Université de Montréal

Characterization of *Actinobacillus pleuropneumoniae* antiviral effect against porcine reproductive and respiratory syndrome virus in porcine alveolar macrophages.

par

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RÉSUMÉ

Le syndrome reproducteur et respiratoire porcin (SRRP) est la maladie infectieuse la plus économiquement importante de l'industrie porcine. Une étude récente a démontré que le surnageant de culture d'*Actinobacillus pleuropneumoniae* (*App*) inhibe l'infection du virus SRRP (VSRRP) *in vitro* dans des cellules de singe. L'objectif de cette étude est de démontrer l'effet antiviral d'*App* contre le VSRRP dans les cellules cibles du virus *in vivo*: les macrophages alvéolaires porcins (MAPs) et d'étudier les mécanismes spécifiques impliqués lors de l'inhibition virale. Les MAPs ont été traités avec *App,* avant et après l'infection avec le VSRRP. À différents temps post-infection, la réplication et la transcription du génome viral ont été quantifiées. L'expression des interférons (IFN) type I et II, ainsi que le profil protéomique en présence ou absence d'*App* ont été évalués. L'expression de certaines protéines a été confirmée par immunobuvardage et immunofluorescence (IF). Les résultats ont démontré que l'effet antiviral d'*App* n'est pas via l'induction des IFN type I et II. *App* inhibe l'infection virale dans MAPs avant la réplication et la transcription du génome viral, ce qui indique qu'*App* inhibe précocement le cycle réplicatif viral. Le profil protéomique a révélé qu'*App* augmentait l'expression de la cofiline, une protéine qui provoque la dépolymérisation de l'actine. De plus, ce phénomène de dépolymérisation a été confirmé par IF. Le traitement des MAPs avec la cytochalasin D (un composé qui provoque la fragmentation des microfilments) a démontré que comme pour *App*, cette drogue inhibe la réplication virale. Les résultats obtenus suggèrent que l'effet antiviral d'*App* est via l'activation de la cofiline et dépolymérisation de l'actine, affectant probablement l'endocytose du VSRRP**.**

Mot clés : SRRP/ VSRRP/ MAPs/ réplication du VSRRP / cytosquelette d'actine/cofiline/*A. pleuropneumoniae*

ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important infectious disease of swine production. A recent study has demonstrated that the culture supernatant of *Actinobacilus pleuropneumoniae* (*App*) inhibits PRRS virus (PRRSV) infection *in vitro* in a monkey cell line. Following this finding, the objective of this study was to demonstrate the antiviral effect of *App* in the primary target cells of PRRSV *in vivo*: porcine alveolar macrophages (PAM) and to elucidate how *App* inhibits PRRSV replication in PAM. Cells were treated with *App* before and after PRRSV infection. At different times post-infection, viral genome replication and transcription were measured in the presence of *App*. mRNA expression of type I and II interferon (IFN) and the proteomic profile of infected cells treated with *App* were evaluated. The expression of selected proteins was confirmed by immunofluorescence (IFA) and Western blot assays. Results showed that *App* antiviral effect against PRRSV is not via the induction of type I and II IFN expression. Moreover, it was observed that *App* inhibits PRRSV infection in PAM before its genome replication and transcription, indicating that *App* antiviral effect takes place early in PRRSV replication cycle. Proteomic results revealed that *App* increases cofilin, a protein that induces actin filaments depolymerisation in its active form. This depolymerisation phenomenon was further confirmed by IFA. Interestingly, a microfilament-disrupting compound (cytochalasin D) induced the same effect on PRRSV replication than *App* suggesting that *App* antiviral effect against PRRSV takes place via the activation of cofilin and thus actin depolymerisation, which probably affects PRRSV endocytosis.

Key words: PRRS/ PRRSV/ PAM/ PRRSV replication/ actin cytoskeleton/ cofilin/ *A. pleuropneumoniae*

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ABREVIATIONS AND SIGLES

ADE	Antibody-dependent enhancement
AMP	Cyclic adenosine monophosphate
App	*Actinobacillus pleuropneumoniae*
Appwt	*App* wild type strain
AppΔapxICΔapxIIC	*App* mutant strain producing non-active ApxI and ApxII toxins
Apx	*App* toxins
ATCC/VR-2332	American Type Culture Collection/PRRSV reference strain for genotype II
BALF	Bronchoalveolar lavage fluid
BAV3	Bovine adenovirus 3
BHI	Brain heart infusion
BHV-1/4	Bovine herpes virus type 1 and 4
BVDV-1	Bovine viral diarrhea virus type 1
cAMP	Cyclic adenosine monophosphate
$CD4^+$/$CD8^+$/ $CD4^+$-$CD8^+$	T cells $CD4^+$/$CD8^+$/$CD4^+$-$CD8^+$ double positives
CD151	Cluster of Differentiation 151 (PRRSV cellular receptor)
CD163	Cluster of Differentiation 163 (PRRSV cellular receptor)
CFU/mL	Colony-forming units per milliliter
CL2621	African green monkey kidney cell line derived from MA-104

ClpP	Protein member of the caseinolytic protease family
3CLpro	3C-like proteinase domain
CPE	Cytopathic effect
CPV	Canine parvovirus
CREB	cAMP response element-binding
DMEM	Dulbecco's modified Eagle's medium
DMVs	Double membrane vesicles
dpi	Days post-infection
dsRNA	Double-stranded ribonucleic acid
EHV-1	Equine herpes virus type 1
ER	Endoplasmic reticulum
F-actin	Actin filaments
FBS	Foetal bovine serum
Fhu A/B/C/D	Cellular proteins responsible for the transport of ferric hydroxamate
FITC	Fluorescein isothiocyanate
GP	Glycoprotein
gRNA	Genomic RNA
H1N1/H3N2	Influenza A virus subtypes
HPIV-3	Human parainfluenza virus 3
IFA	Immunofluorescence assay

IFN α/β/γ	Interferon alpha/beta/gamma
IgG/IgA	Immunoglobulin G and A
IL	Interleukin
IRF3	IFN regulatory factor 3
ISG15	interferon-stimulated gene 15
ISP-1	IFN-beta promoter stimulator 1
JAK-STAT	Janus kinase- Signal Transducer and Activator of Transcription (Type I IFN signaling pathway)
KINEX ™	Antibody microarray proteomic test
LDH	Lactate dehydrogenase
LDV	Lactate dehydrogenase-elevating virus
LPS	Lipopolysaccharide
LV	Lelystad virus
M	PRRSV membrane protein
MARC-145	African green monkey kidney cell line derived from MA-104
MBHPP147	Name of *App* mutant
MDCK	Madin-Darby canine kidney cells
MEM	Minimum essential medium
MHC	Major histocompatibility complex class
MLV	Modified-live virus
MOI	Multiply of infection

N	PRRSV nucleocapsid protein
NAbs	Neutralizing antibodies
NVSL	PRRSV genotype II strain
β-NAD	Beta-nicotinamide adenine dinucleotide
NendoU	Nidovirus endoribonuclease
NFκB	Transcriptional regulator nuclear factor-κB
NOD1/NOD2	Nucleotide-binding oligomerization domain-containing protein 1/2
NPTr	Newborn pig trachea cells
nsps	Non-structural proteins
ORFs	Open reading frames
PAM	Porcine alveolar macrophages
PBS	Phosphate buffer saline solution
PCP α/β	Papain-like cysteine proteinase domains alpha/beta
PCV2	Porcine circovirus 2
PFA	Paraformaldehyde
PGA	Poly-N-acetylglucosamine
PL2pro	Chymotrypsin-like cysteine protease
Poly I:C	Polyinosinic–polycytidylic acid potassium salt
PRDC	Porcine respiratory disease complex
PRF	Programmed ribosomal frameshifting

PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
RdRp	RNA-dependent RNA polymerase
RTC	Replication-transcription complex
S4074	*App* serotype 1 reference strain
SD	Standard deviation
sg mRNA	Sub-genomic messenger RNAs
SJPL	St-Jude porcine lung cells
ssRNA	Single-stranded RNA
$TCID_{50}$/mL	Tissue culture infectious dose 50 % per mL
TNF-α	Tumor necrosis factor alpha
TRSs	Transcription regulatory sequences

Dedicated to my God, my husband, my son and my parents

for their incomparable love, patience, help and encouragement

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INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a worldwide endemic infectious disease which causes significant economic losses in the swine industry (1). The etiological agent, PRRS virus (PRRSV), is an enveloped and single-stranded positive sense RNA virus of approximately 15 kb that encodes for at least 10 open reading frames (ORFs) (2-5). PRRSV is classified in the order *Nidovirales*, family *Arteriviridae* and genus *Arterivirus,* which also includes the lactate dehydrogenase-elevating virus of mice, equine arteritis virus and simian hemorrhagic fever virus (6, 7). PRRSV isolates are divided into two genotypes, where the Lelystad virus in Europe and ATCC VR-2332 in North America are the reference strains for genotype I and II, respectively (6, 8, 9).

PRRSV has a very narrow cell tropism both *in vivo* and *in vitro*. *In vivo,* PRRSV has preference for cells of monocyte/macrophage lineage, especially the fully differentiated macrophages of lungs, lymphoid organs and placenta (10-12). Porcine alveolar macrophages (PAM) constitutes the main *in vivo* target cells of PRRSV and primary PAM has been extensively used for *in vitro* study of host cell infection (6, 11, 13, 14). Two continuous cell lines, from monkey origin, are the only cells able to fully support PRRSV replication *in vitro*: the African green monkey kidney cell line MA-104 and derivatives such as MARC-145 and CL2621 (15) and the newly reported St-Jude porcine lung (SJPL) cells (16, 17).

Following PRRSV entry and release of the viral genome into the cytoplasm, the PRRSV ORF1 is translated and the resulting non-structural proteins (nsps) trigger the formation of the replication-transcription complex, which is associated with double membrane vesicles and supports genome replication and transcription process (18-20). The genome replication is produced by the continuous synthesis of negative (-) full-length RNA strands using as template the genomic RNA (gRNA), then the (-) RNA strands will lead the formation of new gRNAs (21). The genome transcription process is named to the synthesis of a nested set of six sub-genomic mRNAs (sg mRNAs). According with a model proposed by Sawicki and colleagues (22), the generation of these sg mRNAs is through a discontinuous RNA synthesis process, where (-) sg RNA strands are produced and then are used as template for the synthesis of the sg mRNAs.

The actin cytoskeleton plays an important role in PRRSV life cycle inside cells. It has been reported that PRRSV entry into PAM is via clathrin-mediated endocytosis and that this process is microfilament-dependent (13). The use of cytochalasin D (a microfilament-disrupting compound) inhibited PRRSV primary and secondary infection in MARC-145 cell line (23). Moreover, it was observed that in PRRSV infected cells there were less actin filaments (F-actin) expression, than in the adjacent untreated cells suggesting that PRRSV can modulate the actin cytoskeleton to favor cell infection and that higher F-actin expression correlated with PRRSV resistance (23).

Current management strategies to control PRRS, which include surveillance, severe biosecurity measures, whole herd depopulation and repopulation, herd closure and vaccination, seem to be partially efficient for the control of the disease (24-26). This phenomenon has stimulated the research of novel strategies to successfully control PRRSV infection. Several studies have found natural compounds with antiviral activity against PRRSV such as macrolides (27), N-acetylpenicillamine (28), *Cryptoporus volvatus* extracts (29), morpholino oligomer (30, 31), matrine (32), sodium tanshinone IIA sulfonate (33). However, for the moment there are no effective commercially available drugs to prevent PRRSV infection.

Actinobacillus pleuropneumoniae (*App*) is the etiological agent of porcine pleuropneumonia a worldwide endemic disease (34). *App* is divided into two biotypes, the biotype 1 which is dependent on exogenous beta-nicotinamide adenine dinucleotide (β-NAD) and the biotype 2 which is NAD-independent (35). *App* is divided also into 15 serotypes (1-4, 5a, 5b and 6-15) (34, 36). A recent study performed in our laboratory demonstrated that the culture supernatant of a mutant *App* strain (*AppΔapxICΔapxIIC*), which produces inactive Apx I and II toxins, has a potent antiviral effect against PRRSV (37). This phenomenon of inhibition was observed in the monkey SJPL cell line. Since, PAM are the main *in vivo* target cells of PRRSV, the first objective of this study was to demonstrate the *App* supernatant antiviral effect against PRRSV in primary cultures of PAM. Results corresponding to this objective were already published [(37), Annexe I] and demonstrated that *App* supernatant efficiently inhibits PRRSV infection in PAM. Based on the literature and according with preliminary results obtained in our laboratory, it is hypothesised that *App* cell culture supernatant modulates cellular(s)

component(s) and by consequence PRRSV infection is blocked. Then, the second objective of this study is to determine the possible mechanisms involved in the viral inhibition.

CHAPTER I: LITERATURE REVIEW

1. *ACTINOBACILLUS PLEUROPNEUMONIAE*

HISTORY

In 1957, the first case of porcine pleuropneumonia was reported in United States by Pattison and colleagues (38) and the bacteria associated with the pneumonic lesions was firstly classified in the genus *Haemophilus*. Afterwards, in 1983 it was reclassified, since DNA studies revealed that this pathogen belonged to the genus *Actinobacillus* of the *Pasteurellaceae* family together with the bacteria of the genus *Haemophilus*, *Pasteurella* and *Mannheimia.* Thus, *Actinobacillus pleuropneumoniae* (*App*) is the etiologic agent of porcine pleuropneumonia a worldwide endemic disease, which affects pigs of all ages and causes considerable economic losses (34).

CLASSIFICATION

App is a non-motile and a facultative anaerobic Gram-negative encapsulated coccobacillus (39). *App* strains are classified into two biotypes, where the biotype 1 is dependent on exogenous beta-nicotinamide adenine dinucleotide (β-NAD), whereas the biotype 2 is able to synthesize this component by itself (35). Based on the surface polysaccharides this bacterium has been divided into 15 serotypes (1-4, 5a, 5b and 6-15) (34, 36). All serotypes can induce the disease but with differences in virulence (40). *App* is also positive to the CAMP (Christie Atkins Munch-Petersen) test (41).

VIRULENCE FACTORS

The lower respiratory tract is the preferential site of infection of *App*, since it binds the ciliated cells of the terminal bronchiole and alveolar epithelial cells. There are three important steps during *App* infection that allow the apparition of the disease: the colonization, the evasion of the host's defense mechanisms and host tissue damages (42). Different virulence factors have been identified to participate in each of these stages.

Lipopolysaccharides

Several studies have confirmed that the lipopolysaccharide (LPS) is necessary in *App* adherence to the respiratory epithelium (43-45). However, others have postulated that this stage in bacterial pathogenesis is probably LPS-independent (46). The LPS is formed of three

regions: the lipid A, the LPS core and the O-antigen (47). Provost and collaborators demonstrated that a LPS core mutant decreased adherence to host cells, showing its critical role in adhesion (48). According with a proposed multiple-step adhesion mechanism, firstly the O-antigen of the LPS may interact with the phosphatidylethanolamine (a host membrane phospholipid) by a low-affinity binding, then, a stronger union to the respiratory tract is made by the interaction of the LPS core and/or surface proteins (a 55 kDa protein, type 4 fimbriae (will be discussed below)) to other host cell receptors (49). The lipid A of the LPS is able to bind the porcine hemoglobin and by this way the bacteria acquires the iron for its growth (50). The LPS is being associated also to the formation of lesions, since it was demonstrated that the LPS outer core interacts with ApxI and ApxII toxins and this interaction may enhance *App* cytotoxicity and virulence (51).

