Université de Montréal

IDENTIFICATION OF A NEW CELL LINE PERMISSIVE TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS REPLICATION

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IDENTIFICATION OF A NEW CELL LINE PERMISSIVE TO PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS REPLICATION

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RÉSUMÉ

Le syndrome reproducteur et respiratoire porcin (SRRP) est une des maladies les plus dévastatrices économiquement pour l'industrie mondiale du porc. L'agent étiologique du SRRP est le virus du SRRP (VSRRP) lequel est connu pour avoir une spécificité d'hôte très restreinte et pour sa transmission par voie aerosol. Les antigènes et les ARN du VSRRP ont été trouvés dans des cellules épithéliales du tractus respiratoire de porcs infectés par le virus. L'interaction entre les macrophages alvéolaires porcins (PAMs) et le VSRRP a été démontrée comme jouant un rôle important dans l'infection causée par le virus. Malgré cela, l'interaction prenant place entre les cellules épithéliales du tractus respiratoire porcin et le virus ne devrait pas être négligée. Jusqu'à présent, la réplication du VSRRP *in vitro* dans des cellules épithéliales du tractus respiratoire porcin n'a pas été conduite avec succès et les tentatives pour le faire ont échoué. Une nouvelle lignée de cellules épithéliales de poumon de porc (SJPL) est maintenant disponible et sera utilisée dans cette étude afin de déterminer si elle est permissive à la réplication du VSRRP et si elle peut être un modèle approprié pour l'étude de la pathogénèse virale du VSRRP. L'expérimentation a démontré que cette nouvelle lignée cellulaire était permissive à l'infection et à la réplication du VSRRP. Afin de corroborer ces résultats, la cinétique de réplication du virus à été effectuée avec les cellules MARC-145 et SJPL. Aucune différence significative dans la production virale totale n'a été trouvée entre les deux lignées cellulaires. Les cellules SJPL ont permis la réplication de plusieurs souches Nord-Américaines du VSRRP, quoiqu'elles sont légèrement moins efficaces que les cellules MARC-145 pour l'isolement du virus. De plus, les cellules SJPL sont phénotypiquement différentes des cellules MARC-145. Plus précisément, les cellules SJPL sont plus sensibles à l'activation par le VSRRP des pro-caspases 3/7 et plusieurs inducteurs apoptotiques. Elles ont également montré de 8 à 16 fois plus de sensibilité à l'effet antiviral causé par l'IFN-α sur la réplication du virus contrairement aux cellules MARC-145. Ces résultats démontrent que les cellules SJPL pourraient représenter un substitut intéressant aux cellules MARC-145 pour la production d'antigènes pour un vaccin anti-VSRRP. Également, dû à leurs origines (poumon de l'hôte naturel), elles pourraient s'avérer être un modèle *in vitro* plus approprié pour l'étude de la pathogénèse du VSRRP.

Mots clés : Virus du syndrome reproducteur et respiratoire porcin; VSRRP; cellule épithéliale de poumon de porc; SJPL; réplication virale; cellule permissive

ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically devastating diseases for the pig industry worldwide. The etiological agent of PRRS is the PRRS virus (PRRSV), which is known to have a very restricted host specifity and to be airborne transmitted. PRRSV RNAs and antigens were found in epithelial cells of the respiratory tract of swine in PRRSV-infected pigs. Even if the interaction between porcine alveolar macrophages (PAMs) and PRRSV plays an important role in the PRRSV infection, the role of the interaction between epithelial cells of the swine respiratory tract and PRRSV should not been neglected. However, no epithelial cells of the swine respiratory tract have been demonstrated to allow PRRSV replication *in vitro* and attempts to generate such a cell line have failed. The goal of this study is to determine whether epithelial cells of the swine respiratory tract are permissive to PRRSV replication and are a suitable model for studying the viral pathogenesis of PRRSV. We have discovered that the SJPL cell line, an epithelial cell line of the respiratory tract of swine, is permissive to PRRSV infection and replication. To corroborate these results, PRRSV replication kinetics were evaluated in a subclone of the African green monkey kidney MA104 cells (MARC-145), which has been known to be fully permissive to PRRSV infection and replication, and in SJPL cells. No significant difference was found between the two cell lines for overall viral production. Moreover, the SJPL cells were able to permit the replication of several PRRSV North-American strains but they were slightly less efficient for virus isolation than MARC-145 cells. In addition, SJPL is phenotypically different from MARC-145. Specifically, the SJPL cells were more sensitive to procaspases 3/7 activation by PRRSV and several apoptotic inducers compared to MARC-145 cells. In addition, the SJPL cells showed 8 to 16 times more sensitivity to the antiviral effect of IFN-α against PRRSV replication than MARC-145 cells. Altogether, the SJPL cells could be an interesting substitute to MARC-145 cells for PRRSV vaccine antigen production, and could be a more relevant *in vitro* model, because of their origin (lung of the natural host), to study the pathogenesis of PRRSV.

Key words: Porcine reproductive and respiratory syndrome virus; PRRSV; porcine lung epithelial cell; SJPL; virus replication; cell permissiveness

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LIST OF ABBREVIATIONS

aa	Amino acids
ASFV	African swine fever virus,
Cap	Capsid protein
CMI response	Cell mediated immune response
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
EAV	Equine arteritis virus
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
IFA	Immunofluorescence assay
IPMA	Immunoperoxidase monolayer assay
IFN-γ/α	Interferon-gamma/alpha
IHC	Immunohistochemistry
ISH	*In situ* hybridization
LDV	Lactate dehydrogenase-elevating virus
LV	Lelystad virus
MAb	Monoclonal antibody
MARC-145	African green monkey kidney- derived MA-104 cell
MLV	Modified live Virus
MOI	Multiplicity of infection
MSD	Mystery swine disease
NAb	Neutralizing antibody
nm	Nano meter
ORF	Open reading frame
PAM	Porcine alveolar macrophage
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PCV-2	Porcine circovirus type 2
PEARS	Porcine epidemic abortion and respiratory syndrome
PFA	Paraformaldehyde
PHFS	Porcine high fever syndrome
pi	Post-infection/inoculation
PRDC	Porcine respiratory disease complex
PRRSV	Porcine reproductive and respiratory syndrome virus
RNA	Ribonucleic acid
SJPL	Saint-Jude porcine lung cells
SHFV	Simian hemorrhagic fever virus
SIRS	Swine infertility and respiratory syndrome
SIV	Swine influenza virus
SPF	Specific pathogen free
TBS	Tris buffered saline
$TCID_{50}$	Tissue culture infectious dose with a 50% end point
TNF-α	Tumor necrosis factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UTR	Untranslated region
VI	Virus isolation

To my parents,
my brothers and sisters

For believing in me
and
For supporting me

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These last two years will definitely stay engraved in my memory for the rest of my life, and that is due to all the great people that surrounded me and supported me in this journey. First and foremost, I would like to express my sincere gratitude to Dr Carl A Gagnon, professor of University of Montreal, who has been my supervisor since the beginning of my study for giving me the opportunity to live this experience in his laboratory amongst the great people I am now proud to call my friends. He provided me with a great deal of helpful suggestions, important advice and constant encouragement during the course of this work. He also provided me with great insight and knowledge of the research world. He inspired me with his great kindness and endless patience.

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I. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically devastating diseases for the pig industry worldwide (Garner et al., 2001; Neumann et al., 2005; Pejsak et al., 1997). The disease was first reported in the United States in 1987 (Keffaber, 1989; Loula, 1991) and in Europe in the early 1990s (OIE, 1992). Since then, it has spread throughout the world and has caused huge economic losses in swine industry. The etiologic agent, PRRS virus (PRRSV) was identified by investigators in the Netherlands and USA in 1991 (Benfield et al., 1992; Wensvoort et al., 1991b). PRRS has become a well-recognized global swine disease (Albina, 1997; Botner et al., 1994; Hopper et al., 1992; Kuwahara et al., 1994; Tian et al., 2007). In the recent years, new PRRSV variants emerged in Vietnam and China causing unprecedented large-scale outbreaks and catastrophic clinical syndromes (Feng et al., 2008; Tian et al., 2007).

PRRSV is believed to replicate in specific cells both *in vivo* and *in vitro*. The presence of PRRSV antigens and RNAs has been shown in different cells types *in vivo* by immunohistochemistry (IHC) or *in situ* hybridization (ISH) (Halbur et al., 1995a; Magar et al., 1993; Pol et al., 1991; Rossow et al., 1996; Sur et al., 1997). *In vitro*, PRRSV replicates in primary cultures of PAMs as well as freshly isolated blood monocytes or monocytic derived dendritic cells (Voicu et al., 1994; Wang et al., 2007; Wensvoort et al., 1991b). Only two other non-porcine permissive cell lines permit the replication of PRRSV, the MARC-145 and CL2621 cells (subclones of MA104 monkey kidney cell line) (Bautista et al., 1993; Benfield et al., 1992; Kim et al., 1993) which are routinely used for *in vitro* propagation of PRRSV and for large scale production of PRRSV vaccine. It is well known that the respiratory tract is the primary route of PRRSV infection and transmission, and intranasal inoculation was used for experimental infections to support this idea (Brockmeier et al., 2000; Magar et al., 1995; Meredith, 1993; Wensvoort et al., 1992). Since PRRSV antigens could be found in the epithelial cells of the respiratory tract of infected swine, it can be speculated that these cells may favor the propagation of PRRSV *in vitro*. However, to our best knowledge, until now, no epithelial cell of the respiratory tract of swine has been reported to be permissive to PRRSV replication *in vitro*.

The goals of the present study were: (1) to determine if the PRRSV natural cell host of the respiratory tract of swine, the epithelial cells, could support PRRSV replication *in vitro*; (2) to establish a new *in vitro* PRRSV permissive cell model for studying the viral pathogenesis of PRRSV.

II. LITERATURE REVIEW

1. Disease history and terminology

In the late 1980's, catastrophic outbreaks of a previously unrecognized disease in pigs were reported in the United States (Keffaber, 1989; Loula, 1991) where it became widespread, with subsequent extension into Canada (Bilodeau et al., 1991). First described in herds in North Carolina, the syndrome included severe reproductive losses, extensive postweaning pneumonia, reduction of growth performance, and increased mortality (Hill, 1990). In the absence of a recognized cause, the name "Mystery Swine Disease" (MSD) came into common usage (Hill, 1990). In Europe, clinical outbreaks notably similar to MSD were reported in November 1990 near Munster, Germany (OIE, 1992), in the Netherlands in January 1991 and in Belgium in March 1991 (OIE, 1992), but no link was found between outbreaks in Germany and MSD in the U.S. (Anon, 1991). Subsequently, disease was found in Spain (Plana et al., 1992), Great Britain (Edwards et al., 1992), France (Baron et al., 1992), Denmark (Botner et al., 1994), Poland (Pejsak and Markowska-Daniel, 1996) and Czech Republic (Valicek et al., 1997). In Asia, outbreaks occurred in Japan in 1988 (Hirose et al., 1995), in Taiwan in 1991 (Chang et al., 1993) and in China in 1995 (Tong and Qiu, 2003). Thus, the pandemic had spread to most of the major swine producing countries of the world during a short period of time. Initially, a variety of etiologies for MSD were proposed (Bane and Hall, 1990; Daniels, 1990; Hoeffling, 1990; Joo, 1988; Joo, 1990; Quaife, 1989; Reotutar, 1989). In Canada, a new subtype of Influenza A virus was isolated from piglets suffering from severe respiratory disease and added to the list as a possible agent of MSD (Dea et al., 1992; Elazhary et al., 1991). Identifying the etiology was complicated by the fact that one or more of the suspected pathogens, as well as other infectious agents, were commonly isolated from cases of MSD. The lack of a specific etiologic agent combined with various clinical signs led to the use of several disease names, such as blue ear disease (Paton et al., 1991; Wensvoort et al., 1991a), mystery swine disease (MSD) (Hill, 1990; Reotutar, 1989), porcine epidemic abortion and respiratory syndrome (PEARS) (Pol et al., 1991; Terpstra et al., 1991), swine infertility and respiratory syndrome (SIRS) (Benfield et al., 1992; Christianson et al., 1992; Collins et al., 1992), pig plaque (Keffaber, 1989) and new pig disease (Meredith, 1992). One virus first isolated in the Netherlands (Wensvoort et al., 1991b)

was designated Lelystad (LV) and later another virus isolated from sick swine by a team of researchers from South Dakota State University, the University of Minnesota, and Boehringer Ingelheim Animal Health was named Swine Infertility and Respiratory Syndrome (SIRS) virus. Both virus isolates were shown to induce reproductive failure and respiratory signs under experimental conditions (Collins et al., 1992; Terpstra et al., 1991), but in May of 1992, participants at the International Symposium on SIRS in Minneapolis, Minnesota, chose to name the disease the porcine reproductive and respiratory syndrome (PRRS), and since then the agent has been referred to as the PRRS virus (PRRSV).

Today, PRRS is endemic in the global swine producing countries and has become one of the most important pathogens causing economic losses in the swine industries (Albina, 1997; Blaha, 2000; Neumann et al., 2005). PRRSV was diagnosed in Africa for the first time in June 2004 following outbreaks in Western Cape Province, South Africa (OIE, 2005b). Serologic tests did not identify additional infected sites at that time but new outbreaks were identified in October 2005 (OIE, 2005a) and again in August 2007 (Beltran-Alcrudo et al., 2007). Chile is on the verge of becoming the first country to eradicate PRRSV. Chilean producers are currently in the process of culling all sows that were present at the time of infection (Anon, 2007). Sweden claimed to be free of PRRS until 2007 when the disease was recognized as an emerging disease (Carlsson et al., 2009). Most recently, new PRRSV variants emerged and circulated in Vietnam and China causing unprecedented large-scale outbreak and catastrophic clinical syndromes (Feng et al., 2008; Tian et al., 2007; Zhou et al., 2008). Some countries, including Switzerland, New Zealand, and Australia, claim to be free of the disease (Cannon et al., 1998; Elvander et al., 1997; Garner et al., 1996; Motha et al., 1997).

2. Clinical manifestation

PRRS is characterized by anorexia, fever and abortion late in gestation, premature births, stillbirths, and mummified fetuses. However, the two most prevalent clinical signs are severe reproductive failure in sows and gilts (characterized by late-term abortions, an increased number of stillborns, mummified and weak-born pigs) (Bilodeau et al., 1991; Christianson et al., 1992; Keffaber, 1989; Pol et al., 1991; Terpstra et al., 1991) and respiratory problems in pigs of all ages associated with a

non-specific lymphomononuclear interstitial pneumonitis (Bilodeau et al., 1991; Collins et al., 1992; Halbur et al., 1995b; Halbur et al., 1996b; Rossow et al., 1994). Furthermore, the intensity of the disease appears to vary among isolates and variation in PRRSV virulence has been observed in experimentally infected animals (Halbur et al., 1995b; Mengeling et al., 1996). Studies showed that pigs experimentally infected with different isolates developed major differences in clinical disease, rectal temperatures, and gross and histological lung lesions; mildly virulent isolate infections induced transient pyrexia, dyspnea and tachypnea, whereas highly virulent isolate infections exhibited labored breathing, pyrexia, lethargy, and anorexia (Halbur et al., 1995a; Halbur et al., 1995b; Halbur et al., 1996b). Moreover, highly virulent isolates of PRRSV infection resulted in longer periods of viremia, increased severity of clinical signs and mortality, and significantly higher viral loads in blood and tissues (Johnson et al., 2004). Several other factors such as animal age and bacterial co-infection can influence virus replication and clinical signs. Infection of younger animals showed a longer viremia, as well as higher excretion rates and replication rates in macrophages compared to the older pigs (Thanawongnuwech et al., 1998; van der Linden et al., 2003). Additionally, certain bacteria appeared to enhance the duration and severity of PRRSV induced clinical signs (Brockmeier et al., 2000; Thacker et al., 1999). Host immune status may also affect the severity of the clinical signs. Previous exposure to PRRSV can prevent the development of PRRS clinical signs by subsequent infection with the homologous PRRSV (Shibata et al., 2000).

3. Etiology

3.1. Taxonomy

The first PRRSV isolates obtained in Europe and North America were designated Lelystad and ATCC VR-2332 respectively. Now PRRSV is divided into two distinct genotypes, the European (EU) type (or type I) and North American (NA) type (or type II). The EU and NA genotypes of PRRSV share only 63% nucleotide (nt) homology (Allende et al., 1999; Collins et al., 1992; Meulenberg et al., 1993; Nelsen et al., 1999). Although distinct genetically and antigenically, both types exhibit the same genome organization and nearly the same pathogenesis. PRRSV is an enveloped, single-stranded, positive-sense RNA virus classifying in the *Arteriviridae* family

within the genus *Arterivirus,* along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV), because of their similar morphology, genome organization, transcription strategy, macrophage tropism, the ability to induce prolonged viremia and persistent infections (Benfield et al., 1992; Cavanagh, 1997; Plagemann and Moennig, 1992). The family *Arteriviridae*, *Toroviridae*, and *Coronaviridae* are the members of a single established order, *Nidovirales* (Cavanagh, 1997) (Figure 1).

