Université de Montréal

IN VITRO AND *IN VIVO* VIRULENCE EVALUATION OF THE NEW GENOTYPE OF PORCINE CIRCOVIRUS TYPE 2 AND IDENTIFICATION OF A NEW CELL LINE PERMISSIVE TO VIRUS REPLICATION

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IN VITRO AND *IN VIVO* VIRULENCE EVALUATION OF THE NEW GENOTYPE OF PORCINE CIRCOVIRUS TYPE 2 AND IDENTIFICATION OF A NEW CELL LINE PERMISSIVE TO VIRUS REPLICATION

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RÉSUMÉ

Vers la fin de l'année 2004, la population porcine canadienne a connu une recrudescence marquée du syndrome de dépérissement en post-severage (SDPS), problème que l'on avait associé à l'émergence d'un nouveau génotype de *Circovirus porcin de type 2* (PCV-2b) jusque là inconnu en Amérique du Nord. Ainsi, il est devenu important de démontrer que la souche de PCV-2b circulant dans les élevages porcins canadiens est plus virulente que l'ancienne souche (PCV-2a). Des clones plasmidiques infectieux de l'ancien génotype (PCV-2a) et du nouveau génotype (PCV-2b) viral ont été conçus (pPCV-2a et pPCV-2b). L'efficacité de la lignée cellulaire épithéliale porcine de rein (PK-15) transfectée par pPCV-2a et par pPCV-2b a permis un cycle complet de réplication virale. Par la suite, la production de particules infectieuses de PCV-2 a été évaluée durant huit passages cellulaires consécutifs. Ainsi, dix fois plus de particules virales infectieuses de PCV-2b ont été recueillies au 8^{e} passage par rapport au PCV-2a. Plus tard la lignée cellulaire épithéliale trachéale de porcelets nouveau-nés (NPTr) s'est révélée comme étant permissive au PCV-2 et pouvant par conséquent permettre la production de particules virales infectieuses. Cette découverte est très importante car jusqu'à maintenant, il n'y avait, à notre connaissance, qu'un seul type de lignée cellulaire immortalisée (cellules épithéliales porcines de rein) qui permet la production de particules virales infectieuses, suite à l'infection virale.

Donc, pour corroborer les résultats précédents, les cinétiques de réplication virale de PCV-2 ont été évaluées chez les cellules PK-15 et NPTr. La cinétique de réplication virale de PCV-2b s'est montrée considérablement plus efficace que celle de PCV-2a dans les deux lignées cellulaires. Aucune différence significative par rapport à l'efficacité de la réplication virale n'a été observée entre les deux lignées cellulaires. En conclusion, PCV-2b est approximativement 6 à 10 fois plus efficace que PCV-2a pour la synthèse de particules virales infectieuses dans deux lignées cellulaires épithéliales porcines. De plus, la lignée cellulaire NPTr pourrait servir en guise de nouveau modèle *in vitro* pour l'étude de la pathogénèse du PCV-2. Par conséquent, l'efficacité de réplication virale de PCV-2b en comparaison à celle de PCV-2a pourrait, au moins en partie, justifier la recrudescence marquée du SDPS au Canada au cours des dernières années.

MOTS CLÉS : SDPS, cellules NPTr, réplication virale, génotypes de PCV-2, circovirus porcin.

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 unknown in North America. Thus, it became important to demonstrate that the PCV-2b strain circulating in Canadian swine herds is more virulent than the older PCV-2a strain. Infectious DNA clones of PCV-2a and PCV-2b were constructed (pPCV-2a and pPCV-2b). The efficacy of pPCV-2a and pPCV-2b transfected porcine kidney epithelial cell line (PK-15) to allow a complete virus replication cycle and production of PCV-2 infectious particles was evaluated during eight consecutive cell passages. Ten times more PCV-2b infectious viral particles were recovered at the 8^{th} cell passage compared to PCV-2a. Later on, a new porcine cell line, the newborn piglet tracheal (NPTr) epithelial cell line, was found to be permissive to PCV-2 and was consequently able to permit infectious viral particles production. This finding is interesting because until now, there was only one type of immortalized cell line (the porcine kidney epithelial cells) known to permit infectious viral particles production.

Thus, to corroborate early results, the PCV-2a and PCV-2b replication kinetics were evaluated in PK-15 and NPTr cells. PCV-2b virus replication kinetic was significantly more efficient in both cell lines compared to PCV-2a. No differences in virus replication efficiency could be found between both cell lines. In conclusion, PCV-2b is about 6 to 10 times more efficient than the PCV-2a for the synthesis of infectious viral particles in two porcine epithelial cell lines. Moreover, the NPTr cell line could be used as a new *in vitro* model for studying the PCV-2 pathogenesis. Consequently, the virus replication efficiency of PCV-2b compared to PCV-2a could, at least in part, explain the increase in the occurrence of PCVAD in Canada during the recent years.

KEYWORDS: PMWS, NPTr cells, virus replication, PCV-2 genotypes, porcine circovirus

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LIST OF ABBREVIATIONS

aa	Amino acids
A. pleuropneumoniae	*Actinobacillus pleuropneumoniae*
BFDV	Beak and feather disease virus
CaCV	Canary circovirus
Cap	Capsid protein
CAV	Chicken anemia virus
CDCD	Cesarean-derived colostrum-deprived
ConA	Concanavalin A
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
DuCV	Duck circovirus
FBS	Fetal bovine serum
FiCV	Finch circovirus
GoCV	Goose circovirus
GuCV	Gull circovirus
IFA	Immunofluorescence assay
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
ISH	*In situ* hybridization
KHL/IFCA	Keyhole limpet haemocyanin in incomplete Freund's adjuvant

LPS	Lipopolysaccharide
M. hyopneumoniae	*Mycroplasma hyopneumoniae*
MOI	Multiplicity of infection
mrtqPCR	Multiplex real-time quantitative polymerase chain reaction
NPTr	Newborn piglet tracheal epithelial cell line
OR	Odd ratio
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV-2	Porcine circovirus – type 2
PCV-2a	Porcine circovirus – type 2 (genotype 2a)
PCV-2b	Porcine circovirus – type 2 (genotype 2b)
PCVAD	Porcine Circovirus Associated Disease
PDNS	Porcine Dermatitis and Nephropathy Syndrome
PERV	Porcine endogenous retrovirus
PEPSCAN	Peptide scanning
PFA	Paraformaldehyde
PiCV	Pigeon circovirus
PK-15	Porcine kidney epithelial cell line
PMWS	Postweaning Multisystemic Wasting Syndrome
pPCV-2a	Infectious DNA clone of PCV-2a
pPCV-2b	Infectious DNA clone of PCV-2b

PPV	Porcine parvovirus
PRDC	Porcine Respiratory Disease Complex
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
rcf	Relative centrifugal force
Rep	Replicase
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
S. suis	*Streptococcus suis*
SDPS	Syndrome de dépérissement en post-severage
SIV	Swine influenza virus
SK	Swine kidney epithelial cell line
SPF	Specific pathogen free
ss	Single-stranded

To my parents, my kids,
and my wife

For believing in me
and
For supporting me

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I. INTRODUCTION

Postweaning Multisystemic Wasting Syndrome (PMWS) is a swine disease that was first recognized in Canada in 1991 [39, 71]. Afterwards, the syndrome was reported worldwide [8, 10, 24, 128, 147, 151]. The primary etiological agent responsible for PMWS has been identified as a circovirus named *Porcine circovirus-2* (PCV-2; family *Circoviridae*, genus *Circovirus*) [8, 52, 70, 106].

At the end of 2004, the swine industry in the province of Québec, Canada, experienced a significant increase in death rate related to PMWS [42]. It was hypothesized that this emerging problem was caused by the presence of a new type of circulating PCV-2 strain. This was confirmed by sequencing the entire viral genome of several PCV-2 strains originated from PMWS-affected herds [63]. Interestingly, this newly recovered PCV-2 genotype (named PCV-2b in comparison with the previously circulating genotype named PCV-2a) had already been reported in Asia and Europe [46] and has also been recently reported in 2007 in the United States [34, 79]. The appearance of the new PCV-2b genotype in Canada could explain the death rate increase related to PMWS, but this relationship has to be confirmed. Consequently, one of the main objectives of the swine industry and health scientists is to establish if there is a virulence variation between both PCV-2 genotypes (PCV-2a and PCV-2b).

The pathogenesis of PCV-2 infection and major cell types that support PCV-2 replication are poorly understood. The presence of a PCV-2 antigen and nucleic acids has been shown in different cell types *in vivo* by immunohistochemistry (IHC) or *in situ* hybridization (ISH). *In vitro*, the only immortalized cell line that support PCV-2 replication is porcine kidney epithelial cells [10, 106, 111, 142], and PCV-2 isolation and production is routinely performed using porcine kidney (PK-15) and swine kidney (SK) epithelial cell lines [10, 142]. Nonetheless, it is a good assumption to believe that kidney cells are not the first infected cells that favor propagation of the virus in the organism. The oro-nasal route is considered the most likely and frequent route of PCV-2 transmission and experimental infection studies which have mainly used the intranasal route of inoculation support that idea [16, 53, 85, 128, 142].

Consequently, the goal of the present study is therefore: (1) to demonstrate that the PCV-2b strain that circulates in Canadian swine herds is more virulent *in vitro* and *in vivo* than the PCV-2a strain; (2) to establish if epithelial cells of the respiratory tract of swine

could support PCV-2 replication *in vitro*, and (3) to develop a diagnostic test that could rapidly and efficiently differentiate both genotypes.

II. LITERATURE REVIEW

POSTWEANING MULTISYSTEMIC WASTING SYNDROM – PMWS

1. History and definition

Postweaning Multisystemic Wasting Syndrome (PMWS) is a swine disease that was first recognized in Canada in 1991 [39, 71]. Afterwards, the syndrome was reported worldwide [3, 10, 24, 128, 147, 151]. The primary etiological agent responsible for PMWS has been identified as a circovirus named *Porcine circovirus-2* (PCV-2; family *Circoviridae*, genus *Circovirus*) [70].

PMWS is considered a multifactorial disease in pigs, in which a necessary factor is the presence of PCV-2. Since PCV-2 is ubiquitous in the pig population and infection doesn't equate to disease, a definition for PMWS was proposed [158]. Based on this definition, a diagnosis of PMWS requires (1) the presence of clinical signs such as wasting, weight loss and respiratory disease, (2) the presence of the hallmark PCV-2-associated microscopic lesions (lymphoid depletion and/or histiocytic replacement of follicles in lymphoid tissues or both), and (3) PCV-2 antigen or nucleic acids associated with the microscopic lesions as determined by immunohistochemistry (IHC) or *in situ* hybridization (ISH) [158].

2. Disease terminology

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), granulomatous enteritis, reproductive failure and necrotizing lymphadenitis [25, 72]. In addition, PCV-2 has also been associated with myocarditis and vasculitis in growing pigs, hepatitis, CNS disease, and exudative epidermitis [25, 72, 128]. Consequently, the term "porcine circovirus associated disease

(PCVAD)" is now accepted to describe the syndromes in which PCV-2 plays a role. The extent of the involvement of PCV-2 in swine diseases other than PMWS is currently poorly understood. PCV-2 infection is widespread and essentially all pig herds are infected with PCV-2 but relatively few have PCVAD.

3. Postweaning Multisystemic Wasting Syndrome - PMWS

The disease usually 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 [71, 158]. Depletion of lymphocytes in the lymphoid follicles and their replacement by macrophages are the hallmark lesions observed in this syndrome [10, 106]. PMWS has six fundamental clinical signs: wasting, dyspnea, enlarged lymph nodes, diarrhea, pallor, and jaundice [72]. While all the fundamental clinical signs may not be noted in a single pig, affected farms will present the majority of them over a period of time. Other clinical signs include coughing, fever, gastric ulceration, meningitis and sudden death [72]. Macroscopic lesions associated with PCV-2 infection include generalized lymphadenopathy, rubbery lungs with mottling and increased firmness, enlarged spleen and enlarged kidneys [39]. Characteristic microscopic lesions associated with PCV-2 infection and PMWS include lymphoid depletion and histiocytic replacement of follicles in lymphoid tissues. The lymphocellular depletion affects both lymphoid follicles and parafollicular zones [140]. Mild-to-severe granulomatous inflammation in lymphoid and other tissues also is commonly observed [11, 158]. Syncytial cells can be seen frequently, especially in lymph nodes, Peyer's patches and lamina propria of the intestinal villi [140]. Macrophages in affected lymphoid tissues may contain sharply demarcated, spherical, basophilic cytoplasmic inclusion bodies [39, 140]. The inclusions are either large and single or smaller and multiple with groups of up to 12 inclusions [140].

PMWS has been reported from almost all swine producing countries. Morbidity due to PMWS reportedly varies from as little as 4% to as high as 30% and mortality in affected pigs is typically 70 to 80% [11, 39, 71, 99, 139]. There are two forms of PMWS: an

endemic and an epidemic form. The endemic form is seen in North America, where PMWS usually results in low grade but persistent death losses. Rarely, 3 to 4 fold increase in postweaning mortality can be observed. The epidemic form, which is characterized by persistent mortality, appeared to be observed primarily in Europe but based on recent evidence, has become increasingly observed in eastern Canada [22, 23, 42, 47, 63] and North Carolina. Pork producers in Canada and the U.S. have experienced a devastating disease in growing pigs which is manifested as wasting and pneumonia and resulting in persistent mortality of 20-40% of the pigs between 10 weeks and market age (25 weeks) [128].

4. Porcine Dermatitis and Nephropathy Syndrome - PDNS

PDNS is clinically characterized by acute onset of skin lesions (raised purple skin lesions progressing to multifocal raised red scabs with black centers most prominent on the rear legs), fever and lethargy, and is almost always fatal [25]. Macroscopically, there are enlarged tan waxy kidneys with white foci and streaks [25]. Microscopically, there is systemic vasculitis with dermal and epidermal necrosis, and necrotizing and fibrinous glomerulonephritis [25]. The microscopic hallmark lesions of PDNS, generalized vasculitis and glomerulonephritis, are suggestive of a type III hypersensitivity reaction which is characterized by deposition of antigen-antibody aggregates or immune complexes on certain tissue sites [36, 141, 159].

There are two forms of PDNS described: the sporadic form and the epizootic form. With the sporadic form, the mortality is rarely above 0.5%. The epizootic form was first observed in 1999 in England, when there was a sudden, marked increase of PDNS cases [160, 161]. The within-herd mortality was reported to range from 0.25-20% and similar observations were made in the Netherlands [51, 171] . Investigations into this "outbreak" found that there was a clear temporal association of PMWS and PDNS; PDNS cases usually followed PMWS cases on the same farms. Studies have determined that the mean age of pigs affected by PMWS ranges from 6 to 14 weeks whereas the mean age of pigs affected by PDNS ranges from 12 to 16 weeks [69]. Similar observations were made in

Korea [37], and the authors of that study speculated that the presence of both PMWS and PDNS in the same herd but in different age groups was probably due to different strains of PCV-2 or varying susceptibility of the pigs [37]. A recent case-control study investigating PDNS in the Netherlands found that there was a significant association of high antibody titers to PCV-2 and the development of PDNS [171]. The authors were not able to show the PCV-2 antigen by IHC in all of the PDNS cases but they were able to confirm the presence of PCV-2 by polymerase chain reaction (PCR) in all cases of PDNS. Importantly, the authors were able to show that the porcine parvovirus or Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) nucleic acids were not present in many of the PDNS cases as determined by PCR [171]. A study comparing PCV-2 serum load in PMWS and PDNS cases found that PDNS cases had significantly lower numbers of PCV-2 in serum compared to healthy, subclinical PCV-2-infected pigs [119].

PORCINE CIRCOVIRUS TYPE 2

1. History

Porcine circovirus (PCV) was first recognized as a contaminant of the continuous porcine kidney cell line (PK-15) (ATCC-CCL31) in 1974 in Germany and described as picornavirus-like virus [163]. Under experimental conditions, the PCV-PK15-isolate did not induce disease in pigs [7, 164]. In the late 1990's, PCV was associated with a newly-emerged disease syndrome in pigs described as "postweaning multisystemic wasting syndrome" (PMWS) [3]. Sequence analysis of the PMWS-associated PCV revealed differences compared to the earlier described PCV [3, 8, 10, 52, 70, 106, 114]. In order to distinguish the pathogenic PMWS-associated PCV from the non-pathogenic PCV, the pathogenic type was designated porcine circovirus type 2 (PCV-2) and the non-pathogenic type as porcine circovirus type 1 (PCV-1).

2. Taxonomy

Both, PCV-1 and PCV-2 are members of the *Circoviridae* family [135, 168]. The *Circoviridae* family is divided into the genera *Circovirus* [*Circo* indicates that the genome of the virus has a circular conformation] and *Gyrovirus* [*Gyro* is a derivation from the Greek work "gyrus" meaning "ring" or "circuit"] (Fig. 1). The genus *Circovirus* contains the following species: psittacine beak and feather disease virus (BFDV), canary circovirus (CaCV), goose circovirus (GoCV), pigeon circovirus (PiCV), PCV-1 and PCV-2 [77, 131, 137, 166-168]. The species tentatively placed in the genus *Circovirus* are duck circovirus (DuCV), finch circovirus (FiCV), and gull circovirus (GuCV) (Fig. 1). The genus *Gyrovirus* contains only one virus, the chicken anemia virus (CAV) [168] (Fig. 1).