Capsular polysaccharides

Cruijsen and colleagues demonstrated that *App* reduces the phagocytic activity of porcine alveolar macrophages (PAM) *in vitro* by inducing cell lysis, which causes viable bacteria liberation (52). Among the factors that may contribute to *App* survival, the capsular polysaccharides should play an important role. It was demonstrated that encapsulated *App* strains were resistant to complement-mediated killing, whereas non-capsulated strains were killed (53, 54). Moreover, there is a direct association between the type and the amount of *App* capsular polysaccharides and its virulence *in vivo* (55). Capsular polysaccharides are not involved in *App* adherence, since it was observed that a capsule deficient mutant adheres more efficiently to cells than the wild type strain. However, the capsule can mask the adhesins, at least in part, affecting indirectly the adherence (56).

Apx toxins

App repeats-in-toxins (RTX) exoproteins (ApxI, ApxII, ApxIII and Apx IV) are involved in the induction of pulmonary lesions (57, 58). ApxI is the most haemolytic and cytotoxic toxin for alveolar macrophages and neutrophils and induces apoptosis in PAM cells (57, 59-61), ApxII is weakly haemolytic and moderately cytotoxic (34, 60, 62), ApxIII is non-haemolytic, highly cytotoxic and has a pro-apoptotic activity (63) and Apx IV is only secreted *in vivo* and is essential for *App* full virulence (64, 65).

Iron-uptake systems

The lower respiratory tract is limited in supplying the essential nutrients for bacterial growth (34). However, *App* is able to use the host transferrin (66-68) and hemoglobin (50, 69) and exogenous siderophores (70) as sources of iron for its growth. *App* binds to transferrin through two proteins present on its surface of approximately 60 and 100 kDa, where the 100 kDa protein is a transmembrane protein that may form a channel allowing the transport of iron across the outer membrane (67, 71). The hemoglobin receptors are two outer membrane proteins of approximately 75 and 105 kDa, where the 75 kDa protein can also bind hemin (47). The last iron-acquisition system is mediated by the uptake of exogenous siderophores such as the hydroxamate siderophore ferrichrome. There are four genes implicated in the ferric hydroxamate uptake, which are located in a single operon. These genes encode for the outer membrane protein FhuA, which is the receptor for ferrichrome, the FhuD protein is responsible for the translocation of ferric hydroxamate from the outer to the inner membrane and FhuC and FhuB proteins are cytoplasmic-membrane-associated proteins, which are components of an ABC transporter that internalizes the ferric hydroxamate (72).

Biofilm formation

Several studies have demonstrated that *App* has the ability to form biofilm. It is believed that biofilm is necessary for bacteria colonization (34, 73-76). The polysaccharide poly-N-acetylglucosamine (PGA) was observed to be involved in biofilm formation and probably functions as the major biofilm adhesin in *App* (73, 76). Moreover, Buettner and collaborators showed that a mutant *App* strain, deficient in biofilm formation, had a reduction in its virulence (77). A transcriptomic study revealed that, after contact of *App* with the newborn pig trachea (NPTr) cell line, genes involved in biofilm biosynthesis were up-regulated (78).

Other outer membrane proteins

Fimbriae is a bacterial surface structure that is believed to be involved in *App* adhesion since it was demonstrated that the type 4 fimbriae was induced by contact of *App* with the host cells *in vitro* and *in vivo* (79). A 55 kDa outer membrane protein was identified and is postulated to be implicated in the adhesion to the host alveolar epithelial cells (80). Additionally, another

surface protein of 60 kDa was identified, which is able to adhere to porcine collagen and fibrinogen (81).

Secreted proteases

The *App* secreted proteases can be considered as another virulence factor because it was demonstrated that they can degrade the immunoglobulin A and G (IgA, IgG) and the hemoglobin from porcine, human and bovine origin (82, 83). It was observed in another study that a 24 kDa *App* zinc metalloprotease can degrade actin protein *in vitro* (84). In addition there is another *App* protease that was described, the ClpP (member of the Clp (caseinolytic protease family)), to be important in virulence regulation (85).

IMMUNE RESPONSES

App pathobiology includes pulmonary lesions which are characterized by the presence of macrophages, granulocytes, lymphocytes, hemorrhages, necrosis, etc (42). Cruijsen and colleagues compared PAM and polymorphonuclear leukocytes (PMN) abilities to phagocytize and kill *App in vitro* (52). It was observed that both cells were able to phagocytize the bacteria. However, PAM was unable to kill the intracellular bacteria compared to PMN, which killed 95% of the ingested *App*. There are two possible explanations to this phenomenon in PAM 1) cytolysin produced by *App* might affects the cellular killing mechanisms or 2) the phagocytised *App* can cause the impairment of reactive oxygen species synthesis (which have a potential bactericidal capacity), allowing then, the releasing of viable bacteria.

Cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1beta (IL-1β), IL-1α, IL-6 and IL-8 were produced in experimentally infected pigs with *App* (86, 87). The overexpression of these proinflammatory mediators in response to *App* infection are probably involved in the pulmonary lesions associated with the disease (88). Additionally, the expression of IL-10 and IL-12 was detected also in experimentally infected pigs and it was suggested that they are involved in *App* pathogenesis (89). Benga and colleagues detected different amounts of interferon gamma (IFN-γ) in plasma and in bronchoalveolar lavage fluid (BALF) during the infection (90). Moreover, it was observed that the increase of IFN-γ was associated with an increase in the severity of the disease. However, this increase of IFN-γ was

dependant on pig breeds, where the porcine breeds not showing any increase of IFN-γ were more resistant to the disease (90).

2. PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

HISTORY

In the United States, in 1987, a new emerging "mystery swine disease" of unknown etiology causing reproductive failure and neonatal severe pneumonia was reported (91, 92). A similar syndrome was after recognized in Europe in 1990 (93, 94). The causative agent was firstly isolated in the Netherlands using porcine alveolar macrophages and designated as Lelystad virus (LV) (8). Shortly after, it was isolated in North America using gnotobiotic pigs and designated as American Type Culture Collection (ATCC) VR-2332 virus (9). In 1992, the disease was named porcine reproductive and respiratory syndrome (PRRS), according with the symptoms and the observed clinical signs. The PRRS etiological agent, PRRS virus (PRRSV), has spread worldwide in the last decades. In 2006, a highly pathogenic PRRS virus (PRRSV) strain emerged in China and Vietnam, which caused an atypical PRRS outbreak (95). At present, PRRS is a worldwide endemic disease causing significant economic losses in swine production, since it can provoke a severe pneumonia in growing and finishing pigs (1).

TAXONOMY

PRRSV is an enveloped and single-stranded (ss) positive sense RNA virus classified in the order *Nidovirales*, family *Arteriviridae* and genus *Arterivirus,* which also includes the lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV) and simian hemorrhagic fever virus (6, 7). PRRSV isolates are divided into two genotypes, where LV and ATCC VR-2332 are considered the reference strains for PRRSV genotypes I and II, respectively (6, 8). The two genotypes share approximately 60% nucleotide identity (96, 97). Additionally, within the same genotype exists high genetic variabilities (98-101).

MORPHOLOGY

PRRSV virions are pleomorphic with form varying from spherical to oval shape. As observed by cryoelectron tomography, virions size range between 50 to 65 nm with an internal core of around 40 nm in diameter (102). The virion outer membrane is smooth and is formed by a

lipid bilayer, which has protrusions that probably correspond to ectodomains formed by the envelope proteins (102). The nucleocapsid core is formed by the nucleocapsid protein (N) and the viral RNA and it's been suggested to have an asymmetric and helical organization (102, 103). Virions survival and stability are dependent on the pH and temperature. For instance, it was demonstrated that at pH 7.5, the virus was stable for a long period at -20°C and -70°C. The virions half-life is 50 hours at 4°C and pH 6.25 while at a high temperature (37°C) and pH 6.0, it is 6.5 hours (104). Besides, the addition of lipid solvents such as chloroform reduced the virion infectivity from 10^5 $TCID_{50}$/mL (tissue culture infectious dose 50 % per mL) to < 10^1 $TCID_{50}$/mL (6).

GENOME ORGANIZATION

PRRSV genome is approximately 15 kb in length, encodes for at least 10 open reading frames (ORFs) and is capped at the 5' end and polyadenylated at the 3' tail (3-5, 105) (Figure 1). The ORF1a and ORF1b, which comprise almost the three-quarters of the total genome, encodes for 14 non-structural proteins (nsps) (103). The nsps are synthesized as polyproteins (pp1a and pp1ab) obtained from ORF1a and ORF1b, respectively (20). The pp1ab is expressed from a −1 programmed ribosomal frameshifting (PRF) in the ORF1a/ORF1b overlap region (106). The polyproteins are then processed to lead the formation of the 14 nsps: nsp1α, nsp1β, nsp2-6, nsp7α, nsp7β and nsp8-12 (39, 107). The nsp1α, nsp1β, nsp2 and nsp4 encode the viral proteases responsible for polyprotein processing (20, 108, 109). The nsps are also implicated in viral RNA replication, sub-genomic (sg) mRNA transcription and translation. Recently, other PRF (not illustrated in Figure 1) was found that allows the access to a short transframe (TF) ORF, that overlaps the nsp2-encoding region of ORF1a in the +1 frame and it is translated by −2 PRF, yielding the expression of nsp2TF protein (105). The PRRSV structural proteins encoded by the ORF 2 to 7 are synthesized from a nested set of six sg mRNAs by a process of discontinuous RNA synthesis (110). The sg mRNAs are structurally polycistronic with the exception of the sg mRNA7, but are presumed to be functionally monocistronic, with the exception of the sg mRNAs 2 and 5, that are believed to be bicistronic (4, 5, 111). All the sg mRNAs are 3' co-terminal and also shares a 5' leader sequence, which is identical to the 5' end of the genome (108, 112, 113). ORFs 2a, 3 - 5 encode for the glycoproteins (GP) 2a, 3, 4 and 5, respectively. ORF6 and ORF 7 encode for the membrane protein (M) and the N protein,

respectively (2, 108). ORF2b is fully inside the ORF2a and encode for the non-glycosylated protein E (114). The recently discovered ORF5a, which overlaps the 5' end of the ORF5, encode for the ORF5a protein (4, 5).

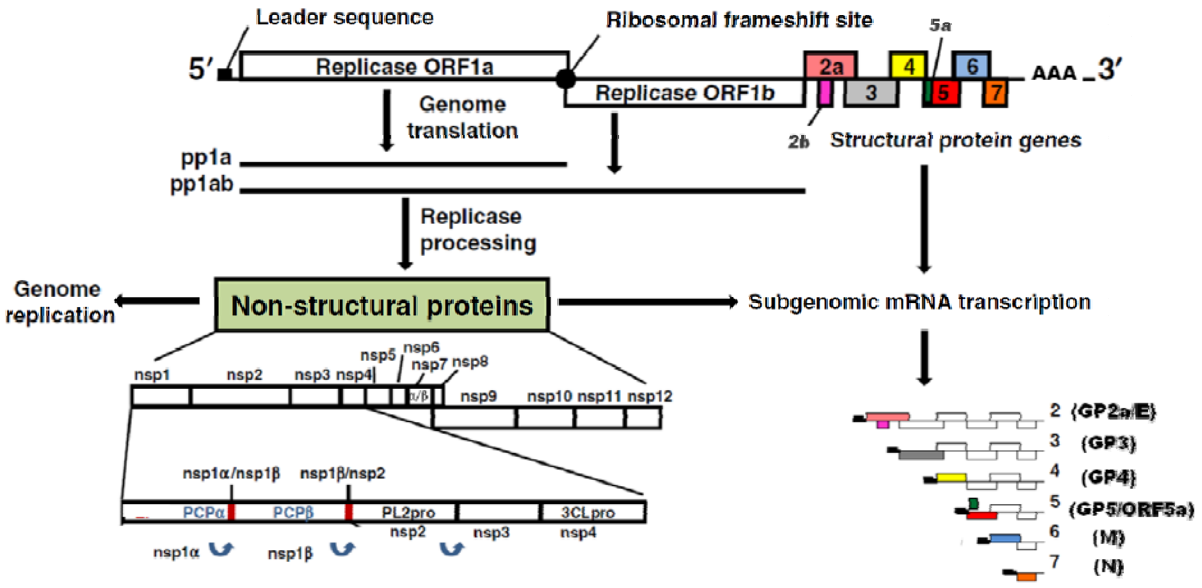

Figure 1: Schematic representation of PRRSV genome organization.

Adapted from Music and Gagnon (115). The top of the figure represents the PRRSV complete genome from ORF1 to ORF7. The leader sequence is represented by a black rectangle and the ribosomal frame shift (between ORF1a and ORF1b) is illustrated as a black circle. The 14 nsps resulted from the proteolytic cleavage of the two polyproteins (pp1a and pp1ab) are represented as well as the four proteases responsible for it: the papain-like cysteine proteinase domains (PCPα and PCPβ) located in nsp1α and nsp1β, respectively, the chymotrypsin-like cysteine protease domain (PL2pro) presented in nsp2 and the main serine proteinase, 3C-like proteinase domain (3CLpro), located in nsp4 (108, 116). PCPα, PCPβ and PL2pro cleave the junction between nsp1α/nsp1β, nsp1β/nsp2 and nsp2/nsp3, respectively and 3CLpro is responsible for the liberation of the remainder nsps (nsp3 to nsp12). The sg mRNAs (2-7) are 3' co-terminal and also contain a common leader sequence in the 5' end. The translated proteins form each sg mRNA are represented between parenthesis.

PRRSV NON-STRUCTURAL PROTEINS

Table 1 describes the principal functions and the most important generalities of the non-structural proteins.

Table 1: PRRSV non-structural proteins characteristics and functions.

Adapted from Music and Gagnon (115).

Nsps	Genes	Characteristics and functions
nsp1	ORF1a	Proteolytic activities (PCPα and PCPβ) (20); zinc finger domain involved in sg mRNAs synthesis (19); virion biogenesis (19); type I interferon suppression (39, 113); induce antibody specific immune response together with nsp2 and nsp7 (113, 117, 118).
nsp2		The largest and the most variable nsp (96, 97); ideal marker for monitoring genetic variation and for developing differential diagnostic tests; proteolytic activity (PL2pro) (20, 115, 119); member of the ovarian tumor protease superfamily (113, 120, 121); type I IFN antagonist (122-124); formation of double membrane vesicles together with nsp3 and nsp5 (18, 125, 126).
nsp3		No specific functions have been attributed
nsp4		Proteolytic activity (3CLpro) (127, 128); type I IFN inhibition (129).
nsp5		No specific functions have been attributed

nsp6		No specific functions have been attributed
nsp7		Cleaved by 3CLpro in nsp7α and nsp7β (109); genome synthesis and translation of viral proteins (130).
nsp8		No specific functions have been attributed
nsp9	**ORF1b**	RNA-dependent RNA polymerase (RdRp); virus transcription and replication (131, 132).
nsp10		Helicase; zinc-binding domain (133, 134); ATPase activity *in vitro* (135, 136).
nsp11		Nidovirus endoribonuclease (NendoU), which is considered the genetic marker of *Nidovirales* order (133, 137, 138); type I IFN inhibition (139).
nsp12		No specific functions have been attributed

PRRSV STRUCTURAL PROTEINS

The PRRSV structural proteins are classified into major or minor structural proteins, based in their abundance into the virion. GP2a, E, GP3 and GP4 are considered the minor structural envelope proteins and GP5 and M are the major structural envelope components. N protein is the sole component of the viral nucleocapsid. Table 2 summarises the main characteristics and functions of PRRSV structural proteins.

Table 2: PRRSV structural proteins characteristics and functions.

Adapted from Music and Gagnon (115).