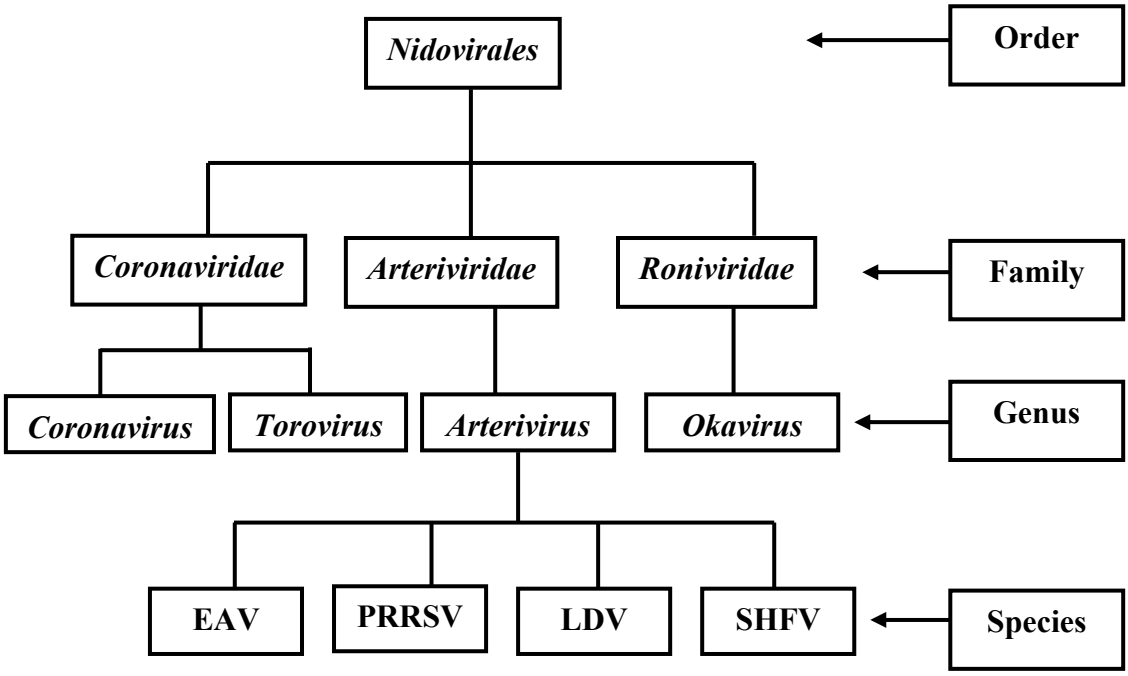

Figure 1. *Nidovirales* order classification (Cavanagh, 1997)

3.2. Viral genomic organization

As described in the earlier studies, mature PRRSV virions contain a spherical icosahedral capsid core of 20-30 nm in diameter, which is surrounded by a lipid envelope containing the viral membrane proteins, yielding a relatively smooth spherical virion of about 60 nm in diameter (Benfield et al., 1992; Dea et al., 2000; Doan and Dokland, 2003a; Doan and Dokland, 2003b). Recently, Spilman et al. (Spilman et al., 2009) described the structure of PRRSV virions based on cryo-electron microscopy (EM) analysis and tomographic reconstruction of virions grown in MARC-145 cells. They reported that the virus has a pleomorphic morphology, a

spherical to oval shape with a size ranging from about 50 to 65 nm, a hollow, layered core of around 40 nm diameter and a smooth outer surface studded with a few envelope protein complexes. The structural analysis indicated that the PRRSV core consists of an helical nucleocapsid wrapped up into a hollow ball (Spilman et al., 2009), contrary to previous studies (Benfield et al., 1992; Dea et al., 2000; Doan and Dokland, 2003a; Doan and Dokland, 2003b). These results were not surprising since other members of the *Nidovirales,* such as *Coronavirus*, are known to possess a helicoidal capsid (Figure 2). The 5'-capped and 3'-polyadenylated viral genome is approximately 15 kb in length (Meulenberg et al., 1993; Snijder and Meulenberg, 1998; Wootton et al., 2000). The viral genome contains nine known overlapping open reading frames (ORFs), designated ORF1a, ORF1b, ORF2a, ORF2b, and ORFs3 through 7(from the 5' to 3'end of the genome), which are transcribed into a nested set of subgenomic mRNAs (sg mRNAs) as shown in Figure 3 (Dea et al., 2000; Meulenberg et al., 1993; Wootton et al., 2000; Wu et al., 2001). The replicase-associated genes which occupy approximately 75% of the viral genome, ORF1a and ORF1b, code for polyproteins pp1a and pp1ab by ribosomal frame shifting, and these proteins are directly translated from the incoming genomic viral RNAs (Snijder and Meulenberg, 1998). The pp1a is predicted to be cleaved at eight sites to form nine nonstructural proteins (nsp): nsp1α, nsp1β, and nsp2 to nsp8 (den Boon et al., 1995; Snijder and Meulenberg, 1998). Proteolytic cleavage of the ORF1b portion of the pp1ab generates products of nsp9 through nsp12 (van Dinten et al., 1996). The 13 nonstructural proteins (nsp) are believed to be involved in genome replication and transcription (Bautista et al., 2002; van Dinten et al., 1999). The C-terminus of ORF1a overlaps the N-terminus of ORF1b by 16 nucleotides. A heptanucleotide slippery sequence, UUUAAAC, located just upstream of the UAG stop codon of ORF1a, and a pseudo-knot structure downstream of the slippery sequence is believed to be essential for the expression of ORF1b of PRRSV via a mechanism of ribosomal frame-shifting (Allende et al., 1999; Meulenberg et al., 1993; Nelsen et al., 1999). The 3' end of the genome (ORFs2 through 7) encodes four glycosylated membrane associated proteins GP2a, GP3, GP4, GP5 (encoded by sg mRNAs 2a, 3-5), two unglycosylated membrane proteins E and M (encoded by sg mRNAs 2b and 6), and a nucleocapsid protein (N) (encoded by sg mRNA 7) (Table 1) (Bautista et al., 1996; Mardassi et al., 1996; Meng et al., 1995a; Meulenberg and Petersen-den Besten, 1996; Meulenberg et

al., 1995; Mounir et al., 1995; Snijder and Meulenberg, 1998; Wu et al., 2001; Wu et al., 2005). Three N-glycosylated minor envelope proteins (GP2a, GP3, and GP4) form heterotrimers by disulfide linkage (Wissink et al., 2005). The nature of GP3 is still controversial, as there are conflicting data regarding its presence as a constituent of the envelope of virus particles. It has been convincingly demonstrated that GP3 is a 45- to 50-kDa structural protein of the PRRSV LV (type I or European) strain (van Nieuwstadt et al., 1996). However, the GP3 has been reported as being a non-structural protein of the PRRSV type II IAF-Klop strain, with a subset of viral GP3 being released into the cell culture medium as a non-virion associated and membrane-free form (Gonin et al., 1998; Mardassi et al., 1998). In the recent years, accumulated data have suggested that GP3 is a structural protein of the PRRSV NA type (Cancel-Tirado et al., 2004; Jiang et al., 2008). Most recently, it was reported that GP3 is a minor structural component of the PRRSV type II (FL12 strain) virion, similar to what has been previously described for PRRSV type I (de Lima et al., 2009). The N protein is not N-glycosylated, although it contains 1 or 2 potential N-glycosylation sites (Meulenberg et al., 1995). All structural proteins are translated from a nested set of 3'-coterminal subgenomic mRNAs, which contain a common leader sequence (Meulenberg et al., 1995; Snijder and Meulenberg, 1998; Wu et al., 2001).

Table I. Comparison of ORFs2 to 7 encoded protein between NA and EU strains of PRRSV

Coding gene	proteins	No. of aa residues		Predicted Mr(kDa)		Apparent Mr(kDa)		N-Glycosylation used sites	
		EU	NA	EU	NA	EU	NA	EU	NA
ORF2a	GP2a	249	256	28.4	29.5	29-30	27-29	2	2
ORF2b	E	70	73	7.8	8.2	10	10	0	0
ORF3	GP3	265	254	30.6	29.0	45–50	42–45	7	7
ORF4	GP4	183	178	20.0	19.6	31–35	31–35	4	4
ORF5	GP5	201	200	22.4	22.4	25	24-26	2	2–5
ORF6	M	173	174	18.9	19.1	18	19	0	0
ORF7	N	128	123	13.8	13.6	15	14-15	0	0

Adapted from Dea S., et al., 2000 with some modifications (Wu et al., 2001). EU: European strains, NA: North American strains, E protein was previously called GP2b

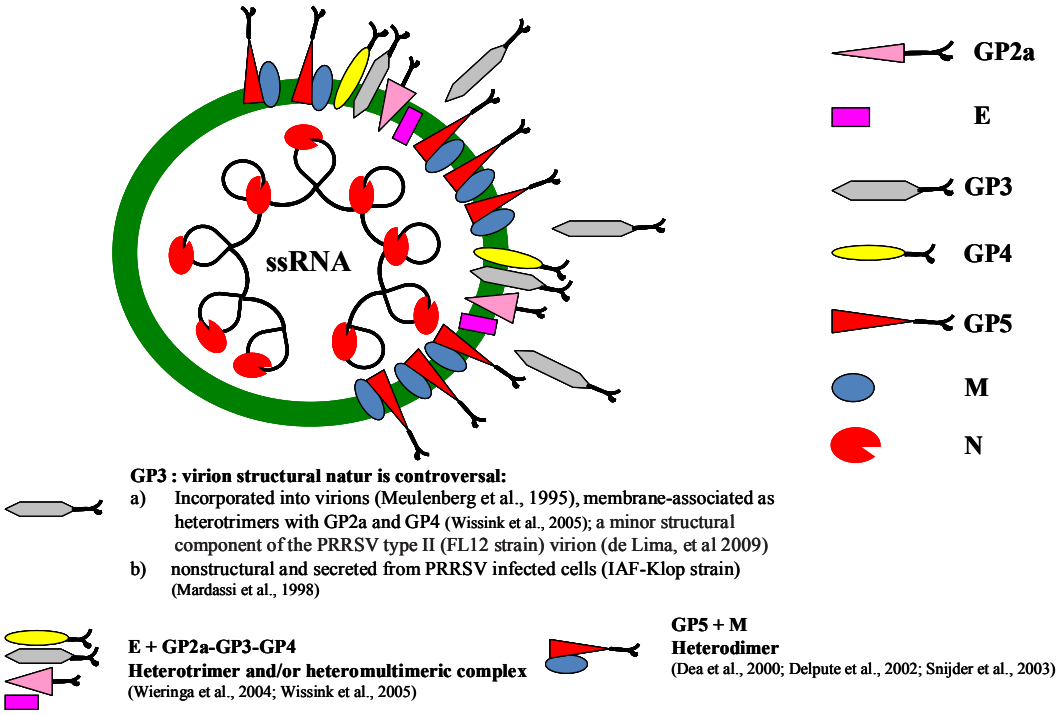

Figure 2. Schematic representation of the virion of PRRSV. The virion is spherical to oval in shape, enveloped, and possesses a non-segmented single-strand RNA genome that is encapsidated by the nucleocapsid protein (N), yielding a helicoidal capsid structure. (Kindly provided by Nedzad Music with modifications).

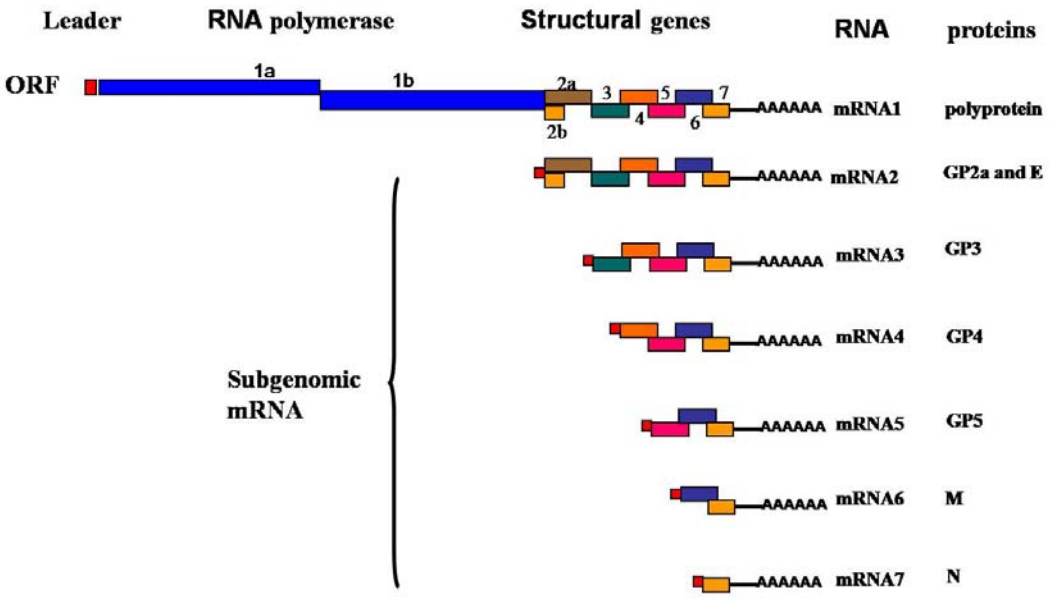

Figure 3. PRRSV genomic organizations (Dea et al., 2000; Meulenberg et al., 1993; Wootton et al., 2000; Wu et al., 2001).

3.3. Virus biological and physical properties

Being an enveloped virus, infectivity of PRRSV outside of the host is affected by temperature, pH and exposure to detergents. It has been reported that infectivity of PRRSV was unchanged after 1 month incubation at 4^{o}C or 4 months at -70^{o}C (Benfield et al., 1992). However, the infectivity decreases with increasing temperature. Specifically, infectivity was reduced 50% after incubation for 12 hours at 37^{o}C and was completely inactivated after 48 hours of incubation at 37^{o}C and 45 minutes incubation at 56^{o}C (Benfield et al., 1992). The PRRSV remains stable at pHs ranging from 6.5 to 7.5 (Bloemraad et al., 1994). Detergents are effective at reducing infectivity of the virus and lipid solvents such as chloroform and ether are particularly efficient at disrupting the viral envelope and inactivating the virion (Benfield et al., 1992). The virus survives in water for up to 11 days, but drying quickly inactivates it (Benfield et al., 1999). Buoyant densities of the infectious viral particles are 1.13–1.15 g/ml in sucrose and 1.18–1.19 g/ml in CsCl (Benfield et al., 1992; Mardassi et al., 1994a; Wensvoort, 1993).

3.4. Virus genetic variation

As for other envelope RNA viruses, a high degree of genomic variability has been reported for the *Arterivirus* (Snijder and Meulenberg, 1998), including PRRSV (Mardassi et al., 1994b; Meng et al., 1995a; Meng et al., 1995b; Murtaugh et al., 1995; Nelsen et al., 1999). Sequence comparisons have shown that there are significant genetic differences between the prototype strains from North America (ATCC VR-2332) and Europe (Lelystad virus - LV) (Meulenberg et al., 1993; Murtaugh et al., 1995; Nelsen et al., 1999), which share only about 63% nucleotide identity (Allende et al., 1999; Nelsen et al., 1999). At the beginning of the global PRRSV epidemic, EU types were detected only in Europe, while US types were restricted to North and Central America (Andreyev et al., 1997) and Asia (Shibata et al., 1996). Now, EU type PRRSV has been found in the US (Fang et al., 2004; Ropp et al., 2004), while US type has been introduced to Europe through the use of a live vaccine (Botner et al., 1999; Botner et al., 1997; Nielsen et al., 2001; Nielsen et al., 2002; Storgaard et al., 1999). Until now, the EU type strain has never been reported in the field in Canada.

phylogenetic analysis places the most recent common ancestor (MRCA) for the EU and US genotypes at at least 100 years back in time (Forsberg, 2005; Hanada et al., 2005; Stadejek et al., 2002), providing strong support for the hypothesis that EU and US viruses evolved in parallel in North America and Europe prior to their cotemporal species jump into pigs and emergence as clinical entities in the later 1980s. Originally, EU genotype viruses were thought to form a very homogeneous, 'Lelystad-like' group (Drew et al., 1997; Le Gall et al., 1998; Suarez et al., 1996b; Wensvoort et al., 1991b). Recently, the view that EU genotype viruses are genetically homogeneous was challenged by the studies of unusually diverse EU genotype PRRSV strains, first in Denmark (Oleksiewicz et al., 2000) and later in Italy (Forsberg et al., 2002), the Czech Republic (Indik et al., 2000), Poland (Stadejek et al., 2002), Spain (Mateu et al., 2003), Germany and the Netherlands (Pesch et al., 2005) and even Thailand (Thanawongnuwech et al., 2004a).

Genetic analyses have shown the existence of two major virus genotypes, the EU and the NA, with extensive genetic variability both within and between these genotypes. The leader sequence of PRRSV strains varies significantly. The 190 bp leader sequence of ATCC VR-2332 strain is 31 bp shorter than that of LV, and possesses a sequence identity of 61% with LV (Nelsen et al., 1999). The leader sequence of another NA strain, the 16244B strain, is 189 bp in length and also differs considerably in nucleotide sequence compared to LV (Allende et al., 1999). Like the leader sequence, the ORF1 gene sequence also differs extensively between the U.S. and the European strains (Allende et al., 1999; Nelsen et al., 1999). The ORF1a of ATCC VR-2332 strain shares only about 55% nucleotide sequence identity when compared to LV. ORF1b is more conserved than ORF1a and shares about 63% nucleotide sequence identity compared to LV.