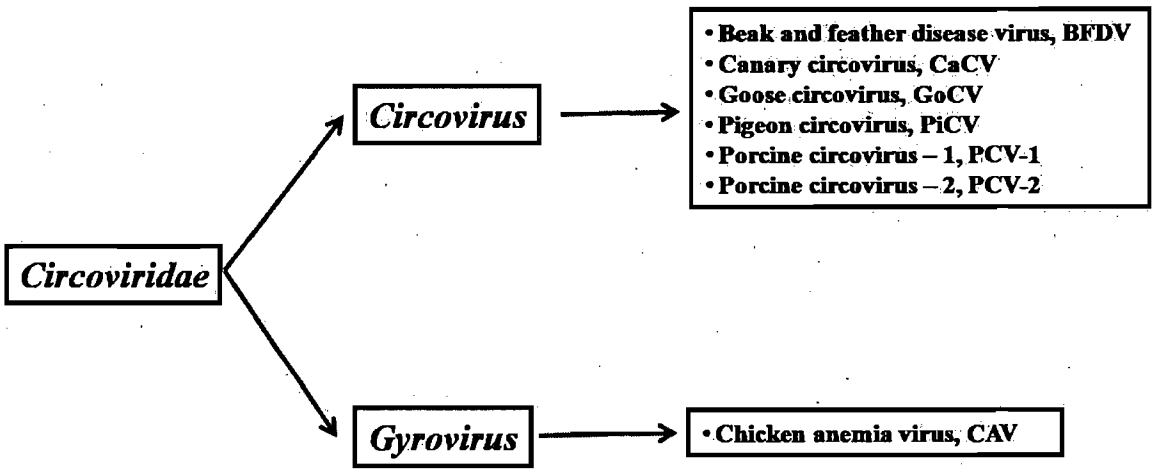

Figure 1. *Circoviridae* family classification.

3. Characterization of PCV-2

Viruses that belong to the *Circoviridae* family have characteristic virions that exhibit icosahedral symmetry and do not posses an envelope. The genomes are covalently closed, circular single-stranded (ss) DNAs, which range in size from 1.7 to 2.3 kb. The genome organization of CAV is negative sense, whereas those of the other circoviruses are ambisense [168]. CAV, PCV-2, and BFDV were found to have an icosahedral T=1 structure containing 60 capsid protein molecules arranged in 12 pentamer clustered units [41]. Circoviruses are host-specific or exhibit a narrow host range and the majority of those reported infect avian species [168]. Subclinical infections are common; however, circovirus infections are associated with clinical disease in some cases such as Chicken Anemia Virus, psittacine BFDV, PiCV disease and PCVAD in pigs. Circovirus infections in all species cause varying degrees of lymphoid depletion and are thought to be immunosuppressive [168].

PCV are among the smallest known mammalian viruses with a diameter of around 17 nm [164]. It is composed of coat protein subunits assembled in 12 pentameric units [41]. The genome of PCV-1 and PCV-2 share 68–76% nt-sequence similarity [105], whereas PCV-1 and PCV-2 intragenotype isolates share 97–99 and 94.6–99% sequence identity, respectively [46, 55]. PCV-2 genome contains three main open reading frames (ORFs)

(Fig. 2), which are the ORF1 (nt 398-1342) encoding the replicase protein (Rep); the ORF2 (nt 1381-314) encoding the immunogenic capsid protein (Cap); and the newly characterized protein encoded by ORF3 (nt 1018-704) which appears to contribute to virus-induced apoptosis of host infected cells [32, 33, 95, 104, 116]. The nucleotide positions refer to those on the putative virus encapsidated genomic ssDNA, and a schematic representation of the positions of these ORFs is shown in Fig 2. The intra-type amino acid homology between the Reps of PCV-1 and PCV-2 is approximately 99.4 and 95.7%, respectively, while the intra-type amino acid homology between the capsid proteins of PCV-1 and PCV-2 is 94 and 90%, respectively [55]. Thus, the capsid genes exhibit more sequence divergence than the Rep genes. In addition to ORF1, ORF2, and ORF3, it is predicted that there are another four potential ORFs (Fig. 2) encoding proteins larger than 5kDa [106].

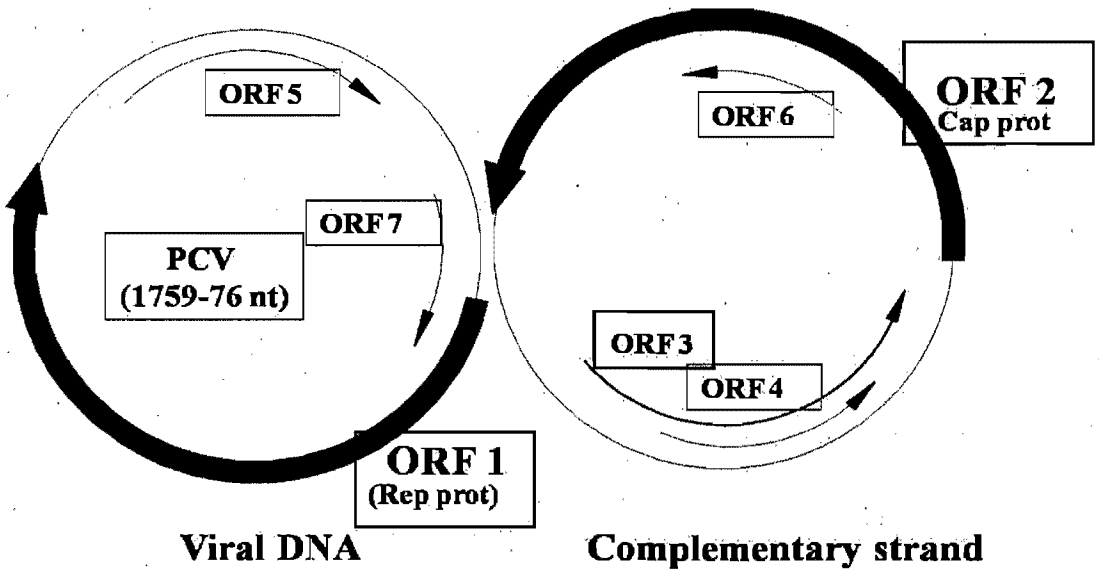

Figure 2. PCV genome organization: overlapping and ambissence ORFs. Adapted from *Meehan et al.* (1998).

identity for the capsid protein. One strain identified in a herd without PMWS was found to be 100.0% homologous to a PCV-2 from a PMWS herd [92]. In 2004, 31 pigs originating from 13 PMWS-affected herds and 25 pigs from 10 PMWS free herds were used for a comparative study, and 38 PCV-2 isolates were sequenced [46]. All the isolates shared 94.2-100% nucleotide identity. A wider nucleotide diversity was observed in the PCV-2 isolates originating from PMWS free herds compared with isolates from PMWS herds; however, residues found to be specific to non-PMWS strains were also found in PMWS strains and no molecular marker of virulence in PMWS strains could be identified [46]. In a case control study done in 2002, PCV-2 was not only found in PMWS cases but also in 62.5% of the control cases. Sequencing and genetic comparison revealed no differences between 5 PMWS-associated PCV-2 isolates and 4 PCV-2 isolates recovered from cases not associated with PMWS [133].

In contrast, by comparing the ORF2 of Taiwanese PCV-2 isolates associated with PMWS, PDNS, nervous signs, abortion, a small number of residue difference associated with the different clinical conditions was found [170]. Research on the construction and characterization of 2 chimeric infectious DNA clones of PCV-1 and PCV-2 has provided further insight into the virus virulence factors [59]. The chimeric PCV-1–2 DNA clone contained the PCV-2 capsid gene cloned in the backbone of the non-pathogenic PCV-1. The chimeric PCV-1–2 virus induced a strong and specific antibody response to the pathogenic PCV-2 capsid antigen and was attenuated (minimal to no lesions, low level and reduced length of viremia, low or nondetectable levels of viral antigen in lymphoid tissues) when inoculated into pigs [59]. In another study, two amino acid mutations occurred in the capsid protein of PCV-2 after 120 serial passages in cell culture and resulted in attenuation of the virus *in vivo* [58]. Significant differences were observed in the PCV-2 genomic copy numbers in serum, and the gross and microscopic lesions in pigs inoculated with the wild-type PCV isolate were more severe than those inoculated with the passage 120 PCV-2 isolate [58]. This study confirmed that minimal changes in the genome of a PCV-2 isolate can markedly alter the virulence of PCV-2 viruses. Possible differences in virulence among Midwestern US field isolates of PCV-2 with 98.9% nucleic acid and 96.7% amino acid sequence identities in ORF2 were investigated in an *in vivo* study reported in 2006 [127]. The *in vivo* study using SPF pigs confirmed that PCV-2 isolates with minimal genomic

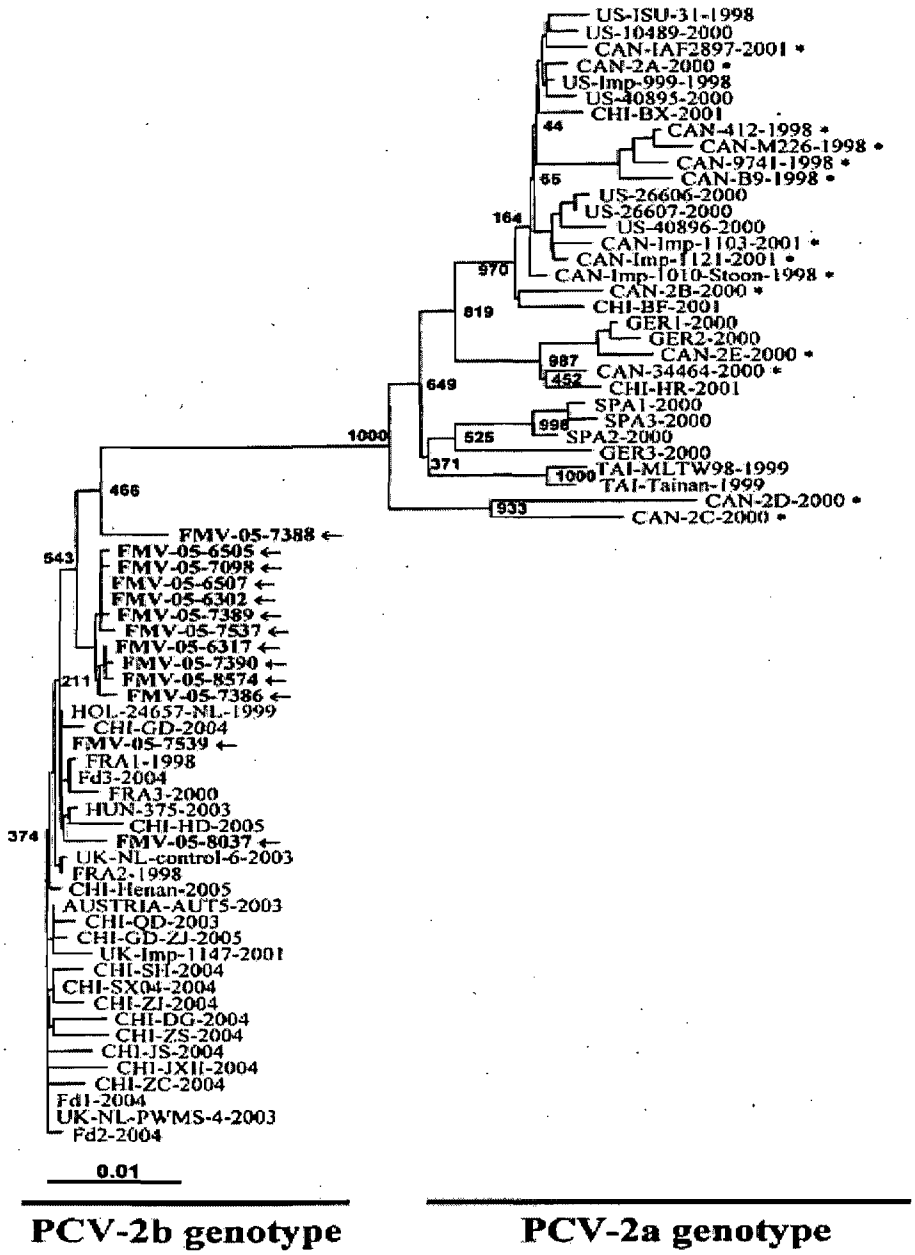

Figure 3. Phylogenetic analysis of the complete genome of porcine circovirus 2 (PCV-2) strains. An unrooted neighbor-joining tree was constructed from aligned nucleic acid sequences of 27 Canadian reference strains (including the newly described 13 sequences identified with an arrow and the older sequences identified with an asterix) and 43 sequences found in GenBank. Taken from *Gagnon et al.* (2007).

4. Biologic and Physical Properties

PCV-1 is stable at pH 3, at 56°C and at 70°C for 15 minutes, and is resistant to inactivation after exposure to chloroform [6]. The buoyant density of PCV-1 in CsCl is 1.37 g per cm^3 [163], and it is 1.36-1.37 g per ml CsCl [6]. The sedimentation coefficient (S) was determined to be 57S when compared with the sedimentation coefficient of a bovine enterovirus [6].

PCV-2 is readily isolated from tissue samples that have been stored at -70°C [52]. PCV-2 was shown to be resistant to some disinfectants (Nolvasan®, DC&R®, Weladol®, or ethanol) but virus titers were significantly reduced by sodium hydroxide, Virkon® S, and others [143].

5. Replication and Mechanisms of Virus Entry into the Cells

All viruses of the *Circoviridae* family are thought to have a similar replication strategy. A circular, double stranded replicative form of DNA intermediate is produced using host cell DNA polymerases during the S phase of cell division [165, 168]. The replicative form serves as template for generation of viral ssDNA using the rolling circle replication mechanism [168]. Viral DNA intermediates are generated in the nuclei and require host cell enzymes for completion of the replication cycle [165]. Virions are assembled in both nuclei and cytoplasm and released from infected cells in the absence of viral cytopathic effects.

Other authors studied the binding characteristics of PCV-2. In the porcine monocytic line 3D4/31, PCV-2 enters predominantly via clathrin-mediated endocytosis and requires an acidic environment for infection [110]. The PCV-2 capsid protein is expressed between 6 to 12 hours post inoculation and nuclear relocation occurred around 12 to 24 hours post inoculation in PK-15 cells. In porcine alveolar macrophages or fetal cardiomyocytes, nuclear localized antigens appeared approximately at 48 hours post inoculation and in fewer cells [108]. Treatment of PK-15 cell cultures with IFN-γ causes a 20 times higher production of PCV-2 progeny [109]. The enhancing effect of interferon-

gamma (IFN-γ) on PCV-2 infection was found to be due to increased internalization of PCV-2 virion-like particles. Expressions of PCV-2 proteins in infected cells were not altered by IFN-γ treatment [109].

During productive infection of PK-15 cells, nine mRNA as capsid RNA, five Rep-associated RNAs (*Rep*, *Rep'*, *Rep3a*, *Rep3b*, and *Rep3c*), and three NS-associated RNAs (*NS515*, *NS672*, and *NS0*) are synthesized by PCV-2 [32]. It was demonstrated that Rep and Rep' are essential for PCV-2 replication [33]. PCV-1 and PCV-2 were found to differ from each other in expression levels of NS and *Rep3c*-associated RNAs [31].

6. PCV-2 isolates

6.1. PCV-2 genetic variation

Molecular studies to determine the genetic variation of PCV-2 found that minor branches of PCV-2 were associated with geographic origin rather than with differences in virulence [55, 103]. The PCV-2 isolates from cases of PDNS and abortions were closely related to a PMWS-associated PCV-2, further establishing the apparent genetic stability of PCV-2 [107]. Four dominant immunoreactive areas are identified by PEPSCAN analysis within ORF2 [101]. In 2002, 34 Eastern Canadian PCV-2 isolates recovered from pigs with various clinical conditions such as PMWS, PRRS, generalized tremors, erysipelas, gastric ulcer, nervous signs, arthritis, and no clinical signs, were sequenced, and the obtained sequences compared to 36 published sequences. Sequence analysis indicated that all the isolates were closely related [91]. Three major regions of amino acid heterogeneity were identified among PCV-2 isolates, and two of the regions corresponded to two of the immunoreactive areas described before [101]. Comparison of three immunodominant regions, however, revealed no link between the capsid protein variation and the pathogenicity of isolates [91]. In addition, PCV-2 from PMWS-affected herds was compared to PCV-2 from herds non-affected by PMWS (healthy pigs) [92]. It was found that closely related strains in 6 different herds (4 with PMWS and 2 without) sharing at least 99.4% of their nucleotide-sequence identity and more than 98.7% of their amino-acid

differences can differ significantly in virulence as measured by levels of virus load in serum and tissues and severity of PCV2-associated lesions [127].

6.2. The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada

Restriction fragment length polymorphism (RFLP) preliminary results suggest the appearance in 2004 of a new PCV-2 genotype in the province of Ontario (Canada) which seems to be related to PCV-2b genotype [21, 47]. Furthermore, it is interesting to observe that the appearance of a new type of circulating PCV-2 strains (seemed to coincide with the increases of death rate and PMWS across Canada and particularly in Québec's swine herds [42, 47]. Sequence analysis of the entire viral genome of PCV-2 Canadian strains has permitted to establish the appearance of a new type of circulating PCV-2 strains in Canada [63]. These new strains have been classified in PCV-2b genotype (Fig. 3). Moreover, it is now obvious that the PCV-2b genotype strains are more prevalent across Canada than the PCV-2a genotype strains [63]. The most variable protein between PCV-2a and PCV-2b genotypes is the Cap protein with an aa (amino acids) sequence identity varying between 88% to 94%. The two other known proteins to be expressed by PCV-2 were less variable than Cap protein with a aa sequence identity between the two genotypes varying from 96% to 99% for Rep protein and from 92% to 98% for ORF3 protein [63].

Interestingly, this newly recovered PCV-2 genotype (named PCV-2b in comparison with the previously circulating genotype named PCV-2a) had already been reported in Asia and Europe [46] and has also been recently reported in 2007 in the United States [34, 81]. Now, an international consensus has been established in regards to the recognition of a least two major PCV-2 genotypes circulating worldwide [34, 50, 63, 67, 120]. Recently, a nomenclature has been proposed, one that takes into account the classification of Gagnon et al (2007) and identified the two major genotypes (PCV-2a and PCV-2b) as well as a third one (PCV-2c) which included only three 80's isolates that had never been reported later on [154].

