual meeting of the American Association of Swine Practitioners, p. 503. Quebec City, Canada.

27. Harding JC: 2004, The clinical expression and emergence of porcine circovirus 2. Vet Microbiol 98:131–135.

28. Hesse R, Kerrigan M, Rowland RR: 2008, Evidence for recombination between PCV2a and PCV2b in the field. Virus Res 132:201–207.

29. Higgins R, Gottschalk M: 1999, Streptococcal diseases. *In*: Diseases of swine, eds. D'Allaire S, Mengeling WL, Taylor DJ, 8th ed., pp. 563–578. Iowa State University Press, Ames, IA.

30. Horlen KP, Schneider P, Anderson J, et al.: 2007, A cluster of farms experiencing severe porcine circovirus associated disease: clinical features and association with the PCV2b genotype. J Swine Health Prod 15:270–278.

1 31. Hosmer DW, Lemeshow S: 1989, Applied logistic regression. John Wiley & Sons, New
2 York, NY.
3 32. Kim J, Chae C: 2004, A comparison of virus isolation, polymerase chain reaction,
4 immunohistochemistry, and in situ hybridization for the detection of porcine circovirus 2
5 and porcine parvovirus in experimentally and naturally coinfected pigs. J Vet Diagn Invest
6 16:45-50.
7 33. Larochelle R, Magar R, D'Allaire S: 2002, Genetic characterization and phylogenetic
8 analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical
9 conditions. Virus Res 90:101–112.
10 34. Lee CS, Moon HJ, Yang JS, et al.: 2007, Multiplex PCR for the simultaneous detection of
11 pseudorabies virus, porcine cytomegalovirus, and porcine circovirus in pigs. J Virol
12 Methods 139:39–43.
13 35. Liu J, Chen I, Du Q, et al.: 2006, The ORF3 protein of porcine circovirus type 2 is involved
14 in viral pathogenesis in vivo. J Virol 80:5065–5073.
15 36. Liu J, Chen I, Kwang J: 2005, Characterization of a previously unidentified viral protein in
16 porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. J Virol
17 79:8262–8274.
18 37. Ma CM, Hon CC, Lam TY, et al.: 2007, Evidence for recombination in natural populations
19 of porcine circovirus type 2 in Hong Kong and mainland China. J Gen Virol 88:1733–1737.
20 38. Mankertz A, Hillenbrand B: 2001, Replication of porcine circovirus type 1 requires two
21 proteins encoded by the viral rep gene. Virology 279:429–438.
22 39. McCullagh P: 1980, Regression models for ordinal data. J Royal Stat Soc Series B 42:109–
23 142.

1 40. McNeilly F, McNair I, O'Connor M, et al.: 2002, Evaluation of a porcine circovirus type 2-
2 specific antigen-capture enzyme-linked immunosorbent assay for the diagnosis of
3 postweaning multisystemic wasting syndrome in pigs: comparison with virus isolation,
4 immunohistochemistry, and the polymerase chain reaction. J Vet Diagn Invest 14:106-112.
5 41. Meehan BM, McNeilly F, Todd D, et al.: 1998, Characterization of novel circovirus DNAs
6 associated with wasting syndromes in pigs. J Gen Virol 79(Pt 9):2171–2179.
7 42. Nawagitgul P, Morozov I, Bolin SR, et al.: 2000, Open reading frame 2 of porcine circovirus
8 type 2 encodes a major capsid protein. J Gen Virol 81:2281–2287.
9 43. Olvera A, Cortey M, Segales J: 2007, Molecular evolution of porcine circovirus type 2
10 genomes: phylogeny and clonality. Virology 357:175–185.
11 44. Olvera A, Sibila M, Calsamiglia M, et al.: 2004, Comparison of porcine circovirus type 2
12 load in serum quantified by a real time PCR in postweaning multisystemic wasting
13 syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs. J Virol
14 Methods 117:75–80.
15 45. Opriessnig T, Thacker EL, Yu S, et al.: 2004, Experimental reproduction of postweaning
16 multisystemic wasting syndrome in pigs by dual infection with *Mycoplasma*
17 *hyopneumoniae* and porcine circovirus type 2. Vet Pathol 41:624–640.
18 46. Ouardani M, Wilson L, Jette R, et al.: 1999, Multiplex PCR for detection and typing of
19 porcine circoviruses. J Clin Microbiol 37:3917–3924.
20 47. Payment P, Trudel M: 1989, Manuel de techniques virologiques. Presses de l'Université du
21 Québec, Québec, Canada. In French.