Structural proteins	Genes	Characteristics and functions
GP2a	ORF2a	Contains 2 two highly conserved putative N-linked glycosylation sites (140); incorporated into virions as a multimeric (GP2a, E, GP3 and GP4) complex; essential for virus infectivity (141); interacts with the cellular receptor CD163 (142, 143); involved in PRRSV uncoating; apoptosis inhibition (144).
E	ORF2b	Unglycosylated and myristoylated structural protein (111); incorporated into virions as a multimeric complex (141); essential for virus infectivity (141, 145); possesses ion-channel like properties and may function as a viroporin in the envelope (145); involved in genome released into the cytoplasm.
GP3	ORF3	One of the most variable PRRSV proteins; highly glycosylated that contains seven predicted N-glycosilation sites (2); its membrane topology is strain dependent (146); highly antigenic and involved in the glycan shielding process (147); incorporated into virions as a multimeric complex; essential for virus infectivity (141).
GP4	ORF4	Highly glycosylated protein; key protein in the formation of the multimeric complex incorporated into virions (141); essential for virus infectivity; mediates interaction between the multimeric complex and GP5 (142); interacts with the cellular receptor CD163 (142, 143); involved in PRRSV uncoating; induce neutralizing antibodies and cell-mediated immune responses (148-151).
GP5	ORF5	The major PRRSV GP and the most variable structural protein with a variable number of potential N-glycosylation sites (140);

		covalent association of GP5 and M is crucial for virus assembly (152, 153); involved in virus entry into the cells and in apoptosis; neutralizing antibodies are predominantly directed to GP5 (154, 155); involved in glycan shielding process (147).
ORF5a	ORF5a	Overlaps the ORF5 in its 5' end; essential for virus viability (4, 5, 156); cannot protect animals from PRRSV infection (157).
M	ORF6	Unglycosylated and the most conserved structural protein; involved in virus assembly and budding (2, 115, 152); GP5/M heterodimer is crucial for virus infectivity (152, 153).
N	ORF7	Unglycosylated, small and highly basic protein (115, 140); the sole component of the viral capsid and interacts with itself by covalent and non covalent interactions to form a homodimer (2, 152); highly immunogenic and is used in diagnostic procedures to detect the presence of the disease (149, 158); localised in the cytoplasm and in the nucleus and nucleolus (159); type I IFN inhibition (160).

CELLULAR TROPISM

PRRSV has a very narrow cell tropism both *in vivo* and *in vitro. In vivo,* PRRSV has high preferences for cells of monocyte/macrophage lineage, especially the fully differentiated macrophages of lungs, lymphoid organs and placenta (10-12). It was also reported that porcine dendritic cells support PRRSV infection, however in those studies monocyte-derived dendritic cells and bone marrow-derived dendritic cells were used, those may differ from the primary dendritic cells (161-164). In naturally infected pigs PRRSV antigens were found in bronchiolar epithelial cells (165). However this finding is contradictory with the result obtained by Teifke and collaborators, which demonstrated that PAM are the only pulmonary target cells of PRRSV (166). In fact, PAM constitutes the main *in vivo* target cells of PRRSV and primary PAM has been extensively used for *in vitro* study of host cell infection. These

cells are of myeloid origin, which circulate in the blood as monocytes and are differentiated into macrophages that reside in tissues (167). PAM cells are members of the mononuclear phagocyte system of the lung and they are able to protect the respiratory tract from invasion of foreign pathogens (by phagocytosis; bactericidal activity; cytotoxicity; cytokines production; activation of T cells) (168, 169). However, ingestion of virus (ex. PRRSV) by PAM allows viral infection and the subsequent functional impairment of the cells (161, 170, 171).

In addition, there are only two continuous cell lines, from monkey origin, that are able to fully support PRRSV replication: the African green monkey kidney cell line MA-104 and its derivatives such as MARC-145 and CL2621 (15) and the newly reported SJPL cells (16, 17). In the literature, it has been reported that non permissive continuous cell lines were able to support PRRSV replication after the introduction of the PRRSV receptors: CD163 or sialoadhesin (172, 173).

PRRSV LIFE CYCLE IN CELLS

In this part, each stage of PRRSV replication cycle inside cells and the cellular components involved in it will be described. Figure 2 summarizes all the steps of the virus replication cycle.

PRRSV entry

PRRSV entry may differ between PAM and MARC-145 cells since the cellular receptors required for it are different. In PAM, a recent review has proposed a possible model for PRRSV entry by integrating the major findings about PRRSV entry into PAM (164). According with the model proposed PRRSV first interacts with the heparan sulphate on the macrophage surface. Then, PRRSV GP5/M heterodimer interacts with PAM sialoadhesin in a much stable way, through the sialic acid-binding domain present in the macrophage sialoadhesin and the sialic acids present on the heterodimer. This is followed by internalization of the virus-receptor complex via a process of clathrin-mediated endocytosis. This process was demonstrated to be dependent on actin cytoskeleton, since the use of cytochalasin D, a microfilament-disrupting compound, blocked virus entry (13). Subsequently, the viral genome is released (will be explained below), from the early endosome, into the cytoplasm (164),

showing its ability to escape prematurely from the endocytic pathway, by evading its degradation in the lysosome (174). In MARC-145 cells, the sialoadhesin is not present (14) and the sialic acids on the virion surface are not essential for the entry (175). It's believed that the virus firstly bind to a heparin-like molecule (176), then will be internalized also via a mechanism of clathrin-mediated endocytosis, since it was demonstrated that cytochalasin D can inhibit PRRSV primary and secondary infections in this cell type (23). However recent studies demonstrated that cholesterol was involved in virus entry and release and also suggested that PRRSV entry in MARC-145 cells could be via a lipid-raft-dependent endocytosis (177, 178). Moreover, the vimentin protein, an intermediate filament, can interact with the PRRSV N protein and is suggested to mediate transport of virus inside the cells, together with other components of the intermediate filaments (179, 180).

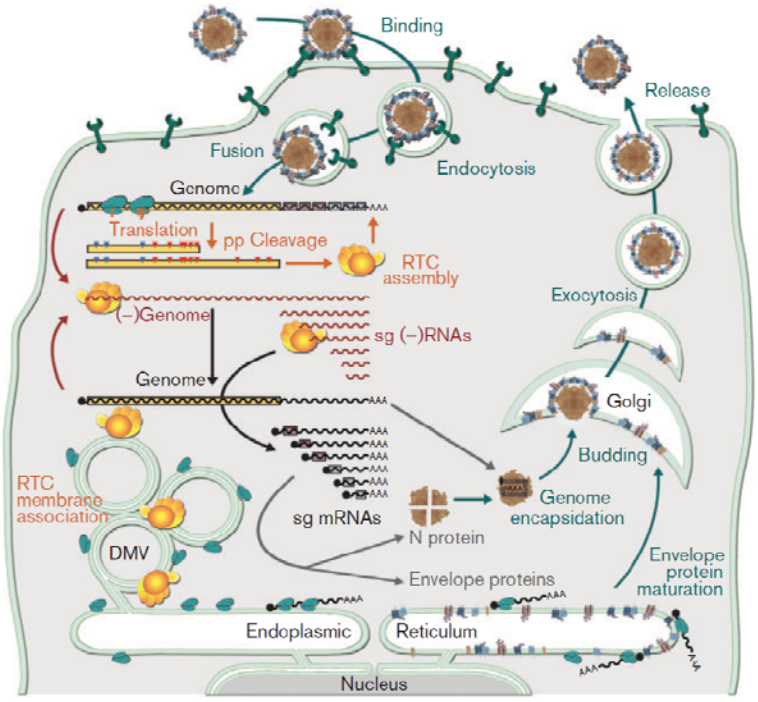

Figure 2: Arterivirus replication cycle inside cells**.**

Taken from Snijder and collaborators (113). Followed entry via receptor-mediated endocytosis the viral genome is released from the early endosome to the cytoplasm. There the ORF1a and ORF1b are translated to lead the formation of the polyproteins and the subsequent mature nsps are formed, which trigger the formation of the replication-transcription complex (RTC), which

is associated with double membrane vesicles (DMVs). RTC supports genome replication and transcription. The sg mRNAs are translated to obtain the different structural proteins. Once the new genomic RNAs (gRNAs) and the structural proteins are synthesised, the virus is assembled. First, genome encapsidation is triggered and then, the nucleocapsid buds to the smooth ER/Golgi complex (where the envelope proteins are retained) to get the viral envelope. Then, the new viral particles are accumulated into vesicles and are released by exocytosis.

PRRSV uncoating

This stage is when the viral RNA genome is released from the early endosome to the cytoplasm. According with the model proposed by Van Breedam and colleagues (164), this process is critically dependent on the acidic pH of the endosome and on the interaction with the CD163 receptor (13, 173). GP2 and GP4 are the structural proteins responsible for this interaction with the scavenger receptor (142). Additionally, it was demonstrated that aspartic protease cathepsin E is involved in PRRSV uncoating stage (181). In MARC-145, PRRSV uncoating was clearly demonstrated to be also dependant on endosome acidification (182). CD151, a host cellular protein, interacts with PRRSV 3' untranslated region (UTR) RNA and may be involved in viral envelope fusion with the endosome (183). CD163 is also necessary for PRRSV infection in MARC-145 cells (173). However, the exact action mechanism of both receptors is yet unknown.

Genome replication and transcription

This stage of PRRSV replication cycle is produced in double membrane vesicles (DMVs) present in the cytoplasm and recently was suggested that probably they are autophagosome-like DMVs (184, 185). Then, once the gRNA is released into the cytoplasm, it will act as an mRNA and the host translational machinery will translate the ORF1a and ORF1b to obtain the polyproteins, which are then cleaved by the internal proteases to obtain the mature nsps. Subsequently, these nsps trigger the formation of the RTC that is associated with the DMVs, which are the presumably site of PRRSV replication and transcription (18, 19). The RTC directs both genome replication and transcription. The replication includes the continuous synthesis, by the RNA-dependent RNA polymerase (RdRp), of negative (-) full-length RNA strands using as template the gRNA and then these (-) RNA strands will lead the formation of

new gRNAs (Figure 3). The transcription process is the synthesis of a nested set of six sg mRNA, where all these sg mRNAs are 5'- and 3'-coterminal with the gRNA (112, 186). Then, the question of how these sg mRNAs are formed, has been subjected to many hypotheses and the most accepted is the model proposed by Sawicki and Sawicki (22), which is probably common within *Nidovirales* order. This model proposes that the sg mRNAs are synthesized from (-) sg RNA strands, which are produced by a discontinuous RNA synthesis process (Figure 3). Conserved transcription regulatory sequences (TRSs) are found preceding each structural protein ORFs termed as body TRSs in the gRNA. The same sequence is also present at the 3' end of the leader sequence (5' end of gRNA) and is denominated as leader TRS (187). The synthesis of (-) sg RNA strands begin at the 3' end of the gRNA, then the elongation (by the RdRp) of the nascent (-) sg RNA strand will follow until the first body TRS appears. Subsequently, the synthesis is attenuated and the nascent (-) sg RNA strand, which carry in its 3' end the complementary sequence to the body TRS, is relocated to the 5' end of the gRNA. There, the complementary body TRS sequence of the nascent (-) sg RNA strand and the leader TRS sequence will be complementary and the nascent (-) sg RNA strand will be elongated by copying the 5' end of the gRNA. Finally, the complete (-) sg RNA strands will serve as template for the synthesis of the sg mRNAs. These sg mRNAs are then translated to form the different structural proteins.

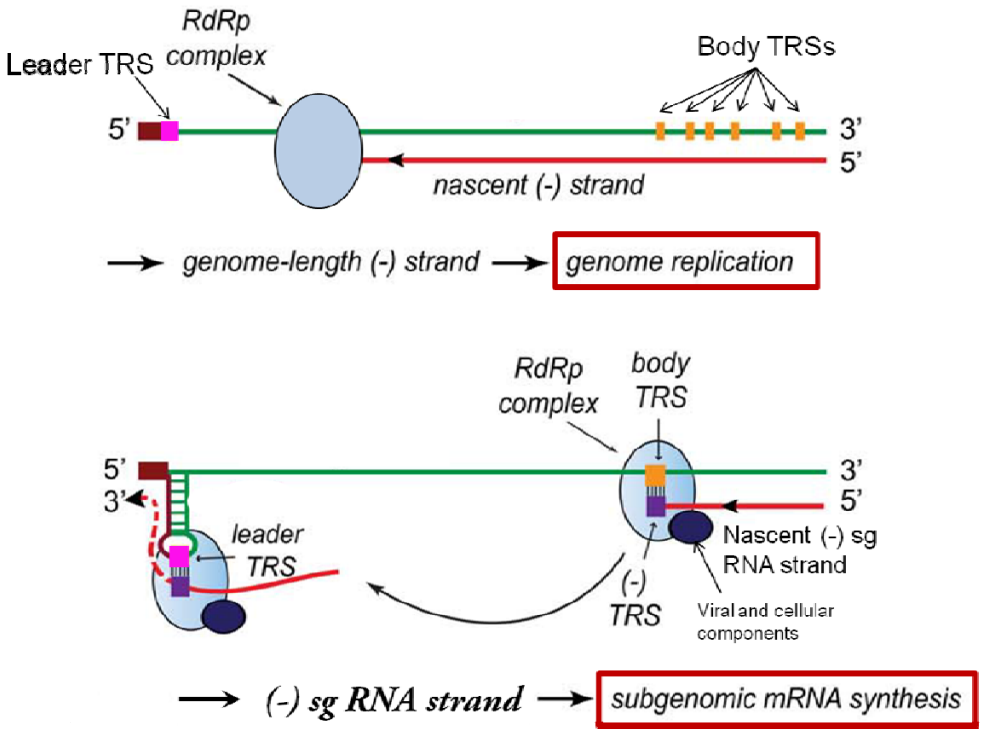

Figure 3: Model for *Arterivirus* genome replication and transcription.

Adapted from Nedialkova and colleagues (21). The genome replication (top of the figure) occurs by a continuous RNA synthesis process (leaded by the RdRp complex), where the new gRNAs are obtained from (-) full-length RNA strands. The second part of the figure represents the discontinuous synthesis of the sg mRNAs, which are obtained from (-) sg RNA strands. The extension of the (-) sg RNA strands begins at the 3' end of the gRNA and is attenuated when the body TRSs appear (yellow rectangle). The nascent (-) sg RNA strand, porting in its 3' end the complementary sequence of the body TRS (violet rectangle), is transferred to the 5' end of the gRNA and will form complementary base pairs with the leader TRS (pink rectangle). Following this union, the elongation of the nascent (-) sg RNA strand is finished and is used as template for the synthesis of the sg mRNAs.

Virion assembly

Virion assembly is believed to begin in the replication site, where de genome and the N protein should interact to form the nucleocapsid (188) and finished in the endoplasmic reticulum (ER) or Golgi complex, where the envelope proteins are retained (113, 189, 190). Therefore, the preformed nucleocapsid is wrapped by the smooth ER/Golgi complex to acquire the viral envelope, a process known as budding. Although the exact mechanism of

virus assembly is not known yet, the formation of GP5/M heterodimer is believed to be determinant in this stage (153, 191).

Virus released

It is beleived that PRRSV virions leave the infected cells by exocytosis (2, 192). The new formed viral particles are accumulated into intracellular vesicles and finally are released by exocytosis (193). This last stage in PRRSV life cycle was demonstrated to be also dependant on cellular cytoskeleton (23).

VIRAL PATHOGENESIS *IN VIVO*

PRRSV can be transmitted horizontally (between infected and naïve animals) and vertically (from sows to the fetuses) and also via the semen of infected boars (194-198). When PRRSV enters the organism (via intranasal, oro-nasal or intramuscular route), it first replicates in the respiratory tract, probably in PAM (198). Afterwards, viremia is developed, as observed in inoculated young pigs (1 to 2 months old), between 3 to 14 days post infection (dpi). After this time, PRRSV persistence (though a "smoldering" infection, where the virus replicates at a low level) was detected in lung lymph nodes and tonsil tissues up to 156 dpi (199). In most cases, the infection is cleared by 156 dpi or shortly after. In young or growing and finishing pigs, the clinical sigs are mainly anorexia, lethargy, cutaneous hyperemia, dyspnea, reduced weight gain and an increase in mortality from secondary infections (200). In infected pregnant sows, PRRSV probably enters into the endometrium during viremia, which probably passes through the placenta and then, can infect fetuses (197, 201). It was demonstrated that the congenital infection is mainly restricted to the end of gestation, probably because there are high number of PRRSV susceptible cells in placenta, late in the gestation (202). The reproductive failure is characterized by late-term abortions, premature farrowings, stillborn fetuses, mummified fetuses and live weak born pigs (203). The molecular bases of PRRSV pathogenesis are not clear at all. However, several studies have demonstrated that PRRSV replication induces apoptosis in infected and in bystander uninfected cells both *in vivo* and *in vitro*. For instance, it was demonstrated that PRRSV induces cell death by apoptosis in the endometrium and placenta in late gestation (201), which probably can justify the reproductive failure associate with the disease. Moreover, PRRSV induces apoptosis in PAM and in pulmonary intravascular

macrophages (PIM) and is able to interfere with the macrophage phagocytic activity, leading the organism susceptible for opportunistic secondary infections (204). In infected MARC-145 cells, it was demonstrated that PRRSV induces, early in infection, antiapoptotic mechanisms, probably to favor its replication, but later cells die by apoptosis (144, 205).