PATHOGENESIS

1. Introduction

The pathogenesis of PCV-2 infection and the major cell types that support PCV-2 replication are poorly understood. The involvement of the immune system in the pathogenesis of PMWS seems central (Fig. 4). Two facts support this idea: 1) the existence of extensive lymphoid lesions [45] and 2) the association of PMWS with several secondary or opportunistic infections [49, 128]. Nevertheless, there are a number of questions that remain to be answered: What are the main virus producing cells? How does the immune response contribute to the development of the syndrome? Why, if most pigs on a farm are infected, only some of them become ill?

2. Transmission of PCV-2

Transmission of PCV-2 is thought to occur through direct contact via oronasal (Fig. 4), fecal and urinary routes [17, 100]. Direct contact with pigs inoculated with PCV-2 42 days previously resulted in virus transmission to 3 of 3 control cesarean-derived, colostrums-deprived (CDCD) pigs [17]. PCV-2 shedding in experimentally infected CDCD pigs was identified by polymerase chain reaction (PCR) in oropharyngeal swabs, nasal swabs, and feces [157]. In another study, the authors quantified PCV-2 DNA in tonsillar, nasal, tracheo-bronchial, urinary and fecal swabs of pigs with and without PMWS. The authors were able to detect PCV-2 DNA in a high percentage of the samples and concluded that PCV-2 is most likely excreted through respiratory secretions, oral secretions, urine and feces of both PMWS-affected and clinically-healthy pigs, with higher viral loads in the PMWS-affected pigs [153]. Vertical transmission has been demonstrated to occur in individual sows in the field [89, 118] and experimentally [80].

3. Cellular distribution of PCV-2 and cell-tissue tropisms

The target cells that support PCV-2 replication are poorly understood. The presence of PCV-2 antigen and nucleic acids has been shown in different cell types *in vivo* (Fig. 4) by IHC or ISH [11, 158]. A recent study found that antigen presenting cells in general, and not only macrophages stained positive by IHC for PCV-2 antigen [35]. In contrast, PCV-2 antigen in lymphocytes was only sporadically detected. In thymus, PCV-2 was only detected in few histiocytic cells in the medulla suggesting that thymocytes and T cells might be more resistant to PCV-2 infection [35]. Immunophenotyping of the target cells of PCV-2 replication *in vivo* has shown that the susceptible cell population in the pig depends on the stage of development of the host at the time of infection. In fetuses, infected cells were identified as cardiomyocytes, hepatocytes and macrophages during early gestation and mainly macrophages towards the end of gestation [144]. In the majority of unweaned piglets, low to moderate PCV-2-replication was demonstrated in macrophages [145]. A variety of cell types (Fig. 4) have also been shown to contain PCV-2 antigens and/or nucleic acid such as: enterocytes, renal and alveolar epithelial cells, vascular endothelial cells, pancreatic acinar and ductular cells, lymphocytes, smooth muscle cells, fibroblasts and germinal epithelial cells [45, 126, 173, 174].

3.1. PCV-2 and Macrophages

Viruses that replicate in the monocyte/macrophage lineage such as porcine parvovirus (PPV) [9, 122] and porcine reproductive and respiratory virus (PRRSV) [12, 74, 142], have been shown to increase the replication of PCV-2 in coinfected pigs and increase the incidence of PMWS. Despite the presence of PCV-2 in macrophages and dendritic cells, recent *in vitro* studies suggest that monocytic cells may not represent the primary target for PCV-2 replication [65]. Monocytes and macrophages were tested for the ability to support PCV-2-replication *in vitro*. PCV-2 replication in these cell types was not observed; however, PCV-2 was not degraded and was stored in the cytoplasm of the cells [65]. Similarly, no evidence of *in vitro* virus replication in dendritic cells was found [169];

however, PCV-2 did persist in dendritic cells without loss of infectivity or the induction of cell death. It has been speculated that because of their migratory capacity, dendritic cells can provide a potent vehicle for transport of the virus throughout the host without the need for replication [169]. *In vitro* studies showed that PCV-2 antigens could only be detected in the cytoplasm of PCV-2-inoculated monocyte/macrophage lineage cells, including swine alveolar macrophage without virus replication being detected [26, 27]. Nonetheless, two swine primary cell lines (alveolar macrophages and peripheral blood mononuclear cells - PBMC) have been demonstrated to allow PCV-2 infectious viral particles production when they were stimulated by bacterial lipopolysaccharide (LPS) or by concanavalin A (ConA) [28, 94].

3.2. PCV-2 and Porcine Kidney epithelial cell line (PK-15)

Until now, swine kidney cells (like PK-15 cells) have been the only immortalized cells shown to be permissive to PCV-2 *in vitro*, meaning that they allow the production of infectious viral particles following their infection with PCV-2 [106, 142]. On the other hand, other primary and immortalized cell lines were shown to be susceptible to PCV-2, meaning the cells could be infected and allow viral protein expression but without allowing infectious viral particles production [26, 28, 65, 110-112, 169]. PK-15 cells support PCV-2 replication *in vitro*, and these cells are routinely used for virus isolation and vaccine production. PCV-2-induced cytopathic effect is typically not observed, and to determine viral replication, immunofluorescent or immunoperoxidase staining has to be performed. Since PCV-2 does not encode its own polymerase, the replication of PCV-2, as for other circoviruses, depends on the cell polymerase present in the cells nucleus during the S phase of the cell cycle [165]. Consequently, glucosamine treatment of the PK-15 cells has shown to be effective in increasing PCV-2 replication [165].

3.3. Lymphoid tissues

Lymphocyte depletion together with histiocytic infiltration are the most characteristic features of PMWS [8, 39, 45, 52]. The reduction of lymphocytes (Fig. 4) might be due to reduced production in the bone marrow, reduced proliferation in secondary lymphoid tissues, or due to increased loss of lymphocytes in the bone marrow or peripheral blood or secondary lymphoid tissues via necrosis or apoptosis. The severity of lymph node depletion can be graded as initial, intermediate, and end stage [146]. Studies on pigs with naturally-acquired PMWS revealed that the absence of follicles and depletion of lymphocytes was associated with a reduction in the number of interfollicular dendritic cells and interdigitating cells as well as a reduction or absence of B cells and CD4+ T lymphocytes [146]. By IHC characterization of PCV-2-associated lesions in naturally-PMWS-affected pigs, Chianini et al. (2003) [35] found increased numbers of macrophages and partial loss and redistribution of antigen presenting cells throughout lymphoid tissues when compared to healthy control pigs. Decrease of proliferation of both, lymphoid and medulla-like tissues in the initial stage of PMWS, but not in the intermediate or final stage, has been reported [35].

Depletion in B-cell associated areas of lymph nodes has been linked to apoptosis induced by the virus [156]. In contrast, when inguinal lymph nodes of pigs with naturally acquired PMWS were investigated, it was found that decreased cell proliferation (and not increased apoptosis) seemed to be the most important variable leading to lymphoid depletion in PMWS [102]. The tissues from experimentally PCV-2 infected pigs were investigated by TUNEL staining and a positive signal was found only within the cytoplasm of virus-positive phagocytic mononuclear cells. It was concluded that apoptosis is not the primary mechanism of lymphoid depletion and hepatocyte loss in PMWS [87].

3.4. Blood

The hemogram of pigs with PMWS (Fig. 4) shows significant alterations [44, 148]. In wasted pigs, the number of lymphocytes is significantly decreased (especially of $CD8^+$

and B cell subsets) and monocytes and neutrophils are clearly increased, with an inversion of the ratio lymphocyte/neutrophil [44, 148]. A typical profile may be 60-70% neutrophils and 30-40% lymphocytes, while their theoretical numbers should be reverse [148, 149]. However, the total number of leukocytes is not altered. Pigs with PMWS usually have normocytic hypochromic anemia with a slight increase in the total number of red blood cells [44, 148].

4. Cytokine profiles in PMWS diseased pigs

Pigs with PMWS have altered cytokines mRNA expression patterns (Fig 4.) in different lymphoid tissues [44]. These alterations consist of an over expression of IL-10 mRNA in thymus and INF-γ mRNA in tonsil. The IL-10 mRNA over expression has been associated to thymic depletion and atrophy in the diseased animals [44]. A decrease in the mRNA expression was seen for IL-2 in the spleen, IL-4 in tonsils and lymph nodes, IL-12p40 in both spleen and inguinal lymph nodes and IFN-γ and IL-10 in inguinal lymph nodes [44]. With regard to pro-inflammatory cytokines, it has been reported that IL-8 mRNA levels are high in tissues with slight or moderate lesions and with small amount of virus, while it is low in tissues with severe lesions [44].

On the other hand, *in vitro* PCV-2 alone modifies the cytokine responses of PBMC (Fig. 4) from healthy pigs when stimulated with mitogens such as phytohemaglutinin (PHA) or superantigens [43].

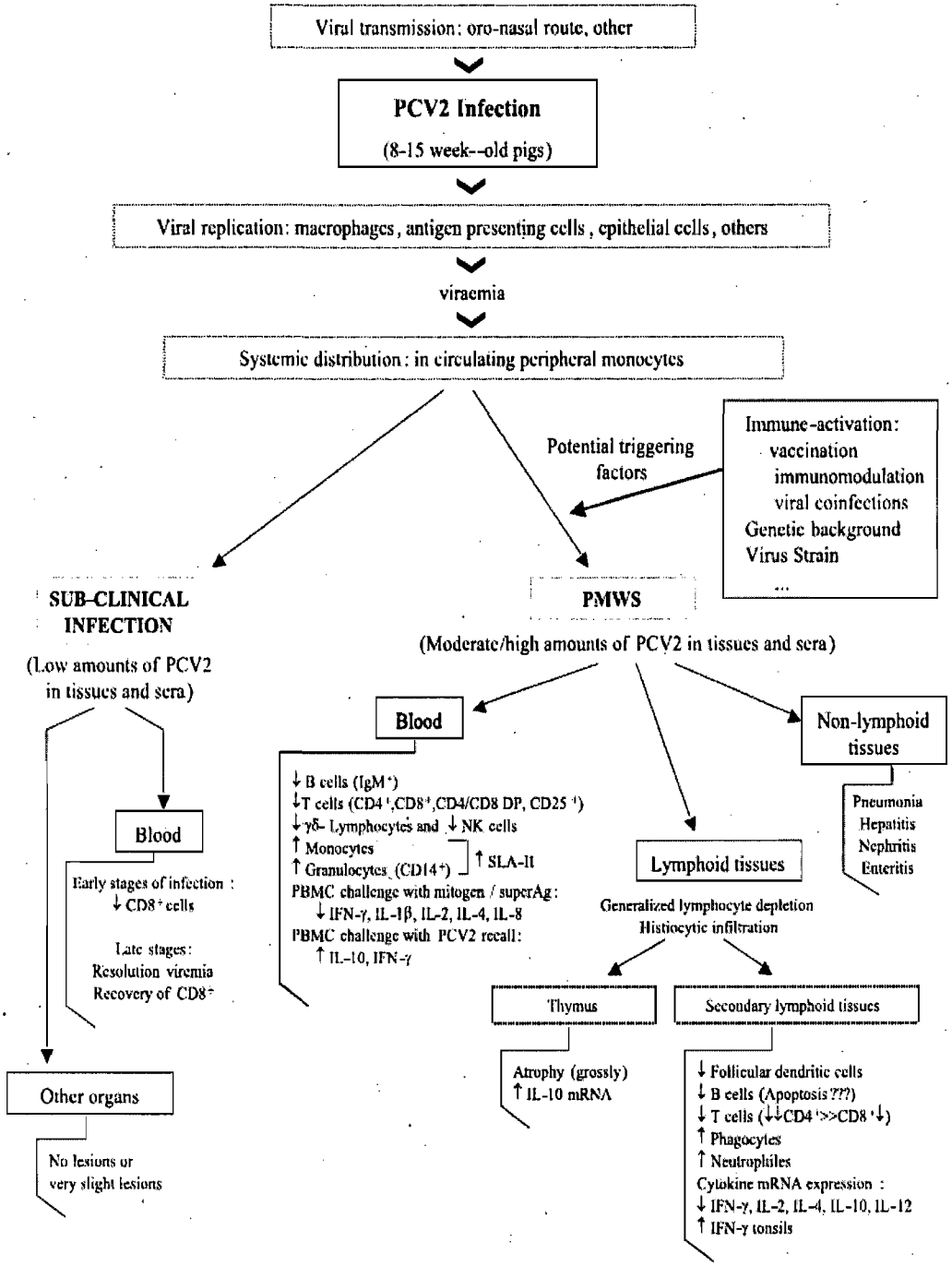

Figure 4. Pathogenesis of PMWS caused by PCV-2. Proposed outline of the pathogenesis including immunologic aspects of PMWS in clinical and sub-clinical PCV-2 infections of piglets. Taken from *Darwich et al.* (2004).

5. Risk factors involved in PMWS development

Evidence from the field supports the thought that PMWS is multifactorial in causality and that not all pigs that are infected with PCV-2 will develop clinical PMWS [128]. Factors that are currently thought to influence the outcome of PCV-2 infection can be broken down (Fig. 5) into 4 main areas of focus: virus, host, co-infections and immune modulation. Experimental work has confirmed that at least these 4 components are the key building blocks, identified to date, in the PMWS models [128].

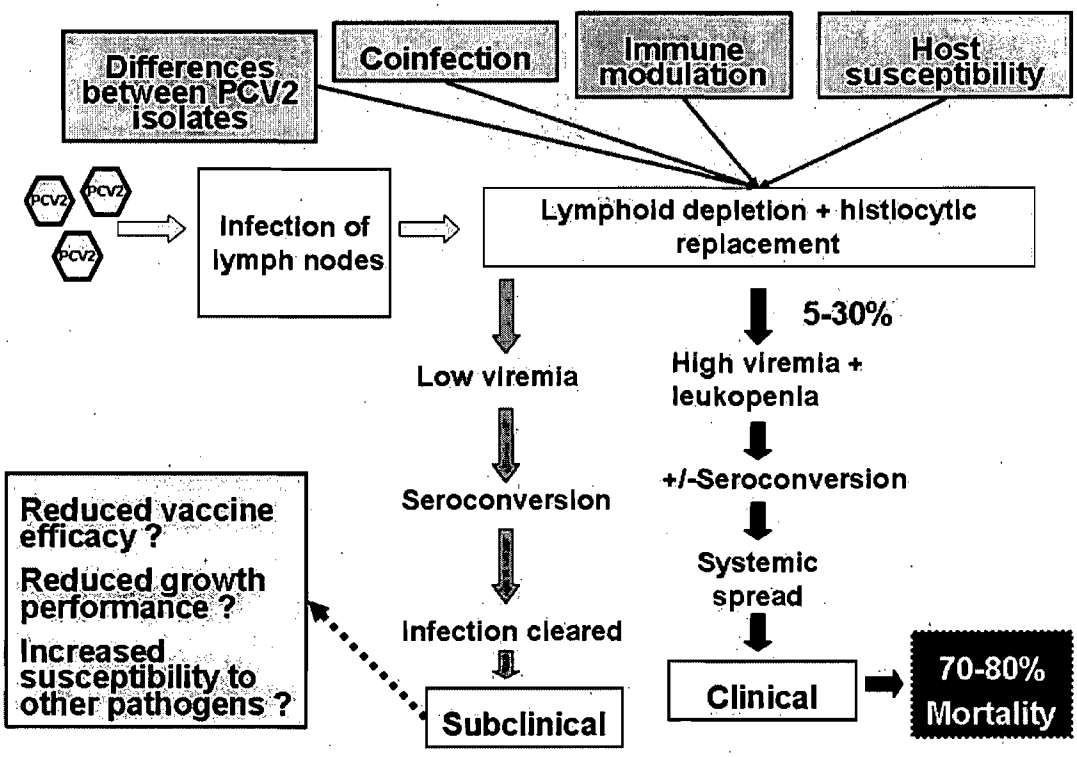

Figure 5. Co-factors involved in the disease development. A current model of the understanding of the progression of PCV-2 infection. Taken from from *Opriessnig et al.* (2007)

Genetic analyses and sequence comparison of **PCV-2 isolates** to date have failed to fully explain differences in clinical manifestations. Then, one of the main objectives of the swine industry and health scientists is to establish if there is a virulence variation between both PCV-2 genotypes (PCV-2a and PCV-2b).

Host susceptibility and its role in PMWS development are poorly understood. Pigs of all breeds seem to be susceptible to PCV-2 infection and clinical PMWS has been observed in a wide variety of purebred and crossbred pigs. Host susceptibility and its effect on the outcome of PCV-2 infection were recently investigated in a controlled pilot project [124]. Three breeds were compared in this study: Duroc, Landrace and Large White. The incidence of systemic PMWS based on gross and microscopic lesions was 0% (0/23) in Durocs, 15.8% (3/19) in Landrace, and 0% (0/21) in Large White [124]. The purebred Landrace pigs used in this experiment were clearly more susceptible to PMWS diseases as measured by the severity of clinical signs and microscopic lesions associated with PCV-2 [124]. A recent *in vitro* study investigating the replication patterns of PCV-2 in pulmonary alveolar macrophages found clear differences between macrophages derived from different conventional crossbred pigs suggestive of differences in susceptibility to PMWS [109].

The primary etiological agent responsible for PMWS has been identified as a PCV-2 [70]. The course of PCV-2 infection that will lead to the occurrence of the disease has been associated with the **presence of other pathogens** such as *Mycoplasma hyopneumoniae*, *Porcine parvovirus* (PPV), *Porcine reproductive and respiratory syndrome virus* (PRRSV), and Swine influenza virus (SIV) [128]. Experimental coinfection of pigs with PCV-2 and other viruses such a PPV [9, 14, 81, 85, 122], PRRSV [12, 74, 142] or bacteria such as *Mycoplasma hyopneumoniae* [123] has been shown to enhance the amount of PCV-2 viral load and PCV-2-associated lesions, and to increase the incidence of PMWS.