Marked differences were also found between EU and NA isolates in some structural genes (Kapur et al., 1996; Murtaugh et al., 1995). GP5 is the most variable structural protein (Mardassi et al., 1995; Meng et al., 1995b) with the highest degree of diversity within one genotype. Among NA isolates, nucleotide homology of the GP5 coding region was found to be 90% or even less (Andreyev et al., 1997; Dee et al., 2001; Meng, 2000) and from 51 to 59% when NA viruses are compared to LV virus (Andreyev et al., 1997; Kapur et al., 1996; Meng et al., 1994; Murtaugh et al., 1995). N protein encoding region (ORF7) is highly conserved among NA isolates, with 95 to 100% amino acid homology, but a comparison of NA viruses and LV revealed only 57

to 59% amino acid homology (Meng et al., 1995a; Murtaugh et al., 1995). M (Matrix) protein encoding region (ORF6) is the most conserved gene among NA isolates, with 96% to 100% amino acid (aa) identity, and is the most conserved gene between NA and EU isolates, with 70 to 81% identity (Kapur et al., 1996; Meng et al., 1995b; Murtaugh et al., 1995).

Based on sequence analysis, the degree of aa identity amongst the NA PRRSV isolates varied from 91 to 99% for GP2 (GP2a and E), 86 to 98% for GP3, and 92 to 99% for GP4 (Kapur et al., 1996; Mardassi et al., 1995; Meng et al., 1995b; Morozov et al., 1995). A comparison of LV with isolate ATCC VR-2332 revealed aa identities of 63, 76, 58, and 68% for GP2a, E, 3, and 4, (Murtaugh et al., 1995; Ropp et al., 2004). With approximately 54 to 60% aa identity between the NA and EU isolates of PRRSV, GP3 is regarded as the second most variable protein amongst PRRSV strains (Mardassi et al., 1995; Murtaugh et al., 1995), with most of the variations located at the N-terminus. In fact, only 29% aa identity is found within the 35 most N-terminal residues between strains from the two continents. Interestingly, despite these extensive aa changes, the potential N-linked glycosylation sites, as well as the general hydropathy profiles of the ORF3 product, are highly conserved. In addition, the GP3 of the NA strains have a C-terminal deletion of 12 aa compared to LV (Mardassi et al., 1995; Meng et al., 1995b; Morozov et al., 1995). According to a study using UK PRRSV isolates, the ORF3 product has a hydrophilic hypervariable region proximate to the C-terminal region that overlaps with ORF4, resulting in a hypervariable region located at the N-terminal extremity of GP4 (Drew et al., 1997; Katz et al., 1995).

4. Pathogenesis

4.1. Virus entry into susceptible cells

PRRSV cell interactions and how the virus enters the cells were first reported in 1996 (Kreutz and Ackermann, 1996). It was speculated that since the direct fusion of the PRRSV envelope with the cellular membrane was not observed at any time, PRRSV entry most probably occurs by receptor-mediated endocytosis. In 1998, this hypothesis was confirmed (Duan et al., 1998) and a PRRSV receptor was identified on PAM by generation of PAM-specific monoclonal antibodies. Now it is generally

believed that despite the very restricted cell tropism of PRRSV, the virus is able to replicate in several non-permissive cell lines upon transfection of its viral RNA. Cell tropism is determined by the presence or absence of specific receptors on the cell surface or other proteins involved in virus entry (Kreutz, 1998; Meulenberg et al., 1998).

So far, several viral receptor candidates or viral binding proteins for PRRSV have been described, including heparan sulphate for binding and sialoadhesin (CD169) for internalization on macrophages, binding protein vimentin on MARC-145, CD163 on MARC-145 and PAMs and CD151 on MARC-145 (Calvert et al., 2007; Delputte et al., 2002; Kim et al., 2006; Kristiansen et al., 2001; Shanmukhappa et al., 2007; Vanderheijden et al., 2001; Vanderheijden et al., 2003). In addition, a yet unidentified 150 kDa polypeptide doublet and a 210 or 220 kDa glycoprotein, which can be speculated to be sialoadhesin, were found to be involved in PRRSV infection of macrophages (Duan et al., 1998; Wissink et al., 2003). In the current model for PRRSV infection of macrophages, heparan sulfate serves as an attachment factor that binds to viral structural M protein or the M-GP5 complex but is not required for internalization (Delputte et al., 2005; Delputte et al., 2002). Subsequently, PRRSV will engage sialoadhesin in a more stable interaction involving sialic acids present on the virion and the N-terminal sialic acid-binging domain of sialoadhesin, followed by internalization (Delputte et al., 2005; Delputte et al., 2004; Delputte and Nauwynck, 2004; Delputte et al., 2007b; Gorp et al., 2008). Upon internalization, the virus is transported towards an endosomal compartment where a drop in pH is required for proper virus replication (Kreutz and Ackermann, 1996; Nauwynck et al., 1999).

The CD163, a cellular protein in the scavenger receptor cystein rich (SRCR) super family and a type I membrane glycoprotein, has been described functioning as the macrophage receptor for hemoglobin-haptoglobin complex binding and as a cellular receptor for PRRSV infection (Calvert et al., 2007; Kristiansen et al., 2001) . CD163 was also essential in PRRSV infection of MARC-145 cells and rendered many non-permissive cells susceptible to PRRSV infection upon expression (Calvert et al., 2007). Moreover, a more recent research demonstrated that co-expression of recombinant CD163 and sialoadhesin in non-permissive cells increased virus production 10-100 times compared with cells expressing only CD163, sustaining the requirement of both molecules for efficient PRRSV infection (Gorp et al., 2008).

The CD151 molecule was identified by RNA-ligand screening of a MARC-145 cell expression library to be a PRRSV 3' UTR RNA-binding protein (Shanmukhappa et al., 2007). In several RNA viruses, the interaction between 5' and/or 3' UTR RNA and host cell proteins was already reported to play an important role in virus replication mechanisms, such as the transcription, translation, orientation and transport of viral RNA (Loffler et al., 1997; Yu and Leibowitz, 1995). The CD151 is a member of the tetraspanin superfamily, which has several cellular functions that include cell signaling, cell activation and platelet aggregation (Fitter et al., 1999; Hasegawa et al., 1998; Sincock et al., 1999). Transfection of CD151 rendered BHK-21, a non-susceptible cell line, susceptible to PRRSV infection. The transfection of siRNA against CD151 inhibited PRRSV infection into MARC-145 cells. Additionally, polyclonal anti-CD151 antibody (Ab) completely blocked PRRSV infection into MARC-145 cells (Shanmukhappa et al., 2007). These results suggest that CD151 plays a critical role in PRRSV infection *in vitro*. Since CD151 is a transmembrane protein, it is reasoned that this molecule serves as the entry molecule (Shanmukhappa et al., 2007).

4.2. Virus transmission

It is known that one of the main characteristics of PRRSV is its high transmissibility, which almost certainly contributed markedly to its quick spread around the world. Pigs are susceptible to infection by a number of routes, including oral, intranasal, intramuscular, intraperitoneal, and vaginal routes (Rossow et al., 1994) (Figure 4).

It is well documented that PRRSV can be transmitted by direct contact, through close 'snout to snout' contact between pigs or by contact with organic secretions excreted by infected pigs (Christopher-Hennings et al., 1995; Rossow et al., 1994; Wills et al., 1997a; Wills et al., 1997b; Yoon et al., 1993). In addition, there seems to exist various other indirect ways by which PRRSV can disseminate to a susceptible population, including contaminated fomites (Otake et al., 2002b; Otake et al., 2002c), arthropods (Otake et al., 2002d; Otake et al., 2003) and aerosols (Otake et al., 2002a).

A. Direct transmissions

The PRRSV has been recovered from a variety of porcine secretions and excretions including semen, saliva, feces, and milk and colostrum (Rossow et al.,

1994; Swenson et al., 1994; Wagstrom et al., 2001; Wills et al., 1997b; Yoon et al., 1993). Specifically, infectious PRRSV and PRRSV RNA have been detected in the semen of experimentally infected boars up to 43 and 92 days post-infection, respectively (Christopher-Hennings et al., 1995; Swenson et al., 1994). Fecal shedding remains a highly debated issue; several studies report the presence of PRRSV in feces from 28 to 35 days following experimental infection whereas others report no detection of virus in fecal samples (Wills et al., 1997b; Yoon et al., 1993).

B. Indirect transmissions

Several indirect transmissions by fomites have been identified. Specifically, boots and coveralls have been identified as potential sources of PRRSV transmission to naïve pigs (Otake et al., 2002c). Due to the propensity for PRRSV replication and circulation in the bloodstream, needles have also been recognized as an indirect means of PRRSV transmission between pigs, demonstrating the need for proper needle management (Otake et al., 2003). Mechanical transmission of PRRSV was demonstrated throughout a coordinated sequence of events involving fomites (boots, coolers and containers, shipping parcels, vehicles) (Dee et al., 2003; Dee et al., 2002). However, studies have demonstrated that certain intervention strategies, such as the use of disposable footwear, boot baths, the wearing of gloves and double-bagging products designated for entry into farms significantly reduced the level of PRRSV contamination on the surface of objects and mechanical spread of the virus (Dee et al., 2004).

Even if PRRSV is relatively fragile in the environment, appropriate weather (wind, temperature, humidity etc) may favour the transmission of the virus trough aerosols up to 4.7 km distance (Dee et al., 2009) .

Insects (mosquitoes (Aedes vexans) and houseflies (Musca domestica)) are commonly observed in swine facilities during the summer months and have been shown to mechanically transmit PRRSV from infected to naïve pigs under experimental conditions (Otake et al., 2002d; Otake et al., 2003). Transport of PRRSV by insects throughout an agricultural area has been reported for up to 2.4 km following contact with an infected pig population (Schurrer et al., 2004). However, the significance of these vectors under field conditions, i.e. in commercial pig farms, still needs to be determined.

Previous studies investigated the role of various mammals (rodents, raccoons, dogs, cats, opossums, skunks) and birds (house sparrows and starlings) in the

transmission of PRRSV(Wills et al., 2000b); none were capable of serving as mechanical or biological vectors (Wills et al., 2000b).

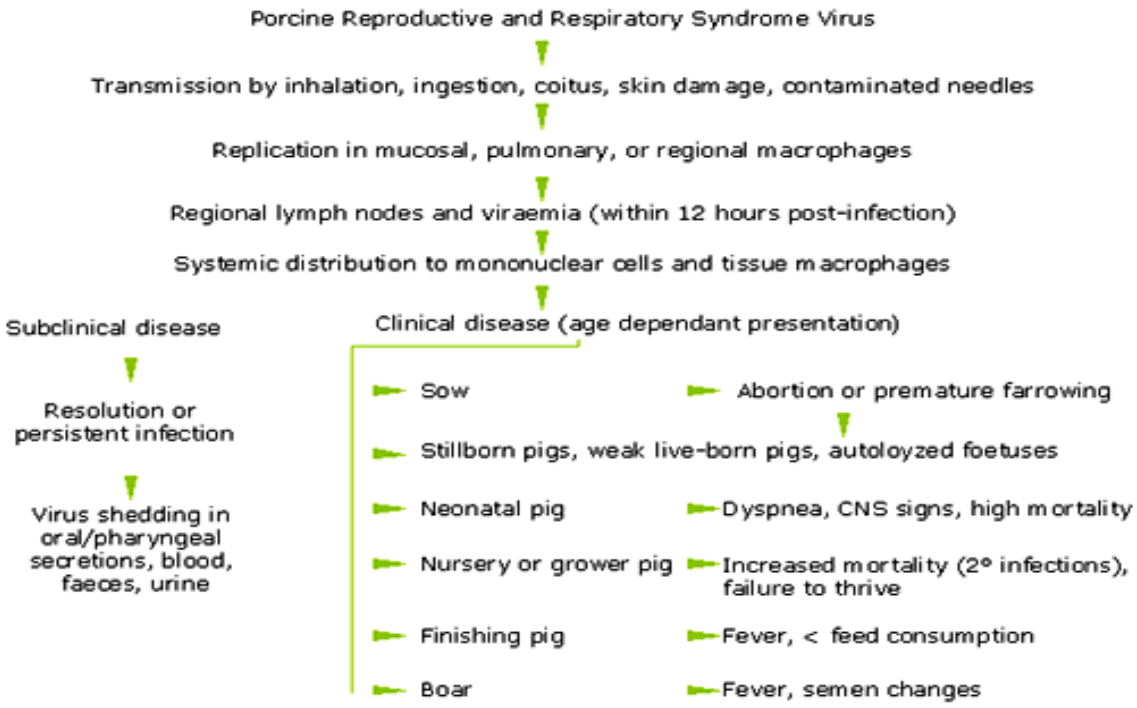

Figure 4. Pathogenesis of PRRSV infection (adapted from www.porcilis-prrs.com)

4.3. PRRSV cell and tissue tropism

PRRSV is generally believed to have a very restricted cell tropism both *in vivo* and *in vitro*. *In vivo*, the virus mainly infects well-differentiated cells of the monocyte-macrophage lineage, in particular porcine alveolar macrophages (PAM), the primary target cells of virus and interstitial macrophages in other tissues such as heart, thymus, spleen and Peyer's patches, hepatic sinusoids, renal medullary interstitium, and adrenal gland (Beyer et al., 2000; Duan et al., 1997; Halbur et al., 1996a; Halbur et al., 1995b). In addition to macrophages, PRRSV RNA and nucleocapsid protein were found by *in situ* hybridization (ISH) in testicular germ cells, endothelial cells in the heart, interdigitating cells in the thymus, dendritic cells in the spleen and Peyer's patches (Halbur et al., 1996a; Sur et al., 1997). In experimentally infected gnotobiotic pigs, PRRSV antigen were found in bronchiolar epithelial cells, arteriolar endothelial cells, monocytes as well as interstitial, alveolar, and intravascular macrophages using an

immnogold-silver immunohistochemical staining (Rossow et al., 1996). The antigens and RNA of European, North American and Korean strains of PRRSV were found in bronchiolar epithelial cells (Pol et al., 1991), epithelium-like cells of alveolar ducts (Magar et al., 1993), and pneumocytes (Cheon et al., 1997; Pol et al., 1991) in the naturally infected pigs, whereas they were not found in these types of cells in the experimentally infected pigs (Teifke et al., 2001). Tissues such as lung, lymphoid tissues, Peyer's patches, and kidney were also the preferred organ targets of PRRSV infection (Haynes et al., 1997; Sur et al., 1996). Studies showed that PRRSV distribution was also isolate or strain-dependent, for instance, more virulent strains had more positive PRRSV cell distribution in more tissues and organs (Haynes et al., 1997). It has been reported that PRRSV can be isolated from the ovary and may be responsible for episodes of female reproductive failure, and PRRSV antigens & RNAs were detected in ovarian follicles in gilts as well (Collins et al., 1992; Kranker et al., 1998; Prieto et al., 1996; Prieto et al., 1997a; Prieto et al., 1997b; Prieto, 1996; Sur et al., 2001).

In vitro, PRRSV can grow in a few cell lines. So far, primary cultures of PAMs as well as freshly isolated blood monocytes or monocytic derived dendritic cells (Voicu et al., 1994; Wang et al., 2007; Wensvoort et al., 1991b) are known to be the only porcine cells that can effectively be used for viral growth. Other non-porcine permissive immortalized cell lines that permit the complete replication cycle of PRRSV are African green monkey kidney cells or derivatives thereof such as MARC-145 or CL2621 (Bautista et al., 1993; Benfield et al., 1992; Kim et al., 1993).

4.4. PRRSV-induced apoptosis

Apoptosis, necrosis/oncosis, autophagy and pyroptosis are now generally recognized distinct processes leading to eukaryotic cell death, with clearly distinguishable morphological and biochemical features (Bergsbaken et al., 2009; Fink and Cookson, 2007; Labbe and Saleh, 2008; Wyllie et al., 1980). However, apoptosis and necrosis/oncosis are better-recognized molecular mechanisms of eukaryotic cell death and can simultaneously occur in tissue or cells exposed to the same stimuli (Shimizu et al., 1996). Whether a cell undergoes apoptosis or not depends on a delicate balance of anti- and pro-apoptotic stimuli (Costers et al., 2008). Mechanistically, apoptosis results from the activity of a distinct subset of caspases (cysteine-dependent

aspartate-specific proteases). Initiator caspases are activated primarily by two mechanisms (Green, 2003): 1) ligation of cell surface death receptors, including the tumor necrosis factor-alpha (TNF-α) receptor and Fas, leads to caspase-8 activation via the extrinsic pathway, and 2) mitochondrial release of cytochrome c activates caspase-9 via the intrinsic pathway. The pathways cross-talk, as caspase-8 can promote cytochrome C release and caspase-9 activation. Both caspase-8 and caspase- 9 activate executioner caspases, including caspase-3, which cleave cellular substrates to produce the features associated with apoptosis. These characteristics include cytoplasmic and nuclear condensation, oligonucleosomal DNA cleavage and maintenance of an intact plasma membrane. Apoptotic cells package their contents into membrane-bound apoptotic bodies and expose surface molecules like phosphatidylserine to target phagocytic uptake and removal such that apoptosis is generally non-inflammatory *in vivo* (Elmore, 2007; Fink and Cookson, 2007). Apoptosis is considered to be an important host defense mechanism that interrupts viral replication and eliminates virus-infected cells (Thomson, 2001). Apoptosis can be induced in virus-infected cells by host immune cells, such as cytotoxic T-lymphocytes and natural killer cells through soluble factors or through direct cell-to-cell contact. In addition, apoptosis can be induced in virus-infected cells as a response to viral replication (Matsumoto et al., 2005; Tanaka et al., 1998). Induction of apoptosis before completion of viral replication would severely limit progeny virus production and virus spread in the host. Consequently, viruses have evolved strategies that inhibit apoptosis during replication, thereby ensuring cell survival until sufficient virus progeny is produced (Teodoro and Branton, 1997; Thomson, 2001). Many viruses have adapted by encoding anti-apoptotic gene products that permit their seemingly undetected replication. Some viruses (e.g., African Swine Fever Virus, ASFV) encode proteins that prevent apoptosis through inactivation of p53 or binding of Bax (Afonso et al., 1996; Brun et al., 1996; Neilan et al., 1993; Revilla et al., 1997; Young et al., 1997). Yet other viruses (such as Baculovirus) possess mechanisms to inhibit apoptosis by expressing caspase inhibitors that interfere with caspase function (Manji and Friesen, 2001). Thus, viruses have evolved numerous mechanisms acting at many different targets to interfere and block apoptosis.