IMMUNE RESPONSES

Innate immune response

During PRRSV infection in PAM, it has been suggested that the virus and the toll-like receptors (TLR) interact. These receptors constitute an early host defense against invading pathogens, since they recognize specific molecular patterns present in the microbes. Stimulation of TLR3, TLR7, TLR8 and TLR9 lead the induction of the type I interferon (IFN) (206), which constitute key cytokines against viruses infections (207). TLR3 is activated by double-stranded (ds) RNA and is well known that during PRRSV genome replication there is formation of dsRNA, then, it is believed that PRRSV eventually interacts with this receptor (149). It has been proposed that PRRSV is able to evade TLR3 signaling pathway in PAM, since it was clearly demonstrated that the induction of the TLR3 using a dsRNA synthetic molecule (poly I: C) increased the level of IFN-alpha (IFN-α), which suppressed PRRSV infection (208), however its susceptibly to IFN-α differ among isolates (209). In contrast, it was observed that PRRSV suppress type I IFN expression in poly I:C treated MARC-145 cells (210). Therefore, PRRSV has developed different strategies to evade the antiviral effects of type I IFN. To date, PRRSV is able to inhibit type I IFN synthesis [by interfering with the functions of ISP-1 (IFN-beta promoter stimulator 1) (139), IRF3 (IFN regulatory factor 3) (129), NFκB (transcriptional regulator nuclear factor-κB) (124, 211) and CREB ((cyclic AMP responsive element binding)-binding protein (CBP)) (212)] and type I IFN signaling [by affecting JAK-STAT signaling pathway (39) and IFN-stimulated response elements such as the ISG15 (interferon-stimulated gene 15) (122, 123)]. Different studies have revealed that the pro-inflammatory cytokines such as TNF- α (213, 214) and IL-6 (215, 216) can be up or downregulated during PRRSV infection and IL-8 is highly expressed (217, 218). Most of the studies, *in vivo* or *in vitro*, demonstrated that PRRSV can induce the mRNA and protein expressions of the pleiotropic IL-10, which is a potent immunosuppressive cytokine that is

believed to play a key role in the immunopathogenesis of PRRSV (170, 219). Moreover, it was recently discovered that PRRSV IL-10 induction depends on NFκB activation and P38 mitogen-activated protein kinase (220).

Adaptive response

PRRSV induces high antibody responses which started at around 5 dpi and can last until 56 dpi and all challenged animals are seroconverting at 14 dpi (215, 221, 222). The antibodies are predominately directed against the glycoproteins, M, N and nsps (nsp1α, nsp1β, nsp2 and nsp7), where N protein induces the strongest response (117, 118, 149, 158). However, most of these are non-neutralizing antibodies (Non-NAbs) and is proposed that they (mainly the antibodies directed against GP5 and N protein) may enhance viral infection by a phenomenon termed as antibody-dependent enhancement (ADE) (223, 224). In ADE, the opsonised virus, by the Non-NAbs, is delivered into the macrophages, which allow virus replication. NAbs appear late in PRRSV infection, around the fourth week pi and the titers are usually low (158, 215). The NAbs are generally directed against the GP3, GP4, GP5 and M, but is believed that GP5 possess the major neutralizing epitope in its ectodomain (148, 149, 225). *In vitro* studies demonstrated that NAbs are able to block PRRSV internalization (14, 226) , however it is not clear why *in vivo* they appear late and theirs titers remain lows. Several hypotheses have been postulated to explain it and one of the most accepted phenomenon is the presence of a decoy epitope in the GP5 ectodomain (225). Two epitopes were detected in GP5 ectodomain, named A and B. A is the immunodominant epitope and B has neutralizing activity and it was proposed that A may act as a decoy epitope, which interferes with the immune response against B and then cause a delay in the apparition of NAbs. The other proposed hypothesis is related with the number of N-glycosylations residues around the neutralizing epitope in GP5, which interfere with the recognition of the epitope by NAbs, a phenomenon known as glycan shielding (147).

The cell-mediated immune response face to PRRSV infection has not been well explored. $CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ T cells have been detected during PRRSV infection and their responses are directed mainly against GP4, GP5, M and N (113, 149). IFN-γ-inducing epitopes have been identified into these structural proteins in addition to nsp2, nsp5, nsp9 and

nsp10 (149, 227, 228). IFN-γ produced by T-cells against PRRSV appears around 8-10 weeks pi and increase gradually after 3-4 months pi or post-vaccination (221). The induced IFN-γ seems to be insufficient to reduce the infection *in vivo*. However, a pre-treatment of MARC-145 and PAM cells with IFN-γ clearly reduced PRRSV infection, probably by the induction of cellular protective immunity (229, 230). Also, it was demonstrated that following PRRSV infection, the expression of MHC II (major histocompatibility complex class II) is decreased (163).

PRRSV CONTROL AND ELIMINATION

PRRSV current management strategies include surveillance, for instance: avoid introduction of contaminated semen into the herd, pig's clinical examination and blood samples analysis, surveillance of pig's production to detect possible reproductive problems, the implantation of severe biosecurity measures within the herd. Once the virus is already inside the farm, different measures have been described to eliminate it, such as: test and removal, whole herd depopulation and repopulation, herd closure and vaccination (25). At present, vaccination partially prevents PRRSV infection. There are two types of commercially available vaccines, the modified-live virus (MLV) vaccine and the killed-virus vaccine (24, 26). Adaptive response against MLV vaccines is weak and late (24, 149). However, they can offer an effective protection in reducing the reproductive and respiratory sigs and lesions associated with the disease (24, 231, 232). Nevertheless, the MLV vaccines efficacy has been questioned since they are genotype-specific vaccines or even strain-specific vaccines, which make them partially ineffective face to heterologous field strains (233). Another aspect that put in doubt the MLV vaccines is their safety, since their reversion to virulence it was clearly proved, through recombination with field isolates (234). The killed-virus vaccines are safe, but less effective or ineffective in inducing protection (26, 235). Additionally, another problem found was that vaccinated pigs cannot be differentiated from pigs naturally infected (133). For these reasons, there are continuous efforts in order to find the perfect safe and effective PRRSV vaccine. In this sense, several alternative vaccines have been created such as bacterial vector vaccines (236), DNA vaccines (237), plant-derived vaccines (238), multistrain vaccines (239), autogenous inactivated PRRSV vaccines (240) and others.

3. BACKGROUND OF THIS THESIS

Together with the current novel vaccine strategies against PRRSV, other researchers have been focusing in finding PRRSV antiviral compounds, which can be an alternative and also effective strategy to prevent or control PRRSV infection. Accordingly, recent published works showed a few natural compounds with antiviral activities against PRRSV, as glycosides, terpenoids, coumarins, isoflavones, peptolides, alkaloids, flavones, macrolides (27), N-acetylpenicillamine (28), sodium tanshinone IIA sulfonate (33), morpholino oligomer (30, 31), flavaspidic acid AB (241), Matrine (32), dietary germanium biotite (242), *Cryptoporus volvatus* extracts (29), etc. Each of these compounds inhibits PRRSV replication differently. For instance, the flavaspidic acid AB inhibits PRRSV internalization and the cell-to-cell transmission, probably by the induction of type I IFN (241). Sun and colleagues demonstrated that Matrine inhibits N protein expression and has antiapoptotic functions (32). Moreover, *Cryptoporus volvatus* extract was demonstrated to inhibit PRRSV infection *in vitro* and *in vivo*, probably by the direct inhibition of PRRSV polymerase (RdRp) activity (29). Despite all these efforts, there are no effective commercially available antiviral drugs to prevent PRRSV infections.

A recent research performed in our laboratory demonstrated that the culture supernatant of a mutant *App* (*AppΔapxICΔapxIIC*) strain has a potent antiviral activity against PRRSV ((37), Annexe I).This strong antiviral effect was observed in the newly discovered SJPL permissive cell line, but was almost ineffective in MARC-145 infected cells. Interestingly, this phenomenon was also observed in the primary target cells of PRRSV, the porcine alveolar macrophages (results corresponding to the first objective of this project). Since it is believed that the bacterial antiviral effect against PRRSV is via the modulation of cellular(s) component(s), this thesis has as second goal to identify the possible mechanisms used by *App* to inhibit PRRSV infection in PAM.

CHAPTER II: ***Actinobacillus pleuropneumoniae*** **blocks porcine reproductive and respiratory syndrome virus replication prior to its genome replication and transcription.**

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This article is in preparation to be submitted to Journal of Virology.

AUTHOR CONTRIBUTIONS

As mentioned in the introduction section, this thesis has two objectives. The results from the first objective, which is to demonstrate the *App* cell culture supernatant antiviral effect against PRRSV in porcine alveolar macrophages (PAM), were already published by Levesque and coworkers (24) (see annexes section, specifically Annexe I: Figures 3 and 4).

The results concerning the second objective of my work that is to identify the possible mechanisms used by *App* cell culture supernatant to inhibit PRRSV infection in PAM cells *in vitro* will be discussed in this article. Also, we have included the SJPL and MARC-145 cells in order to compare the results.

My contribution to this study was in performing almost all tests described in this paper, which include: cell viability and mortality tests, type I and II IFN and β-actin mRNA expressions by qRT-PCR, PRRSV genome replication and transcription kinetics assay by qRT-PCR, western blot, IFA and viral titer determination. Dr. Chantale Provost helped me in interpreting and analysing the Kinex™ Antibody Microarray results and in performing the PAM collecting technique from lungs. I followed the technical advices of Dr. Chantale Provost and my professor's suggestions.

ABSTRACT

Current management strategies are inadequate for long term control of PRRS, which justifies the search of novel strategies to control the disease. Recently, a strong antiviral acitvity of *Actinobacillus pleuropneumoniae* (*App*) cell culture supernantant against PRRSV in PAM and SJPL infected cells was discovered. Following this finding, the objective of the present study was to understand how *App* inhibits PRRSV replication. First, cells were treated with *App* before and after PRRSV infection. At different times post-infections, viral genome replication and transcription were measured in the presence of *App*. Type I and II interferon (IFN) mRNA expression and proteins expression modulation of PRRSV infected PAM cells treated with *App* were evaluated using qRT-PCR and the KINEX™ Microarrays assays, respectively. The expression of some modulated proteins were subsequently, confirmed by immunofluorescence (IFA) and western blot assays. Results showed that type I and II IFN mRNA expressions were not modulated in the presence of *App*. Moreover, it was observed that *App* inhibits PRRSV infection before the first cycle of genome replication and transcription, indicating that *App* antiviral effect against PRRSV take place at an early step during PRRSV infection. The proteomic experiments revealed an increase of cofilin expression (a protein that regulates actin cytoskeleton dynamics) in the presence of *App,* which was further confirmed by western blot. Subsequently, a diminution of actin filaments was demonstrated by IFA. Interestingly, the treatment with cytochalasin D (an actin polymerization inhibitor) revealed the same effect on PRRSV replication than *App* suggesting that *App* antiviral effect against PRRSV may take place via the activation of cofilin which provokes actin depolymerisation and subsequently, probably affects PRRSV endocytosis.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is considered a worldwide endemic disease which causes significant economic losses in pig-producing countries. The causative agent, PRRS virus (PRRSV), belongs to the family *Arteriviridae* of the *Nidovirales* order. PRRSV is an enveloped single-stranded positive sense RNA virus of approximately 15 kb in length that encodes for at least 10 open reading frames (ORFs) (1, 2). PRRSV has a strongly restricted cell tropism for the monocyte–macrophage lineage *in vivo*. The primary target cells for PRRSV infection *in vivo* are the fully differentiated porcine alveolar macrophages (PAM), which are often used for *in vitro* study of host cell infections (3-6). In the literature, the only two continuous cell lines non-genetically modified able to fully replicate PRRSV are: African green monkey kidney cell line MA-104 and its derivatives such as MARC-145 (7) and the newly reported SJPL cells (8, 9).

Following PRRSV entry and release of the viral genome into the cytoplasm, the PRRSV ORF1 is translated and the resulting non-structural proteins trigger the formation of the replication-transcription complex, which is associated with double membrane vesicles and supports genome replication and transcription processes (10-12). The genome replication is produced by the continuous synthesis of negative (-) full-length RNA strands using as template the positive genomic RNA [(+) gRNA], then the (-) RNA strands will lead the formation of new (+) gRNAs (13). The genome transcription process is the synthesis of a nested set of six sub-genomic mRNAs (sg mRNAs). According to a model proposed by Sawicki and colleagues (14), the generation of these sg mRNAs is through a discontinuous RNA synthesis process, where (-) sg RNA strands are produced and then are used as template for the synthesis of the sg mRNAs.

Current management strategies, which focus on the prevention of PRRSV infection (ex. surveillance and removal, whole herd depopulation and repopulation, herd closure (15), etc.) and vaccination using commercially available modified live-attenuated vaccines or autogenous killed vaccines, have usually been demonstrated to be inadequate for long-term control of PRRS (16). This supports the search of novel strategies to control PRRSV infection. Recent published works have reported the discovery of natural compounds that possess antiviral

activities against PRRSV such as macrolides (17), N-acetylpenicillamine (18), *Cryptoporus volvatus* extract (19), morpholino oligomer (16, 20), Matrine (21), sodium tanshinone IIA sulfonate (22). Each of these compounds inhibits PRRSV replication differently. For instance, the flavaspidic acid AB inhibits PRRSV internalization and cell-to-cell virus transmission, probably by the induction of type I IFN (23). Sun and colleagues demonstrated that Matrine inhibits N protein expression and has antiapoptotic functions (21). Moreover, *Cryptoporus volvatus* extract was demonstrated to inhibit PRRSV infection *in vitro* and *in vivo*, probably by the direct inhibition of PRRSV polymerase activity (19). However and despite these efforts, there are no effective commercially available drugs to prevent PRRSV infection.

Recent works performed in our laboratory revealed that the cell culture supernatant of *Actinobacillus pleuropneumoniae* (*App)* mutant strain (*AppΔapxICΔapxIIC*) possesses a strong antiviral activity against PRRSV in SJPL and PAM cells, but this antiviral activity was not observed in MARC-145 cells (24). This was the first report of a bacterial antiviral effect against PRRSV *in vitro*. Thus, the purpose of this study is to elucidate the action mechanism of *App* cell culture supernatant antiviral effect against PRRSV. Results showed that *App* cell culture supernatant blocks PRRSV replication prior to its first genome replication and transcription cycle in PAM and SJPL cells. Following proteomic analyses, data suggest that the early *App* antiviral effect against PRRSV in PAM cells takes place via the activation of cofilin and thus actin depolymerisation, which would probably affect PRRSV endocytosis.