Studies have demonstrated that **immunostimulation** may trigger progression of PCV-2 infection to disease and characteristic lesions of PMWS. The PMWS was reproduced in gnotobiotic pigs stimulated with keyhole limpet hemocyanin in incomplete Freund adjuvant (KLH/ICFA) and inoculated with PCV-2 [4, 86, 88, 121]. A recent study has determined that the timing of vaccination with a commercially available *M. hyopneumoniae* vaccine had an effect on PCV-2 replication and PCV-2-associated lesion

severity. It also confirmed differences in PCV-2-associated lesions among the treatment groups and concluded that no or minimal PCV-2-associated lesions were observed when pigs were vaccinated 2 to 4 weeks prior to expected PCV-2 exposure [125]. A study was conducted to determine if the adjuvants (as opposed to the antigen) in commercial swine vaccines increase replication of PCV-2 and incidence of PCV-2-associated disease. It also considered the possible difference between adjuvants in this regard [78]. Under the conditions of the study, it was found that by the later stages of infection (35 days postinoculation), the pigs vaccinated with the oil-in-water adjuvants had an increased length of PCV-2 viremia, increased amount of PCV-2 in serum and tissue and increased severity of lymphoid depletion compared to pigs vaccinated with the aqueous and aluminum hydroxide products [78].

PMWS is most often observed in pigs between 5 and 12 weeks of **age** with most cases occurring between 6 and 10 weeks of age [128]. Reports of PMWS in adult pigs are rare. PCV-2 associated lesions are typically observed from 14 to 35 days post PCV-2 inoculation in experimental studies [45, 128].

DIAGNOSIS

Generally, diagnosis of viral diseases in swine is based on detection of the virus by culture, PCR, IHC or ISH, and/or detection of virus antibodies by serology. However, diagnosis of PMWS is different from this general diagnostic approach because PCV-2 can be detected in normal healthy pigs [11, 19, 82, 83]. The detection of PCV-2 alone does not necessarily confirm a diagnosis of PMWS. Therefore, the diagnosis of PMWS must meet three criteria: (i) the presence of compatible clinical signs, (ii) the presence of characteristic microscopic lesions, and (iii) the presence of PCV-2 within these lesions [158]. These three criteria separately are not diagnostic of PMWS. Currently, IHC or ISH are considered the gold standard for detecting PCV-2 within characteristic microscopic lesions as part of the diagnosis of PMWS [158].

Based on RFLP and gene sequence comparison results, PCV-2b was suggested to be associated with the mortality rate increase observed in several Canadian swine herds [23]. Consequently, even if no experiment confirms yet if the PCV-2b strains found in Canada are more virulent than previously circulating PCV-2a strains, it became important to develop a low cost (compared to entire viral genome sequencing) diagnostic test that could rapidly and efficiently differentiate both genotypes.

INTERVENTION STRATEGIES

1. Good management practices

Prior to the availability of PCV-2 vaccines in 2006 in North America, successful treatment and control of PMWS had primarily focused on ensuring good production practices that minimize stress, eliminate co-infections or minimize their effect, and eliminate potential triggering factors that induce immune stimulation and trigger progression of PCV-2 infection to PMWS. A 20-point plan to control PCVAD on severely affected farms was proposed [98]. The main points of this plan have been summarized as the 4 golden rules (www.thepigsite.com, accessed April 10, 2007) and include 1) limiting pig-to-pig contact, 2) reduction of stress, 3) good hygiene and 4) good nutrition.

Use of disinfectants in buildings and transport vehicles that have been demonstrated to be efficacious against PCV-2 transmission [143] is recommended. For instance, with sodium hydroxide and Virkon S (Antec International, Sudbury, Suffolk), a reduction of *in vitro* virus titers was observed.

2. PCV-2 vaccines

Evidence to date indicates that the commercial vaccines (Table 1) are a remarkably effective tool to reduce losses in herds and production systems experiencing PMWS in growing pigs. The inactivated, oil-adjuvanted PCV-2-vaccine (CIRCOVAC, Merial Com, Lyon, France) licensed for use in breeding-age animals was the first vaccine on the market and has been used most extensively in Europe (Table 1). CIRCOVAC has been shown to be beneficial in reducing PCV-2 circulation and shedding in the first weeks of life and improving pig health under experimental conditions [29]. During field efficacy studies in Germany and France, the use of CIRCOVAC resulted in a rise in PCV-2 antibody levels in the breeder herds and a concurrent decrease in PMWS rates in the growing pigs [29]. When

used in Canada in 2006, field trials demonstrated that CIRCOVAC significantly ($P<0.05$) decreased mortality (6.4–8.3% prior to vaccination) [132].

A conditionally licensed product (Circumvent^{tm} PCV – Intervet Inc, USA) for use in growing pigs became available to the North American market in April 2006 (Table 1). Investigations including pigs on 21 Canadian farms showed that the mortality rate in vaccinated pigs was lowered by 77.5% when compared with nonvaccinated pigs [68].

SuvaxynH PCV-2 One Dose TM (Fort Dodge Animal Health, Fort Dodge, IA) is the first US Department of Agriculture–approved and fully licensed commercial PCV-2 vaccine in the United States (Table 1). This is the killed version of the live chimeric PCV-1–2 virus [57, 59]. Preliminary results from several large fields studies in the United States demonstrated significantly decreased mortality ($P<0.001$) with the use of the killed chimeric vaccine, accompanied by reduced treatment costs compared to those encountered for nonvaccinated pigs [40].

Preliminary results from field trials using the baculovirus expressed PCV-2 vaccine Ingelvac CIRCOFLEX TM (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) (Table 1) also demonstrated significant reduction in mortality ($P<0.003$) in vaccinated pigs compared to nonvaccinated pigs on four Canadian finishing sites [48].

Table I. Commercial PCV-2 vaccines available in North America.

	Vaccine			
	Ingelvac® CIRCOFLEX™n	**Suvaxyn® PCV-2 One Dose™m**	**CIRCUMVENT™ PCV**	**CIRCOVAC®**
Antigen	PCV-2 ORF2 protein expressed in inactivated baculovirus	Inactivated PCV-1-2 chimera	PCV-2 expressed in inactivated baculovirus	Inactivated PCV-2
Dose	1 mL intramusculary (IM); single dose	2 mL IM; single dose	2 mL IM; 2 injections 3 wks apart	2 mL IM; Primary vaccination: 2 injections 3-4 wks apart, at least 2 wks before mating Revaccination: 1 injection at each gestation, at least 2-4 wks before farrowing
Licensed for	Healthy pigs 3 wks and older	Healthy pigs 4 wks and older	Healthy pigs 3 wks and older	Healthy female breeding-age pigs
Available	United States	United States	United States; Canada	Canada; Europe

Adapted from *Opriessnig et al.* (2007).

EXPERIMENTAL INFECTION MODEL OF PMWS

Experimental PCV2-infection models indicate that PCV-2 is an opportunist, depending on immunostimulation [13, 15, 86] or coinfecting agents like PPV [9, 14, 53, 76, 81, 84, 85, 122, 130], PRRSV [12, 74, 142], or *M. hyopneumoniae* [123] for PCV-2 infection to progress to clinical PMWS. While most research groups have not been able to reproduce clinical disease in pigs inoculated singularly with PCV-2, some groups have succeeded. In early experimental models, piglets inoculated with PCV-2 alone only developed a very mild clinical disease with slight histological lesions characteristic to those of PMWS [9, 16, 100].

A cofactor was needed for immune stimulation to reproduce the disease. This hypothesis arose from experiments using days-old gnotobiotic piglets inoculated with PCV-2 and keyhole limpet haemocyanin in incomplete Freund's adjuvant (KLH/ICFA) [86]. In that experiment, the piglets developed PMWS in higher proportion and with higher severity than the unstimulated animals, which developed a subclinical PCV-2 infection. This hypothesis was further explored under natural conditions. Others authors [88] showed that conventional on-farm pigs inoculated with a parapoxvirus immunomodulator combined with a vaccine against *M. hyopneumoniae* developed more severe clinical signs than pigs to which none of the above was administered and were only naturally exposed to the virus.

Different type of animals models were used in experimental infection, such as gnotobiotic pigs [85], colostrums deprived cesarian derived (CDCD) pigs [17, 74, 134], colostrums deprived (CD) pigs [5, 9, 12] and conventional pigs [1, 16, 90].

Fenaux et al. (2002) investigated the infectivity of an US PCV-2 molecular infectious DNA clone. The infectious clone was directly inoculated into the liver or inguinal lymph nodes and mild PCV-2-associated lesions were observed in 4-weeks-old conventional pigs confirming the role of PCV-2 in PMWS [56].

III. MATERIAL, METHODS & RESULTS

Identification of a new cell line permissive to porcine circovirus type 2 replication: the newborn piglet tracheal cells

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Short running title: NPTr cells are permissive to PCV-2 replication

Abstract

The pathogenesis of *Porcine circovirus type 2* (PCV-2) infection and the major cell types that support PCV-2 replication *in vivo* are still not fully understood. Until now, the porcine kidney cell line (PK-15 and its derivative) was known to be the only immortalized cell line permissive to PCV-2 replication. The goal of this study was to establish if epithelial cells of the respiratory tract of swine could support PCV-2 replication *in vitro*. The newborn piglet tracheal (NPTr) epithelial cells were discovered to be permissive to PCV-2 infection following 1) the detection of the nucleocapsid protein in PCV-2 infected cells and 2) infectious viral particles production. In addition, no difference was observed between the kinetics of PCV-2 infectious viral particles production in NPTr and PK-15 infected cells ($P>0.05$). These results suggest that the epithelial cells of the respiratory tract of swine can be the primary cell target for PCV-2 replication and could subsequently provide a way of entry in swine. In conclusion, the NPTr cell line could be used as a new *in vitro* model for studying PCV-2 pathogenesis.

Key words: NPTr cells, PK-15 cells, PCV-2 replication

Introduction

Postweaning multisystemic wasting syndrome (PMWS) is a swine disease first recognized in Canada in 1991 [15, 28]. Afterwards, the syndrome was reported worldwide [5]. The disease usually affects 5 to 12 weeks 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 [28, 52]. Depletion of lymphocytes in the lymphoid follicles and their replacement by macrophages are the hallmark lesions observed in this syndrome [1, 36]. The etiological agent responsible for PMWS has been identified as a circovirus named *porcine circovirus-2* (PCV-2) [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 respiratory disease complex (PRDC), porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, granulomatous enteritis, necrotizing lymphadenitis, exudative epidermitis and congenital tremor [6, 29]. Consequently, the term "Porcine circovirus-associated disease (PCVAD)" is now accepted to describe the syndromes in which PCV-2 plays a role.

Porcine circovirus (PCV) are small, icosahedral, non-enveloped viruses with a circular single-stranded (ss) DNA genome of 1.7 kb [36, 54]. PCV are among the smallest known mammalian viruses with a diameter of around 17 nm [53]. It is composed of coat protein subunits assembled in 12 pentameric units [16]. PCV-2 genome contains three main open reading frames (ORFs), which are the ORF1 (314 aa) encoding the replicase protein (Rep); the ORF2 (233 aa) encoding the immunogenic capsid protein (Cap); and the newly characterized protein encoded by ORF3 (105 aa) which appears to contribute to virus-induced apoptosis of host infected cells [11, 12, 34, 35, 40]. It is noteworthy that there are at least two PCV-2 genotypes that are presently circulating worldwide but there is no international consensus about the nomenclature that should be used to identify them [13, 24, 27, 41].

The pathogenesis of PCV-2 infection and the major cell types that support PCV-2 replication are poorly understood. The presence of a PCV-2 antigen and nucleic acids has been shown in different cell types *in vivo* by immunohistochemistry (IHC) or *in situ*

hybridization (ISH). It has been suggested that PCV-2 initially replicates in the tonsil [3]. Large amounts of PCV-2 antigen or nucleic acids are found in the cytoplasm of macrophages and dendritic cells replacing lymphocytes in the depleted follicles in lymphoid tissues [2, 52]. In thymus, PCV-2 was only detected in few histiocytic cells in the medulla suggesting that thymocytes and T cells might be resistant to PCV-2 infection [14]. Immunophenotyping of the target cell of PCV-2-replication *in vivo* has shown that the susceptible cell population in the pig depends on the stage of development of the host at the time of infection. In fetuses, infected cells were identified as cardiomyocytes, hepatocytes and macrophages during early gestation and mainly macrophages towards the end of gestation [48]. In the majority of unweaned piglets, low to moderate PCV-2-replication was demonstrated in macrophages [49]. A variety of cell types has also been shown to contain PCV-2 antigens and/or nucleic acid such as: enterocytes, renal and alveolar epithelial cells, vascular endothelial cells, pancreatic acinar and ductular cells, lymphocytes, smooth muscle cells, fibroblasts and germinal epithelial cells [17, 43, 56, 57]. Despite the presence of PCV-2 in macrophages and dendritic cells, *in vitro* studies suggest that monocytic cells may not represent the primary target for PCV-2 replication [26]. Monocytes and macrophages were tested for the ability to support PCV-2 replication *in vitro*. PCV-2 replication in these cell types was not observed; however, PCV-2 was not degraded in the cytoplasm of the cells [26]. Similarly, no evidence of virus replication in dendritic cells *in vitro* was found [55]. However, PCV-2 did persist in dendritic cells without loss of infectivity [55]. It has been speculated that because of their migratory capacity, dendritic cells can provide a vehicle for transport of the virus throughout the host [55]. Porcine monocytic cell line 3D4/31 is susceptible to PCV-2 infection but not permissive to virus replication [38, 39]. Moreover, *in vitro* studies showed that PCV-2 antigens could only be detected in the cytoplasm of PCV-2-inoculated monocyte/macrophage lineage cells, including swine alveolar macrophages without virus replication being detected [7, 8]. Nonetheless, swine alveolar macrophages have been demonstrated to allow PCV-2 replication when they are stimulated by bacterial lipopolysaccharide (LPS), but this result has to be confirmed because of the existence of contradictory reports [9, 22].

Therefore, what are the main virus producing cells? Swine epithelial cells are known to be PCV-2 infected in pigs with naturally occurring PMWS [31, 46]. *In vitro*,

some porcine epithelial cells such as the ST cell line, have been shown to be susceptible to PCV-2 infection but not permissive to virus replication [39]. The PK-15 and SK porcine kidney epithelial cell lines are the only immortalized cell lines known to allow PCV-2 replication [36, 47]. Nonetheless, it is a good assumption to believe that kidney cells are not the first infected cells that favor propagation of the virus in the organism. The oro-nasal route is considered the most likely and frequent route of PCV-2 transmission, and experimental infection studies, which have mainly used the intranasal route of inoculation support that idea [1, 4, 20, 32, 33, 47]. Consequently, the aim of this study was to establish if epithelial cells of the respiratory tract of swine could support PCV-2 replication *in vitro*. In this study, the PCV-2 permissivity of a few swine cell lines was investigated. The present study demonstrates that the newborn piglets tracheal (NPTr) immortalized epithelial cell line [23] is permissive to PCV-2 replication.

Materials and Methods

Virus and cells. The FMV-06-1717 PCV-2 reference strain, used in the present study has been previously characterized as a member of the PCV-2b genotype [25]. This virus was propagated into PK-15A cells, a subclone of PCV-1 noninfected PK-15 cells [45], and the virus was purified and concentrated following ultracentrifugation on a 30% sucrose cushion using the SW28 Beckman Coulter rotor (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada) at 83 000 relative centrifugal force (rcf) during a 5 hr period. The virus pellet was resuspended in 2 mL of a phosphate buffer saline (PBS) containing 2% fetal bovine serum, and aliquots of the virus stocks were then conserved at –70°C for future use. The infectious dose of the virus stock was determined from a 96-well microplate of PK-15A-infected cells following immunofluorescence assay (IFA) [45] by the Kärber method (Payment P, Trudel M: 1989, Manuel de techniques virologiques, Presses de l'Université du Québec, Québec). For the calculation of the virus titer, a well was considered to be positive for PCV-2 if specific fluorescence was detected in cells regardless of the amount of immunofluorescent positive cells. The virus titer was expressed in tissue culture infectious dose 50 per mL ($TCID_{50}$/mL). The titer of the PCV-2 stock used was determined to be 1 x 10^7 $TCID_{50}$/mL. The PK-15A cells were maintained at 37^0C in 5% CO_2 atmosphere in Minimum Essential Medium (MEM) with Earle's salts (Invitrogen Corporation, GibcoBRL, Burlington, ON, Canada), supplemented with 10% fetal bovine serum (FBS) (Wisent Inc, St-Bruno, QC, Canada), 300 U/ml of penicillin , 300 mg/ml of streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 mM HEPES. The NPTr cells (kindly provided by Dr. M. Ferrari) [23] were grown at 37^0C in 5% CO_2 in MEM with Earle's salts (Invitrogen Corporation, Canada), supplemented with 10% fetal bovine serum (FBS) (Wisent Inc, St-Bruno, QC, Canada), 300 U/ml of penicillin, 300 mg/ml of streptomycin, 1mM sodium pyruvate (Invitrogen Corporation, Canada).

Immunofluorescence assay. To titer the PCV-2 virus stock and to determine the PCV-2 permissivity of cell lines, an IFA was performed as previously described with some modifications [45]. Briefly, an amount of 3 x 10^4 NPTr cells were seeded per well in an eight-well LabTek chamber slides (Nalge Nunc International, Rochester, NY, USA) and

cells were immediately infected while in suspension with FMV-06-1717 PCV-2 reference strain at a multiplicity of infection (MOI) of 1. PK-15A cell suspensions were infected simultaneously using the same conditions as positive control. Mock-infected cells were included as negative controls. At 3 days post infection (pi), cells were washed three times with sterile PBS solution and fixed with a solution containing 4% paraformaldehyde (PFA) in PBS for 20 min. Then, the cells were washed three times with PBS and they were permeabilized with a solution containing 0.1% triton X-100 in PBS for 10 min. Finally, cells were rinsed three times with a solution containing 0.02% Tween-20 in PBS (PBS-T). All the PFA fixation steps were done at room temperature. Afterwards, cells were incubated in a solution containing 1% bovine serum in PBS-T for 20 min to block unspecific binding of the antibodies. After this step, cells were incubated with a 1:200 dilution of the polyclonal PCV-2 porcine serum [45] at 37^0C for 90 min. Then, cells were washed three times with a solution containing 1% bovine serum in PBS-T and incubated with a rabbit anti-pig FITC conjugated antibody (MP Biomedicals, Solon, OH, USA) diluted 1:75 at 37^0 C for 60 min. Thereafter, the cells were washed three times with a solution containing 1% bovine serum in PBS-T followed by three washes with PBS only. Then, cells were visualized using a DMI 4000B reverse fluorescence microscope. Photographs of the cells were taking with a DFC 490 digital camera and the images were analyzed using the Leica Application Suite Software, version 2.4.0 (Leica Microsystems).