Several studies demonstrated that PRRSV infection induced apoptosis both *in vitro* and *in vivo* (Choi and Chae, 2002; Kim et al., 2002; Labarque et al., 2003; Miller

and Fox, 2004; Sirinarumitr et al., 1998; Suarez et al., 1996a; Sur et al., 1997; Sur et al., 1998). PRRSV infection of MARC-145 and porcine alveolar macrophages (PAMs) resulted in apoptosis characterized by morphological changes, DNA fragmentation and specific caspase activation. PRRSV induces apoptosis both directly (in infected cells) and indirectly (in bystander cells) and within both infected tissue cultured cells and animals, apoptotic cells are observed and contribute to the pathology observed in the animal (Sirinarumitr et al., 1998).

The evidence of direct induction of apoptosis coming from *in vitro* studies demonstrated that artificial expression of GP5 using viral vectors can induce apoptosis within cell monolayers, but the mechanism was not well elucidated (Gagnon et al., 2003; Suarez et al., 1996a). Expression of PRRSV GP5 (ORF5) gene in cell monolayers using a vaccinia virus expression vector induced apoptosis, while the vaccinia vector alone did not (Suarez et al., 1996a). Gagnon et al. (2003) expressed the GP5 protein using an adenovirus expression system and detected an increase in caspase 3 activity in cell monolayers transfected with recombinant vector (Gagnon et al., 2003). The apoptosis inducing region of GP5 has been mapped to the N-terminal 119 amino acids by Fernandez et al. (Fernandez et al., 2002). Recently, the question of if and when PRRSV modulates apoptosis in PRRSV-infected macrophages was investigated (Costers et al., 2008). This study showed that during a PRRSV infection two oppositely directed sets of reactions are switched on in PRRSV-infected macrophages *in vitro*: at first, reaction favor anti-apoptosis, but finally, PRRSV-infected macrophages die by apoptosis (Costers et al., 2008). Both anti- and pro-apoptotic effects were not only observed in PRRSV-infected macrophages, but also in PRRSV-infected MARC-145 cells (Costers et al., 2008). In conclusion, this study showed that PRRSV replication results in activation of anti- and pro-apoptotic pathways. Early in infection, the balance tends towards anti-apoptosis, whereas late in infection, the balance is driven towards pro-apoptosis. In addition, this study indicates that the ability of PRRSV to modulate apoptosis in the infected cell is intrinsic to the virus, and not dependent on the cell type (Costers et al., 2008). Lee et al. (2007) provided further evidence of apoptosis induced by PRRSV directly in PRRSV-infected MARC-145 cells and ultimately, the authors elucidated that PRRSV induced apoptosis is through a mitochondria-mediated pathway. In summary, these authors demonstrated that (i) PRRSV infection causes characteristic morphological and biochemical changes of apoptosis such as chromatin condensation, DNA fragmentation, externalization of

phosphatidylserine (PS), caspase activation, and poly (ADP-ribose) polymerase (PARP) cleavage; (ii) PRRSV induces caspase-dependent apoptosis and activates both caspase-8 and caspase-9; (iii) a crosstalk between extrinsic and intrinsic pathways existed since caspase-8 activated through ligation of the death ligand with the death receptor, possibly TNFR-α/TNFR1 and Fas/FasL, and mediates caspase-9 activation via Bid cleavage; (iv) PRRSV caused an increased ratio of Bax/Bcl-2 which is followed by the disruption of the mitochondrial transmembrane potential and cytochrome c release; (v) oxidative stress induced by PRRSV is involved in apoptosis; and (vi) PRRSV infection causes secondary necrosis (Lee and Kleiboeker, 2007).

Other studies have shown that PRRSV induces apoptosis mostly in uninfected bystander cells both *in vitro* and *in vivo*. In previous studies, the majority of apoptotic cells were detected in lung lavages, lungs, and lymphoid tissues by using IHC, TUNEL, DNA electrophoresis, and electron microscopy, but upon performing dual-labeling experiments it was concluded that the majority of apoptotic cells were not infected with PRRSV (Choi and Chae, 2002; Kim et al., 2002; Labarque et al., 2003; Miller and Fox, 2004; Sirinarumitr et al., 1998; Suarez et al., 1996a; Sur et al., 1997; Sur et al., 1998). Chang and collaborators have reported that alveolar macrophages from PRRSV-infected pigs showed a significantly increased apoptotic rate (22-34%) compared to porcine circovirus 2 infected alveolar macrophages (3%) (Chang et al., 2005). Given the fact that only 5-10% of alveolar macrophages were PRRSV-infected, theses authors suggested that TNF-α or GP5 released from PRRSV-infected cells caused apoptosis in bystander cells. Another study from the same group demonstrated that increased FasL expression in PRRSV-infected macrophages caused apoptosis in co-cultured swine splenic lymphocytes (Chang et al., 2007).

5. Host immunology

A complex immunological interaction exists between PRRSV and pigs that involves both induction and subversion of host defenses (Murtaugh et al., 2002). Immunization to PRRSV infection is a double-edged sword. On the one hand, PRRSV has a predilection for immune cells and the disease manifestations can be linked directly to changes in the immune system. PRRSV appears to replicate exclusively in cells of the immune lineage, notably macrophages; the direct replication of which may

lead to immunosuppression, precipitate secondary infection and/or mediate disease. On the other hand, the virus stimulates immunity post-infection that protects an animal from re-infection. Thus, the immune system appears to be intimately involved in both the disease process and protection from disease (Molitor et al., 1997). PRRS is one of the most challenging subjects of research in veterinary viral immunology and our current knowledge on the basic mechanisms for PRRSV protective immunity is still fragmentary.

Because porcine alveolar macrophages (PAMs) have a pivotal role for an effective innate and adaptive immune response (Janeway et al., 2005a; Janeway et al., 2005b), any suppression of macrophage function plus a fall in macrophage numbers due to viral infection could increase the host's susceptibility to secondary infections. For the innate immune response, the main role of PAMs is to ingest and subsequently kill pathogens, and release cytokines such as TNTα and IL-1, which can activate pathways of both the innate and adaptive immune response (Fearon and Locksley, 1996; Janeway et al., 2005a; Janeway et al., 2005b). By acting as antigen-presenting cells, macrophages, along with dendritic cells (DCs), also trigger the adaptive immune response (Janeway et al., 2005a). PRRSV can also multiply inside DCs, which can affect the activation of these cells and prevent triggering of the adaptive immune response (Loving et al., 2007; Wang et al., 2007). Several studies have also shown weak and atypical innate immune responses, such as weak IFN-α responses (van Reeth, 1999) and high induction of interleukin(IL)-10 in PRRSV-infected porcine monocytes, macrophages and dendritic cell (Flores-Mendoza et al., 2008; Suradhat et al., 2003) and *in vivo* in PRRSV-infected pigs (Suradhat, 2003; Sutherland et al., 2007; Thanawongnuwech et al., 2004a; Thanawongnuwech et al., 2004b)

Pigs mount a rapid antibody response to infection by PRRSV, which is detectable from day 5 post-infection, but these early antibodies are mainly directed to the N- and M-proteins and are non-neutralizing. Neutralizing antibodies (NAbs) begin to appear between 7-28 days post-infection, but their titers remain low, and substantial variation in the neutralizing antibody response has been reported (Loemba et al., 1996; Plagemann, 2006). Typical titers of serum NAbs, which are considered unusually low in comparison with those induced by other viruses, are between 2 and 12 (Labarque et al., 2000; Loemba et al., 1996). Thus the humoral immune response to PRRSV in pigs is characterized by early production of strong, non-NAbs, which are detected from 5-6

days post-infection (pi), followed by the delayed appearance of neutralizing antibodies between 3 and 4 weeks post-infection, which then persist at low levels (Figure 5) (Lopez and Osorio, 2004).

The protective capacity of NAbs is debated. The early development of non-NAbs and later development of NAbs may have a significant effect on the development of PRRSV persistent infections. It has been shown that non-NAbs enhance viral replication in alveolar macrophages, a phenomenon known as antibody-dependent enhancement (ADE) (Yoon et al., 1996; Yoon et al., 1997). The non-neutralizing humoral response may act as a Trojan horse for PRRSV by coating the virus and enhancing the internalization of viral particles into macrophages (Mateu and Diaz, 2008). In contrast, development of NAbs is not sufficient to completely eliminate the virus (Mateu and Diaz, 2008). Likewise, viremia may be resolved in the absence of detectable levels of neutralizing antibodies (Diaz et al., 2006). Nonetheless, NAbs may play a central role in protecting swine against reinfection with PRRSV since passive transfer of antibodies fully protected pregnant sows against a challenge of virulent PRRSV and blocked transplacental infection (Osorio et al., 2002). Similarly, pigs receiving an amount of NAbs sufficient to reach a serum titer of 8 consistently did not develop viremia, whereas serum titers of 32 produced sterilizing immunity (Lopez and Osorio, 2004; Osorio et al., 2002). However, other authors do not report such a strong correlation between NAbs and the absence of viremia (Jiang et al., 2007a; Jiang et al., 2007b; Jiang et al., 2007c; Plagemann, 2006; Zuckermann et al., 2007). Delay in the neutralizing antibody response to PRRSV has been postulated (in addition to other hypotheses) to be due to the presence of a nearby immunodominant “decoy” epitope (aa 27–30 of GP5), which may evoke a robust, early, and non-protective immune response that masks and/or impairs the response to the major neutralizing epitope (aa 37-45 of GP5) (Ostrowski et al., 2002). An alternative explanation of the peculiar nature of the PRRSV-neutralizing response could be a so-called glycan-shielding phenomenon as proposed for the human and simian immunodeficiency viruses. “Glycan shielding” may be a primary mechanism to explain evasion from NAbs, ensuring *in vivo* persistence of these viruses (Wei et al., 2003). Also, observations using field strains of PRRSV appear to support the role of N-linked glycosylation sites in interfering with the neutralizing antibody response. Spanish PRRSV strains have evolved from 1991 to 2005 and there has been a trend to gain two glycosylation sites in N37 (Asp) and N53 (Asp) flanking the major

neutralizing epitope of GP5 compared to LV strain, consistent with selection of strains inducing weaker NAb responses (Mateu et al., 2006). Together, these findings support the suggestion that natural infection with PRRSV may involve an immune evasion strategy in which few NAbs are produced, and/or large amounts of PRRSV NAbs in sera of PRRSV-infected animals may be unable to react with virions due to blocking or shielding of the neutralizing epitope by the glycan moieties on GP5 (Ansari et al., 2006) .

Cell-mediated immune (CMI) response to PRRSV determined by lymphocyte blastogenesis and adaptive cytokine production is delayed, primarily detectable in the *in vitro* recall response of PBMC around 1-2 weeks after infection (Bassaganya-Riera et al., 2004; Bautista and Molitor, 1997; Charerntantanakul et al., 2006; Lopez Fuertes et al., 1999; Meier et al., 2003; Royaee et al., 2004). Infection with PRRSV has been shown to increase the numbers of various peripheral blood mononuclear leukocyte subsets (Albina et al., 1998b; Diaz et al., 2005), beginning with an increase in $CD^{8\alpha+}$ cells, 1week after infection, followed by an increase in the numbers of $CD4^{+}$ and γδ T cells, 7 weeks post-infection. The latter was shown to coincide with an increase in the number of interferon-γ (INF-γ) producing cells in the peripheral blood (Batista et al., 2004), an indicator for proliferation of cytotoxic cells. Over the same period, the number of effector cells specific for PRRSV was shown to increase, reaching maximal levels at 7 weeks post-infection (Bautista and Molitor, 1997). Protective immunity against PRRSV is not clearly understood. Various studies suggest that the cell-mediated immune response is not sufficient to completely eliminate the virus and to prevent persistent infection (Batista et al., 2004; Lowe et al., 2005; Murtaugh et al., 2002). A delay in the appearance of the cellular immune response suggests that PRRSV infection involves a mechanism of immunosuppression or immunomodulation (Done, 1995; Murtaugh et al., 2002).

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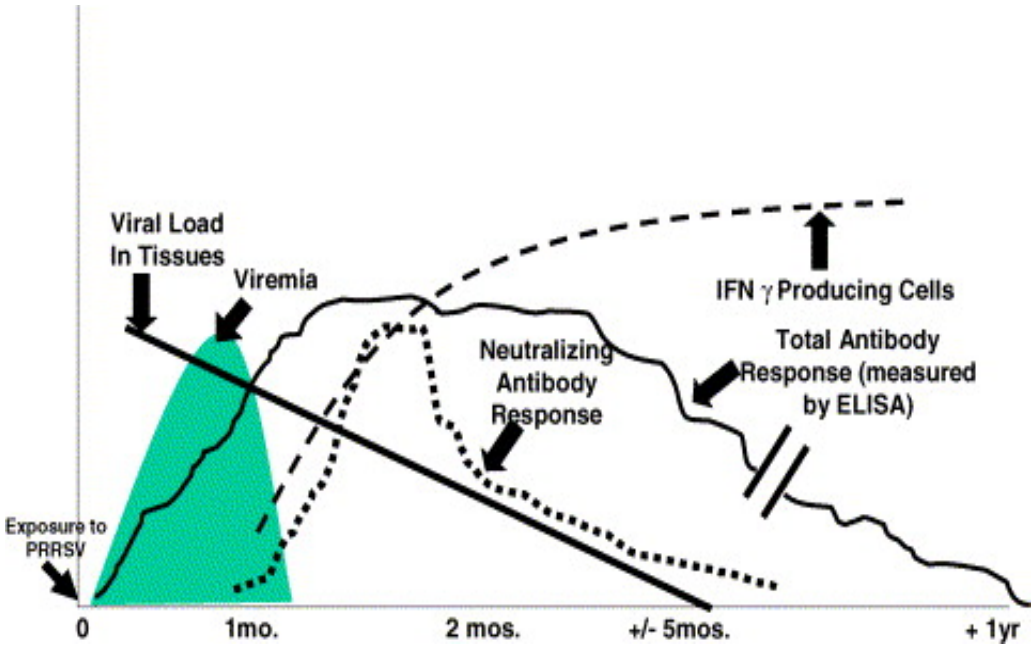

Figure 5. Temporal sequence of events after infection of a pig with PRRSV (Adapted from Lopez and Osorio, 2004).

6. Disease control and eradication

6.1. Control and eradication strategies

The rapid spread and economic impact of PRRS have made it a frequent topic of research, especially regarding its control (Neumann et al., 2005; Zimmerman et al., 1997). The key elements of a PRRS control and eradication program are early disease detection and rapid laboratory confirmation; quick identification of the infected farms and control of the infection through different stamping out strategies. As with many other infectious diseases, the most effective means of control often depends on the use of vaccines as well as the implementation of improved management practices. Regarding the first option, there are currently a few commercially available vaccines. These include modified live virus (MLV) as well as inactivated-virus or killed virus (KV) vaccines. They are all made from cell culture of MARC-145 since MARC-145 cells have been the most convenient for vaccine production up until now. However, the nature of the pig's immune response to PRRSV makes the development of an unquestionably safe as well as highly effective vaccine a formidable challenge.

Consequently, in many affected herds, the development of strategies for control and perhaps eventual eradication of PRRS depends on a thorough knowledge of the epidemiology of the disease and vaccination is only one of several approaches to be considered in designing a control strategy (Prieto and Castro, 2005).

Various control programs have been developed to eliminate the virus from infected farms, but no single program is satisfactory for controlling it in all types of herds. Programs including partial depopulation (Dee et al., 1997), segregated early weaning (Rajic et al., 2001), vaccination with nursery depopulation (Dee et al., 1998), and test and removal (T&R) (Dee and Molitor, 1998; Dee et al., 2000) have been described, and the T&R technique has been applied successfully to some herds.

Vaccination and/or partial or total depopulation strategies, test and removal procedures or acclimatization of incoming pigs has proven efficient in the eradication of PRRS (Dee et al., 2000; Yang et al., 2008). Partial or total depopulation is used as an eradication strategy in many farms (Dee and Joo, 1997). PRRSV was efficiently eliminated from a seedstock breeding farm and a supplying boar stud by a modified test and removal method based on an indirect fluorescent assay (IFA) test to detect antibodies (Dee and Molitor, 1998), and a nested reverse transcriptase-PCR (nRT-PCR) to detect virus nucleic acids (Yang et al., 2008).

Central to the control of PRRS is prevention of the spread of PRRSV within the pig herd. The herd should be stable with a uniform level of immunity throughout the herd, with no PRRSV-negative pigs. In breeding herds, the modified live vaccines have been used as an aid to creating this uniform immunity. Clinical symptoms are reduced and the infection of piglets prior to weaning is prevented (Lopez and Osorio, 2004).