48. Pogranichniy RM, Yoon KJ, Harms PA, et al.: 2002, Case-control study on the association of porcine circovirus type 2 and other swine viral pathogens with postweaning multisystemic wasting syndrome. J Vet Diagn Invest 14:449–456.

49. Racine S, Kheyar A, Gagnon CA, et al.: 2004, Eucaryotic expression of the nucleocapsid protein gene of porcine circovirus type 2 and use of the protein in an indirect immunofluorescence assay for serological diagnosis of postweaning multisystemic wasting syndrome in pigs. Clin Diagn Lab Immunol 11:736–741.

50. Rovira A, Balasch M, Segales J, et al.: 2002, Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J Virol 76:3232–3239.

51. Segales J, Allan GM, Domingo M: 2005, Porcine circovirus diseases. Anim Health Res Rev 6:119–142.

52. Segales J, Calsamiglia M, Rosell C, et al.: 2002, Porcine reproductive and respiratory syndrome virus (PRRSV) infection status in pigs naturally affected with post-weaning multisystemic wasting syndrome (PMWS) in Spain. Vet Microbiol 85:23–30.

53. Sorden SD, Harms PA, Nawagitgul P, et al.: 1999, Development of a polyclonal-antibody-based immunohistochemical method for the detection of type 2 porcine circovirus in formalin-fixed, paraffin-embedded tissue. J Vet Diagn Invest 11:528-530.

54. Tischer I, Gelderblom H, Vettermann W, Koch MA: 1982, A very small porcine virus with circular single-stranded DNA. Nature 295:64–66.

55. Yang ZZ, Habib M, Shuai JB, Fang WH: 2007, Detection of PCV2 DNA by SYBR Green I-based quantitative PCR. J Zhejiang Univ Sci B 8:162–169.

1 **Table 1.** Design of the Porcine circovirus-associated disease compatible (PCVAD/c) score and

2 distribution of clinical cases in the score system.

PCVAD/c score	PCVAD clinical signs	PCVAD histopathological lesions	No. of cases	Percentage
0	No	No	49	40.50
1	Yes	No	5	4.13
2	No	Yes	18	14.87
3	Yes	Yes	49	40.50

3

1 **Table 2.** *Porcine circovirus-2* (PCV-2) genotype classification results based on the new

2 multiplex real-time quantitative polymerase chain reaction assay and their prevalence in Porcine

3 circovirus-associated disease compatible (PCVAD/c) and non compatible cases.*

Genotype classification	Total no. of cases	Distribution by PCVAD/c status	
		Negative†	**Positive‡**
PCV-2a	5 (4.13)	3 (60.00)	2 (40.00)
PCV-2b	112 (92.56)	45 (40.18)	45 (40.18)
PCV-2a and -2b	4 (3.31)	1 (25.00)	2 (50.00)
Total	121	49 (40.50)	49 (40.50)

4 * Numbers in parentheses are percentages.

5 † Based on grade 0 PCVAD/c score (see Table 1).

6 ‡ Based on grade 3 PCVAD/c score (see Table 1).