PCV-2 infectious viral particles production. Initially, two flasks containing 5 x 10^5 PK-15A and NPTr cells each were infected in suspension with FMV-06-1717 PCV-2 reference strain at 0.005 MOI. At 96 hrs pi, one infected flask of each cell type was harvested following three cycles of freeze and thawed at – 70^0C. After centrifugation at 2000 rpm at 4^0C for 10 min, the supernatant containing PCV-2 free virus particles was collected and the amount of virus was determined by Kärber method in PK-15A cells (see above). The remaining infected cells of the other flask were trypsinised, divided and seeded into two new flasks and then cells were reinfected with a 1:6 dilution of the previously collected PCV-2 virus, which will be accounted for a subsequent virus infection cycle. The same protocol was carried out 4 times for subsequent passages. Following each passage mock-infected cells were included as controls.

PCV-2 replication kinetic in PK-15A and NPTr cells. 1 x 10^5 PK-15A and NPTr cells were infected in suspension with FMV-06-1717 PCV-2 reference strain at 1 MOI. At 4 hrs pi, the cells were washed five times with sterile PBS and fresh culture medium was added. At each time post infection (0, 4, 6, 12, 18, 24, 48, 72, 96 and 120 hrs pi), the infected cell cultures were collected and centrifuged at 2000 rpm at 4^0C for 10 min. Then, cell pellets and supernatants were stored at -70^0C until virus titration was realized in PK-15A cells (see above). Before titration, all pellet samples were frozen and thawed three times to free infectious viral particles, and cell debris were discarded after centrifugation at 2000 rpm at 4^0C for 10 min. Mock-infected controls were included in each experiment. All experiments were performed two times in triplicate.

Statistical analyses. The statistical analyses were realized using the GraphPad Prism version 4 software. The paired t test and the regular two-way ANOVA combined with the Bonferroni post-tests models were used to determine if there is statistical significant difference between PK-15A and NPTr cell lines in regards to their efficiency to allow the replication and production of PCV-2 infectious viral particles.

Results

Identification of the NPTr PCV-2 permissive cell line by immunofluorescence assay. In the perspective to find out new cell lines permissive to PCV-2 infection, some porcine cell lines were infected with 1 MOI of PCV-2 (data not shown). Interestingly, only one cell type, the NPTr cells, was found to express PCV-2 antigens when PCV-2 infected (Fig. 1). The NPTr infected cells were shown to contain a high level of PCV-2 Cap antigen since they reacted very strongly to specific porcine polyclonal anti PCV-2 antibody (Fig. 1c). Both intracytoplasmic and intranuclear positive signals were found in PCV-2 NPTr infected cells with a higher amount of antigen detected in the nucleus as illustrated in the inset of Fig. 1c. The ratio of the NPTr infected cells expressing the PCV-2 antigens was very similar to the control PK-15A infected cells (data not shown). As expected, the NPTr mock-infected cells were negative for the expression of PCV-2 antigens (Fig. 1d). Interestingly, following visualization by phase contrast microscopy of PCV-2 NPTr infected cells, no major cytopathic effect (CPE) was observed compared to mock-infected cells (Fig. 1a and 1b). Nonetheless, at 72 hrs pi, differences in the cell morphology could be seen in several NPTr infected cells and showed a marked increases in cell size and particularly in their nucleus size compared to mock-infected cells (Fig. 1a and 1b insets). Furthermore, the NPTr cells were not only permissive to PCV-2b genotype virus (Fig. 1), but were also permissive to PCV-2a genotype virus [24] (data not shown).

PCV-2 infectious viral particles production at different cell passages. Following the confirmation that the NPTr cells were able to express PCV-2 antigen, the efficacy of the NPTr cells to allow a complete virus replication cycle and produce PCV-2 infectious particles had to be determined. The amount of PCV-2 virus production in PK-15A and NPTr infected cells was evaluated during five consecutive passages (Fig. 2). To make sure that the NPTr infected cells were able to produce infectious viral particles, a very low amount of virus was used in the initial inoculum in hope that the virus yields production will increase during subsequent cell passages. Consequently, the amount of PCV-2 in the initial inoculum used to infect NPTr cells at the first passage of the assay following dilution of the virus stock was 4.17×10^2 $TCID_{50}$/mL (Fig. 2). Interestingly, at the first passage,

1.78×10^3 $TCID_{50}$/mL infectious viruses were found in both PK-15A and NPTr infected cell lysates suggesting that infectious viral particles were produced. At subsequent cell passages, the virus yields in both cell lines increased until the 5th passage, at which the amount of PCV-2 virus recovered from cell lysates was 3.16×10^5 $TCID_{50}$/mL for both cell lines. Statistical analysis revealed that there was no significant difference between PCV-2 infectious particles yields from NPTr and PK-15A infected cells ($P=0.2832$) even if a smaller amount of virus was recovered from NPTr infected cells at passage 3 and 4 (Fig. 2).

PCV-2 replication kinetic in PK-15A and NPTr cells. To confirm the previous results in regards to the virus yields obtained from NPTr and PK-15A infected cells at different cell passages, the PCV-2 replication kinetic in both cell lines was compared. At different times pi, the PCV-2 replication kinetic in both cell lines and the amount of virus yields was observed in culture medium (or supernatant) and in cell (or cell pellets). Virus titers obtained in the supernatants and cells at different times pi are illustrated in Fig. 3. Interestingly, the PCV-2 infectious particles production was clearly demonstrated in NPTr cells since the amount of virus recovered at 120 hrs pi was 4465 and 794 times higher compared to 4 hrs pi in cell pellets and the culture medium, respectively. Furthermore, a significant increase in PCV-2 infectious particles production started to appear in both cell lines at 48 hrs pi since the amount of virus recovered was 45 to 316 times higher compared to 4 hrs pi. No statistical significant difference was observed in the overall PCV-2 replication kinetic in PK-15A and NPTr cells ($P>0.05$) since the virus yields in the culture medium and cell fractions were very similar with one exception at 72 hrs pi where a significant difference between the virus yields in PK-15A and NPTr cell pellets was observed ($P<0.001$). Interestingly, at 72 hrs pi and until the end of the experiment, the amount of PCV-2 recovered in each cell line from the cell fractions was 10 to 20 times higher compared to the virus recovered from the supernatant ($P<0.001$) (Fig. 3), suggesting that PCV-2 was weakly released from infected cells.

Discussion

Since PCV-2 transmission is considered to occur most likely via the oro-nasal route and because epithelial cells of the respiratory tract were found to be positive by ISH and IHC [17], it was logical to evaluate if cell lines from the respiratory tract could permit the replication of the virus even if until now, swine kidney cells have been the only immortalized cells shown to be permissive to PCV-2 replication *in vitro* [36, 47]. Consequently, different cell lines were evaluated for their capacity to allow the replication of PCV-2. Surprisingly, the newly established NPTr cell line [23] was found to be permissive to PCV-2 replication (Fig. 1, 2, and 3). Moreover, the NPTr cells were statistically identical to PK-15 cells in regards to their efficacy to produce infectious viral particles (Fig. 3) suggesting that NPTr cells could replace the PK-15 cells in terms of vaccine production. Unfortunately, Ferrari et al (2003) [23] have suggested that those cells could not be used for vaccine production according to the U.S. Food and Drug Administration (FDA) guidelines since they contain porcine endogenous retrovirus (PERV) and they possess transforming activity. Interestingly, the NPTr cells were already known to permit the replication of several swine viruses such as pseudorabies virus, pig parvovirus, hog cholera virus, transmissible gastroenteritis virus of swine, encephalomyocarditis virus, swine vesicular disease virus and enteroviruses [23]. Furthermore, the NPTr cells are permissive to a variety of sub-types of influenza virus from human, swine, avian and horse origins. Consequently, from now on, PCV-2 can be added to this exhaustive list of viruses that can replicate in NPTr cells. Noteworthy, the NPTr cells can be a useful tool for virus isolation in a virology diagnostic laboratory since they permit the replication of a large variety of swine viruses [23].

In addition to the experiment illustrated in Fig. 1, PCV-2 NPTr infected cells were fixed and stained at different times pi (24, 48, 72, 96 and 120 hrs pi) (data not shown). It was noticed that the expression of the PCV-2 antigens began at 24 hrs pi. Between 48 hrs to 96 hrs pi, an increased number of PCV-2 positive cells was observed. At 96 hrs pi and until the end of the experiment, the observed number of PCV-2 positive cells was at a constant maximum level, a phenomenon that appeared 24 hrs after what has been previously reported by others [10, 37, 58]. In addition, at 96 hrs pi and until the end of the experiment,

an increased number of detached cells was observed. It is possible that the cells may have died following PCV-2 infection as previously described with PK-15 cells [37, 58], but further experiments will have to be conducted to determine if detached cells are dying cells.

In an earlier study of Yu et al. (2007) [56], following an evaluation of microscopic lesions, it was suggested that PCV-2 replication begins in the lymph nodes at the nearest site of virus entry and infection. Moreover, their results suggest that the lymph nodes near the infection site may also play an important role in PCV-2 persistence in the infected host. On the other hand, the present study suggests that epithelial cells of the respiratory tract of swine may potentially be more involved in the pathogenesis of the viral infection such as virus entry and proliferation than previously thought since it was clearly demonstrated that PCV-2 could replicate in an epithelial cell line of the respiratory tract of swine like the NPTr cells (Fig. 3). Moreover, it is consistent with the fact that one of the most frequent clinical signs observed in PMWS cases are related to respiratory problems [6, 15, 19, 30, 44, 50, 51]. Consequently, these results suggest that, *in vivo*, the epithelial cells of the respiratory tract of swine could be the primary cell target for PCV-2 replication but further experiments will have to be conducted to support this affirmation.

As reported by Gagnon et al. (2008) [25], logistic regression analyses revealed that PCV-2 increased the odds ratio of isolating two 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. Others have also reported that the odds ratio to find *S. suis* increased significantly in PCV-2 positive animals [18]. In addition, PCV-2 has been reported to support and exacerbate the establishment of secondary bacterial infections such as *Mycoplasma hyopneumoniae* [18, 21, 42]. The fact that epithelial cells of the respiratory tract of swine are permissive to PCV-2 replication may favor the establishment of secondary bacterial infections which in return could be the cofactors necessary for the development of PMWS. Work is now in progress to determine if PCV-2 has an impact in regards to the susceptibility of NPTr cells to secondary bacterial infections. But most importantly, the present finding of the NPTr cells being permissive to PCV-2 replication can lead to the establishment of a new interesting *in vitro* model for studying the pathogenesis of PCV-2.

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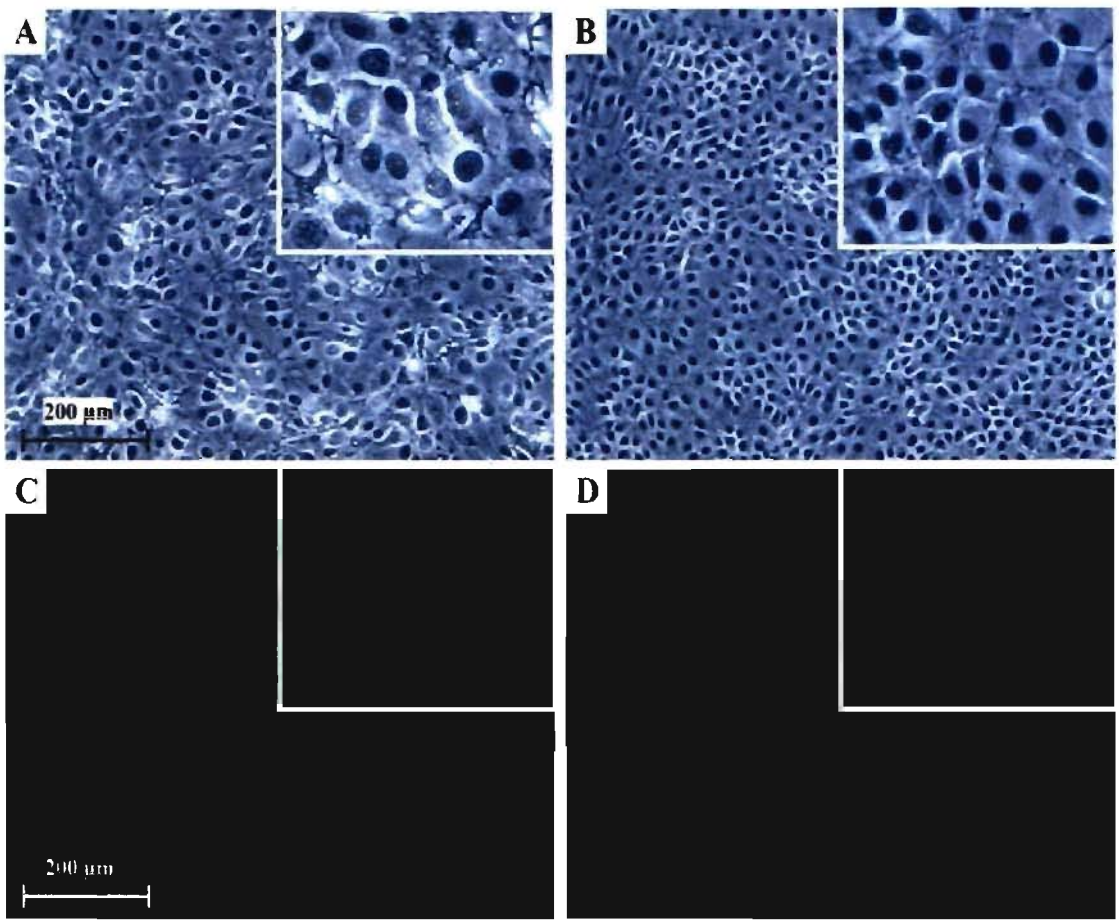

Figure 1. Detection of NPTr PCV-2 infected cells by immunofluorescence assay. NPTr cells were fixed at 72 hrs pi with a 4% PFA solution followed by incubation with porcine polyclonal anti-PCV-2 serum (Racine et al., 2004) and stained with anti-porcine FITC-conjugated secondary antibody. Panel A and C: NPTr PCV-2 infected cells with 1 MOI. Panel B and D: mock-infected NPTr cells. Upper panels (A and B): NPTr cells visualized by phase contrast microscopy. Lower panels (C and D): same NPTr cells of panels A and B, respectively, visualized following IFA. A higher magnification of the cells is shown in inset of each panel.

Figure 2. PCV-2 infectious particles production at different cell passages

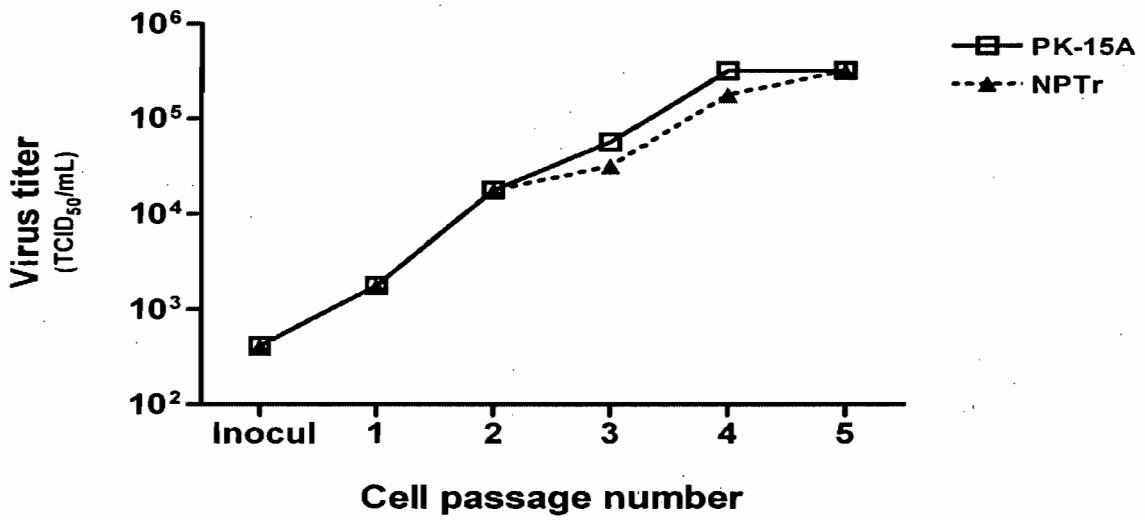

Figure 2. PCV-2 infectious particles production in PK-15A and NPTr cells following five consecutive passages. PCV-2 was serially passaged in PK-15A and NPTr cells as described in the Material and Methods section of the manuscript. The amount of the infectious viral particles recovered after each passage was determined in PK-15A cells by the Kärber method following IFA to detect PCV-2 infected cells. The initial inoculum (inocul) used to infect both cell lines was 4.17×10^2 $TCID_{50}$/mL. No significant difference was found between PCV-2 infectious particles yields from NPTr and PK-15A infected cells (P=0.2832).