Limitations of T&R have been documented (Dee and Molitor, 1998; Dee et al., 2000), and include a high degree of labor involved in testing an entire herd, and diagnostic costs that approach US $10.00/tested sow. Furthermore, a high accurate test is required to reduce the impact of animal removal on the productivity and profitability of the farm (Dee and Molitor, 1998; Dee et al., 2000). Depopulation is expensive and it is only effective if strict biosecurity is applied and if all the pig farms in the affected region are following the same strategy. Therefore, a combination of depopulation and vaccination is an interesting alternative option for control.

6.2. Treatments and prevention

In the acute disease phase, when PRRSV first enters the farm it is important to cover the period at risk, which is usually six to eight weeks, with in-feed antibiotics and water medication. The broad-spectrum antibiotics, tetracyclines, trimethoprim/sulpha, or synthetic penicillins are the medication of choice to treat secondary infections.

Vaccination is a common procedure to minimize economic losses associated with this pathogen and to prevent reinfection, and vaccines have been proven to be effective in experimental trials (Opriessnig et al., 2005) and field studies (in a preferentially homologous rather than heterologous PRRSV strain-specific manner) (Mavromatis et al., 1999; Sornsen et al., 1998). Nonetheless, the efficacy of these currently used vaccines is somewhat controversial and it is generally well accepted that there is a need for improvement in their safety and efficacy. MLV vaccines are still able to cause viremia and thus can spread to other pigs, as reported in Denmark (Botner et al., 1997; Madsen et al., 1998). The MLV vaccines do not prevent reinfection, and even the field virus does not induce a lifelong immunity. Furthermore, MLV vaccines do not allow serological discrimination between vaccinated and naturally infected pigs. The efficacy of PRRSV KV vaccines is less than ideal. The vaccines induce poor CMI response and do not induce an antibody response (measured by IDEXX ELISA) (Bassaganya-Riera et al., 2004; Piras et al., 2005; Zuckermann et al., 2007). Furthermore, due to the PRRSV genetic diversity and quasispecies (Goldberg et al., 2003; Rowland et al., 1999), PRRSV vaccine failures are not uncommon in the field, and vaccine efficacy is far from being universal and complete. Likewise, due to the continuous mutations affecting the viral genome, the PRRSV has the ability to persist in herds for long periods of time (Allende et al., 2000; Goldberg et al., 2003). This persistence and variability pose serious challenges for the diagnosis and control of PRRSV that might be further complicated by reversion of live vaccine viruses into the ancestor wild-type virus and recombination of viruses in the field (Botner et al., 1997; Meng, 2000; Nielsen et al., 2001; Storgaard et al., 1999). Overall, the characteristics of PRRSV increase the difficulties of PRRS prevention.

6.2.1. PRRS Vaccines

PRRSV MLV vaccine is recommended for use in sows and gilts for the reduction of viremia or reproductive failure and in piglets for the reduction of viremia or respiratory disease. The vaccines are efficacious but induce delayed antibody and cell mediated immune (CMI) responses (Charerntantanakul et al., 2006; Foss et al., 2002; Meier et al., 2004). PRRSV KV vaccines are recommended for use in sows and gilts for the reduction of reproductive failure and disorders.

In comparison, MLV vaccines based on American and European-type viruses were originally developed for the control of PRRS in growing pigs. Ingelvac PRRS ATP (Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) vaccine based on atypical PRRS parent strain is a non-adjuvanted vaccine licensed for use in 3-18 weeks old animals. In experimental and field trails, vaccination reduced gross lung pathology in 9/9 trials and had from 54%- 97% reduction of gross lesions as compared to unvaccinated controls (significance level $P<0.05$) (Bulletin of Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO).

Porcilis PRRS (Intervet/Schering-Plough Animal Health Wim de Körverstraat, Netherlands) vaccine containing European DV strain is a Diluvac Forte adjuvanted vaccine and is authorized for use in both fattening and breeding pigs. Field trails reported a mortality, a reduction daily weight gain and as being proved “safe” in vaccinated pigs (http://www.porcilis-prrs.com/spc-porcilis-prrs.asp). Experimental and field studies have demonstrated that the vaccination of young pigs confers protection against clinical disease but not against infection (Gorcyca et al., 1995; Labarque et al., 2004; Mavromatis et al., 1999; Mengeling et al., 2003; van Woensel et al., 1998).

Several experimental PRRSV vaccines have been developed to improve existing vaccines. These include DNA, recombinant peptide, and synthetic peptide vaccines. DNA vaccines were demonstrated to induce antibody and CMI responses and have some efficacy in protecting pigs from developing viremia and respiratory diseases (Pirzadeh and Dea, 1998; Rompato et al., 2006; Xue et al., 2004) Recombinant and synthetic peptide vaccines are not as potent and efficacious as MLV and DNA vaccines. They require numerous injections, and yet do not confer protection (Charerntantanakul et al., 2006; Pirzadeh and Dea, 1998).

Recent interest in improving immune response to PRRSV vaccines is the

utilization of vaccine adjuvants. Several kinds of vaccine adjuvants such as cytokines, chemical reagents, and bacterial products, have been studied for their ability to potentiate immune response to PRRSV vaccines. These vaccine adjuvants possess either T helper 1 (Th1) or Th2 inducing properties and some of them also possess innate immune stimulatory property (i.e. APC activation and pro-inflammatory cytokine production) (Charerntantanakul, 2009). These adjuvants have been tried in commercial PRRSV MLV vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim, St. Joseph, MO)(Charerntantanakul et al., 2006; Foss et al., 2002; Meier et al., 2004; Royaee et al., 2004), in-house KV vaccine (Linghua et al., 2007; Linghua et al., 2006; Zhang et al., 2007), DNA vaccine (Rompato et al., 2006; Xue et al., 2004), and recombinant and synthetic peptide vaccine (Charerntantanakul et al., 2006; Hyland et al., 2004). However, only some of them resulted in enhanced immune response or increased vaccine efficacy. Future studies are, therefore, required to seek new vaccine adjuvants that can potentiate immunogenicity and protective efficacy of PRRSV vaccines.

7. Diagnosis of PRRSV infection

A diagnosis of PRRSV infection is based on typical clinical signs, seroconversion, characteristic microscopic lesions and the demonstration of PRRSV genome and antigens by virus isolation, *in situ* hybridization (ISH) and reverse transcriptase polymerase chain reaction (RT-PCR) methods.

Serologic tests used in the diagnosis of PRRSV infection include the indirect fluorescent assay (IFA) test, serum neutralization (SN) test, immunoperoxidase monolayer assay (IPMA), and enzyme-linked immunosorbent assays (ELISA) such as commercially available kits (HerdChek-PRRSV, IDEXX Laboratories Inc., Westbrook, ME)(Collins et al., 1996).

In addition to the serologic tests such as ELISA, molecular biology diagnostic assays such as RT- PCR are the most frequent assays used for diagnosis of PRRSV. RT-PCR is better used for samples that cannot be used in cell cultures such as semen and samples in which PRRSV infectivity has been reduced, such as autolytic tissue. Restriction fragment length polymorphism analysis of PCR-amplified products was developed for the differentiation of field and vaccine PRRSV isolates (Wesley et al.,

1998). Recently, real-time PCR for quantification of PRRSV in naturally infected and challenged pigs was also reported (Chung et al., 2005) and reverse transcription loop-mediated isothermal amplification assay (RT-LAMP) was developed for rapid PRRSV detection (Li et al., 2009).

The correlation between the onset of clinical signs and pathological lesions, PCR positivity and other diagnostic techniques gives stronger evidence of PRRSV infections.

III. MATERIALS, METHODS & RESULTS

Identification of a new porcine lung epithelial cell line permissive to porcine reproductive and respiratory syndrome virus infection and replication

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Abstract

Airborne transmitted pathogens, such as porcine reproductive and respiratory syndrome virus (PRRSV), need to interact with host cells of the respiratory tract in order to be able to enter and disseminate in the host organism. Primary porcine alveolar macrophages (PAM) and immortalized MARC-145 (a monkey kidney cell) cell lines are known to be permissive to PRRSV replication *in vitro*. MARC-145 cells are the more suitable cells for large-scale virus production *in vitro*. However, no epithelial cell of the respiratory tract of swine had been reported to be permissive to PRRSV infection and replication *in vitro*. The goal of this study was to determine if epithelial cells of the respiratory tract of swine could support PRRSV replication *in vitro*. Interestingly, an epithelial cell line of the respiratory tract of swine, the SJPL, was found to be permissive to PRRSV infection and replication. Following PRRSV replication, the amount of infectious PRRSV particles produced in infected SJPL cells compared to infected MARC-145 cells was similar. The SJPL cells were able to permit the replication of several PRRSV North-American strains but they were slightly less efficient for virus isolation than MARC-145 cells. In addition, the SJPL cells were 8 to 16 times more sensitive to the antiviral effect of IFN-α against PRRSV replication than MARC-145 cells. In conclusion, the SJPL cells could be an interesting substitute to MARC-145 cells for PRRSV vaccine antigens production and they could be a more relevant *in vitro* model because of their origin (lung of the natural host) to study the pathogenesis of PRRSV.

Key words: Porcine reproductive and respiratory syndrome virus; PRRSV; porcine lung epithelial cell; SJPL; virus replication; cell permissiveness

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is present worldwide and is one of the most economically important infectious diseases of swine production (Neumann et al., 2005). PRRS disease was first described in the United States in 1987 (Keffaber, 1989; Loula, 1991) and a few years later in the Netherlands (Wensvoort et al., 1991). The disease has many clinical manifestations but the two most prevalent are severe reproductive failure in sows and gilts (characterized by late-term abortions, an increased number of stillborns, mummified and weak-born pigs) (Albina, 1997; Keffaber, 1989) and respiratory problems in pigs of all ages associated with a non-specific lymphomononuclear interstitial pneumonitis (Albina, 1997; Keffaber, 1989; Rossow et al., 1994).

The etiological agent, porcine reproductive and respiratory syndrome virus (PRRSV) was identified in 1991 by investigators in the Netherlands and shortly after in the USA (Benfield et al., 1992; Collins et al., 1992; Wensvoort et al., 1991). The PRRSV is an enveloped, single-stranded positive sense RNA virus, approximately 50–65 nm in diameter classified in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* along with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus of mice (LDV), and simian hemorrhagic fever virus (SHFV) (Benfield et al., 1992; Cavanagh, 1997)**.** PRRSV genome is approximately 15 kb in length. The viral RNA genome is capped at the 5' end and polyadenylated at the 3' end and encodes at least nine open reading frames (ORFs) (Dea et al., 2000), each of which is expressed via the generation of a 3'-coterminal nested set of subgenomic (sg) mRNAs (Gorbalenya et al., 2006). The virus is genetically, antigenically, and pathogenically heterogeneous (Dea et al., 2000; Meng, 2000). Currently, PRRSV isolates are divided into two distinct genotypes, the European genotype (EU) or type I represented by the Lelystad virus (LV) and the North American genotype (NA) or type II represented by the ATCC VR-2332 strain (Hanada et al., 2005).

PRRSV is known to have a very restricted cell tropism both *in vivo* and *in vitro*. *In vivo*, the virus mainly infects well-differentiated cells of the monocyte-macrophage lineage, in particular porcine alveolar macrophages (PAMs), the primary target cells of virus and interstitial macrophages in other tissues such as heart, thymus, spleen and Peyer's patches, hepatic sinusoids, renal medullary interstitium, and adrenal gland (Beyer et al., 2000; Duan et al., 1997; Halbur et al., 1995; Halbur et al., 1996). In

addition to macrophages, PRRSV RNA and nucleocapsid protein (N) were found by *in situ* hybridization (ISH) in testicular germ cells, endothelial cells in the heart, interdigitating cells in the thymus, dendritic cells in the spleen and Peyer's patches (Halbur et al., 1995; Sur et al., 1997). In experimentally infected gnotobiotic pigs, PRRSV antigens were found in bronchiolar epithelial cells, arteriolar endothelial cells, monocytes as well as interstitial, alveolar, and intravascular macrophages using an immunogold-silver immunohistochemical staining (Rossow et al., 1996). PRRSV RNAs and antigens were also found in bronchiolar epithelial cells (Pol et al., 1991), epithelium-like cells of alveolar ducts (Magar et al., 1993), and pneumocytes (Cheon et al., 1997; Pol et al., 1991) in the naturally infected pigs whereas it was not found in these types of cells in the experimentally infected pig (Teifke et al., 2001). Tissues such as lung, lymphoid tissues, Peyer's patches, and kidney were also the preferable organ targets of PRRSV infection (Haynes et al., 1997; Sur et al., 1996). PRRSV distribution is also strain-dependent (Haynes et al., 1997).

In vitro, PRRSV was originally isolated on primary cultures of PAMs (Wensvoort et al., 1991) and so far, these cells as well as freshly isolated blood monocytes or monocytic derived dendritic cells (Voicu et al., 1994; Wang et al., 2007), remain the only porcine cells that can effectively be used for viral propagation since they could be infected by the virus and allow its replication. Only two other non-porcine permissive immortalized cell lines permit the complete replication cycle of PRRSV, the MARC-145 and CL2621 cells (subclones of MA104 monkey kidney cell line) (Bautista et al., 1993; Benfield et al., 1992; Kim et al., 1993), which are routinely used for *in vitro* propagation of PRRSV and for large-scale production of PRRSV vaccine strains.

PRRSV can be airborne transmitted over long distances (Dee et al., 2009). Airborne transmitted pathogens need to interact with host cells of the respiratory tract such as epithelial cells and alveolar macrophages in order to be able to enter and disseminate in the host organism. If PRRSV is airborne transmitted and PRRSV antigens and viral RNA can be detected in epithelial cells of the respiratory tract of infected pigs, than we can speculate that in addition to the alveolar macrophages, epithelial cells of respiratory tract could be permissive to PRRSV replication *in vitro*. Nonetheless, no immortalized epithelial cell of the respiratory tract of swine had been previously reported to be permissive to PRRSV infection and replication *in vitro* and attempts to find such cells have previously failed (Ferrari et al., 2003; Huang et al.,

2009; Wensvoort et al., 1991). The goal of this study was to determine whether immortalized epithelial cells of the respiratory tract of swine could support PRRSV replication *in vitro* and eventually if they could be used as a more suitable model for studying the pathogenesis of PRRSV and as an alternative method for PRRSV vaccine antigens production compared to MARC-145 cells.

2. Materials and methods

2.1. Cells and viruses

MARC-145 cells, which is a subclone of the African green monkey kidney MA104 cells that is highly permissive for PRRSV (Kim et al., 1993), were maintained as previously described (Kheyar et al., 2005). The porcine lung epithelial cell line (SJPL) was kindly provided by Dr R.G. Webster (St. Jude Children's Hospital, Memphis, TN, USA) (Seo et al., 2001). This cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, GibcoBRL, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Wisent Inc, St-Bruno, QC, Canada), 1% sodium pyruvate, 1% L-glutamine, 1.4% MEM nonessential amino acids, 300U/mL of penicillin, 300 mg/mL of streptomycin and 1% antibiotic-antimycotic solution (Invitrogen Corporation, GibcoBRL) as previously described (Seo et al., 2001). The newborn pig trachea epithelial cell line (NPTr), kindly provided by Dr. M. Ferrari (Instituto Zooprofilattico Sperimental, Brescia, Italy), was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, GibcoBRL) supplemented with 10% FBS (Wisent Inc), 1% sodium pyruvate, 1% L-glutamine, 1.4% MEM nonessential amino acids, 1% antibiotic-antimycotic solution, 300U/mL of penicillin and 300 mg/mL of streptomycin (Invitrogen Corporation, GibcoBRL) as previously described (Ferrari et al., 2003). The PK15A (porcine kidney) cells were used as a control to assess the infectivity of the porcine circovirus type 2 (PCV-2). The PK15A cells, a subclone of PCV noninfected PK15 cells were maintained as previously described (Gagnon et al., 2008) in Earle's minimal essential medium (MEM) (Invitrogen Corporation, GibcoBRL), supplemented with 10% FBS (Wisent Inc), 300 U/mL of penicillin, 300 mg/mL of streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2.5 mg/mL of amphotericin B, and 10 mM HEPES buffer (Invitrogen Corporation, GibcoBRL). All cell lines were cultured at 37°C in 5% CO_2 atmosphere.

The PRRSV strain used to establish the permissiveness of the SJPL cells was the MARC-145 cells adapted IAF-Klop North American reference strain (Gagnon et al., 2003). The PRRSV virus stock was obtained following three cycles of freeze-thaw of PRRSV IAF-Klop infected MARC-145 cells. Afterward, the virus was purified following a 3.5 hrs period of ultracentrifugation on a 30% sucrose cushion (in a TBS

solution: 50mM tris pH7.5, 150mM NaCl) using the SW28 Beckman Coulter rotor at 83,000 relative centrifugal force (rcf). The virus pellets were resuspended in 0.5 mL of PBS and aliquots of the virus stock were then conserved at –70°C for future use. The infectious dose of the virus stock was calculated from a 96-well microplate of MARC-145 infected cells by the Kärber method as previously described (Gagnon et al., 2008). Virus titers were expressed in tissue culture infectious dose 50 per mL ($TCID_{50}$/mL). The FMV-06-1717 PCV-2b strain (Gagnon et al., 2008) has been used in the coinfection experiment. The PCV-2 viral stock has been produced as previously described (Gagnon et al., 2008).