7

1 **Table 3.** Odds ratios of having a Porcine circovirus-associated disease compatible (PCVAD/c)

2 score greater than zero, as a function of *Porcine circovirus-2* (PCV-2) viral load, and result of

3 immunohistochemistry (IHC) testing.*

Scenario	IHC result	PCV-2/vl	Odds ratio	95% confidence interval
1	Negative	With 1 log-scale unit increase	4.08	2.66–6.25
2	Positive	Without increase compared to reference case	3.52×10^4	2.72×10^1–4.57×10^7
3	Positive	With 1 log-scale unit increase	5.45×10^4	6.93×10^1–4.29×10^7

4 * PCV-2/vl = logarithm of *Porcine circovirus-2* viral load. Odds ratios were determined using

5 the following reference case: negative IHC result and PCV-2/vl equal 0.

6

1 **Table 4.** Type of effect and statistical significance of the Porcine circovirus-associated disease

2 compatible (PCVAD/c) score, *Porcine circovirus-2* (PCV-2) viral load, and the presence and

3 viral load of *Porcine respiratory and reproductive syndrome virus* (PRRSV) on the odds of

4 isolating common swine pathogens.*

Pathogen	Prevalence in PCVAD/c grade 3 vs. grade 0 (%/%)	Model P-value	Odds ratio† (P-value, type-3 analysis)			
			PCVAD‡	PCV-2/vl	PRRSV	PRRSV/vl
Actinobacillus pleuropneumoniae	8.16/0	0.0112	NS	1.887 (0,0583)	NS	NS
Arcanobacterium pyogenes	12.24/18.37	NS	NS	NS	NS	NS
Bordetella bronchiseptica	8.16/16.33	0.0590	NS	0.779 (0,0393)	NS	1.305 (0,1267)
Brachyspira hyodysenteriae	2.04/6.12	NS	NS	NS	NS	NS
Escherichia coli	16.33/36.73	0.0108	NS	0.829 (0,0473)	0.161 (0,0478)	1.479 (0,1377)
Haemophilus parasuis	24.49/24.49	0.0052	NS	NS	NS	1.443 (0.0065)
Mycoplasma hyopneumoniae	6.12/10.20	0.0088	7.800 (0,0127)§	NS	NS	NS
Pasteurella multocida	42.86/34.69	0.0009	NS	NS	NS	1.474 (0,0014)
Salmonella spp.	2.04/6.12	0.1085	NS	0.672 (0,1568)	NS	NS
Staphylococcus hyicus	12.24/18.37	NS	NS	NS	NS	NS
Streptococcus suis						
All serotypes	63.27/53.06	0.1085	NS	NS	NS	1.203 (0,1131)
Pathogenic serotypes¦	67.74/37.04	0.0172	NS	1.369 (0,0223)	NS	NS
Swine influenza virus	12.24/8.16	NS	NS	NS	NS	NS

5 * NS = nonsignificant; PCV-2/vl = logarithm of *Porcine circovirus-2* viral load; PRRSV/vl =

6 logarithm of *Porcine reproductive and respiratory syndrome virus* viral load.

7 † Odds ratio values lower than 1 indicate decreased risk of isolation and values greater than 1

8 indicate increased risk of isolation.

9 ‡ Statistical association was made with the PCVAD/c grade score as described in Table 1.

10 § A significant odds ratio to recover *Mycoplasma hyopneumoniae* was made only with

11 PCVAD/c grade score of 2.

12 ¦ *Streptococcus suis* pathogenic serotypes (1/2, 1, 2, 3, 4, and 7) commonly associated with

13 clinical disease.