Figure 3. PCV-2 replication kinetics

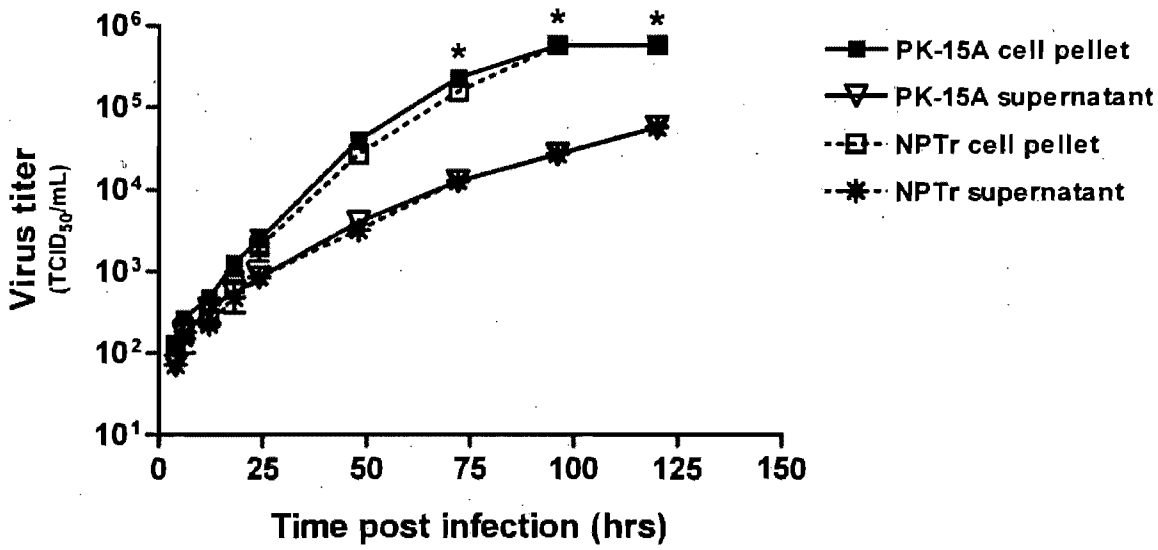

Figure 3. PCV-2 replication kinetics in PK-15A and NPTr cells. PK-15A and NPTr cells were infected with 1 MOI of PCV-2. At different times pi, the amount of PCV-2 recovered from PK-15A and NPTr infected cells was determined in culture medium (or supernatant) and in cell (or cell pellet). A significant increase in PCV-2 infectious particles production started to appear in both cell lines at 48 hrs pi. No statistical significant difference was observed in the overall PCV-2 replication kinetic between PK-15A and NPTr cells ($P>0.05$). Asterix represent a statistical significant difference ($P<0.001$) between the amount of virus recovered from the cell culture medium and the cell pellet.

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Differential virus replication efficiency of porcine circovirus *2a* and *2b* in porcine trachea and kidney epithelial cell lines

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Short running title: PCV-2b is more efficient than PCV-2a *in vitro*

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 unknown in North America. Thus, it became important to demonstrate that PCV-2b strain circulating in Canadian swine herds is more virulent than older PCV-2a strain. Infectious DNA clones of PCV-2a and PCV-2b were constructed (pPCV-2a and pPCV-2b). The efficacy of pPCV-2a and pPCV-2b transfected PK-15 cells to allow a complete virus replication cycle and production of PCV-2 infectious particles was evaluated during eight consecutive cell passages. Ten times more PCV-2b infectious viral particles were recovered at the 8^{th} cell passage compared to PCV-2a. To corroborate this result, the PCV-2a and PCV-2b replication kinetics were evaluated in porcine kidney (PK-15) and newborn piglet tracheal (NPTr) epithelial cell lines. PCV-2b virus replication kinetic was significantly more efficient in both cell lines compared to PCV-2a. In conclusion, PCV-2b is about 6 to 10 times more efficient than the PCV-2a for the synthesis of infectious viral particles in 2 porcine epithelial cell lines. Moreover, since PCV-2 transmission occurs most likely via the oro-nasal route, the fact that PCV-2b replicates more efficiently in tracheal epithelial cells than PCV-2a may contribute significantly to the spreading of the virus in the organism at its entry site. Consequently, the virus replication efficiency of PCV-2b compared to PCV-2a could easily explain the increased occurrence of PCVAD in Canada during the recent years.

Introduction

Postweaning multisystemic wasting syndrome (PMWS) is a swine disease that was first recognized in Canada in 1991 [10, 24]. Afterwards, the syndrome was reported worldwide [1, 2, 6, 37, 41, 42]. The disease usually 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 [24, 45]. The primary etiological agent responsible for PMWS has been identified as a circovirus named *Porcine circovirus-2* (PCV-2; family *Circoviridae*, genus *Circovirus*) [1, 15, 23, 31]. Nonetheless, the course of PCV-2 infection that will lead to the occurrence of the disease has been associated with the presence of other pathogens such as *M. hyopneumoniae*, *Porcine parvovirus* (PPV), *Porcine reproductive and respiratory syndrome virus* (PRRSV), and Swine influenza virus (SIV) [13, 16, 35, 36, 39]. Furthermore, the disease appearance following PCV-2 infection is known to be more complex than initially established because PCV-2 can play a pivotal role in several syndromes [7, 25]. 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, Canada, experienced a significant increase in death rate related to PCVAD [11]. It was hypothesized that this emerging problem was caused by the presence of a new type of circulating PCV-2 strain. This was confirmed by sequencing the entire viral genome of several PCV-2 strains originating from PMWS-affected herd [20] . Interestingly, this newly recovered PCV-2 genotype (named PCV-2b in comparison with the previously circulating genotype named PCV-2a) had already been reported in Asia and Europe [12] and had also been recently reported in 2007 in the United States [8, 27]. Now, an international consensus has been established in regards to the recognition of at least 2 major PCV-2 genotypes circulating worldwide [8, 14, 20, 22, 34] but there is no universal nomenclature accepted to describe them. Recently, a nomenclature has been proposed, one that takes into account the classification of Gagnon and collaborators (2007) and identifies the two major genotypes

(PCV-2a and PCV-2b) as well as a third one (PCV-2c) including only three 80's isolates that have never been reported afterwards [44].

The appearance of the new PCV-2b genotype in Canada could explain the death rate increase related to PMWS, but this relationship has to be confirmed. Consequently, one of the main objectives of the swine industry and health scientists is to establish if there is virulence variation between both PCV-2 genotypes (PCV-2a and PCV-2b).

The aim of the present study was to demonstrate that PCV-2b strain that circulates in Canadian swine herds is more virulent *in vitro* than PCV-2a strain. Thus, the virus replication efficiency of PCV-2a and 2b in porcine kidney epithelial cell line (PK-15) was evaluated. Noteworthy, the newborn piglet tracheal (NPTr) epithelial cell line [19] was recently discovered to be permissive to PCV-2 replication [33]. Consequently, the virus replication kinetics of PCV-2a and 2b was also evaluated in NPTr cells. The present study demonstrates that PCV-2b is 6 to 10 times more efficient than PCV-2a for infectious viral particles production in two porcine epithelial cell lines.

Materials and Methods

Cells. The PK-15A cells [40], a subclone of PK-15 PCV-1 non infected cells, were maintained at 37^0C in 5% CO_2 atmosphere in Minimum Essential Medium (MEM) with Earle's salts (Invitrogen Corporation, GibcoBRL, Burlington, ON, Canada), supplemented with 10% fetal bovine serum (FBS) (Wisent Inc, St-Bruno, QC, Canada), 300 U/mL of penicillin, 300 mg/mL of streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10 mM HEPES (Invitrogen Corporation, GibcoBRL, Burlington, ON, Canada). The NPTr cells [19] were grown at 37^0C in 5% CO_2 in MEM with Earle's salts, supplemented with 10% FBS, 300 U/mL of penicillin, 300 mg/mL of streptomycin, 1mM sodium pyruvate.

Construction of PCV-2a and 2b infectious DNA clones. Viral DNA genome extraction was performed from tissue samples (lungs and lymph nodes) submitted at the molecular diagnostic laboratory of the Faculté de médecine vétérinaire (FMV) of the Université de Montréal using the Qiagen QIAamp DNA Mini Kit (Qiagen, Valencia, CA) as previously described [21]. The PCV-2 genotype identification was performed with the multiplex real-time quantitative polymerase chain reaction (mrtqPCR) assay, as previously described [21] and the strains classification was confirmed following sequencing of the entire viral genome followed by sequence analyses as previously described [20]. A strain from each genotype (PCV-2a: FMV-07-0039 and PCV-2b: FMV-06-0732) was selected for the construction of PCV-2 infectious DNA clones. The construction of PCV-2a and 2b infectious DNA clones was done using the method of Fenaux et al. (2002) [17] with minor modifications. Briefly, the extracted DNA was amplified by PCR with PfuUltra High-Fidelity DNA Polymerase (Stratagene, La Jolla, California, USA). The PCR products encompassing the entire viral genome of both strains were produced using the following primers: F-PCVSAC2 (5'-TCCCCGCGGGCTGGCTGAACTTTTGAAAGT-3'), R-PCVSAC2 (5'-TCCCCGCGGAAATTTCTGACAAACGTTACA-3') and R-PCVKPNI (5'-CGGGGTACCAAATTTCTGACAAACGTTACAGGGTAC-3'). Then, to construct infectious DNA clones containing two copies of the entire PCV-2 viral genome, a two steps approach was used. In the first step the PCR product containing the complete PCV-2 viral

genome, amplified by F-PCVSAC2 and R-PCVKPNI was cloned into the pCR-Blunt plasmid vector using the Zero Blunt PCR Cloning Kit (Invitrogen Canada Inc, Burlington, Ontario, Canada) according to the manufacturer's instructions. Similarly, the PCR product containing the complete PCV-2 viral genome amplified by F-PCVSAC2 and R-PCVSAC2 was cloned into the pCR-Blunt plasmid vector. At the 2nd step, the full-length PCV-2 genomic DNA was extracted from the recombinant pCR-Blunt: F-PCVSAC2/R-PCVSAC2 plasmid following *Sac*II restriction enzyme digestion and cloned at the *Sac*II site of the recombinant pCR-Blunt: F-PCVSAC2/R-PCVKPNI plasmid to obtain recombinant plasmids containing two copies of the DNA viral genome. The orientation of the two copies of the DNA viral genome and the integrity of the PCV-2 infectious DNA clones (pPCV-2a and pPCV-2b) were confirmed by PCR, restriction fragment length polymorphisms and sequencing.

PK-15A cells transfection with PCV-2 infectious DNA clones. A monolayer of PK-15A cells at 50% - 70% confluency were trypsinized and centrifuged at 1000 rpm for 5 min. The cell pellet was washed twice with sterile phosphate-buffered saline (PBS), and 2×10^6 cells were resuspended in 0.2 mL of electroporation buffer (growth media without serum). The cells were mixed with 10 μg of DNA of each infectious DNA clones then transfected using a Gene Pulser Xcell Electroporation System (BIO RAD Laboratories Inc, Life Science Research Division, Mississauga, Ontario, CA). The cells were pulsed twice with the following settings: 425V, 0Ω, and 25μF [30] (exponential decay protocol was used with a 0.2 cm Gene Pulser cuvette). Mock-transfected cells with empty pCR-Blunt vector were included as controls. The cells were washed twice with sterile PBS buffer and seeded in two culture flasks. A flask of mock-transfected cells with empty pCR-Blunt vector was included as a negative control.

Generation of PCV-2a and PCV-2b infectious virus stocks. Following cell transfections, the viruses produced were serially propagated into PK-15A cells and were purified and concentrated following ultracentrifugation on a 30% sucrose cushion using the SW28 Beckman Coulter rotor (Beckman Coulter Canada Inc., Mississauga, Ontario, Canada) at 25 000 rpm for 5 hrs. The virus pellets were resuspended in 2 mL of a

phosphate buffer saline (PBS) containing 2% fetal bovine serum, and aliquots of the virus stocks were then conserved at –70°C for future use. The infectious dose of the virus stocks were determined from a 96-well microplate of PK-15A-infected cells following immunofluorescence assay (IFA) [40] by the Kärber method as previously described [21]. For the calculation of the virus titer, a well was considered to be PCV-2 positive if specific fluorescence was detected in cells. The virus titer was expressed in tissue culture infectious dose 50 per mL ($TCID_{50}$/mL).

Immunofluorescence assay. To determine the *in vitro* infectivity of the molecular DNA infectious clones and to titer the PCV-2 virus stocks, an IFA was performed as previously describe with minor modifications [40]. Briefly, to determine the *in vitro* infectivity of the molecular DNA infectious clones, transfected cells (see above) were cultivated on eight-well LabTek chamber slides (Nalge Nunc International, Rochester, NY, USA). An amount of 3 x 10^4 PK-15A transfected cells were seeded per well. Mock-transfected cells with non-recombinant pCR-Blunt vector were included as a negative control. The transfected cells were fixed at 72 hrs post transfection with a solution of 4% paraformaldehyde (PFA) in PBS for 20 min. Then, the cells were washed three times with PBS and they were permeabilized with a solution containing 0.1% triton X-100 in PBS for 10 min. After incubation with a blocking solution (1% bovine serum in PBS-T for 20 min), cells were incubated with a 1:200 dilution of the polyclonal PCV-2 porcine serum [40] at 37^0C for 90 min. Then, cells were washed three times with a solution containing 1% bovine serum in PBS-T and incubated with a rabbit anti-pig FITC conjugate antibody (MP Biomedicals, Solon, OH, USA) diluted 1:75 at 37^0 C for 60 min. After three washing steps, cells were visualized using a DMI 4000B reverse fluorescence microscope. Photographs of the cells were taking with a DFC 490 digital camera and the images were analyzed using the Leica Application Suite Software, version 2.4.0 (Leica Microsystems).

PCV-2 infectious viral particles production efficiency. Following transfections of 2x10^6 PK-15A cells with each infectious DNA clones, cells were divided and seeded in two 25 cm^2 culture flasks. At 96 hrs post infection (pi), one transfected-flask with each PCV-2 genotype was harvested following three cycles of freeze and thaw at – 70^0C. After

centrifugation at 2000 rpm at 4^0C for 10 min, the amount of PCV-2 infectious virus particles contained in the supernatant was determined, as described above. The remaining cells of the 2nd flask were trypsinised and seeded in two new 25 cm^2 flasks and reinfected with a 1:6 dilution of the previously collected PCV-2 virus. The same protocol was carried out 7 times for subsequent passages. Mock-transfected cells were included as controls.

PCV-2 replication kinetics in PK-15A and NPTr cells. 1 x 10^5 PK-15A and NPTr cells were infected in suspension with 0.5 MOI of PCV-2a and 2b produced from the infectious DNA clones. Following a 4 hrs absorption period, the virus inoculum was removed and cells were washed five times with PBS before adding fresh culture medium. At different times post infection (0, 4, 6, 12, 18, 24, 48, 72, 96 and 120 hrs pi), the infected cell cultures were collected, centrifuged at 2000 rpm at 4^0C for 10 min, then cell pellets and supernatants were stored at -70^0C until virus titration. Before titration, all cell pellets and supernatants were frozen and thawed three times to free infectious viral particles, and cell debris were discarded after centrifugation at 2000 rpm at 4^0C for 10 min. Mock-infected cells were included in each experiment as control. All experiments were performed two times in triplicate.

Statistical analyses. The statistical analyses were realized using the GraphPad Prism version 4 software. The regular two-way ANOVA combined with the Bonferroni post-tests models were used to determine if there were statistically significant differences between PCV-2a and 2b in regards to their efficiency to replicate in PK-15A and NPTr cell lines.

Results

Construction of PCV-2a and PCV-2b infectious DNA clones. The complete genomes of both PCV-2 viruses were amplified by PCR. Two copies of the PCV-2 genomes were ligated in tandem into the pCR-Blunt plasmid vector to produce the PCV-2a and PCV-2b infectious DNA clones (named pPCV-2a and pPCV-2b, respectively). The ability of pPCV-2a and pPCV-2b to express the viral capsid protein (Cap) was determined *in vitro* following transfection of PK-15A cells with pPCV-2a and pPCV-2b and its detection by IFA. Both intracytoplasmic and intranuclear positive signals were found in pPCV-2a and pPCV-2b transfected PK-15A cells with a higher amount of antigen detected in the nucleus as illustrated in Fig. 1. The ratio of the PK-15A transfected cells expressing the PCV-2 antigens was very similar for both genotypes (Fig. 1). The PK-15A cells transfected with the empty pCR-Blunt vector remained negative for PCV-2 antigen by IFA (Fig. 1).

PCV-2 infectious viral particles production efficiency. Following the confirmation that the pPCV-2a and pPCV-2b transfected PK-15A cells were able to express PCV-2 proteins, the efficacy of the transfected PK-15A cells to allow a complete virus replication cycle and infectious viral particles production was determined. The amount of PCV-2 virus produced in PK-15A transfected cells was evaluated during eight consecutive cell passages (Fig. 2). At the beginning, the cells were transfected with same amount of PCV-2 DNA infectious clones (10 μg). At the first cell passage (i.e. transfected cells), 10^3 $TCID_{50}$/mL infectious viruses were recovered from PK-15A transfected cells for both genotypes (Fig. 2). Furthermore, it was noticed that the production of PCV-2 infectious viral particles was less efficient during the 2nd and 3rd cell passages. However, at the 4th cell passage, the amount of PCV-2 infectious viral particles recovered was identical to the amount recovered at the first cell passage and started to increase at that point until the end of the experiment, i.e. until the 8th cell passage, for both PCV-2 genotypes. At the 8th cell passage, the amount of PCV-2 virus recovered from cell lysates was 10^4 $TCID_{50}$/mL and 10^5 $TCID_{50}$/mL for PCV-2a and PCV-2b, respectively. Consequently, 10 times more PCV-2b infectious viral particles were recovered at the 8th cell passage compared to PCV-2a

(Fig. 2). Furthermore, this latest result was confirmed by IFA in PK-15A infected cells with PCV-2a and 2b cell lysates of the 8^{th} cell passage (Fig. 3). As illustrated in Fig. 3, the amount of cells expressing the Cap protein was higher in PCV-2b infected cells compared to PCV-2a infected cells when the same amount of inoculum was used. In addition, the cells infected with the PCV-2b 8^{th} cell passage lysate 10^{-2} dilution has a similar number of IFA positive cells than the 10^{-1} dilution of PCV-2a 8^{th} cell passage lysate infected cells (Fig. 3), which furthermore indicated that the PCV-2b virus production was higher compared to PCV-2a virus production. The PK-15A mock infected cells remained negative for the expression of PCV-2 antigen (Fig. 3).