RESULTS

Impact of *App* cell culture supernatant on cells viability and mortality

In order to evaluate the impact of *App* cell culture supernatant on cell viability and mortality, PRRSV PAM infected cells were incubated during 48 hours in the presence or absence of *App* cell culture supernatant. The viability test, based on an enzymatic reaction that will occur only in metabolically active cells, showed no statistically significant differences between the negative control and the *App* cell culture supernatant treated cells as illustrated in Figure 1A. PRRSV infected cells had the lowest cell survival compared to all the other treatments. PRRSV PAM infected cells and treated with *App* cell culture supernatant demonstrated higher cell survival compared to *App* non-treated PRRSV infected cells. Opposite results were

observed in the mortality test (Figure 1B), which measures the LDH released in death-lysed cells. Negative control and *App* cell culture supernatant treated cells had low mortality rate. PRRSV infected cells had the highest cell mortality. PRRSV infected cells treated with *App* cell culture supernatant had a significant lower mortality rate compare to *App* non-treated PRRSV infected cells. Results showed that *App* cell culture supernatant did not affect PAM cells integrity and metabolism and counteracts PRRSV effect.

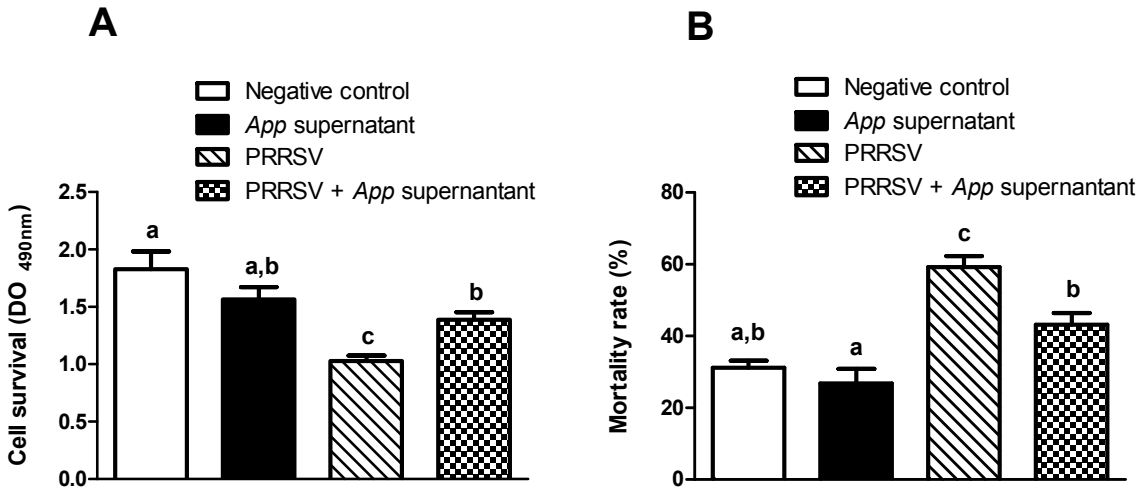

Figure 1: PAM viability and mortality in the presence of *App* cell culture supernatant.

PAM cells were infected with PRRSV IAF-Klop strain (MOI 0.5) during 4 hours followed by the addition of *App* cell culture supernatant. At 52 hours pi the cell viability (A) and mortality (B) were determined. Bars labelled with different superscripts letters within the same assay indicates that these sets of data are statistically different (P <0.05).

Type I IFN and IFN-γ mRNAs relative expression in *App* treated cells.

Specific qRT-PCR assays were performed to determine if the *App* cell culture supernatant was inducing the expression of type I and II IFN mRNAs because they are known as potent antiviral molecules against PRRSV infection (29-31). Results showed that at 52 hours pi, *App* cell culture supernatant only induced a basal level of IFN-β and IFN-γ mRNAs expression, which were similar to the expression level found in the negative control, while IFN-α mRNA expression was only slightly increase compared to the negative control (Figure 2). A

statistically significant decrease in IFN-α and IFN-β mRNA relative expressions were observed in PAM infected cells treated with the bacterial supernatant compared to PRRSV infected cells alone, which can be the consequence of PRRSV replication reduction caused by *App* treatment.

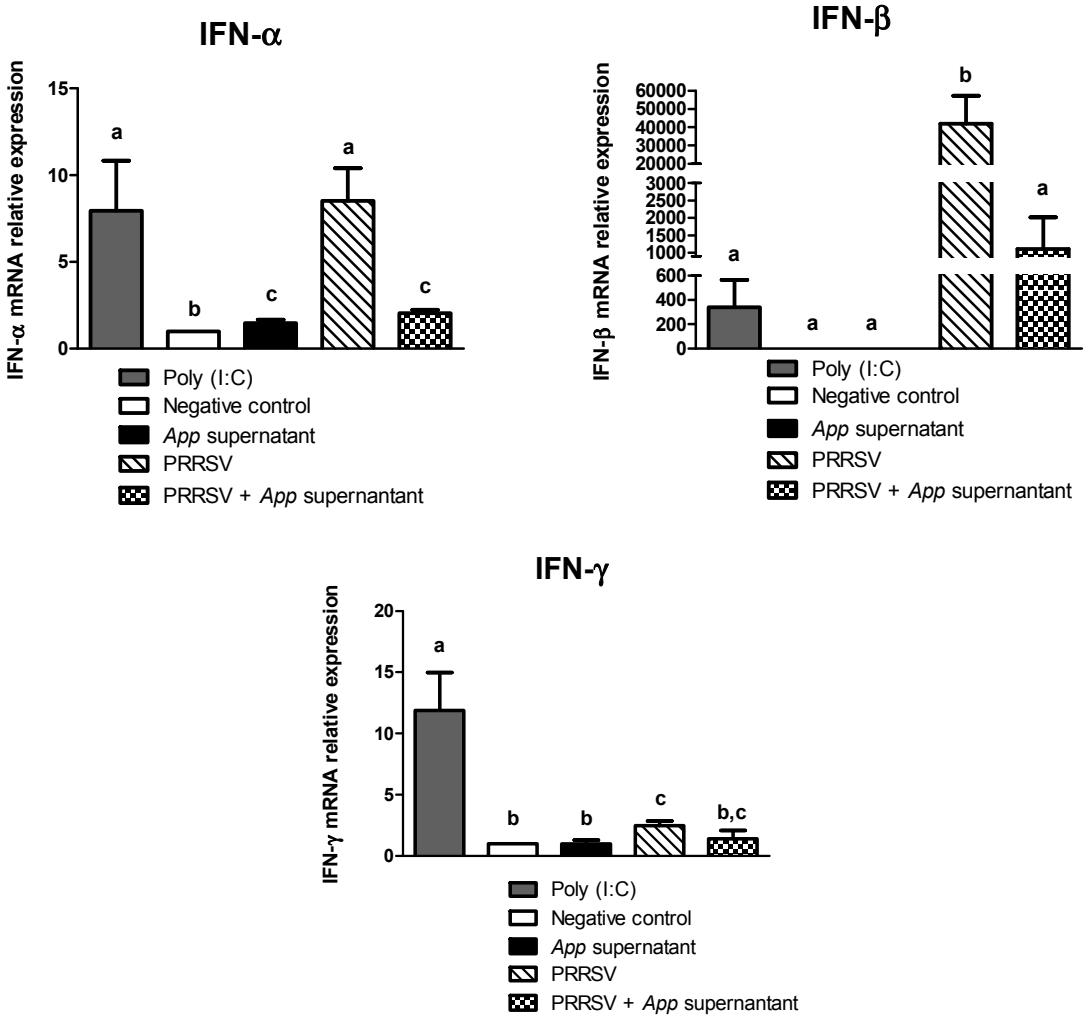

Figure 2: Type I and II IFN mRNAs relative expression in PAM cells treated with *App* cell culture supernatant.

mRNA relative expression of IFN-α, IFN-β and IFN-γ was determined at 52 hours pi in presence or absence of App cell culture supernatant. Transfected cells with Poly (I:C) were included as positive control. Bars labelled with different superscripts letters within the same assay indicates that these sets of data are statistically different (P <0.05).

PRRSV genome replication and transcription kinetics in the presence of *App* cell culture supernatant.

PRRSV genome replication and transcription were studied to pinpoint where the *App* antiviral effect occurs in the PRRSV replication cycle. The (+) gRNA and the sg mRNAs copies/mL were quantified by qRT-PCR through time during 52 hours using both infection protocols. In PRRSV PAM infected cells, starting between 8-16 hours pi, an increase in (+) gRNA copies/mL was observed reaching a plateau at 32 hours pi (Figure 3). However, in PAM PRRSV infected and *App* supernatant treated cells no increase in (+) gRNA copies/mL was detected. Moreover, in the presence of *App* cell culture supernatant, a statistically significant diminution in (+) gRNA copies/mL from 28 to 52 hours pi was observed when compared with the data at 4 hours pi. Similarly to genome replication results, PRRSV sg mRNAs copies/mL began to rise between 8-16 hours pi in PRRSV infected cells reaching a plateau at 32 hours pi and no increase in the presence of *App* cell culture supernatant was observed. Also, a statistically significant decrease in sg mRNAs copies/mL from 24-52 hours pi was observed when compared with to 4 hours pi, in PRRSV infected cells treated with *App* supernatant (Figure 3). Similarly to PAM cells, an increase in (+) gRNA and sg mRNAs copies/mL between 8-16 hours pi (Figure 3) in PRRSV SJPL infected cells was observed compared to PRRSV infected and *App* treated cells. However, a statistically significant increase was observed in the presence of the bacterial supernatant in (+) gRNA and sg mRNA copies/mL from 38 to 52 hours pi and from 24 to 52 hours pi, respectively, when compared to 4 hours pi. In MARC-145 cells, no significant differences were obtained between the infected cells treated or not with *App* cell culture supernatant in both genome replication and transcription assays (Figure 3). These results clearly showed that *App* cell culture supernatant inhibits PRRSV infection before the first cycle of PRRSV genome replication/transcription, in PAM and SJPL cells. In addition, similar results were obtained in the three cell types using both infections protocols (data not shown).

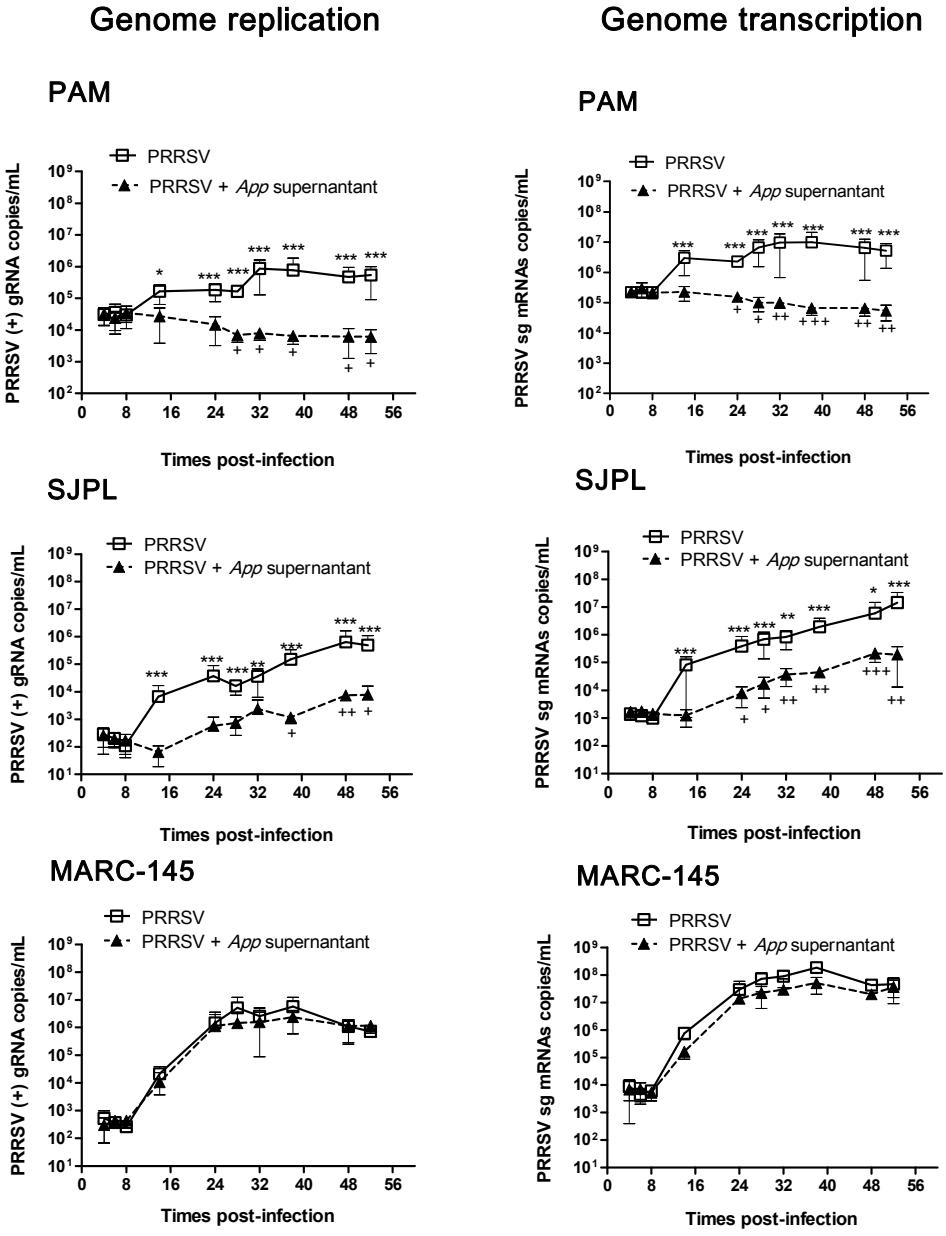

Figure 3: PRRSV genome replication and transcription kinetics assays in infected cells treated with *App* cell culture supernatant.

Cells were pre-treated with the bacterial antiviral during 2 hours followed by PRRSV infection during 4 hours and after freshly bacterial supernatant was added. At different times post-infection (pi) the PRRSV (+) gRNA and sg mRNAs copies/mL were calculated in PAM, SJPL

and MARC-145. The two-way ANOVA statistical analyse was performed using the raw data (Ct values). The asterisk means that in the same time point both groups are statistically different (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The t tests statistical analyse, within the experimental group of PAM and SJPL infected cells treated with *App,* was performed in order to compare all the times respect to time 4 hours pi ($^{+}P < 0.05$).

Proteomic Microarray study

A proteomic microarray (Kinexus (KAM)) was performed in PAM infected and/or *App* treated cells at 52 hours pi. Z-ratios were used to compare changes between the control and treated samples. Z-ratios were calculated between: 1) all treatments compared to the negative control, 2) PRRSV infected cells treated with *App* cell culture supernatant compared to cells treated with *App* cell culture supernatant alone and 3) PRRSV infected cells treated with *App* cell culture supernatant compared to PRRSV infected cells. The analysis report obtained by Kinexus includes all the proteins analysed that were modulated or not and also a shortlist which resume the protein modulation events (i.e. expression and/or phosphorylation state) that are recommended for follow-up. After sorting out the Z-ratios results, the mostly modulated proteins were put together and listed in Table 1. For instance, proteins implicated in cell cycle regulation such as tumor suppressor protein p53 (32) and cyclin-dependent protein-serine kinase 1/2 (CDK1/2)(33) were downexpressed or in cell division such as retinoblastoma-associated protein 1 (Rb) (34) was overexpressed in PRRSV infected PAM cells treated with *App* supernatant compared to PRRSV infected cells alone. NF-kappa-B p65 nuclear transcription factor, which is implicated in many biological processes, such as cell growth (35) and apoptosis (36), was overexpressed mostly in PAM infected cells in the presence of the bacterial supernatant compared to negative control. The protein SET (I2PP2A), involved in apoptosis (37), was also overexpressed in PRRSV infected PAM treated with *App* compared to PRRSV infected cells. As shown in Table 1, total cofilin 1 (a protein that regulates actin cytoskeleton dynamics) was overexpressed in PRRSV infected cells treated with the *App* cell culture supernatant in comparison to PRRSV infected cells, *App* treated cells and negative control cells.

Table 1: Z-ratios of the most modulated cell proteins by PRRSV and *App* supernatant.