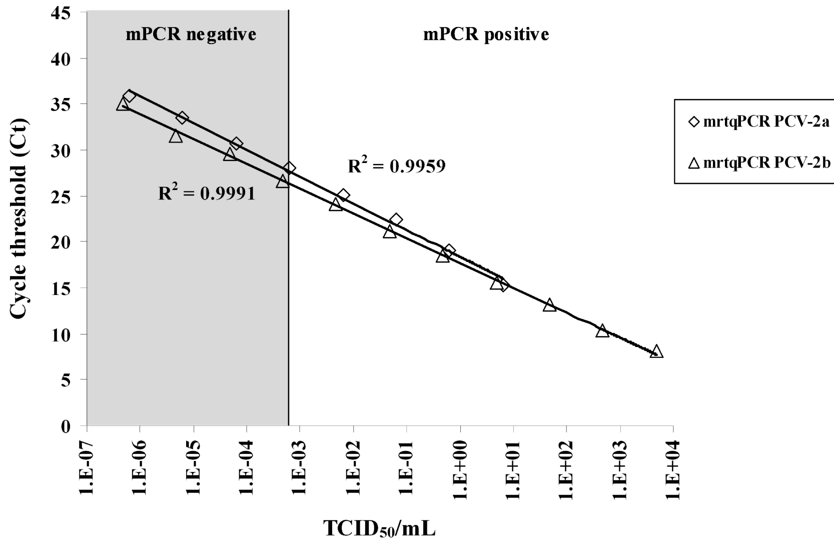
14

Figure legends

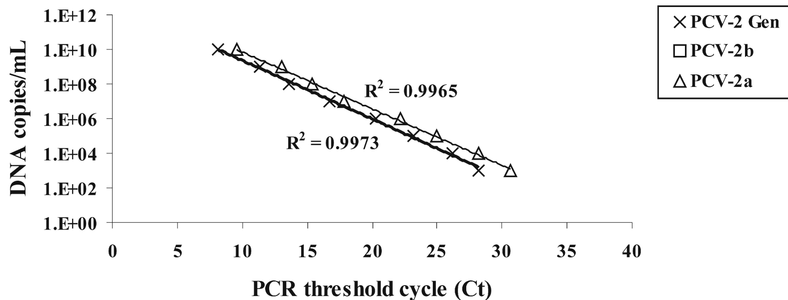
Figure 1. Comparison of the sensitivity of the multiplex real-time quantitative polymerase chain reaction (mrtqPCR) assay to that of a conventional multiplex PCR assay (mPCR). Ten-fold serial dilutions of *Porcine circovirus-2* genotypes 2a and 2b (PCV-2a [strain IAF 2897] and -2b [strain FMV-06-1717]) were used as templates in the mrtqPCR and mPCR assays. The R^2 curve values are indicated next to their corresponding curve in the figure. The mPCR was carried out as previously reported.[44] The mrtqPCR and mPCR assays were done in triplicate and similar results were obtained. Therefore, to improve the clarity of the figure, standard deviation results have been omitted. Ct = threshold cycle value obtained with the mrtqPCR assay; mrtqPCR PCV-2a (◊) = PCR results obtained with the Circo-2a-Probe; mrtqPCR PCV-2b (Δ) = PCR results obtained with the Circo-2b-Probe.

Figure 2. Differentiation of *Porcine circovirus-2* (PCV-2) genotypes by the multiplex real-time quantitative polymerase chain reaction (mrtqPCR) assay. Ten-fold serial dilutions of PCV-2 recombinant plasmids (pCR2.1/PCV-2a and pCR2.1/PCV-2b) were used as templates in the mrtqPCR assay. The R^2 curve values are indicated next to their corresponding curve in the figure. **A,** pCR2.1/PCV-2a was used as template. Negative results (>37 Ct) were obtained for PCV-2b detection. **B,** pCR2.1/PCV-2b was used as template. Negative results (>37 Ct) were obtained for PCV-2a detection. **C,** both pCR2.1/PCV-2a and pCR2.1/PCV-2b were used as template. The mrtqPCR assay was done in triplicate and similar results were obtained. Therefore, to improve the clarity of the figure, standard deviation results have been omitted. Ct = threshold cycle value obtained with the mrtqPCR assay; PCV-2 Gen (×) = PCR results obtained with the Circo-Gen-Probe; PCV-2a (Δ) = PCR results obtained with the Circo-2a-Probe; PCV-2b (□) = PCR results obtained with the Circo-2b-Probe.