2.2. Immunofluorescence assay (IFA)

The presence of PRRSV antigens in infected cells was determined by an immunofluorescence assay (IFA). Briefly, cells were infected with IAF-Klop PRRSV strain at a multiplicity of infection (MOI) of 1. Then, infected cells were fixed at 72 hrs post-infection (pi) with a 4% paraformaldehyde (PFA) solution prepared as previously described (Ausubel et al., 2002). Mock-infected cells were included as negative controls. After an incubation period of 30 minutes at room temperature, the PFA solution was removed and cells were washed three times with a phosphate buffer saline solution (PBS). Then, cells were incubated during 10 minutes at room temperature with a PBS solution containing 1% Triton X-100. After removing the Triton X-100 solution, the cells were washed three times with a PBS-Tween 20 solution (PBS containing 0.02% Tween 20). After the permeabilization procedure, cells were incubated 30 minutes with PBS containing 0.2% Tween 20 and 1% Fetal Bovine Serum Albumin. Then, the α7 rabbit monospecific antisera (a specific anti-N PRRSV protein antibody) (Gagnon et al., 2003) was diluted 1/200 in the washing buffer and added to the cells and incubated at room temperature for a 30 minutes period. Cells were then washed and were incubated for 30 minutes with the washing buffer containing a 1/160 dilution of anti-rabbit specific antisera FITC conjugated (Sigma-Aldrich Inc., St-Louis, USA). Finally, cells were visualized using a DMI 4000B reverse fluorescence microscope, image of the cells were taking with a DFC 490 digital camera and the image were analyzed using the Leica Application Suite Software, version 2.4.0 (Leica Microsystems Inc., Richmond Hill, Canada). The same techniques was used to detect the capsid protein (cap) of PCV-2 in the coinfection

experiment, except that a specific polyclonal pig serum was used as the primary antibodies (diluted 1/200) (Racine et al., 2004) and an anti-swine PE conjugated antibody (Abcam Inc., Cambridge, USA) was used as the secondary antibodies (diluted as suggested by the manufacturer).

2.3. Virus production during multiple cell passages

Twenty-five cm^2 Flasks (Corning Inc., NY, USA) were seeded with 10^6 MARC-145 or SJPL cells and those cells were infected with 0.005 MOI of IAF-Klop PRRSV strain. The cytopathic effect (CPE) was observed by light microscopy every day until the end of the experiment at 5 days pi. Then, cells with their supernatants were subjected to three cycles of freeze-thaw at -70°C and the virus stock solutions were kept at -70°C for future use. Four subsequent viral passages in MARC-145 and SJPL cells were done as previously described except that a dilution of 1/20 of the previous viral stock solutions was used for cell infection. Mock-infected cells were included as controls in each passage. The amount of virus production at each passage was calculated from a 96-well microplate of MARC-145 infected cells by the Kärber method and the results were expressed in tissue culture infectious dose 50 per 10^6 infected cells ($TCID_{50}/10^6$ cells).

2.4. Virus replication kinetics assay

10^5 MARC-145 and SJPL cells were infected with PRRSV IAF-Klop strain using an MOI of 1. The inoculums were removed after 4hrs of incubation. Cells were washed three times with culture medium and fresh cell culture medium was added. At different times point post infection (0, 4, 9, 12, 18, 24, 48, 72, 96 and 120hrs p.i.), both supernatants (cell culture medium) and cell pellets (cells) were collected after centrifugation. Then cell pellets and supernatants were stored at -70°C until used. Three cycles of freeze-thaw were performed to release infectious viral particles from the supernatants and cell pellets. Afterwards, supernatants and cell pellets were centrifuged at 4000 rpm at 4°C during 10 min to remove cellular debris and virus titration was performed in MARC-145 cells as described above. Mock-infected cells were included in each experiment as controls. All experiments were repeated two times in triplicate.

2.5. Virus isolation

Virus isolation (VI) was attempted from 22 swine samples (lung and lymph nodes tissues) submitted from October 2007 to September 2008 to the Veterinary virology diagnostic laboratory of the Veterinary college of the Université de Montréal. Those samples originate from 3 to 10 weeks old animals housed in different Canadian farms and they were submitted for different reasons such as PRRSV outbreaks, porcine circovirus associated disease outbreaks, or others health problems. Three of the submitted samples were PRRSV negative by a commercially available real-time PCR diagnostic assay (Tetracore Inc., Rockville, MD, USA) and the amount of infectious PRRSV contained in the 19 real-time PCR positives cases was determined using the same assay as previously described (Gagnon et al., 2008). For VI, about 1-2 cm^3 of pool of tissue samples were homogenized and resuspended in 9 mL of culture medium without FBS. Then, three cycles of freeze-thaw at -70°C were performed and tissues homogenates were centrifuged at 4000 rpm for 10 min. The supernatants were collected and filtered (with a filter size of 0.2 micron). Following the sample treatment, cell culture media of confluent MARC-145 and SJPL cell monolayers of 25 cm^2 flasks (around 10^6 cells) were removed and 1 mL of filtered sample was added with 1 mL of culture medium without FBS. Afterwards, the cells were incubated at 37°C with 5% CO_2 atmosphere for a 4 hrs absorption period. Then, the virus inocula were removed and cells were washed three times with a sterile PBS solution. Six mL of fresh culture medium were added and cells were incubated for 5 days. Then, three cycles of freeze-thaw were realized at -70°C and cell lysates were centrifuged at 4000 rpm for 10 min. Supernatants of cell lysates were collected and used for a subsequent cell infection cycle. Briefly, 1 mL of the cell lysate supernatants was resuspended in 1 mL of cell culture medium and inoculated to a freshly prepared cell culture. Then, after a 4 hrs absorption period, 4 mL of cell culture medium was added onto cells and cells were incubated at 37°C with 5% CO_2 atmosphere for 5 days. This new infection step was done for three consecutive times. At the fourth passage, the VI status was confirmed by the presence of CPE and a positive IFA result. To further characterise the PRRSV strains that were isolated from both cell lines, PCR products encompassing the ORF5 gene were obtained from tissues and fourth VI cell passages, and subsequently sequenced. Sequences were analyzed using the CLUSTAL W alignment method of the

BioEdit sequence alignment editor version 7.0.9 software (Ibis Therapeutics, Carlsbad, CA, USA).

2.6. Apoptosis

Confluent monolayers of MARC-145 and SJPL cells were infected with PRRSV IAF-Klop strain at 0.5 MOI or were incubated with a mix of apoptotic inducers (500 µg/mL actinomycin D, 60 nM vinblastine sulfate, 100 µg/mL cycloheximide and 40 µg/mL puromycin 2HCl; Biomol Research Laboratories Inc., Plymouth meeting, PA, USA) as positive controls. Cellular changes associated with the infection or the inducers were respectively visualized at 72 hrs pi and 24 hrs post-incubation under a light microscope (Leica Microsystems Inc.). At this time, cells were disrupted in a lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT and 100 µM EDTA) for 5 minutes followed by sonication (Sonifier S-450A, Branson, Danbury, CT, USA). Then, protein concentrations were measured by a Bradford assay following the manufacturer's instructions (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). Subsequently, apoptosis was assessed by detecting the activation of procaspases 3/7 as described by Gagnon *et al.* (2003), with minor modifications. Briefly, a volume of cell lysate corresponding to 50 µg of total cell protein was added to the assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 100 µM EDTA and 10% glycerol). Then, specific substrate for caspases 3/7, the Ac-DEVD-AFC fluorogenic substrate (Biomol Research Laboratories Inc.), was added at a final concentration of 200 µM and the rate of fluorescence released was monitored with a 96-well plate fluorometer (Synergy HT, Biotek, Winooski, VT, USA). The results were expressed as relative fluorescence released (relative fluorescence units or RFU) per second per µg of cell lysates.

2.7. Inhibition of PRRSV replication

10^5 MARC-145, SJPL and PK15A cells were infected in suspension with an infectious dose of 1 MOI of IAF-Klop PRRSV and FMV-06-1717 PCV-2b strains individually and simultaneously to determine if PCV-2 could inhibit the replication of PRRSV in SJPL as previously reported in MARC-145 cells (Chang et al., 2005). The infected cells were seeded in 24 wells plates. The CPE was evaluated at different times

post-infection and the cells were fixed with PFA at 96 hrs pi and IFA was realized to detect the expression of viral proteins of both viruses. To determine the amount of porcine IFN-α that is able to inhibit the replication of PRRSV in permissive cell lines, 10^4 MARC-145 and SJPL cells were seeded into wells of 96-well tissue culture plates (Corning Incorporated) and incubated overnight. The cells were then infected with the PRRSV IAF-Klop strain at an infectious dose of 0.5 MOI in a culture medium without FBS and incubated during 4 hrs. The culture medium was then removed and replaced by a complete medium (ie with 10% FBS) with different concentrations of porcine IFN-α (PBL, New Jersey, USA) and incubated during 5 days. Then, the development of CPE was monitored and an IFA was realized as described above for the detection of PRRSV proteins expression in infected cells. All the experiments were done in duplicate.

2.8. Statistical analysis

A two-way ANOVA model, followed by Bonferroni *post-hoc* tests (Graphpad PRISM Version 4.0 software) were used to determine if a statistically significant difference exists between MARC-145 and SJPL cell lines in regards to the amount of PRRSV produced after multiple cell passages and procaspases 3/7 activation by PRRSV. Differences were considered statistically significant with a $p<0.05$. For the viral replication kinetics experiment, the time-course of $TCID_{50}$ measured from the cell pellets and supernatants was analyzed with SAS Version 9.1 software. The following linear mixed-effect model for repeated measurements was solved using restricted maximum-likelihood estimation (Littell et al., 2006): $Y_{ijkl} = \mu + \alpha_i + \beta_j + \tau_k + (\alpha\cdot\beta)_{ij} + (\alpha\cdot\tau)_{ik} + (\beta\cdot\tau)_{jk} + (\alpha\cdot\beta\cdot\tau)_{ijk} + R\cdot(\alpha\cdot\beta)_{ijl} + e_{ijkl}$. Where Y_{ijkl} is the measured $TCID_{50}$; μ is the grand mean; cell line (α_i), type of analytical matrix (β_j), and sampling time (τ_k) are fixed factors; the experiment replicate (R_l) is a random effect; and e_{ijkl} is the random error term. As indicated in the equation above, this statistical model included all dual and triple interactions between the fixed-effect factors, and the random-effect factor R_l was nested within cell and analytical matrix. The strategy for covariance structure modeling proposed by Littell et al (2000) was used. Briefly, the model was estimated first with a free covariance structure. After inspecting the estimated covariance matrix, the model was estimated anew with more parsimonious

covariance models (e.g., compound symmetry, first-order autoregressive), which structure resembled that of the unstructured covariance matrix. The heterogeneous first-order autoregressive covariance model was selected because it fitted best to the empirical covariance matrix, as determined with the Akaike information criterion (Littell et al., 2000). Least-square means were used to assess differences between the two cell lines at each time and for each type of analytical matrix (i.e., cell pellets or supernatants), using Bonferroni-adjusted significance thresholds. The areas under the time-$TCID_{50}$ curves (AUC) were calculated for each cell*matrix*replicate in order to obtain estimates of total viral production for each cell line following the 120 hrs duration of the experiment.

3. Results

3.1. SJPL cells susceptibility to PRRSV

In order to evaluate the susceptibility of epithelial cells of the respiratory tract of swine in regards to PRRSV, two epithelial cell lines, the NPTr and SJPL cells, were inoculated with PRRSV IAF-Klop strain at 1 MOI. As previously reported, the NPTr cells were not permissive to PRRSV (data not shown) (Ferrari et al., 2003). Surprisingly, the PRRSV-infected SJPL cells developed a very light CPE at 72 hrs pi compared to mock infected cells as illustrated in Figure 1, which suggested the replication of PRRSV. The amount of CPE observed in SJPL cells increases over time but it has been always significantly smaller compared to PRRSV-infected MARC-145 cells (data not shown and Figure 1). To confirm the PRRSV proteins expression in infected SJPL cells, an IFA was performed. Interestingly, the PRRSV N protein was detected in PRRSV-infected SJPL cells (Figure 1) which indicate that PRRSV was able to express at least the N viral protein. Most of the IFA positive cells have positive signal localized in the cytoplasm (Figure 1) such as what have been previously reported for infected MARC-145 cells (Magar et al., 1995).

3.2. Infectious PRRSV particle production in infected SJPL cells

To establish if SJPL cells allow full PRRSV replication cycle and infectious particles production after being in contact with infectious virions, the amount of infectious PRRSV particles produced by infected SJPL cells was evaluated during five consecutive passages. As illustrated in Figure 2, the amount of infectious virus yield from the inoculum ($10^{3.3}$ $TCID_{50}/10^6$ cells) compared to the first passage in SJPL cells ($10^{6.6}$ $TCID_{50}/10^6$ cells) increased around 2000 times which clearly indicates that SJPL permits the production of infectious viral particles. The amount of virus yield was maintained during subsequent passages which further indicates that infectious PRRSV particles are produced (Figure 2). However, the overall production of infectious particles in SJPL cells compared to MARC-145 cells do not seems to be significantly different ($P>0.05$).

In order to determine the efficiency of PRRSV production in SJPL cells compared to MARC-145 cells, a replication kinetics experiment has been conducted

where the amounts of infectious viruses produced in the cell culture medium (supernatant) and in the cells (cell pellet) were evaluated at different times pi (Figure 3). The repeated-measures linear mixed model for analyzing the time-course of $TCID_{50}$ in cell pellets and supernatants (Figure 3) revealed a significant effect of time ($P<0.0001$): on average, viral titers significantly increased by 18 h after inoculation, peaked by 48 h and decreased afterwards to values that significantly differed with respect to the first measurement time ($P<0.0001$). Three significant dual interactions of fixed-effect variables were recorded: cell*matrix ($P=0.0224$), cell*time ($P=0.0006$), and matrix*time ($P<0.0001$). With respect to the cell*matrix interaction, the viral titers of SJPL supernatants did not differ significantly from the ones of their cell pellets (Bonferroni-adjusted least-square difference; $P=1$), but the ones of MARC-145 supernatants were 0.23 ± 0.08 $TCID_{50}$ units higher than the ones of their cell pellets, an almost significant difference ($P=0.0628$). The viral titers of the SJPL cell pellets were 0.22 ± 0.08 $TCID_{50}$ units lower than the ones of MARC-145 cell pellets ($P=0.0806$), but those of the SJPL supernatants were 0.51 ± 0.08 $TCID_{50}$ units lower than the ones of MARC-145 supernatants ($P<0.01$). With respect to the cell*time interaction, least-square differences of viral titers for a cell line with respect to its peak value recorded at 48 h revealed that the viral production of SJPL cells was highest between 24 and 96 h, viral titers being significantly lower at all other times ($P<0.0001$). In contrast, the peak viral production of MARC-145 cells lasted between 24 and 48 h, with viral titers being significantly lower at the all other sampling times ($P\leq0.0273$). With respect to the matrix*time interaction (with cell-specific differences taken into account), least-square differences in viral titer between supernatants and cell pellets were significant during the first 9 h of incubation ($P<0.0001$) and the 48 h to 120 h interval ($P\leq0.0002$). Thus, more infectious viral particles could be collected at 48 hrs pi and later on from the culture medium of both infected cells compared to their respective cell pellets (around 10 times more). Moreover, the AUC of time-$TCID_{50}$ curves results indicated that the overall estimation of PRRSV infectious virion production of the SJPL cells averaged 98% of those of MARC-145 cells for cell pellets and 90% for supernatants.

3.3. Virus isolation efficiency

From the 19 PRRSV real-time PCR positive cases, 11 PRRSV isolates could be obtained using MARC-145 cells compared to 8 with SJPL cells (Table 1).

Consequently, the VI efficiency with MARC-145 and SJPL cells were 58% and 42%, respectively, suggesting that MARC-145 could be slightly more suitable for PRRSV VI from clinical samples. In addition, all VI positive cases with SJPL cells were also VI positive with MARC-145 cells. Interestingly, when the amount of PRRSV was higher than 500 $TCID_{50}$ of PRRSV/gram of tissue, the VI efficiency was also very high for both cell lines. More precisely, the VI efficiency for tissues that have > 500 $TCID_{50}$ of PRRSV/gram was 100% and 88% for MARC-145 and SJPL cells, respectively (Table 1). To further characterize the PRRSV strains that were isolated, the ORF5 gene of five cases that were both VI positive with MARC-145 and SJPL cells were sequenced. Sequence analyses revealed that all PRRSV strains are NA type isolates (data not shown). Interestingly, the nucleotide (nt) identities between the tissues and the fourth cell passage in both cell lines of each cases were 100% identical indicating that the same PRRSV strains, that were identified initially in the tissues, were isolated. Moreover, at the fourth cell passage, the ORF5 sequences of viruses isolated from each porcine tissue homogenate in SJPL and MARC-145 cells were 100% identical which suggests that SJPL cells allow the isolation of the same strains as those isolated with MARC-145 cells. Sequence analyses also revealed genetic variability between strains that were isolated from each porcine tissue homogenate with SJPL cells (86.4% to 93.2% nt identities) and compared to the PRRSV reference strain IAF-Klop (88.3% to 91.0% nt identities).

3.4. SJPL cells susceptibility to PRRSV apoptosis

As illustrated in Figure 4, the SJPL cells are more sensitive to procaspases 3/7 activation by PRRSV and several apoptotic inducers since the level of SJPL procaspases 3/7 activation was 2.7 to 4.4 times higher compared to MARC-145 cells. In addition, activation of procaspases 3/7 in PRRSV-infected MARC-145 and SJPL cells was 3.5 to 6.2 times higher ($p<0.05$), respectively, compared to noninfected cells (Figure 4). Even if procaspases 3/7 activation was higher in SJPL cells, at the time the cells were disrupted (24 hrs and 72 hrs pi), the CPE was very mild in SJPL cells compared to MARC-145 cells since at least 60% of the PRRSV-infected MARC-145 cells visualized by light microscopy showed CPE (data not shown and Figure 1).