Target Protein Name	Phospho Site (Human)[a]	Z-ratio (*App* + PRRSV *vs* PRRSV)[b]	Z-ratio (*App* + PRRSV *vs* APP)	Z-ratio (*App vs* Neg)	Z-ratio (PRRSV *vs* Neg)	Z-ratio (*App* + PRRSV *vs* Neg)
p53	S392	-1.50	-[c]	-	1.04	-
CDK1/2	T14+Y15	-1.74	-	-	1.50	-
CDK1 (CDC2)	Pan-specific	-2.76	-2.27	-	-	-2.23
Rb	T821	1.53	-	-	-	-
NFkappaB p65	S529	1.07	1.62	1.90	2.05	3.30
I2PP2A	Pan-specific	1.69	-	1.24	-	1.63
CREB1	S129+S133	1.33	-	-	-	-
Catenin b1	Pan-specific	-1.82	-	-	-	-
Smad2/3	Pan-specific	2.85	-	-	-	-
Cofilin 1	Pan-specific	1.01	1.37	-	-	1.28
Actin	Pan-specific	-	1.06	-	-	-
LIMK1	Pan-specific	-	-1.98	-	-	-
RONa	Pan-specific	1.32	-	-	-1.30	-
SOD (Mn)	Pan-specific	1.30	-	-	-	-
HO1	Pan-specific	-	1.75	-	-	1.55
MEK3/6 (MAP2K3/6)	S218/S207	-	1.63	-	-	1.34
Hsc70	Pan-specific	-	-1.66	-	-	-
Hsp105	Pan-specific	-	-1.94	1.31	-	-

[a] KAM tracks both protein expression (with pan-specific antibodies) and phosphorylation (with phospho-site-specific antibodies). The phosphorylation sites detected with the phospho-site antibodies are numbered corresponding to the human sequences.

[b] Z-ratios reveal the largest changes between the control and treated samples. Positive and negative Z-ratios mean a protein over- or down-expressed compared to the specific experimental groups.

[c] -: non-modulated proteins.

Actin cytoskeleton modulation in *App* treated cells

According to previous reports, *App* has been shown to degrade actin *in vitro* (38). Furthermore, PRRSV needs an intact actin cytoskeleton for cell infection and replication (5, 28). In order to confirm KAM results, western blot analysis were performed to detect total and phosphorylated cofilin (P-cofilin) at 52 hours pi. Thus, in order to confirm the antibodies microarray (KAM) results and the involvement of cytoskeleton in the *App* antiviral effect, western blot analyses were performed to detect total and phosphorylated cofilin (P-cofilin) at 52 hours pi using the three PRRSV permissive cell lines. Western blot analyses revealed that in PRRSV infected PAM cells treated with *App* cell culture supernatant, the total cofilin relative density was higher (1.52) compared to PRRSV infected cells (1.06), in *App* cell culture supernatant treated cells (0.83) and in untreated cells (0.63) (Figure 4 and Table 2). Interestingly, the relative density of P-cofilin was lower (0.3) in PAM infected cells treated with the bacterial supernatant and also in PRRSV infected cells (0.54) compared to the other experimental groups (Figure 4 and Table 2). Since total cofilin was mostly increased in PAM infected cells treated with the bacterial supernatant antiviral and P-cofilin was lowered, then it is easy to conclude that there is relatively more active cofilin in this experimental group compared to others treatments. In SJPL cell line, the P-cofilin relative density was lowered in PRRSV infected cells treated (0.68) or not (0.3) with the bacterial antiviral as in PAM. However there was no difference in total cofilin expression between treatments (Figure 4 and Table 2). In MARC-145 cells, similarly to SJPL cells, the total cofilin protein level did not differ between treatments (Figure 4 and Table 2). However, in a surprising way, it was observed that in MARC-145 infected cells treated with *App* cell culture supernatant there was more P-cofilin (relative density of 1.74) than in the other treatments (Figure 4 and Table 2), which differs to those results obtained in PAM and SJPL.

Since cofilin is known to provoke F-actin depolymerisation in its active form (dephosphorylated) and the previous presented results indicated differences in cofilin active form level between treatments, the next step was to study the F-actin expression in the three cell types at 52 hours pi. A decrease in F-actin fluorescence intensity in *App* cell culture supernatant treated PAM cells was observed, but this diminution was more remarkable in PRRSV infected cells treated with *App* supernatant (Figure 5A). In SJPL cell line, IFA revealed also a decreased in F-actin fluorescence in SJPL *App* cell culture supernatant treated cells compared to the untreated cells (Figure 5B). Interestingly, in MARC-145, no marked differences were detected between the *App* supernatant treated or untreated cells (Figure 5B). Following these findings, the β-actin mRNA and protein expressions were studied in PAM, SJPL and MARC-145 cells. These results highly suggest that actin cytoskeleton is involved in *App* cell culture supernatant antiviral effect. In addition, as showed in Figure S1, in supplemental information, the β-actin mRNA and the protein expression levels were unaltered between treatments in each cell types.

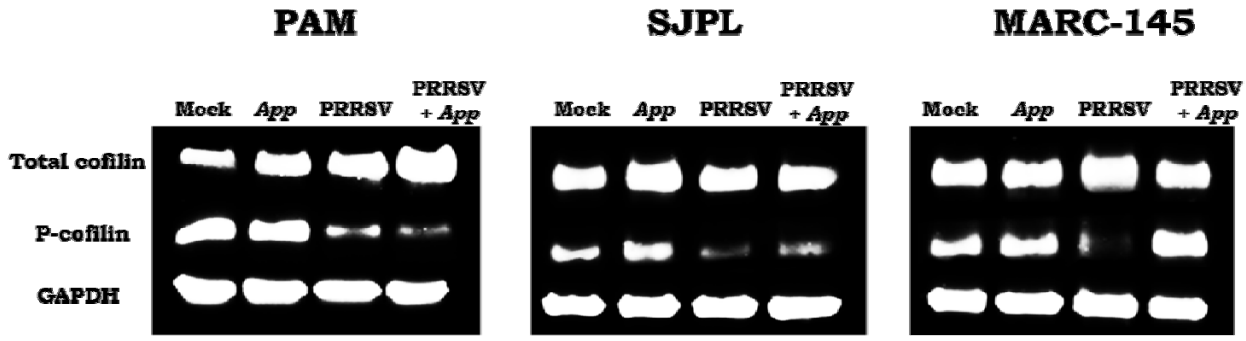

Figure 4: Cofilin expression level in PRRSV infected cells treated or not with *App* cell culture supernatant.

At 52 hours pi, protein extracts were obtained and western blot analysis were performed to detect total cofilin and its inactive form (phosphorylated) in PAM, SJPL and MARC-145 cells. GAPDH was included as an internal control.

Table 2: Total cofilin and P-cofilin relative densities in PAM, SJPL and MARC-145 cells.

	Relative density [a]	
PAM	**Total cofilin**	**P-cofilin**
Negative control	0.63	1.55
App cell culture supernatant	0.83	1.52
PRRSV	1.06	0.54
PRRSV+ *App* cell culture supernatant	1.52	0.30

	Relative density	
SJPL	**Total cofilin**	**P-cofilin**
Negative control	0.96	1.39
App cell culture supernatant	1.09	1.69
PRRSV	1.01	0.30
PRRSV+ *App* cell culture supernatant	0.93	0.68

MARC-145	**Total cofilin**	**P-cofilin**
Negative control	0.97	1.03
App cell culture supernatant	0.98	1.10
PRRSV	1.00	0.19
PRRSV+ *App* cell culture supernatant	0.97	1.74

[a] Image J program was used to calculate the total cofilin and P-cofilin relative densities from the images represented in the Figure 4.

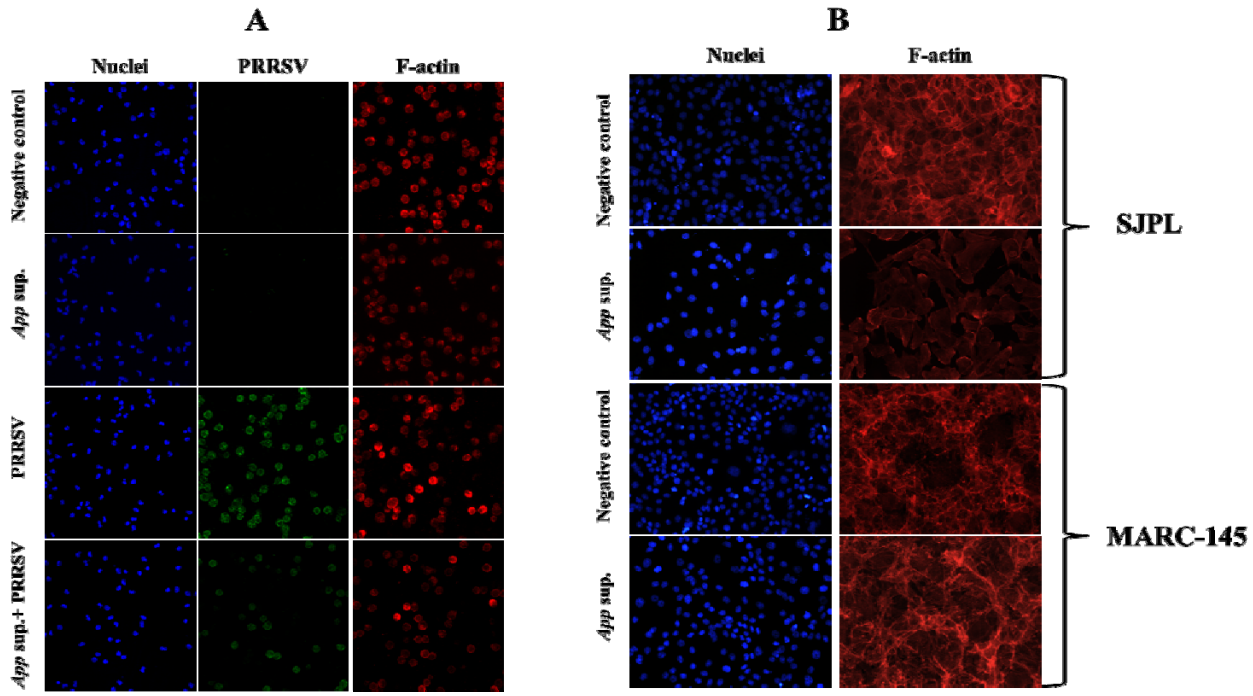

Figure 5: F-actin expression in the presence of *App* cell culture supernatant.

At 52 hours pi, cells were fixed to perform IFA. A: Confocal microscopy projection images in PAM, DAPI: nuclei staining (bleu), FITC: PRRSV detection (green), Alexa Fluor® 594 phalloidin: F-actin (red). Pictures were taken at 200X magnification. B: IFA images of SJPL and MARC-145 cells treated or not with the bacterial supernatant. Pictures were taken at 100X magnification.

Infectious viral particles production in PRRSV-infected cells treated with cytochalasin D.

The microfilament disrupt compound, cytochalasin D, effect on PRRSV infection in PAM and MARC-145 cells is already known (5, 28). However, it was important to test its effect in our experimental conditions and with the cells that have not been previously tested, i.e SJPL. For this purpose, PAM, SJPL and MARC-145 cells were infected and treated with 3µM of cytochalasin D using both infection protocols. At 52 hours pi the infectious viral particles produced were determined. As illustrated in Figure 6, the amount of infectious virions in PAM infected cells in the presence of cytochalasin D (2.2 log10 $TCID_{50}$/mL) was significantly lower

than in non-treated infected cells (5.5 log10 $TCID_{50}$/mL) with a difference of 3.3 log10 (P<0.05). In addition, cytochalasin D completely blocked PRRSV infection in SJPL cells with at least a 5 X 10^5 times infectious titer reduction (P<0.05) (Figure 6). Interestingly, in MARC-145 cells, cytochalasin D did not inhibit PRRSV infection with virus titers of 6.5 log10 and 6.8 log10 $TCID_{50}$/mL in cytochalasin D treated and untreated cells, respectively, (Figure 6). Moreover, in PAM and SJPL cells, there is a complete inhibition of PRRSV infection in the presence of cytochalasin D because the amount of infectious virions obtained in infected cells treated with this compound, was lower or equal to the infectious PRRSV particles measured at 4 hours pi, which is considered to be the amount of particles attached and/or entered into the cells [2.6 and ≤1.5 log10 $TCID_{50}$/mL in PAM and SJPL, respectively) (Figure 6). In addition, with both infection protocols (cytochalasin D added 2 hours before or 4 hours after PRRSV infection) similar results were obtained (data not shown). These results clearly showed that cytochalasin D inhibits PRRSV infection in PAM and SJPL cells, but not in MARC-145 cells such as *App* cell culture supernatant.

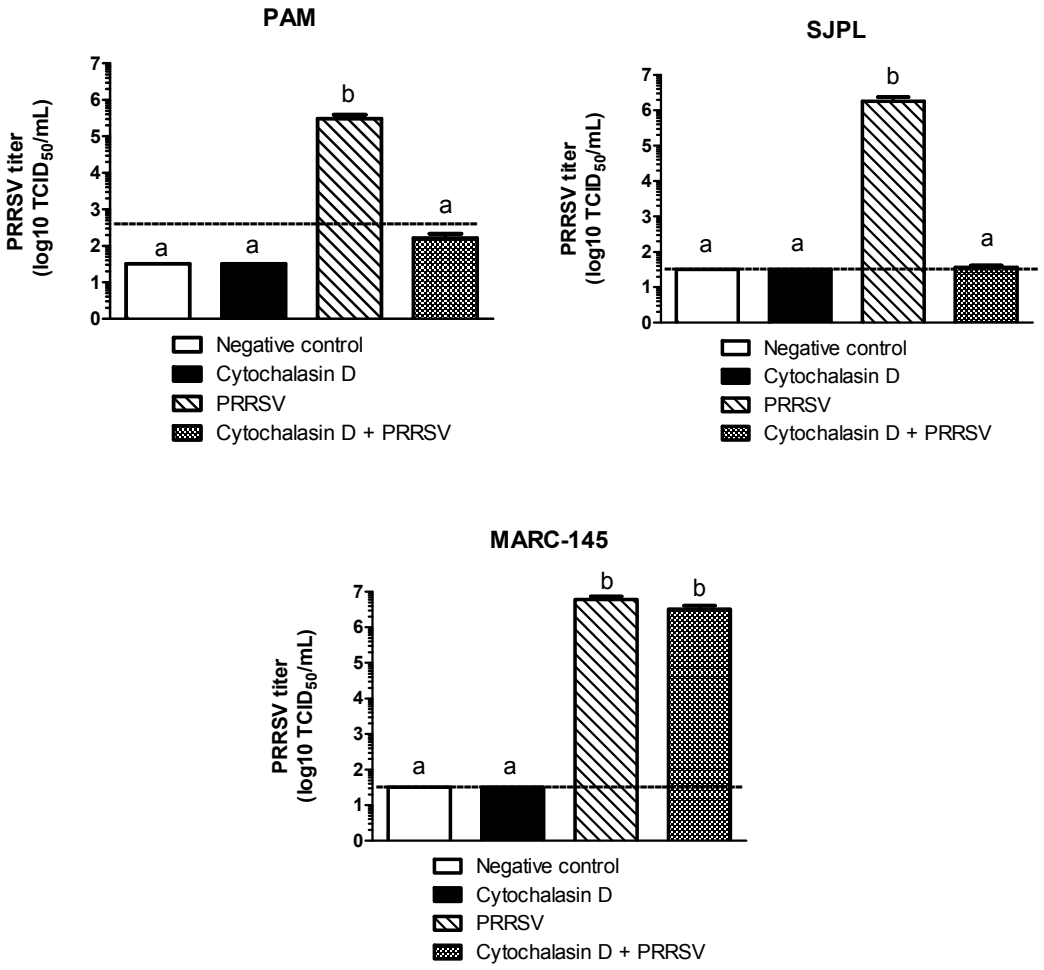

Figure 6: Infectious viral particles production in PRRSV-infected PAM, SJPL and MARC-145 cells treated with cytochalasin D.