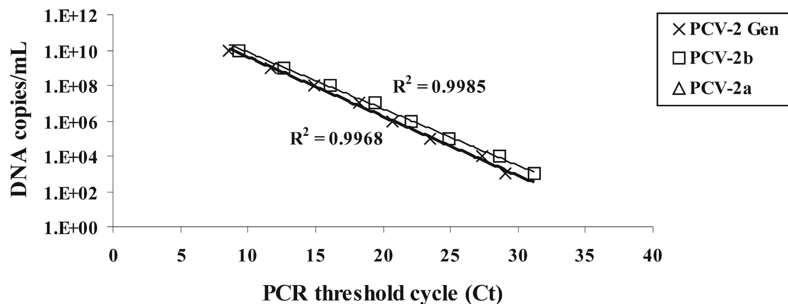
1 **Figure 3.** *Porcine circovirus-2* (PCV-2) quantification: infectious particles versus DNA viral
2 genome copies. Ten-fold serial dilutions of known amounts of PCV-2b reference virus (FMV-
3 06-1717) and of PCV-2b recombinant plasmid (pCR2.1/PCV-2b) were used as DNA templates
4 in the multiplex real-time quantitative polymerase chain reaction (mrtqPCR) assay. The amount
5 of PCV-2b virus was expressed in $TCID_{50}$/mL and the amount of pCR2.1/PCV-2b was
6 expressed in number of DNA copies/mL. The R^2 curve values and the equations of the curve are
7 indicated next to their corresponding curve in the figure. The data obtained in regards to PCV-2a
8 virus and recombinant PCV-2a plasmid were not shown since the curves were overlapping with
9 those already present in the figure. Ct = threshold cycle value obtained with the mrtqPCR assay.
10 **Figure 4.** Relationship between the logarithms of the *Porcine circovirus-2* (PCV-2) and *Porcine*
11 *respiratory and reproductive syndrome virus* (PRRSV) viral loads, and the Porcine circovirus-
12 associated disease compatible (PCVAD/c) grade score classification. **A,** the amount of PCV-2
13 found in submitted tissues (lungs and lymph nodes) was evaluated using the multiplex real-time
14 quantitative polymerase chain reaction (mrtqPCR) assay and was expressed as DNA copies/gram
15 of tissue. **B,** the amount of PRRSV found in submitted tissues (lungs and lymph nodes) was
16 evaluated using a PRRSV real-time PCR diagnostic kit,[h] as described in the materials and
17 methods section of the manuscript, and was expressed in $TCID_{50}$/gram of tissue. All PRRSV-
18 negative cases were not included in panel B. Moreover, each case submitted to the diagnostic
19 laboratory was classified following their immunohistochemistry (IHC) results. PCV-2/vl = PCV-
20 2 viral load; PRRSV/vl = PRRSV viral load. (Note: to improve clarity, the cases with negative
21 IHC results had their PCVAD/c scores shifted upwards by 0.06, and cases with positive IHC
22 results had their PCVAD/c scores shifted upwards by 0.12.)


mrtqPCR
results
45
40
35
30
25
20
15
10
5
0
Cycle threshold (Ct)
mPCR negative
mPCR positive
R2 = 0.9959
R2 = 0.9991
mrtqPCR PCV-2a
mrtqPCR PCV-2b
1.E-07
1.E-06
1.E-05
1.E-04
1.E-03
1.E-02
1.E-01
1.E+00
1.E+01
1.E+02
1.E+03
1.E+04
TCID50/mL

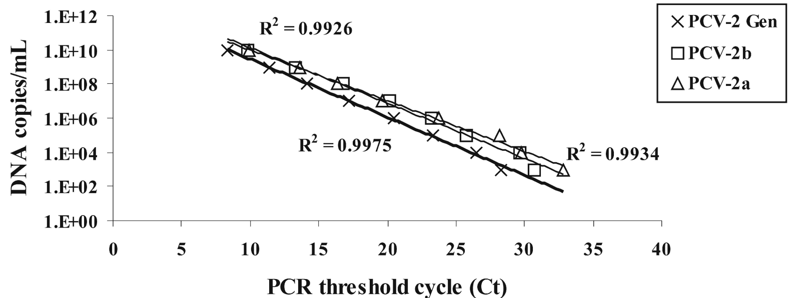
A)