3.5. Inhibition of PRRSV replication

Coinfection of PRRSV-infected MARC-145 cells with PCV-2 has previously been reported to cause the inhibition of PRRSV replication (Chang et al., 2005). Furthermore, this inhibition was associated with the presence of IFN-α that was contained in the PCV-2 viral stock (Chang et al., 2005). Thus, a similar result was obtained since the presence of PCV-2 was able to block the replication of PRRSV in infected MARC-145 cells (Table 2). Surprisingly, the presence of PCV-2 did not block the replication of PRRSV in infected SJPL cells using the same experimental conditions (Table 2). Then, to evaluate the impact of IFN-α in regards to PRRSV replication, different amounts of IFN-α were added in the cell culture media of both cell lines. Interestingly, the minimal concentration of IFN-α needed to have an antiviral effect against PRRSV replication was 8 to 16 times lower in infected SJPL cells (between 3.13 to 6.25 U/mL) compared to infected MARC-145 cells (between 50 to 78.13 U/mL) (Table 3).

4. Discussion

Previous attempts to find porcine immortalized cell lines into which PRRSV infectious particles could bind, enter and complete a full virus replication cycle including virion production, such as epithelial cell line of the respiratory tract, have failed (Ferrari et al., 2003; Huang et al., 2009; Wensvoort et al., 1991). In the present study, a new immortalized porcine epithelial cell line of the respiratory tract of swine, the SJPL cells (Seo et al., 2001), was found to be permissive to PRRSV infection and replication (Figures 1,2 and 3). The SJPL cells have been known to be permissive to a variety of sub-types of influenza virus from human, swine, avian and horse origins (Seo et al., 2001). From now on, PRRSV can be added to the list of viruses that can replicate in SJPL cells. To our knowledge, it is the first time that an immortalized epithelial cell line of the respiratory tract of swine has been reported to be permissive to PRRSV infection and replication *in vitro*. Interestingly, the amount of infectious virus produced in infected SJPL cells was at least 90% as high as the amount produced in infected MARC-145 cells, and their viral production peak was lasting longer than the one of MARC-145 cells, suggesting that the SJPL cells could easily replace the MARC-145 cells (and related cells that derivate from MA104 cells) in a large scale PRRSV live or killed vaccine production. Furthermore, several PRRSV strains of the NA genotype that possess various ORF5 sequences could be isolated from pig samples and were able to replicate in SJPL cells (Table 1) indicating that SJPL cells could also be used for autogenous vaccines production. In addition to the experiments that have been conducted with NA PRRSV strains, we have also conducted some experiments with an EU reference strain, the Lelystad virus (LV) (data not shown). Interestingly, LV SJPL infected cells could also express the N viral protein, which have been detected by IFA, and permit the production of PRRSV infectious particles (data not shown). As indicated in Table 1, viruses could be isolated from pig tissue samples on three occasions only in MARC-145 and not SJPL cells when the amount of PRRSV in tissue was at its lowest, suggesting that MARC-145 cells could be more sensitive for VI than SJPL cells. Further experiments will have to be conducted to ascertain this latest finding. Nonetheless, the SJPL cells were able to allow the replication of several PRRSV NA ORF5 genomic variants and the LV reference strain (data not shown and Table 1) indicating that at least these cells are permissive to a wide spectrum of PRRSV isolates.

Phenotypically, the SJPL cells are distinguishable from the MARC-145 cells (Figure 4, Tables 1,2 and 3). As reported previously by Chang et al. (2005), PRRSV was not able to replicate in PCV-2 and PRRSV co-infected MARC-145 cells (Table 2) and this phenomenon could be related to the presence of IFN-α in the PCV-2 viral stock that was used to realize the experiments. Moreover, other reports have previously demonstrated the antiviral effect of IFN-α in regards to PRRSV (Brockmeier et al., 2009). Interestingly, PRRSV was able to replicate in PCV-2 and PRRSV co-infected SJPL cells (Table 2) suggesting that SJPL cells could be less responsive to the antiviral effect of IFN-α. Consequently, different amount of IFN-α were added in the cell culture media to evaluate its antiviral effect in regards to both cell lines. It was found that SJPL cells are more responsive to the antiviral effect of IFN-α than MARC-145 cells (Table 3) suggesting that another undetermined mechanism was involved in the inhibition of PRRSV replication by PCV-2 in infected MARC-145 cells. Noteworthy, no PCV-2 capsid protein expression could be detected in both PCV-2 infected SJPL and MARC-145 cell lines indicating that PCV-2 do not replicate in those cells.

Many studies have demonstrated that PRRSV induces apoptosis both *in vitro* and *in vivo* (Choi and Chae, 2002; Kim et al., 2002; Labarque et al., 2003; Miller and Fox, 2004; Sirinarumitr et al., 1998; Suarez et al., 1996; Sur et al., 1997; Sur et al., 1998) and several techniques have been used to demonstrate this phenomenon such as procaspase 3 activation in PRRSV IAF-Klop infected MARC-145 cells (Gagnon et al., 2003). At 72 hrs pi, the CPE visualized by light microscopy in PRRSV-infected SJPL cells was very mild compared to infected MARC-145 cells (Figure 1) and over time, the appearance of CPE in infected SJPL cells was significantly delay compared to infected MARC-145 cells (data not shown). Consequently, it could be expected that the amount of caspase 3 in infected MARC-145 will be higher compared to infected SJPL cells. Surprisingly, the opposite situation was observed indicating that SJPL cells are more suited for procaspases 3/7 activation than MARC-145 cells even if only mild CPE was observed in SJPL cells following PRRSV infection or incubation with apoptotic inducers (Figure 4). This latest result clearly demonstrates that SJPL cells are phenotypically completely different from MARC-145 cells and that the level of procaspases 3/7 activation induces by PRRSV is not related to the level of CPE that could be observed by light microscopy. In fact, other cell death mechanisms have been

reported to occur in PRRSV-infected cells such as necrosis (Costers et al., 2008; Wang et al., 2007).

As mentioned by Huang et al. (2009), even if MARC-145 cell line is permissive for PRRSV propagation *in vitro*, it is not of pig origin, and thus PRRSV infection in the MARC-145 cells may not reflect the actual virus–host interactions. It is further supported by the fact that SJPL cells are phenotypically different from MARC-145 cells (Figures 1 and 4, Tables 2 and 3). Thus, an immortalized epithelial cell line of the respiratory tract of swine, such as SJPL cells, could be more convenient to use than primary cell cultures, for obvious reasons, and could be a more relevant *in vitro* model than MARC-145 cells to study the pathogenesis of PRRSV. Furthermore, PRRSV is an important pathogen of the respiratory tract of swine that favor secondary bacterial and virus infections (Rossow, 1998). Even if PRRSV interaction with alveolar macrophages plays a crucial role in the virus pathogenesis, its interaction with the epithelial cells of the respiratory tract of swine should not be neglected because they have been proven to be infected *in vivo* (Pol et al., 1991; Rossow et al., 1996). Thus, studying the PRRSV-SJPL interactions should give us new insight in regards to the viral pathogenesis of PRRSV.

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Figure 1. Detection of the N viral protein in PRRSV infected SJPL cells by an immunofluorescence assay

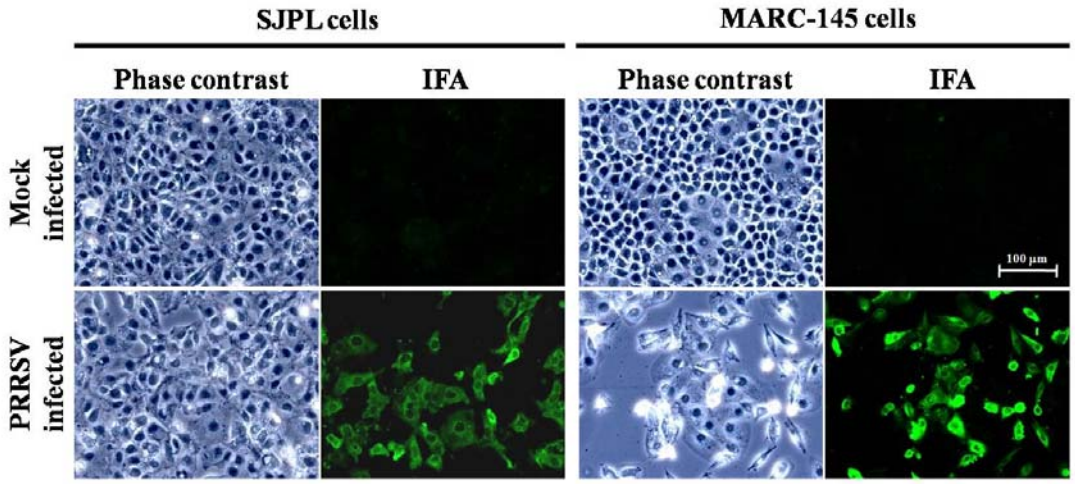

Figure 1. Detection of the N viral protein in PRRSV-infected SJPL cells by an immunofluorescence assay. The IFA was done as described in the materials and methods section. Mock-infected cells are illustrated as control in the upper panels. Cells infected at 1 MOI with PRRSV IAF-Klop reference strain are illustrated in lower panels. Cells were visualized with visible light (phase contrast) and UV (IFA).

Figure 2. PRRSV infectious particle production in infected SJPL cells following five consecutive cell passages

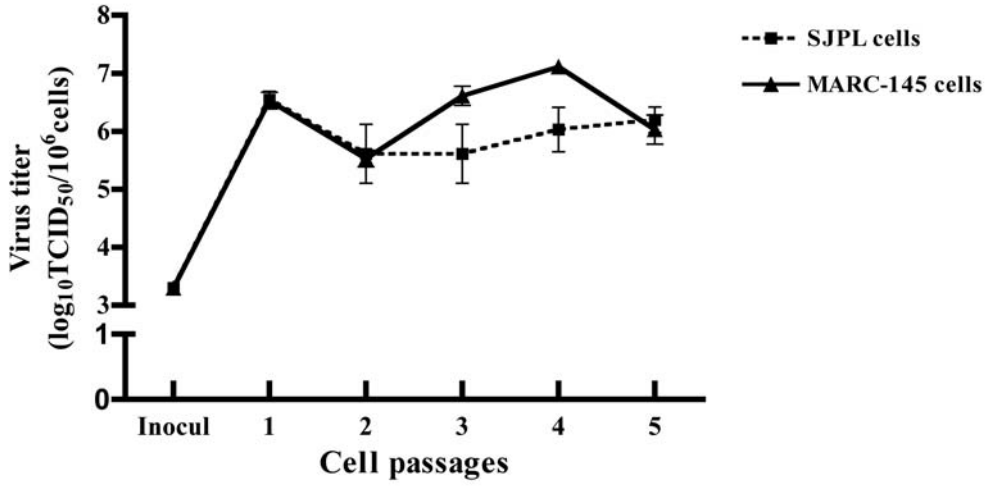

Figure 2. PRRSV infectious particles production in infected SJPL cells following five consecutive passages. PRRSV IAF-Klop strain was serially passaged in MARC-145 and SJPL cells as described in the materials and methods section. The amount of the infectious viral particles recovered after each passage was determined in MARC-145 cells. The virus titers were expressed as $TCID_{50}$ per 10^6 cells. The initial viral inoculum (inocul) used to infect both cell lines was $10^{3.3}$ $TCID_{50}/10^6$cells.

Figure 3. PRRSV replication kinetic in SJPL infected cells

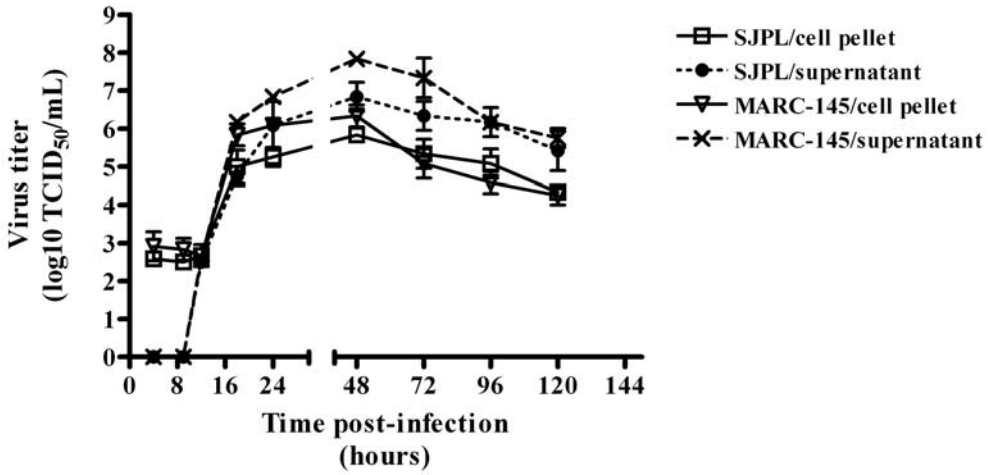

Figure 3. PRRSV replication kinetics in infected SJPL cells. MARC-145 and SJPL cells were infected at 1 MOI with PRRSV IAF-Klop strain. At different time pi, the infectious viruses recovered from the cell culture medium (or supernatant) and the cells (or cell pellet) were titrated in MARC-145 cells. Experiment was done in triplicate.

Figure 4. Procaspases 3/7 activation in PRRSV infected SJPL cells

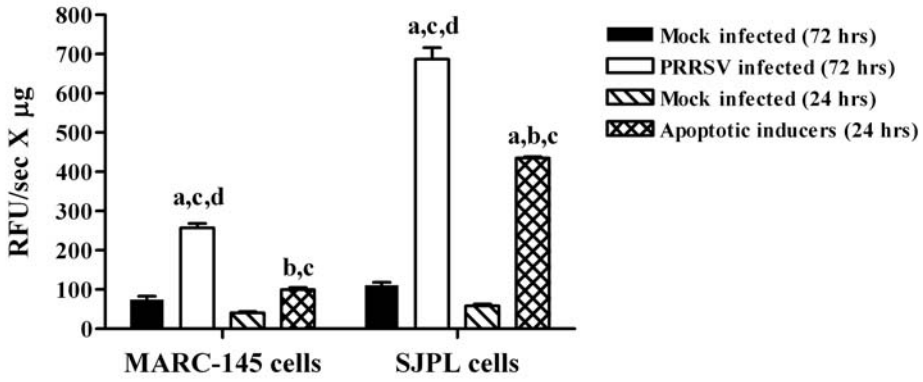

Figure 4. Procaspases 3/7 activation in PRRSV-infected SJPL cells. MARC-145 and SJPL cells were infected at 0.5 MOI with PRRSV IAF-Klop strain or incubated with a combination of four apoptotic inducers (actinomycin D, vinblastine sulfate, cycloheximide and puromycin) as a positive control. At 24 hrs post-incubation with the apoptotic inducers, MARC-145 cells have developed high CPE level compared to SJPL cells which showed low to mild CPE. At 72 hrs, PRRSV-infected cells were disrupted for the detection of caspase 3 using a specific fluorogenic substrate. The results were expressed as relative fluorescence released (relative fluorescence units or RFU) per second per µg of cell lysates. Mock infected cells were used as control and the experiment was done in triplicate. a,c,d: statistically different ($P<0.05$) from mock infected cells (24 and 72hrs pi) and apoptotic inducers treated cells; b,c: statistically different ($P<0.05$) from PRRSV and mock infected cells (24 hrs); a,b,c: statistically different ($P<0.05$) from PRRSV and mock infected cells (24 and 72hrs pi).

Table 1. PRRS virus isolation efficiency from swine samples using SJPL cells compared to MARC-145 cells.

Amount of PRRS virus in tissues[a] ($TCID_{50}$/g)	**Cell lines**	
	MARC-145 *(number isolated/number tested)* [b]	**SJPL** *(number isolated/number tested)*
0-100[c]	*0 / 7*	*0 / 7*
101-500	*2 / 6*	*0 / 6*
501-2500	*7 / 7*	*6 / 7*
2501-40000	*2 / 2*	*2 / 2*
Total:	*11 / 22*	*8 / 22*

[a]The PRRSV was quantified as previously described using a real-time PCR assay (Gagnon et al., 2008).

[b]The virus isolation were attempted with both cell lines using the same swine tissue homogenates.

[c]Three cases out of 7 were real-time PCR negative for the presence of PRRSV.

Table I. PRRSV virus isolation efficiency from swine samples using SJPL cells compared to MARC-145 cells

Table 2. Inhibition of PRRSV infection in PCV-2 co-infected cells

Cells	Presence of cytopathic effect and detection of PRRSV antigens by immunofluorescence			
	Uninfected cells	Infected cells		
		PRRSV	PCV-2b	PRRSV + PCV-2b
MARC-145	*neg*	*pos*	*neg*	*neg*
SJPL	*neg*	*pos*	*neg*	*pos*

neg: negative for CPE and PRRSV antigens; pos: positive for CPE and PRRSV antigens.
As a control, PK15A cells were infected with all virus combinations and the cells were only PCV-2 antigens positive by IFA when PCV-2b virus was present.
Note: the experiment was done in duplicate.