Cells were pre-treated with 3 µM of cytochalasin D during 2 hours followed by PRRSV IAF-Klop strain infection during 4 hours period. At 52 hours pi, viral titers were determined by the Kärber method and were expressed in $TCID_{50}$/mL. The dotted lines represent the intracellular infectious PRRSV particles at 4 hours pi. Log10 $TCID_{50}$/mL $\leq$ 1.5 is established when no cytopathic effect is observed. Bars labelled with different superscripts letters within a cell line indicate that these sets of data are statistically different (P <0.05).

***App* cell culture supernatant antiviral effect against others PRRSV strains in MARC-145 cells.**

Levesque and colleagues have clearly demonstrated that the *App* cell culture supernatant antiviral effect against PRRSV is irrelevant in MARC-145 cells when compared to PAM and SJPL response (24). Thus, in order to determine if the *App* antiviral effect against PRRSV in MARC-145 cells was PRRSV strain dependent, virus titers for different PRRSV strains was calculated at 52 hours pi. Results demonstrated that the amount of infectious virions in PRRSV LV infected cells treated with *App* supernatant (5.7 log10 $TCID_{50}$/mL) was significantly lower than in PRRSV LV infected cells (6.8 log10 $TCID_{50}$/mL) with an approximately difference of 1 log10 $TCID_{50}$/mL ($P<0.05$) (Figure 7). Viral titers obtained with PRRSV NVSL strain demonstrated that the bacterial supernatant significantly reduces the amount of infectious virions to 5.4 log10 $TCID_{50}$/mL compared to PRRSV NVSL infected cells alone (6.9 log10 $TCID_{50}$/mL) (Figure 7). The highest *App* supernatant antiviral effect was observed to be against PRRSV FMV09-11SS278 strain. A 4.1 and 6.6 log10 $TCID_{50}$/mL was obtained in PRRSV FMV09-11SS278 infected cells treated or not with *App* supernatant, respectively, with a significant difference of 2.5 log10 $TCID_{50}$/mL ($P<0.05$).

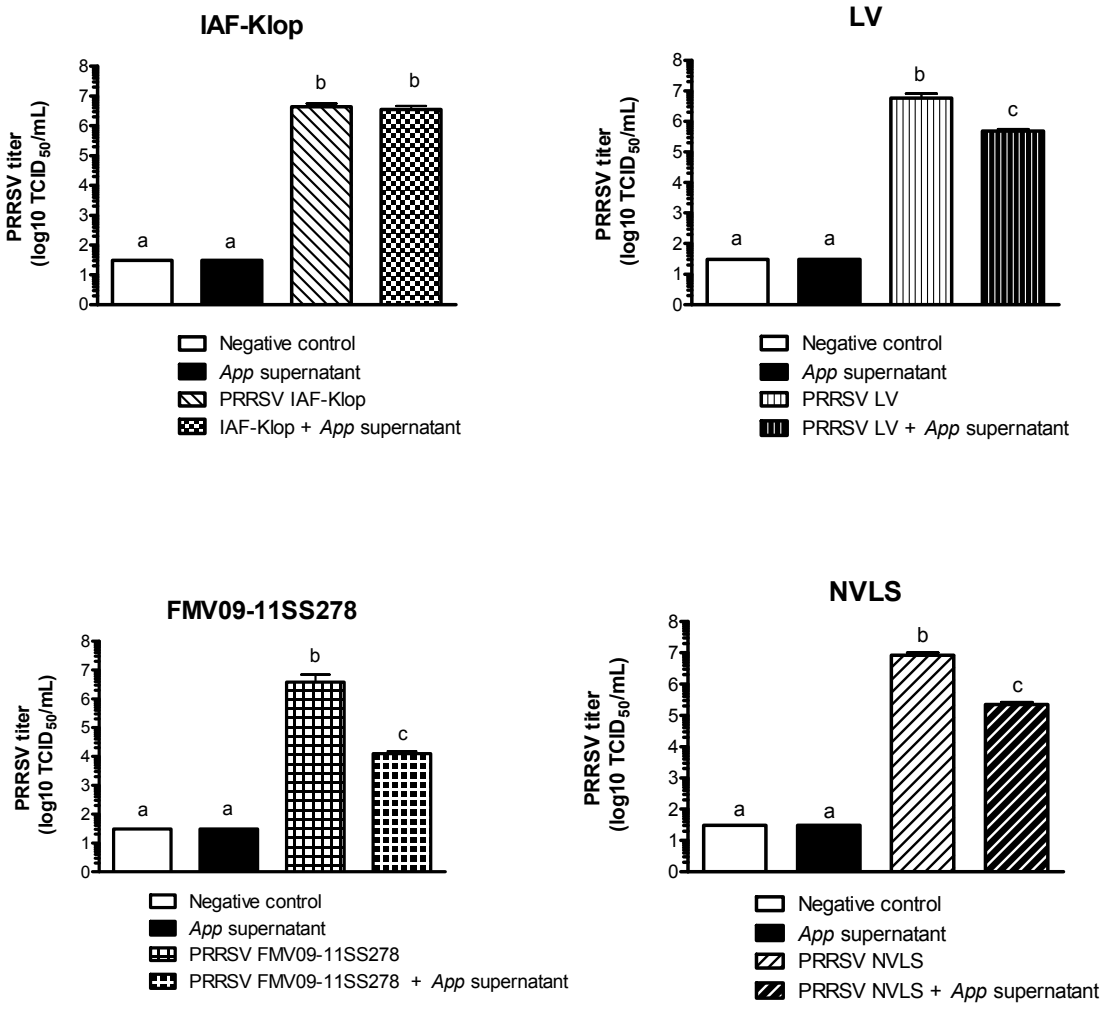

Figure 7: *App* supernatant antiviral activity against others PRRSV strains in MARC-145 cells.

MARC-145 cells were infected with PRRSV LV, FMV09-11SS278 and NVSL strains (MOI 0.5) during 4 hours followed by the addition of *App* cell culture supernatant. Viral titers were determined by the Kärber method at 52 hours pi and were expressed in $TCID_{50}$/mL. PRRSV IAF-Klop MARC-145 infected cells were used as control. Bars labelled with different superscripts letters within the same assay indicates that these sets of data are statistically different ($P < 0.05$).

DISCUSSION

The newly discovered antiviral activity of *App* supernatant against PRRSV was shown to be effective in the SJPL and PAM cells (24) but not in MARC-145 cells. In this previous study, it was also demonstrated that other viruses such as equine herpes virus type 1, swine influenza H1N1 and H3N2 infection could be inhibited by the *App* cell culture supernatant in the SJPL cells but to a significant much lower extend compared to PRRSV. Interestingly, bovine adenovirus 3, bovine herpes virus type 1 and bovine viral diarrhea virus type 1 infections were not affected by the *App* cell culture supernatant, indicating that SJPL cells are metabolically active and able to support virus replication. Unfortunately, since the impact of *App* cell culture supernatant on PAM cells viability and mortality was unknown, it was important to establish the PAM cells status following *App* supernatant treatment. Results indicate that the bacterial supernatant did not induce cell death, since similar results were obtained between the untreated cells and *App* cell culture supernatant treated cells (Figure1). In addition, it was observed that in PRRSV infected cells treated with *App* antiviral, there was a significant increase of cell survival and a significant decrease in mortality rate compared to PRRSV infected cells. This can be the consequence of the PRRSV lower replication due to the antiviral effect of *App* cell culture supernatant. Taken together, these results demonstrated that PAM *App* treated cells are viable and metabolically active to support virus infection and, that the observed antiviral effect of the bacterial supernatant is not due to cell mortality.

In order to identify the *App* antiviral mechanism of action, the expression of type I and II IFN was determined because those cytokines are very important in the host antiviral immune response (39, 40), even though it is well known that PRRSV has developed strategies to evade their antiviral effects (41-44). It was observed that PRRSV infected PAM cells treated with *App* cell culture supernatant has not increased type I IFN and IFN-γ mRNA relative expressions compared to *App* supernatant treated and untreated cells (Figure 2), indicating that *App* antiviral effect is not via the induction of those cytokines. Lévesque and collaborators also observed that the bacterial supernatant did not induce type I IFN expressions in the SJPL cell line (24). However, an induction in type II IFN was detected in SJPL cells, which suggest that *App* antiviral effect might be via the induction of IFN-γ. These results also suggest that *App* antiviral effect could occur via different mechanisms that are cell type dependant.

Moreover, it was showed that mRNA relative expression of type I IFN was decrease in PAM PRRSV infected cells treated with the bacterial supernatant compared to PRRSV infected cells alone, which it is supposed to be due to the bacterial antiviral effect.

Garcia-Cuellar and colleagues have demonstrated that an *App* secreted 24kDa cloned Zn-metalloprotease is able to degrade actin protein *in vitro* (38). On the other hand, different studies have revealed the important role of actin cytoskeleton on PRRSV infection (5, 6, 28, 45). Therefore, followings these previous findings, it is more likely that *App* cell culture supernatant antiviral effect was specifically directed against the cells which subsequently induced PRRSV infection inhibition. All stages of virus replication cycle are dependent on host cell machinery. For instance, 1) PRRSV entry occurs via receptor-mediated endocytosis and this process was demonstrated to be microfilament dependent (5, 28); 2) PRRSV uncoating is known to be dependent on acidic pH of the early endosomes and also involved cellular proteases (5, 46, 47); 3) PRRSV genome replication/transcription is believe to be produced in autophagosome-like double-membrane vesicles (48, 49); etc. In order to identify at which PRRSV replication cycle step the bacterial antiviral effect occurs, the PRRSV genome replication and transcription were evaluated. Results clearly demonstrate that *App* antiviral effect against PRRSV takes place prior to the first cycle of genome replication and transcription. The fact that during the first 4 to 8 hours pi similar results were obtained between infected cells treated or not with *App* supernatant using both PRRSV infection protocols (*App* treatment prior or after PRRSV infection) (Figure 3), indicated that at least PRRSV attachment to cells is not inhibited by *App* treatment because it's well known that this process in PAM reaches a maximum at one hour pi (5). Overall, the viral genome replication/transcription kinetics results clearly indicate that *App* cell culture supernatant PRRSV antiviral effect takes place at least during the entry, uncoating or during the formation of the replication/transcription complex. Unfortunately, at the moment, it is not possible to specify at which of these three virus replication steps, the *App* antiviral effect occurs. Otherwise, the *App* antiviral effects seem more efficient in PAM compared to SJPL cells. The gRNA replication was entirely inhibited in PAM cells whereas in SJPL cells, a small but statistically significant increase of (+) gRNA and sg mRNAs copies was observed over time (Figure 3) suggesting that in these cells, few PRRSV particles can achieve a complete

replication cycle. Both cell types are phenotypically different and this may explain these observations. A recent study performed by Provost and colleagues has demonstrated that PRRSV receptors harbour in both cell types are different, since in SJPL cell line, only the CD151 receptor was identified, but not the CD163 and sialoadhesin as in PAM (9). Further studies should be conducted in order to identify PRRSV entry mediators and to know in details about PRRSV replication cycle in the new SJPL cells infection model. At the moment, there is no data to explain why MARC-145 cellular response in regards to *App* antiviral action is different. The major difference known between MARC-145 and PAM cells in regards to PRRSV replication cycle is the virus entry into the cell. PAM and MARC-145 cells PRRSV entry mediators are different confirming that virus entry differs between the two cell types. For instance, in MARC-145 cells, contrary to PAM, the sialoadhesin is absent (6) and the sialic acids present in the virion are not essential for infectivity (50). It was also reported that cholesterol is critical for PRRSV entry in MARC-145 cells and also suggested that PRRSV entry could be via a lipid-raft-dependent endocytosis (51, 52). Therefore, the MARC-145 adapted IAF-Klop strain can use a completely different entry mechanism to that in PAM, which makes PRRSV infection in this cell type resistant to *App* antiviral effect.

The use of specific antibodies expanded in a microarray is an effective and convenient method for tracking specific proteins and their phosphorylation states that could be involved in a cellular response. As mentioned previously, both pathogens interact with the actin cytoskeleton and Kinexus microarray results revealed that cofilin 1 and LIMK1 (proteins implied in actin pathway) were modulated. Consequently, the actin cytoskeleton modulation was investigated. It has been established that cofilin severing activity induces F-actin free ends accessible for actin polymerization and depolymerisation (53, 54). Cofilin has two states: unphosphorylated and phosphorylated, where only the active cofilin (unphosphorylated form) is able to bind F-actin and promote depolymerisation (53). LIMK can be finding in two states, the phosphorylated LIMK being active and is assumed to deactivate cofilin following its phosphorylation (54, 55). In Kinexus (Table 1) total cofilin and LIMK proteins were over and downexpressed, respectively. Thus, these results suggested a possible involvement of the actin cytoskeleton in the *App* antiviral activity.

Actin cytoskeleton is involved in many RNA and DNA virus replication cycle (56-59). During PRRSV infection, the microfilaments are a critical component necessary for PRRSV primary and secondary infection (5, 28). It was observed more active cofilin in PAM infected cells treated with the bacterial supernatant than in the others experimental groups (Figure 4 and Table 2). This finding suggests the possible modulation of F-actin, which was further analysed by IFA. In fact, the confocal microscopy images revealed that F-actin fluorescence intensity was decreased in *App* cell culture supernatant treated cells, but the decrease was more pronounced in PAM infected cells treated with the bacterial antiviral (Figure 5 A). A previous study suggested that a negative correlation between the F-actin expression level and PRRSV infection exists (28), indicating that probably PRRSV decreases F-actin to favour its infection. Therefore, it is possible that PRRSV establishes the F-actin quantities needed for its infection but when certain low and high thresholds are exceeded, PRRSV infection is inhibited. For that reason, it is believed that probably when PRRSV infected cells are treated with *App* cell culture supernatant, there is a detectable increase of active cofilin that will subsequently induce F-actin depolymerisation and thereafter PRRSV infection inhibition. Interestingly, a decreased of P-cofilin combined with a reduction of F-actin in SJPL cells treated with *App* suggest that the antiviral mechanism in both PAM and SJPL might be similar. Interestingly, in MARC-145 PRRSV infected cells treated with the bacterial antiviral more P-cofilin was detected than in the others experimental groups (Figure 4 and Table 2) and it was observed by IFA that there is no F-actin depolymerisation in the presence of *App* cell culture supernatant compared to negative cells (Figure 5B). Therefore, since MARC-145 cellular response face to *App* antiviral differs from PAM and SJPL cells, these results highly suggest that cofilin is a possible cellular target of *App* cell culture supernatant. Moreover, the fact that β-actin mRNA and protein expression levels were unaltered between treatments in the three cell types (Figure S1), suggest that the observed F-actin depolymerisation phenomenon in PAM and SJPL is not due to the metalloprotease secreted by *App* that degrade β-actin *in vitro* (38) but rather due to cofilin (54).