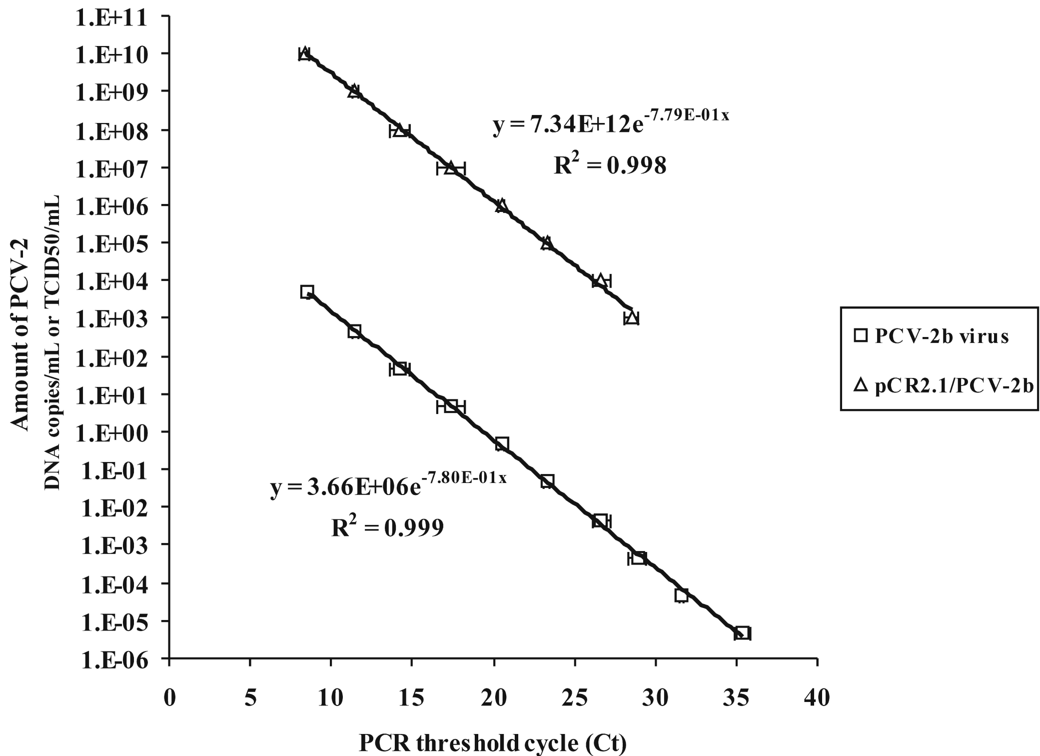


B)



C)





Amount of PCV-2
DNA copies/mL or TCID50/mL
1.E+11
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
1.E-05
1.E-06
0
5
10
15
20
25
30
35
40
PCR threshold cycle (Ct)
y = 7.34E+12e^-7.79E-01x
R^2 = 0.998
y = 3.66E+06e^-7.80E-01x
R^2 = 0.999
PCV-2b virus
pCR2.1/PCV-2b

A)

PCVAD/c score

3
2
1
0

3.5 4.5 5.5 6.5 7.5 8.5 9.5 10.5 11.5

PCV-2/vl

× IHC untested

○ Negative IHC

● Positive IHC

PCVAD average score

multivariate polytomous logistic regression: P=0.0161

B)

PCVAD/c score

3
2
1
0

-2 -1 0 1 2 3 4 5

PRRSV/vl

× IHC untested

○ Negative IHC

● Positive IHC

PCVAD average score

multivariate polytomous logistic regression: P>0.9