PCV-2 replication kinetics in PK-15A and NPTr cells. To confirm the previous results, the PCV-2a and PCV-2b replication kinetics were evaluated in PK-15 and NPTr cells. Consequently, the amounts of virus yields in cell culture medium (or supernatants) and in cells (or cell pellets) were determined at different times pi (Fig. 4). PCV-2 infectious particles production was clearly demonstrated in both porcine epithelial cell lines since the amount of virus recovered in PK-15A infected cells at 120 hrs pi was 790 and 3702 times higher compared at 4 hrs pi in cell pellets for PCV-2a and PCV-2b, respectively (Fig. 4). Similarly, the amount of virus recovered in NPTr infected cells at 120 hrs pi was 1000 and 4444 times higher compared to 4 hrs pi in cell pellets for PCV-2a and PCV-2b, respectively (Fig. 4). At 72 hrs pi and until the end of the experiment, the amount of PCV-2a and PCV-2b recovered from the cell fractions in both cell lines was 10 times higher compared to the amount of viruses recovered from the supernatant or cell culture medium ($P<0.001$) (Fig. 4), suggesting that PCV-2 was weakly released from infected cells. Overall, the amount of PCV-2b recovered in both infected cell lines was 3, 6 and 6 times higher compared to PCV-2a infected cells at 72, 96 and 120 hrs pi, respectively ($P<0.001$) (Fig. 4), indicating that PCV-2b was more efficient compared to PCV-2a in regards to virus replication and subsequently for infectious viral particles production. In addition, there is no significant difference in the amount of the virus yields for both PCV-2 genotypes between both cell lines ($P>0.05$) (Fig. 4).

Discussion

Beside genomic analysis results, it is the first time that phenotypic difference between PCV-2a and PCV-2b viruses such as *in vitro* virus replication kinetic is reported. In the present study, PCV-2b virus has been shown to be 6 to 10 times more efficient than PCV-2a for infectious viral particles production (Figures 2 and 4). Moreover, this latest result has been obtained in 2 permissive porcine cell lines, the PK-15 and the newly discovered NPTr tracheal epithelial cells [33], which further indicates that this phenotypic characteristic could possibly be applied to several other porcine cell types beside kidney cells. Since PCV-2 transmission is considered to occur most likely via the oro-nasal route, it was interesting to found out that PCV-2b virus replicates more efficiently in NPTr cells, which may contribute significantly to the spreading of the virus in the organism at its entry site. Several studies have shown a correlation between PCV-2 viral loads with the severity of histopathological lesions and disease expression [3, 9, 21, 26, 28, 29, 43]. Thus, it is conceivable to correlate this finding with the fact that if virus replication of PCV-2b is more efficient then it should increase the tissues viral load and subsequently favor the appearance of PCVAD. In addition, it is interesting to pin point that PCV-2b has appeared at the same time than the PCVAD prevalence increases in Canada [4, 5, 11]. Moreover, a recent retrospective study showed a statistically significant PCVAD frequency increase of characteristic histological lesions observed in lymph node, spleen, lung, small intestine, colon, and kidney, in PCV-2b infected animals compared to pigs infected with PCV-2a strains [5]. As an example, there is 9.33 times more chances to observe depletion of lymphoid follicles in lymph node of animals infected with PCV-2b strains compared to animals infected with PCV-2a strains [5]. In addition, viral burden in lymph node, which has been estimated with immunohistochemistry (IHC) staining, was also significantly increased in pigs infected with the PCV-2b strains compared to PCV-2a strains [5]. Because these results were derived from a retrospective analysis of clinical cases submitted to Canadian diagnostic laboratories, the conclusion may be biased but it strongly suggests that PCV-2b virus is more virulent than PCV-2a virus, and it is supported by the fact that PCV-2b virus replication is more efficient than PCV-2a virus replication (Figures 2 and 4). On the other hand, a recent study has demonstrated no difference in virulence between

PCV-2a and PCV-2b in a conventional pig infection model meaning that the PCVAD outbreaks seen in recent years cannot be explained only by the introduction of more virulent strains of PCV-2 [38]. Interestingly, seqùence analysis has demonstrated that the PCV-2/ORF2 gene sequence in samples collected from Swedish pigs, prior to and during an outbreak of enzootic PMWS, could be classified into three main genogroups designated SG1, SG2 and SG3 [46]. Of particular significance, one of the genogroups (SG1) has never been associated with PMWS in the field whereas another (SG3) was predominantly recovered from farms following outbreaks of PMWS [46]. In addition, sequence analysis revealed that Canadian PCV-2b strains associated with PCVAD outbreaks fall clearly within SG3 genogroup (>98.5% ORF2 nucleotide sequence identities) and further supports the hypothesis of the authors [46].

In a previous study, the replication kinetics of seven different PCV-2 strains were compared in PK-15 cells [32]. It was demonstrated that the production of infectious viruses in PK-15 cells can be more efficient for some strains (like Stoon-1010) compared to other strains (like strain 1121) but only strains of the same genotype (PCV-2a) were compared [32]. Furthermore, the total maximum amount of recovered viruses was 100 lower compared to the present study. Small differences in the genetic code could easily be involved in the differences observed in regards to virus replication efficiency. As an example, it was demonstratred that PCV-2 passaged 120 times in PK-15 cells (VP120) replicated more efficiently than wild-type virus that had been passaged only once (VP1) [18]. Two amino acids mutations were identified in the Cap protein between VP1 and VP120 viruses; proline to alanine at position 110 (P110A) and arginine to serine at position 191 (R191S), which indicate that ORF2 gene could be involved in virus replication efficiency. The most variable gene between the PCV-2a and PCV-2b genotypes is the ORF2 gene encoding the Cap protein, with an amino acid (aa) sequence identity varying from 88% to 94% between genotypes [20].

In conclusion, PCV-2b is about 6 to 10 times more efficient than the PCV-2a for the synthesis of infectious viral particles in two porcine epithelial cell lines. It could easily explain the increased occurrence of PCVAD during the recent years. The fact that PCV-2b virus replication is more efficient in two porcine cell lines emphasizes the importance of the present study. Since only one virus in each PCV-2 genotype has been studied, it will be

interesting to evaluate several strains of each genotype to generalize the present findings. Furthermore, experimental infection of susceptible pigs with PCV-2a and PCV-2b will be necessary to establish with certainty any relationship between the PCVAD severity and PCV-2 genotypes.

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Figure 1

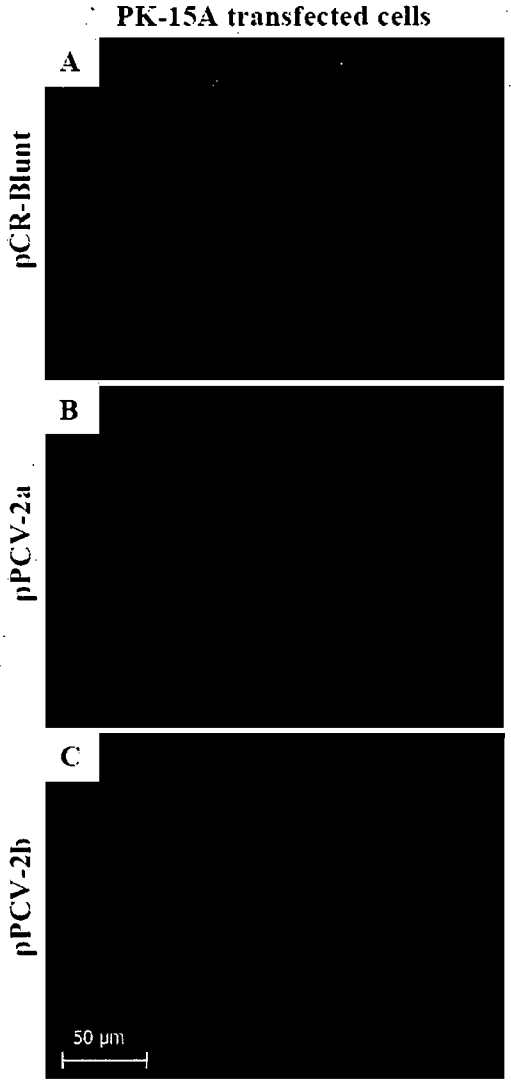

Figure 1. Detection of PCV-2 infectious DNA clones transfected PK-15A cells by immunofluorescence assay. PK-15A cells were transfected with PCV-2a and 2b infectious DNA clones (pPCV-2a in panel B and pPCV-2b in panel C, respectively) or with the empty pCR-Blunt vector (panel A: pCR-Blunt). Transfected cells were fixed at 72 hrs pi with a 4% PFA solution followed by incubation with porcine polyclonal anti-PCV-2 serum and stained with anti-porcine FITC-conjugated secondary antibody. The ratio of the PK-15A transfected cells expressing the PCV-2 antigens was similar for both genotypes. The PK-15A pCR-Blunt transfected cells remained negative for PCV-2 antigen by IFA.

Figure 2

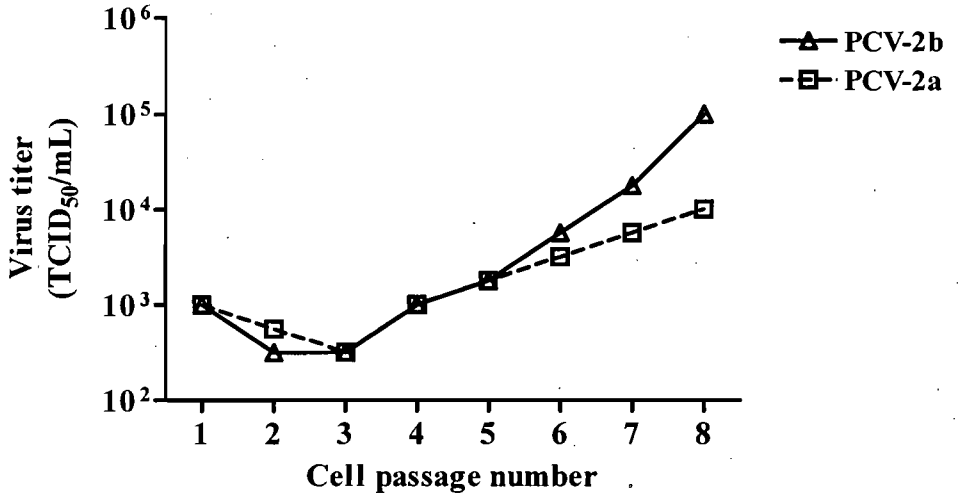

Figure 2. PCV-2 infectious viral particles production in PK-15A transfected cells. At passage number 1, the cells were transfected with same amount (10μg of DNA) of PCV-2 DNA infectious clones (pPCV-2a and pPCV-2b). Then, produced virus (PCV-2a and PCV-2b) was serially passaged in PK-15A cells as described in the Materials and Methods section of the manuscript. The amount of the infectious viral particles recovered after each passage was determined in PK-15A cells by the Kärber method following IFA to detect PCV-2 infected cells. Ten times more PCV-2b infectious viral particles were recovered at the 8th cell passage compared to PCV-2a.

Figure 3

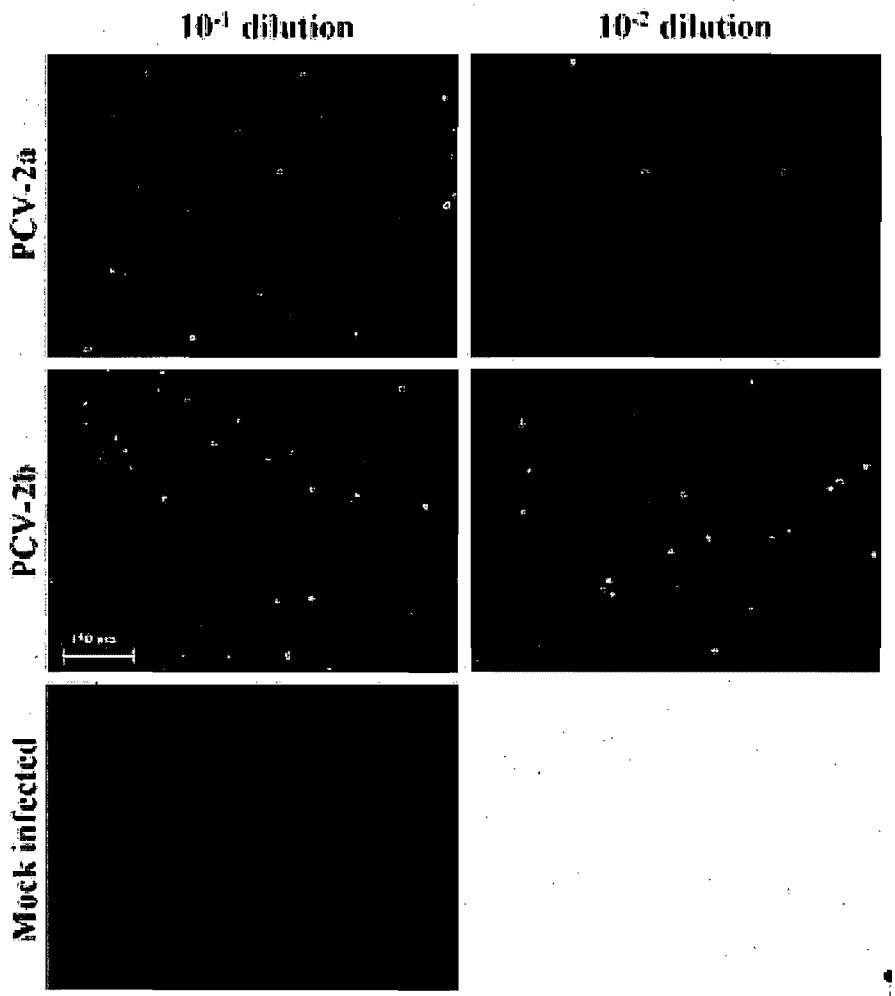

Figure 3. Detection of PK-15A cells infected with the cell lysates of the 8th cell passage. PK-15A cells were fixed at 72 hrs pi with a 4% PFA solution followed by incubation with porcine polyclonal anti-PCV-2 serum and stained with anti-porcine FITC-conjugated secondary antibody. The amount of cells expressing the Cap protein was higher in PCV-2b infected cells compared to PCV-2a infected cells at the same virus dilution inoculums. The cells infected with a 10^{-2} dilution of PCV-2b 8th cell passage lysate has a similar number of IFA positive cells than the cells infected with a 10^{-1} dilution of PCV-2a 8th cell passage lysate. The PK-15A mock infected cells remained negative for the expression of PCV-2 antigen.

Figure 4

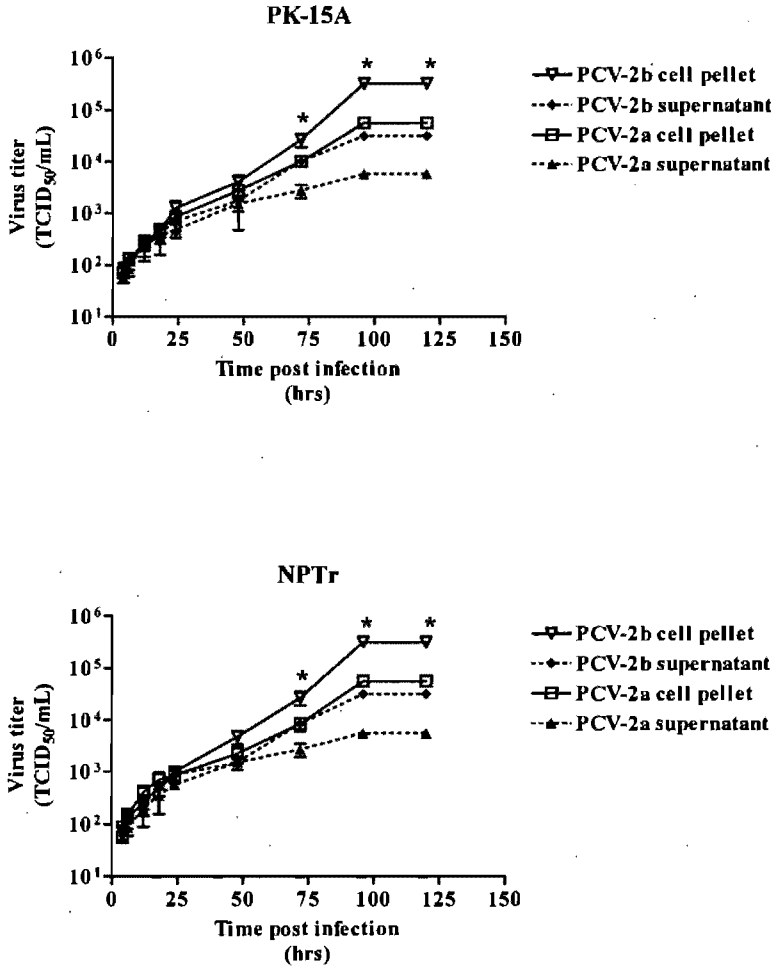

Figure 4. PCV-2 replication kinetics in PK-15A and NPTr infected cells. PK-15A and NPTr cells were infected in suspension with 0.5 MOI of PCV-2a and PCV-2b. At different times pi, the amount of PCV-2 recovered from PK- 15A and NPTr infected cells was determined in culture medium (or supernatant) and in cell (or cell pellet). At 72 hrs pi and until the end of the experiment, the amount of PCV-2a and PCV-2b recovered from the cell fractions in both cell lines was 10 times higher compared to the amount of viruses recovered from the supernatant or cell culture medium ($P<0.001$). The amount of PCV-2b recovered in both cell lines was 3, 6 and 6 times higher compared to PCV-2a at 72, 96 and 120 hrs pi, respectively (*$P<0.001$).