Table II. Inhibition of PRRSV infection in PCV-2 co-infected cells

Table 3. Minimal concentration of IFNα for the inhibition of PRRSV infection

	PRRSV infection inhibition	
Cells	**Cytopathic effect inhibition**	**Immunofluorescence inhibition**
MARC-145	> 6.25, < 12.5[a]	> 50, < 78.13
SJPL	> 0.78, < 1.56	> 3.13, < 6.25

[a]Express in U/μl.

Note: the experiment was done in duplicate.

Table III. Minimal concentration of IFN-α for the inhibition of PRRSV infection

IV. Discussion

In vitro, PRRSV replicates in primary cultures of PAMs and to some extent in porcine peripheral blood monocytes (Delputte et al., 2007a; Duan et al., 1997) and dendritic cells (Chang et al., 2008). Primary PAM and immortalized African green monkey kidney epithelial cells and its derivatives such as MARC-145 are known to be permissive to PRRSV replication in vitro (Benfield et al., 1992; Kim et al., 1993). MARC-145 and CL2621 cells, both derived from the MA104 monkey kidney cell line are also routinely used for in vitro propagation of wild and vaccine strains (Bautista et al., 1993; Benfield et al., 1992; Kim et al., 1993). As mentioned by Huang et al. (2009), although immortalized African green monkey kidney epithelial cells MA-104 and cells derived thereof (MARC-145 and CL2621) are known to be permissive to PRRSV replication, they are neither of pig origin nor monocyte–macrophage lineage (Benfield et al., 1992; Kim et al., 1993) and thus PRRSV infection in the MARC-145 cells may not reflect the actual virus-host interactions. Porcine monocyte/macrophage lineage cells such as PAMs are the target for PRRSV in vivo, and thus are ideal cell models for the immunological and pathogenic study of PRRSV. However, due to laborious isolation procedure, low viability of the primary cells in vitro, unreliable freezing procedures for long-term storage and heterogeneous phenotype of the isolated cells, such studies have their flaws (Huang et al., 2009). Our findings are interesting because they represent the first time that immortalized epithelial cells of the swine respiratory tract are reported to permit infectious PRRS viral particle production. Therefore, SJPL could be a more relevant and convenient model for the immunological and pathogenic study of PRRSV than other models.

In the present study, the susceptibility to PRRSV infection of two new porcine epithelial cell lines of the respiratory tract (NPTr and SJPL) was evaluated. Cells were infected with the IAF-Klop North American reference strain. SJPL developed a mild CPE compared to control (mock infected) at 72hrs post-infection. Following performance of an immunofluorescence assay (IFA), surprisingly, expression of the N protein could be detected only in SJPL infected cells. This result further confirms CPE observations and suggests that SJPL is susceptible to PRRSV infection. Interestingly, the number of positive cells was almost the same compared to infected MARC-145 cells (data not show), probably due to the fact that more affected MARC-145 died and were detached from the cell layer which could have lead to the loss of positive MARC-145 cells (Figure 1). Afterwards, a multiple passage infectious viral particle production experiment was conducted to establish if SJPL cells could allow full

PRRSV replication cycle and progeny virion production after being in contact with infectious virions. The results showed that PRRSV viral infectious particles were produced after 5 consecutive passages in SJPL cells and the amount of viruses produced in infected SJPL cells was similar than in MARC-145 infected cells. It is noteworthy that the viral production from the low level of inocula reached highest levels at the first passage (from a inocula of $10^{3.3}$ $TCID_{50}/10^6$ cells to the first passage of $10^{6.6}$ $TCID_{50}/10^6$ cells) (Figure 2). In order to determine the efficiency of PRRSV production in SJPL cells compared to MARC-145 cells, a replication kinetics experiment was conducted where the amounts of infectious viruses produced in the cell culture medium (supernatants) and in the cells (cell pellets) were evaluated at different times pi. Both cells, MARC-145 and SJPL, were identical with regards to their efficacy to produce infectious viral particles (Figure 3). Following the above experiments, it was concluded that SJLP cells were fully permissive to PRRSV replication.

In order to evaluate the permissiveness of SJPL to LV, the European type of PRRSV replication, similar experiments were conducted as compared to the IAF-Klop strain. Specifically, the susceptibility of two new porcine epithelial cell lines of the respiratory tract (NPTr and SJPL) to LV infection was evaluated. The development of CPE was monitored and N proteins expression was detected by an IFA. Interestingly, SJPL could express the N protein although the positive signal was weaker and the number of positive cells was less compared to IAF-Klop infected cells at 72hrs pi (data not show), possibly due to the fact that the N protein Abs which were used in the experiments are heterologeneous to the LV strain. Infected-SJPL cells also developed mild CPE compared to the control. The viral production efficiency in SJPL was successfully evaluated by a viral replication kinetics experiment. No significant differences were found between the SJPL and MARC-145 cells in regards to overall viral production.

Diagnostic findings indicate a high level of pneumonia, with the isolation of PRRS virus in complex with a wide variety of other microbial agents (Rossow, 1998). PRRSV infection results in secondary infection or predisposes secondary pathogen infections (Brockmeier et al., 2000; Cooper et al., 1995; Wills et al., 2000a). Our finding that epithelial cells of the swine respiratory tract are permissive to PRRSV replication may predispose establishment of secondary virus or bacterial infections which in turn could be the cofactors enhancing clinical signs of the disease syndrome.

Work is now underway to determine if PRRSV has an impact with regards to the susceptibility of SJPL to secondary bacterial infections *in vitro*. Most importantly, our findings provide a new and interesting *in vitro* model for studying the pathogenesis of multifactorial respiratory disease.

In comparison to PCV2, PRRSV alone caused much more adverse effects on MARC-145 and SJPL cells directly or indirectly. As seen in a previous study, PCV2 and PRRSV co-infected MARC-145 and PAMs had a CPE reduction effect compared to PRRSV alone infected cells (Chang et al., 2005). PCV2 is a potent IFN-α inducer and the released IFN-α may contribute to the anti-viral effects to PRRSV in PCV2/PRRSV co-infection in MARC-145 (Chang et al., 2005). PRRSV infection dose not elicit type I interferon expression *in vitro* or *in vivo* (Albina et al., 1998a; Buddaert et al., 1998; van Reeth, 1999). The lack of IFN-α response is significant, since IFN-α-mediated events inhibit PRRSV replication *in vitro* (Albina et al., 1998a; Buddaert et al., 1998) and elevation of IFN-α *in vivo* by preinfection with PRCV substantially attenuates subsequent PRRSV replication (Buddaert et al., 1998). In the present study, PRRSV was not able to replicate in PCV-2 and PRRSV MARC-145 co-infected cells (Table 2) and this phenomenon could be related to the presence of IFN-α in the PCV-2 viral stock that was used to conduct the experiments as reported previously by Chang et al. (2005). Moreover, other reports have previously demonstrated the antiviral effect of IFN-α with regards to PRRSV(Brockmeier et al., 2009; Chang et al., 2005). Interestingly, PRRSV was able to replicate in PCV-2 and PRRSV SJPL co-infected cells (Table 2) suggesting that SJPL cells could be less responsive to the antiviral effect of IFN-α. However, it was found that SJPL cells are more responsive to the antiviral effect of IFN-α than MARC-145 cells (Table 3). It is noteworthy that no PCV-2 capsid protein expression could be detected in both PCV-2 SJPL and MARC-145 infected cell lines indicating that PCV-2 do not replicate in these cells. Previous studies have documented the antiviral effect of IFN-α on PRRSV infection (Buddaert et al., 1998; Lee et al., 2004). Effects similar to the present results were described for monocyte cultures, where IFN-α also reduced or blocked PRRSV infection of macrophages when added before or during inoculation (Delputte et al., 2007a). In contrast, these authors observed that monocytes that were cultivated for 2 days with IFN-α, followed by 1-day cultivation without IFN-α, showed a remarkably higher susceptibility to PRRSV infection (20 fold), up to levels similar to those in

macrophages. The effect of IFN-α receptor sialoadhesin on susceptibility of monocytes to PRRSV infection is determined by the balance between enhancement of infection due to induction of receptor sialoadhesin expression and reduction of infection caused by the antiviral action of IFN-α (Delputte et al., 2007a). Likewise, PRRSV attenuates innate immune responses, evades the antiviral cytokine (IFN-α) response, and blocks IFN-α production in the cytoplasm of infected alveolar macrophages (Mateu and Diaz, 2008). Several mechanisms have been described that are used by such viruses as herpesviruses, influenza virus, and hepatitis C virus to escape from the IFN system, such as inhibitors of IFN production and signaling (Katze et al., 2002; Landolfo et al., 1995; Levy and Garcia-Sastre, 2001). In this study, PRRSV may evade the IFN response in PCV2 and PRRSV coinfected SJPL cells or another undetermined mechanism may have been involved in the inhibition of PRRSV replication by PCV-2 in infected MARC-145 cells. In future studies, it will be interesting to establish if a PRRSV receptor sialoadhesin exists in SJPL and whether IFN-α treatment can induce a PRRSV receptor sialoadhesin expression in SJPL cells which could further enhance SJPL susceptibility to PRRSV infection.

Apoptosis is one of the molecular mechanisms of eukaryotic cell death. PRRSV induces apoptosis both *in vitro* and *in vivo* (Choi and Chae, 2002; Kim et al., 2002; Labarque et al., 2003; Miller and Fox, 2004; Sirinarumitr et al., 1998; Suarez et al., 1996a; Sur et al., 1997; Sur et al., 1998) and several techniques have been used to demonstrate this phenomenon such as procaspase 3 activation in PRRSV IAF-Klop infected MARC-145 cells (Gagnon et al., 2003). For our primary results, at 72 hrs pi, the CPE visualized by light microscopy in PRRSV-infected SJPL cells was very mild compared to infected MARC-145 cells (Figure 1) and over time, the appearance of CPE in SJPL infected cells was significantly delayed as compared to infected MARC-145 cells (data not shown). However, our latest results showed that SJPL had higher level of response to PRRSV infection in regards to apoptotic inducers compared to infected MARC-145 cells (Figure 4). Therefore, the level of procaspases 3/7 activation induces by PRRSV is not related to the level of CPE that could be observed by light microscopy. In fact, some viruses encode proteins that can inhibit apoptosis through inactivation of p53 or binding of Bax and encode anti-apoptotic gene products that permit their seemingly undetected replication (Teodoro and Branton, 1997). Several viruses encode viral homologs of Bcl-2. These homologs can inhibit pro-apoptotic

proteins such as Bax and Bak, which are essential for the activation of apoptosis. Examples of viral Bcl-2 proteins include the Epstein-Barr virus BHRF1 protein and the adenovirus E1B 19K protein(Polster et al., 2004). Some viruses express caspase inhibitors that inhibit caspase activity and an example is the CrmA protein of cowpox viruses. Whilst a number of viruses can block the effects of TNF-α and Fas. For example the M-T2 protein of myxoma viruses can bind TNF-α preventing it from binding the TNF-α receptor and inducing a response (Hay and Kannourakis, 2002). Furthermore, many viruses express p53 inhibitors that can bind p53 and inhibit its transcriptional transactivation activity. Consequently p53 cannot induce apoptosis since it cannot induce the expression of pro-apoptotic proteins. The adenovirus E1B-55K protein and the hepatitis B virus HBx protein are examples of viral proteins that can perform such a function (Wang et al., 1995). Moreover, Costers et al. 2008b have demonstrated that PRRSV infection resulted in activation of two opposite mechanisms, which are anti and pro-apoptotic pathways and that a balance exists between them. Anti-apoptotic effects played a major role in the PRRSV early infection of MARC-145. In present study, statistical analysis has showed that peak viral production in SJPL infected cells last longer time than in MARC-145 infected cells (Figure 3). PRRSV-infected SJPL might undergo more anti-apoptosis than pro-apoptosis compared to PRRSV-infected MARC-145 cells and therefore, a delayed development of CPE was showed in PRRSV-infected SJPL cells.

So far, several viral receptors candidate or viral binding proteins for PRRSV have been described, such as heparan sulphate and sialoadhesin (CD169), binding protein vimentin as well as CD163 and CD151 (Calvert et al., 2007; Delputte et al., 2002; Kim et al., 2006; Kristiansen et al., 2001; Shanmukhappa et al., 2007; Vanderheijden et al., 2001; Vanderheijden et al., 2003). These molecules are known to increase susceptibility of cells to PRRSV infection. But which molecules exist on SJPL cells surface that will increase SJPL cell permissiveness to PRRSV replication. It is strongly recommended to do western blot experiments in order to establish the mechanism involved in the permissiveness of SJPL to PRRSV infection.

Immunological and reverse genetic experiments have shown that viral protein glycosylation greatly influences antigenicity, immunogenicity, and protective efficacy of PRRSV vaccines and other viral vaccines (Abe et al., 2004; Ansari et al., 2006; Chen et al., 2000; Dowling et al., 2007). Moreover, it was documented that the poor,

meager and sluggish neutralizing immune response invoked by PRRSV *in vivo* is, in a great part, due to the phenomenon of "glycan shielding" caused by the sugars that surround the antigenic sites of the surface glycoprotein GP5 of PRRSV (Wei et al., 2003). This shielding by the sugar moieties would preclude the host's antibodies to reach and neutralize the immunogenic epitopes, which like the epitope B described on the GP5 of PRRSV, interact with the viral receptor on the host cell (Ansari et al., 2006). Elimination (through a process called "hypoglycosylation") of selected sugar moieties present on the surface of GP5 dramatically enhances the ability of a PRRSV strain to invoke a more robust response composed of PRRSV-neutralizing Abs (Ansari et al., 2006). Adenoviral-expressed GP5 of PRRSV in 293 cells was different from its authentic viral protein (in PRRSV-infected MARC-145 cells) in terms of viral protein glycosylation (Gagnon et al., 2003) suggesting that viral protein glycosylation is host cell dependent. PRRSV antigens produced in MARC-145 may be different from those produced in SJPL cells in terms of protein glycosylation. Consequently, PRRSV vaccine production in these two different cell lines could affect vaccine efficiency. Further studies are required to identify the viral protein glycosylation difference with regards to both cell lines. Most importantly, because SJPL is a swine lung epithelial cell, it is an ideal cell line for live or killed vaccine production.

V. Conclusion

Porcine reproductive and respiratory syndrome is a major disease that poses a significant threat to the global swine industry. The results from this study will provide a new cell model for better understanding the pathogenesis of PRRSV and a new method for PRRSV vaccine antigen production as well as virus isolation.

A new epithelial cell line derived from the swine respiratory tract, the SJPL cell, is fully permissive to PRRSV infection and replication. The amount of infectious viral particles produced in SJPL infected cells was similar to the amount produced in infected MARC-145 cells suggesting that SJPL cells could easily replace MARC-145 cells in a large scale PRRSV live or killed vaccine antigens production. Virus isolation from swine tissues showed that the new cell line is suitable for isolation of a wide range of ORF5 variants and that MARC-145 cells seem more sensitive for VI than SJPL cells.

Furthermore, SJPL cells showed a distinct phenotype compared to MARC-145 cells. It was found that SJPL cells are more responsive to the anti-viral effect of IFN-α than MARC-145 cells and that the level of procaspases 3/7 activation induced by PRRSV is not related to the level of CPE that could be observed by light microscopy. Since the SJPL cell is from the PRRSV host origin, *in vitro* interaction between SJPL and PRRSV or other respiratory pathogens could represent the real interaction between host and pathogens. This suggests that the SJPL cell is a more ideal or suitable *in vitro* model for studying the pathogenesis of PRRSV than other known permissive cell lines.

It should be noted that the mechanism involved in the permissiveness of SJPL cells to PRRSV infection should be demonstrated. Therefore, in future work, it will be interesting to investigate which receptors or protein molecules are responsible for the permissiveness of SJPL to PRRSV infection using the information acquired during this study and new methodologies. Likewise, regarding the LV, European strain, it is also strongly recommended to do some experiments in regards to SJPL cells.

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Appendix1

Title of conference presentations and posters

J.J. Jia, N. Music, M. Jacques, C.A. Gagnon. (2008): Identification of a new cell line permissive to porcine reproductive and respiratory syndrome virus replication. 89th Annual meeting of the Conference of Research Workers in Animal Diseases (CRWAD). Chicago, Illinois, USA. December 7, 8 and 9, 2008. Abstract 127p, poster.

J.J. Jia, N. Music, M. Jacques, C.A. Gagnon. (2008): Identification of a new cell line permissive to porcine reproductive and respiratory syndrome virus replication. International PRRS Symposium, Chicago, Illinois, USA December 5-6, 2008. Abstract page 32, poster.

J.J. Jia, N. Music, M. Jacques, C.A. Gagnon. (2008): Identification d'une nouvelle lignée cellulaire permissive pour la réplication du virus du syndrome reproducteur et respiratoire porcin (VSRRP). 4e Colloque International Francophone de Microbiologie Animale & 2e Symposium du Centre de Recherche en Infectiologie Porcine (CRIP). St-Hyacinthe, Québec, Canada. September 22-24, 2008. Abstract C02 page 87. Poster.

J.J. Jia, D. Tremblay, S.M. Elahi, N.A. Bryant, D.M. Elton, S. Carman, J.P. Lavoie, J. Elsener, C.A. Gagnon. (2007) Genetic relatedness of recent Canadian equine influenza virus isolates with vaccine strains used in the field. 88th Annual meeting of the Conference of Research Workers in Animal Diseases (CRWAD). Chicago, Illinois, USA. December, 2-4, 2007. Abstract 44P, page 100. Poster.