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IV. DISCUSSION

At the end of 2004, the swine industry in the province of Quebec experienced a significant increase in death rate related to PCVAD. At that time, no statistical analysis supported this observation and no data indicating the extent of the increase in death rate was available. Consequently, an epidemiological survey that included producers (for a total of 245 producers) that annually sold on the market 15% (1 000 000 pigs) of the entire Quebec pig production was conducted to provide valuable information on the severity of the mortality increase [42]. This study included all types of production and revealed an increase of 2.39% in the mortality rate in Quebec pig farms in 2005 (7.57%) compared to 2004 (5.18%). More specifically, weaning-finishing production had a mortality rate average of 7.53% in 2005 compared to 5.31% in 2004. Similarly, finishing production had a mortality rate average of 7.66% in 2005 compared to 4.88% in 2004. Interestingly, 56% of the producers indicated that their production had a clinical, pathologic, or laboratory diagnosis of PCVAD at the time of the survey, which was held at the end of 2005 [42]. It was hypothesized that this emerging problem was caused by the presence of a new type of circulating PCV-2 strain. This was confirmed by sequencing the entire viral genome of several PCV-2 strains originating from PMWS-affected herd [63]. The appearance of the new PCV-2b genotype in Canada could explain the death rate increase related to PCVAD. To confirm this relationship, one general objective and four specific objectives were formulated in this study. The general objective of the present study was to establish whether the new genotype (PCV-2b) is more pathogenic than the old genotype (PCV-2a) and therefore determine whether the increase, since 2004, in the occurrence of the disease associated with PCV-2 (PCVAD) can be explained by the emergence of the new genotype. Specific objectives were: (1) to construct various infectious molecular DNA clones with the genome sequence of new and old genotypes; (2) to evaluate *in vitro* viral replication of the different PCV-2 viruses; (3) to evaluate *in vivo* pathogenesis and (4) to improve diagnostic tests for detection and differentiation of the PCV-2 new genotype from the old one. In our work, we had significant results and progress in three of our objectives. Unfortunately, *in vivo* work did not produce any satisfying results.

Considering the fact that the PCV-2 virus is recognized as a virus whose propagation in cell culture is not very efficient, it was of the utmost importance to proceed in a certain way that would encourage virus production in larger quantities to eventually be

able to perform experimental infections on the piglet. Consequently, the reverse genetics, as described by Fenaux and his collaborators (2002), were the applied method to fulfill the criteria [56]. Two copies of the PCV-2 genomes were ligated in tandem into the pCR-Blunt plasmid vector to produce the PCV-2a and PCV-2b infectious DNA clones as previously described [56] with minor modification, and they were shown to be infectious *in vitro* when transfected into PK-15 cells (article # 2, Fig. 1). The construction of an infectious PCV-2 molecular DNA clone and the demonstration of infection by direct transfection *in vitro* and *in vivo* should be very advantageous for future PCV-2 studies. Using recombinant plasmids constructed *in vitro* to test different regions or genes of PCV-2 for their roles in virus replication and pathogenesis, this *in vitro-in vivo* transfection system will enable us to study the structural and functional relationship of PCV-2 genes. The replication and pathogenesis of PCV-2 can be studied without having to produce infectious virus stocks by propagating PCV-2 in cell cultures. This is advantageous, as serial cell culture passages may select for viral variants [6]. Another advantage of using cloned PCV-2 genomic DNA, instead of live virus, is its relative ease for quantitation of the inoculation dose. The amount of the cloned PCV-2 DNA used for inoculation can be easily determined by a spectrophotometer, whereas the dose of live PCV-2 virus requires infectivity titration in cell cultures and confirmation of infection by IFA. Finally, direct transfection of the cells with cloned PCV-2 plasmid DNA eliminates the problems associated with the presence of other indigenous swine agents that could be present in viral stock.

A new, the newborn piglet tracheal (NPTr) epithelial cell line [61], was found to be permissive to PCV-2 (article # 1, Fig 1). This finding is interesting because until now, there was only one type of immortalized cell line (the porcine kidney epithelial cells) known to permit infectious viral particles production [106, 142]. Both cells, PK-15 and NPTr, were identical in the regards to their efficacy to produce infectious viral particles (article # 1, Fig. 3; article # 2, Fig. 4), suggesting that new cell line could be used for vaccine production. Unfortunately, this remains an open question, because NPTr cells contain porcine endogenous retrovirus (PERV) and this is not allowed by the Food and Drug Administration (FDA) guidelines [61]. Furthemore, the NPTr cells can be a useful tool for virus isolation in a virology diagnostic laboratory since they permit the replication of a large variety of swine viruses [61]. Nonetheless, the NPTr cell line is now the second

immortalized cell line known to allow PCV-2 infectious viral particles production and in that regards, this cell line can be used as a new *in vitro* model to study PCV-2 pathogenesis. Work is now in progress to determine if there is any difference between NPTr and PK-15 cells following PCV-2 infection such as cytokine modulation.

Beside genomic analysis results, it is the first time that phenotypic difference between PCV-2a and PCV-2b viruses such as *in vitro* virus replication kinetic is reported. In the present study, PCV-2b virus has been shown to be 6 to 10 times more efficient than PCV-2a for infectious viral particles production (article # 2, Fig. 2 and 4). The PCV-2 transmission is considered to occur most likely via the oro-nasal route because experimental infection studies, which have mainly used the intranasal route of inoculation, support that idea [128]. Then, it was interesting to find that PCV-2b virus replicates more efficiently than PCV-2a virus in NPTr cells. *In vivo*, it was demonstrated that tracheal epithelial cells of PCVAD sick animals may express a high amount of PCV-2 antigens [20]. Taken together, these results suggest that tracheal epithelial cells could possibly contribute to the spreading of the virus in the organism by increasing the viral load at its entry site, but *in vivo* experiment will have to be conducted to confirm this hypothesis. Several studies have shown a correlation between PCV-2 viral loads with the severity of histopathological lesions and disease expression [18, 64, 73, 90, 152]. Thus, it is conceivable to correlate this finding with the fact that if virus replication of PCV-2b is more efficient, then it should increase the tissues viral load and subsequently favor the appearance of PCVAD. Moreover, a recent retrospective study showed a statistically significant PCVAD frequency increase with characteristic histological lesions observed in PCV-2b infected animals compared to pigs infected with PCV-2a strains [23]. Because these results were derived from a retrospective analysis of clinical cases submitted to Canadian diagnostic laboratories, the conclusion may be biased but it strongly suggests that PCV-2b virus is more virulent than PCV-2a virus, and it is supported by the fact that PCV-2b virus replication is more efficient than PCV-2a virus replication as reported in this study. On the other hand, a recent study has demonstrated no difference in virulence between PCV-2a and PCV-2b in a conventional pig infection model meaning that the PCVAD outbreaks seen in recent years cannot be explained only by the introduction of more virulent strains of PCV-2 [129]. Small differences in the genetic code could easily be involved in the differences

observed in regards to virus replication efficiency. As an example, it was demonstrated that PCV-2 passaged 120 times in PK-15 cells (VP120), replicated more efficiently than a wild-type virus that had been passaged only once (VP1) [58]. Two amino acids mutations were identified in the Cap protein between VP1 and VP120 viruses; proline to alanine at position 110 (P110A) and arginine to serine at position 191 (R191S), which indicates that the ORF2 gene could be involved in virus replication efficiency. In addition, the most variable protein between the PCV-2a and PCV-2b genotypes was the Cap protein, which is encoded by the ORF2 gene, with an amino acid (aa) sequence identity varying from 88% to 94% between genotypes [63].

In order to develop a PMWS infection model, Yorkshire X Landrace SPF (specific pathogen free) weaned piglets of a one week age originated from a maternity recognized to be free of the major porcine pathogens such as PRRSV, *M. hyopneumoniae,* influenzavirus, TGEV / PRCV and PPV were purchased. At their arrival, blood samples were collected and serological and PCR tests were executed to ascertain their health status and confirm the absence of pathogens susceptible to influence the experimental infections. The piglets were housed for three weeks before infection procedure. Two days before the experimental infections, blood samples were taken to establish their negative status in regard to PCV-2.

Experimental infections were conducted as described by Krakowka and al. (2001) and Nielsen and al. (2003) [86, 117]. Therefore, there were four experimental piglet groups, each divided as follows: 1) non-infected piglets, 2) non-infected and injected with KLH/ICFA, 3) piglets infected with PCV-2b alone and 4) piglets infected with PCV-2b and injected with KLH / ICFA. The advantages of KLH/IFCA as an immuno-stimulator which favours PCV-2 infection have already been demonstrated in few experimental infection studies [128]. During the study, the animals were monitored daily for clinical signs of disease. Each week, blood samples were collected, and rectal temperatures and body weight were recorded. The animals were to be humanitarianly sacrificed at the 35th day post-infection. Various organs were taken (lungs, mediastinal and tracheobronchial lymph nodes, intestines, kidneys, liver, spleen) to evaluate and quantify the histopathological lesions as well as to determine the presence of a PCV-2 antigen in those lesions (essential condition to confirm PCVAD).

Unfortunately, *in vivo* work did not produce any satisfying results. Four experimental infections were carried out, each time with minor modifications in the experimental design. The corrections we made were based on our own experience, and on new results and publications in other laboratories. In the end, we were not able to develop a constant model for PMWS disease production. During the four experimental infections, 26 piglets were infected and only one of them has developed PCV-2 characteristic lesions without clinical signs. This was a too low success rate to be considered as an efficient infection model. In conclusion, this project is to be continued and for its success, a more efficient infection model has to be elaborated. The possible solutions are to change the infection protocol: 1) either the animal type such as CDCD, gnotobiotic or 2) by the addition of a cofactor such as PRRSV, PPV, etc.

Following the development of a good infection model, it would have been possible to study the pathogenecity and virulence of PCV-2b compared to PCV-2a in infected animals.

Finally, in this study, a new multiplex real-time quantitative polymerase chain reaction (mrtqPCR) assay was developed to sensitively identify and differentiate PCV-2 viruses in clinical samples [64]. With the exception of the conventional PCR assay developed previously [79], all other PCR assays reported until now did not differentiate PCV-2 genotypes [18, 38, 93, 172]. Moreover, the conventional PCR diagnostic test reported previously [79] required 2 PCR reactions to identify both genotypes whereas only 1 reaction is required in the our mrtqPCR [64]. Previous results indicated that the gene that best distinguishes PCV-2a from PCV-2b was the ORF2, with a nt sequence identity varying from 88–94% between both genotypes [63].

Afterwards, a retrospective epidemiologic survey was conducted using PCV-2 mrtqPCR–positive cases that were submitted to the molecular diagnostic laboratory of the Faculté de médecine vétérinaire (FMV) of the Université de Montréal to determine the prevalence of PCV-2 genotypes and to establish if other viral or bacterial pathogens were associated with the clinical disease. Furthermore, because PCV-2 antigen detection in microscopic lesions by IHC is one of the three criteria used for the diagnosis of PCVAD cases [152, 158], the survey was conducted to determine the relationship between the PCV-2 IHC results and the PCV-2 viral load with PCVAD. The relationship between PCV-2

viral load and clinical expression of PCVAD confirms results previously reported [18, 119]. Higher is the PCV-2 viral load in tissue samples, higher is the probability to have PCVAD [64]. In the present study, the prevalence of PCV-2b virus was very high, as previously reported [63]. In fact, 95.9% of the submitted cases were infected with PCV- 2b virus, 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 [64]. 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. The course of PCV-2 infection that will lead to the occurrence of the disease has been associated with the presence of other pathogens, such as *M. hyopneumoniae*, PPV, PRRSV, and SIV [49, 54, 128, 133]. 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 [64], as previously reported. Similar to the correlation between PCV-2 viral load and PCVAD, there was a significant association between PRRSV viral load and the appearance of PCVAD. In contrast, the multivariate polytomous logistic regression analysis suggested that the presence and viral load of PRRSV had no significant effect on the PCVAD score, when both PCV-2 viral load and IHC result parameters are included in the regression model [64]. This suggests that PRRSV may be less involved as a cofactor responsible for the induction of PCVAD than expected [54, 133]. The most interesting finding was the significantly increased odd ratios (ORs) of isolating two major porcine respiratory pathogenic bacterias, *A. pleuropneumoniae* and *S. suis* pathogenic serotypes (1/2, 1, 2, 3, 4, and 7), as PCV-2 viral load increases. 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 after PCV-2 infection.

V. CONCLUSION

Postweaning multisystemic wasting syndrome (PMWS) is a major disease that poses a significant threat to the economics of global swine industry and more recently in North America. It is expected that results from this study will contribute to better understanding the virulence variation between both PCV-2 genotypes (PCV-2a and PCV-2b) and can help swine industry and health scientists to deal with this important issue.

Results from the first part of this thesis demonstrate that the newborn piglet tracheal (NPTr) immortalized epithelial cell line is permissive to PCV-2 replication. This suggests that the epithelial cells of the respiratory tract of swine can be the primary cell target for PCV-2 replication and could subsequently provide a way of entry in swine. In addition, the NPTr cell line could be used as a new *in vitro* model for studying PCV-2 pathogenesis and can be a useful tool for virus isolation in a virology diagnostic laboratory.

Furthermore, it was shown that the PCV-2b is about 6 to 10 times more efficient than the PCV-2a for the synthesis of infectious viral particles in two porcine epithelial cell lines. This finding could explain, at least in part, the increase in the occurrence of PMWS during recent years. The fact that PCV-2b virus replication is more efficient in two porcine cell lines emphasizes the importance of the present study. Experimental infection of susceptible pigs with PCV-2a and PCV-2b will be necessary to establish with certainty any relationship between the PMWS severity and PCV-2 genotypes.

Moreover, the infectious DNA clones constructed in this study could be used for mutant virus production. This would enable us to study the amino acids (aa), which could be responsible for the pathogenesis alteration between PCV-2b and PCV-2a.

It should be emphasized that *in vivo* experiments have not provided the expected results. Therefore, it is strongly recommended to repeat *in vivo* infection using the experience acquired during this study and other recent studies in order to develop a more efficient experimental infection model.

In the framework of this study, 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 increase the risk of developing PMWS. Relationship established between PCV-2 viral load and clinical expression of PMWS confirms results previously reported. Higher is the PCV-2 viral load

in tissue samples, higher is the probability to have PMWS. In addition, it has been demonstrated that mrtqPCR could replace the IHC assay to confirm PCVAD cases. 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 in the bacterial colonization of the swine respiratory tract.

VI. REFERENCES

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APPENDIX 1

Title of Conference Presentations and Posters

N Music, D Tremblay, J Harel, M.-H. Venne, S.M. Elahi, CA Gagnon : Emergence of a PCV2 genotype unknown to Canada correlates with the increase of PMWS disease in pig farms. (poster presentation). First Annual General Meeting of the Canadian Research Network on Swine infectious Diseases (SIDNet): 2006, Saint-Hyacinthe, Quebec, Canada.

N Music, D Tremblay, J Harel, M.-H. Venne, S.M. Elahy, CA Gagnon: The emergence of a porcine circovirus type 2 (PCV2) genotype previously unknown to Canada. (poster presentation) CRWAD (conference of research workers in animal diseases); 87th Annual Metting: December 3, 4 and 5, 2006 Chicago, Illinois, US.

N Music, D Tremblay, J Harel, M.-H. Venne, S.M. Elahi, CA Gagnon: The emergence of porcine circovirus 2b genotype (PCV-2b) in swine in Canada. (oral presentation) 1st Annual Symposium – CRIP (swine infectious disease research centre): may 28-29 2007, Saint-Hyacinthe, Quebec, Canada.

N Music, J.R.E. del Castillo, J Harel, G Fontaine, D Tremblay, CA Gagnon Development of a multiplex real-time quantitative-PCR assay for detection and differentiation of porcine circovirus 2a and 2b genotypes and its use in an epidemiological survey. (oral presentation) 1st Annual Symposium – CRIP (swine infectious disease research centre): may 28-29 2007, Saint-Hyacinthe, Quebec, Canada

D Tremblay, **N Music**, J.R.E. del Castillo, J Harel, G Fontaine, CA Gagnon Simultaneous detection and differentiation of porcine circovirus 2a and 2b genotypes with a real-time quantitative-PCR assay and its use in an epidemiological survey. (oral presentation) 6th Annual Meeting of Canadian Animal Health Laboratorians Network (CAHLN): June 10-13 2007, Saskatoon, SK, Canada,

N Music, D Tremblay, J.R.E. del Castillo, G Fontaine, J Harel, CA Gagnon Development of a multiplex real-time quantitative-PCR assay for detection and differentiation of porcine circovirus 2a and 2b genotypes and its use in an epidemiological survey. (poster

presentation) International PRRS Symposium 2007, November 30- December 1 2007 Chicago, Illionis, US

N Music, D Tremblay, J.R.E. del Castillo, G Fontaine, J Harel, CA Gagnon Development of a multiplex real-time quantitative-PCR assay for detection and differentiation of porcine circovirus 2a and 2b genotypes and its use in an epidemiological survey. (poster presentation) CRWAD (conference of research workers in animal diseases); 88th Annual Metting : December 2,3, and 4, 2007 Chicago, Illinois, US

JJ Jia, **N Music**, M Jaques, CA Gagnon Identification of a Porcine Lung Epithelial Cell Line permissive to Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) Replication (poster presentation) 4e Colloque international francophone de microbiologie animale: Septembre 21-24, 2008 St-Hyacinthe, Québec, Canada

N Music, M Jaques, CA Gagnon Replication of Porcine circovirus genotype 2b (PCV-2b) is more efficient than genotype 2a (PCV-2a) in two different porcine epithelial permissive cell lines (poster presentation) 4e Colloque international francophone de microbiologie animale: Septembre 21-24, 2008 St-Hyacinthe, Québec, Canada

N Music, M Jaques, CA Gagnon Identification of a new cell line permissive to porcine circovirus type 2 replication: the newborn piglet tracheal cells (oral presentation) 4e Colloque international francophone de microbiologie animale: Septembre 21-24, 2008 St-Hyacinthe, Québec, Canada

APPENDIX 2

Publications

J Vet Diagn Invest 20:545–558 (2008)

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

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

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 PCV-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 epidemiologic 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, the 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 ($P > 0.9$), which suggests 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: Epidemiologic 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, and outbreaks have been 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 etiologic agent responsible for PMWS has been identified as a circovirus particle named *Porcine circovirus-2* (PCV-2; family *Circoviridae*, genus *Circovirus*).[1,19,25] The 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, porcine respiratory disease complex, 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 Quebec 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

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