In order to confirm the involvement of actin cytoskeleton in *App* antiviral effect, the effect of cytochalasin D (a drug that destabilizes actin filaments) on PRRSV replication was determined in the three PRRSV permissive cell models. The use of this drug has been a valuable tool for

investigating the functional roles of actin filaments in cellular processes and in viral pathogenesis (5, 28, 59-64). cytochalasin D was able to inhibit PRRSV replication in PAM and SJPL cells, but not in MARC-145 cells just like *App* cell culture supernatant (Figure 6 compared to Figure 4 from Levesque and colleagues (24)). Cytochalasin D was added before or shortly after few hours pi and both experimental designs were able to inhibit PRRSV replication (data not shown) indicating that PRRSV was able at least to attach to the target cells. Therefore, these results highly suggest that *App* supernatant inhibits PRRSV infection during PRRSV entry via clathrin-mediated endocytosis, since it is well known that this process is actin cytoskeleton dependant (5, 28, 65-67). Conflicting data have been previously reported in regards to the antiviral effect of cytochalasin D against PRRSV in MARC-145 infected cells. In fact, Cafruny and collaborators have demonstrated that cytochalasin D at 1-2 µM concentration was able to inhibit PRRSV primary infection in MARC-145 cells (28). However, in the present study, a higher dose (3 µM) was used which may explain why cytochalasin D was able to inhibit PRRSV replication in MARC-145 infected cells. Noteworthy, this discrepancy can also be the consequence of having used different PRRSV strains in each study. Several studies have demonstrated that PRRSV isolates adaptation process in MARC-145 generates genetic changes, including deletions, insertions or substitutions and is characterised by higher titers, faster growth kinetics making the new adapted isolates less virulent than the wild type (68-72). It would be possible that PRRSV IAF-Klop strain can use an entry mechanism that is actin cytoskeleton independent, in order to successfully replicate in the MARC-145 cells, which perfectly explains why the *App* antiviral effect is inefficient in MARC-145 cells.

Following this hypothesis the *App* supernatant antiviral effect against others PRRSV strains was investigated in MARC-145 infected cells. It was observed that *App* supernatant inhibits PRRSV LV similar to IAF-Klop strain ((24) and Figure 7). However, the bacterial antiviral effect was more effective against PRRSV NVSL and FMV09-11SS278 strains, which confirmed that *App* supernatant antiviral effect is PRRSV strain dependent in MARC-145 cells. Further studies need to be conducted in order to ascertain this hypothesis.

In conclusion, this study clearly demonstrated that *App* cell culture supernatant inhibits PRRSV infection prior to the first cycle of PRRSV genome replication/transcription in PAM

and SJPL, probably via the activation of cofilin, which can provoke actin depolymerisation and subsequently this phenomenon might affects PRRSV endocytosis. Further studies are in progress in order 1) to confirm that *App* cell culture supernatant affects PRRSV entry by endocytosis in PAM and 2) to find the active metabolite(s) present in *App* cell culture supernatant that is responsible for its antiviral effect.

MATERIAL AND METHODS

Cells

MARC-145 cells, a subclone of the African green monkey kidney MA104, and the SJPL cell lines were maintained as previously described (24). The SJPL cell line was kindly provided by Dr R.G. Webster (St. Jude Children's Hospital, Memphis, TN, USA) (8). PAM cells were obtained from lungs of 2 to 14 weeks old pigs as previously described (9, 24) and animals were sacrificed following the ethic protocol 12-Rech-1640 approved by our institutional ethic committee. PAM cells were cultured for 24 hours in complete Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation GibcoBRL, Burlington, ON, CA) prior to assay (24). All cells were cultured and infected at 37°C in 5% CO_2 atmosphere.

Viral and bacterial strains

The PRRSV strain used in this study was the Canadian genotype II reference strain IAF-Klop and the virus stocks were obtained as previously described (9). The *App* strain used in this study was the mutant MBHPP147 from the strain S4074, which is the serotype 1 reference strain. This mutant (*AppΔapxICΔapxIIC*) is known to produce non-active ApxI and ApxII toxins and was kindly provided by Ruud P.A.M. Segers (MSD Animal Health, Boxmeer, The Netherlands). *AppΔapxICΔapxIIC* strain was cultured on brain heart infusion (BHI) broth and/or agar (Invitrogen) supplemented with 15 µg/ml nicotinamide adenine dinucleotide (NAD) at 37°C in 5% CO_2. The cell culture supernatant from *AppΔapxICΔapxIIC* strain was obtained as previously described (24). Briefly, *AppΔapxIΔapxIIC* from an overnight culture grown at an OD_{600nm} of 0.6 were resuspended at a multiple of infection (MOI) of 10:1 in complete cell culture medium, containing NAD, to a concentration of 10^6 CFU/ml and incubated overnight at 37°C in 5% CO_2. Thereafter, the mutant grown in complete cell culture

medium was centrifuged at 4000 rpm for 15 minutes and harvested supernatants were passed through a 0.22 μm filter to remove all residual bacteria. The supernatant was then conserved at -20°C for further usage.

Cells infection

Protocol #1: Cells were infected, as previously described (24), at 0.5 MOI with PRRSV IAF-Klop strain and incubated in DMEM without serum or other additives during four hours, then all non-attached virus were removed following two soft washing step using PBS. Thereafter, the *AppΔapxIΔapxIIC* (*App*) cell culture supernatant or complete medium in the case of controls, were added. In addition, another PRRSV infection protocol was tested to determine if *App* cell culture supernatant had an impact prior PRRSV infection. Protocol #2: Cells were pre-treated with the bacterial supernatant during two hrs, followed by PRRSV infection at 0.5 MOI in DMEM without serum or other additives during four hours, then infected cells were washed and finally *App* cell culture supernatant or the complete medium were added. Both infection protocols were used in all experiments, unless specified.

Cells viability and mortality

A total of $2x10^5$ PAM cells/well were seeded into 96 well-tissue culture plates (Corning, Tewksbury, MA, USA) and incubated for a 24 hours period. Afterwards, cells were infected using the protocol #1 described above and incubated in the presence of *App* cell culture supernatant or complete medium during 48 hours. Cell viability was measured with CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) at 52 hours post-infection (pi). Twenty ul of the CellTiter substrate were added to the cells followed by one hour of incubation at 37°C in 5% CO_2. Cellular mortality was determined using the lactate dehydrogenase (LDH)-measuring CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI, USA). Released LDH in culture supernatants was measured with a 30-minutes coupled enzymatic assay. Mechanically lysed cells were used as 100%-mortality positive control. For both methods non-infected cells were used as a negative control and the absorbance was measured at 490 nm with a SynergyTM HT multi-detection microplate reader (Biotek, Winooski, VT, USA). Both assays were repeated three times.

Type I IFN, IFN gamma (IFN-γ) and β-actin relative mRNA expression.

$4x10^6$ PAM cells/well and $5x10^5$ (SJPL/MARC-145) cells/well seeded in 6 well-tissue culture plates were infected and incubated in the presence of *App* cell culture supernatant or complete medium during 48 hours. As positive control for innate immunity induction PAM were transfected with Polyinosinic–polycytidylic acid potassium salt (Poly (I:C) [50 μg/mL] (Sigma-Aldrich, St. Louis, MO, USA), using polyethylenimine (PEI) [1 μg/μL] (Sigma-Aldrich, St. Louis, MO, USA). Total cellular RNA was extracted from cells using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. RNA quantification was performed using NanoDrop® ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). One μg of total RNA was reverse-transcribed using the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada). The cDNA obtained was amplified using the SsoFast™ EvaGreenW Supermix kit (Bio-rad, Hercules, CA, USA) in the Bio-Rad CFX-96 sequence detector apparatus. The PCR amplification steps used were an enzyme activation step of 3 min at 98°C, followed by 40 cycles of a denaturing step of 2 sec at 98°C and an annealing/extension step of 5 sec at 57°C. The primers pairs used for the amplification of type I IFNs and IFN-γ in PAM were: IFN-α: F 5'-ACTCCATCCTGGCTGTGAGGAAAT-3' and R 5'-TCTGTCTTGCAGGTTTGTGGAGGA-3'; IFN-β: F 5'-CTCTCCTGATGTGTTTCTCC-3' and R 5'-GTTCATCCTATCTTCGAGGC-3'; IFN- γ: F 5'-GAGCCAAATTGTCTCCTTCTAC-3' and R 5'- CGAAGTCATTCAGTTTCCCAG-3'. The β-actin gene amplification was performed using the primers F 5'-ACCACTGGCATTGTCATGGACTCT-3' and R 5'-ATCTTCATGAGGTAGTCGGTCAGG-3' for PAM (porcine origin) and the primers F 5'-GGCATCCATGAAACTACCTTC-3' and R 5'-AGGGCAGTAATCTCCTTCTG-3' for SJPL and MARC-145 cells (monkey origin). Peptidylprolyl isomerase A (PPIA) and beta-2 microglobulin (B2M) were employed as normalizing genes in PAM and SJPL/MARC-145 cells, respectively, and were amplified using the following primers pairs: PPIA: F 5'-TGCAGACAAAGTTCCAAAGACAG-3' and R 5'-GCCACCAGTGCCATTATGG-3'; B2M (9): F 5'-GTGCTATCTCCACGTTTGAG-3' and R 5'-GCTTCGAGTGCAAGAGATTG-3'. All primer sequences were designed from the NCBI

Gen-Bank mRNA sequences using web-based software primerquest from Integrated DNA technologies (http://www.idtdna.com/Scitools/Applications/Primerquest/) unless specified. Uninfected cells were employed as the calibrator reference in the analysis. Differences mRNA quantification between experimental groups were calculated using the $2^{-\Delta\Delta Ct}$ method. Experiments were repeated three times in duplicate.

PRRSV genome replication/transcription kinetics

For this experience, $4x10^6$ PAM cells/well and $5x10^5$ SJPL/MARC-145 cells/well, were plated into 6 well-tissue culture plates (Corning, Tewksbury, MA, USA) and incubated during 24 hours. Cells were infected and incubated in the presence of *App* cell culture supernatant. At different times pi (4, 6, 8, 14, 24, 28, 32, 38, 48 and 52 hours) samples were collected to perform specific qRT-PCR assays. Total RNAs were extracted from cells and quantified as described above. 1.5 µg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, Burlington, ON, CA) following the manufacturer's protocol. The strategy used to determine PRRSV genome replication and transcription has already been published however new primers were designed for this study (25). Briefly, in the RT-PCR were added individually in each sample 2 pmol of the gene-specific reverse primers, PRRSV ORF1: R 5'-AGAAAGCACGTAAGCTCCAGCCAA-3', which allows detecting only the PRRSV (+) gRNA and PRRSV ORF7: R 5'-AGCATCTGGCACAGCTGATTGACT-3' to detect all the viral sg mRNAs (which all contain ORF7 gene sequence). It is important to specify that with the ORF7 reverse primer, all the (+ strand) ORF7 sequence are detected, which include the ORF7 sequence from PRRSV viral genome and all the viral sg mRNAs sequences, explaining its use to quantify genome transcription. As internal control, the housekeeping mRNA from PPIA (in PAM cells samples) and B2M (in SJPL and MARC-145 cells samples) were also performed on the same RNA preparations using the reverse primers described above. The cDNA was treated with 1.5 µg RNase A (Invitrogen, Burlington, ON, Canada) for 30 minutes at 37°C to remove the remaining RNAs, followed by inactivation of RNase A by heating at 95°C for 10 minutes. 2µl of cDNA was amplified using the same reagents and conditions described above. The primers pairs used for amplification were: PRRSV ORF1: F 5'-TGTGAGTTTGACTCGCCAGAGTGT-3' and R 5'-TACAGTCTGCAACAATGCCAAGCC-3', PRRSV ORF7: F 5'-

GCGGCAAGTGATAACCACGCATTT-3' and R 5'-TGCTGCTTGCCGTTGTTATTTGGC-3' and for PPIA and B2M, the primers pairs used are described above. The Ct values obtained were expressed in PRRSV (+) gRNA and PRRSV sg mRNAs copies/mL, for PRRSV genome replication and transcription, respectively. For this purpose, a standard curve was generated. First, the PRRSV viral genome molecular weight was calculated using the PRRSV strain ATCC VR-2332 complete genome sequence (26) and a formula available in Life technologies web site (http://www.lifetechnologies.com/ca/en/home/references/ambion-tech-support/rna-tools-and-calculators/dna-and-rna-molecular-weights-and-conversions/) was used. Afterwards, PRRSV viral genome was purified from the virus stock and its concentration was determined. Ten-fold dilutions of the PRRSV purified RNA was done and RT-qPCR assays were performed as described above to establish the standard curve. All experiments were repeated three times in duplicate.

Proteomic assay

$4x10^6$ PAM cells were infected using the infection protocol #2 and incubated in the presence or absence of *App* cell culture supernatant during 48 hours. 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 follow by sonication (8 × 10 sec pulses on ice, with cooling intervals of 15 seconds) (Sonifier S-450A, Branson, Danbury, CT, USA). Then, total cell protein concentrations were measured by a Bradford assay following the manufacturer's instructions (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). Cell lysates (uninfected cells, *App* supernatant treated cells, virus infected cells and cells treated with both) were sent to Kinexus Bioinformatics Corporation (Vancouver, BC, Canada) to perform the Kinex™ Antibody Microarray (KAM) (http://www.kinexus.ca/). KAM tracks both protein expression (with pan-specific antibodies) and phosphorylation (with phospho-site-specific antibodies). The phosphorylation sites detected with the phospho-site antibodies are those corresponding to the human sequences. In addition, KAM consider as significant a Z ratio of ±1.2-1.5. A negative value infers a decrease in expression or phosphorylation from the control sample, whereas a positive value infers an increase expression compared to the control.

Western blot assays

$4x10^6$ PAM cells and $5x10^5$ SJPL/MARC-145 cells were infected and incubated in the presence or absence of *App* cell culture supernatant. At 52 hours pi, total protein extracts were obtained and quantified as described above. Forty µg of total proteins from each samples were loaded using 4X laemmli buffer and were fractionated in denaturing conditions by electrophoresis on 10% (w/v) SDS-PAGE gels, then transferred onto a nitrocellulose membrane (Bio-rad, Hercules, CA, USA) using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-rad, Hercules, CA, USA). Membranes were blocked with TBS-Tween 20 containing 5% (w/v) BSA (Sigma-Aldrich, St. Louis, MO, USA) or 5% (w/v) non fat dry milk during 2-3 hours at room temperature. Subsequently, they were labelled with a 1:1,000 dilution of rabbit Cofilin antibody (# 3312, Cell Signaling Technology, MA, USA) and with 1:2,500 dilution of mouse monoclonal β-actin antibody (mAbcam 8226, Abcam Inc., MA, USA) and incubated at 4°C overnight. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Thermo scientific, IL, USA) and horseradish peroxidase-conjugated goat anti-mouse (Thermo scientific, IL, USA) at a dilution of 1:3,000 were used as secondary antibodies, respectively. The protein bands were visualized using the SuperSignal® West Dura Extended Duration Substrate (Thermo scientific, IL, USA) in the FUSION-FX Chemiluminescence System (Montreal Biotech Inc., QC, Canada). The same membranes were mild striped using the protocol described in Abcam web site (http://www.abcam.com/index.html?pageconfig=resource&rid=11353&source=pagetrap&viapagetrap=strippingforreprobing) and were re-probed with rabbit GAPDH monoclonal antibody (#5174, Cell Signaling Technology, MA, USA) to confirm equal loading and with the rabbit Phospho-Cofilin antibody (#3311, Cell Signaling Technology, MA, USA) both at a dilution of 1:1,000. Relative density of total cofilin and P-cofilin were calculated with the image processing program Image J. All experiments were repeated two times.

Immunofluorescence assay (IFA) for the detection of PRRSV antigen and F-actin

Cells were seeded in 8 well glass slide Nunc® Lab-Tek® Chamber Slide™ system (Sigma-Aldrich, St. Louis, MO, USA) and were fixed at 52 hours pi, during 30 minutes at room temperature, with a 4% paraformaldehyde (PFA) solution prepared as described previously

(9). Uninfected cells were used as negative control. The IFA assay was performed as describe previously (9). Briefly, the fixed cells were washed with a phosphate buffer saline solution (PBS) and were permeabilized during 10 minutes in a PBS solution containing 1% Triton X-100. Subsequently, they were washed with PBS-Tween 20 (0.02%) and incubated during 30 minutes with PBS containing 0.2% Tween 20 and 1% BSA. Then, cells were incubated with the α7 rabbit monospecific antiserum (a specific anti-N PRRSV protein antibody) diluted 1/200 at 4°C overnight (27). Finally, cells were washed and incubated in 1/160 dilution of anti-rabbit specific antiserum FITC conjugated (Sigma-Aldrich, St. Louis, MO, USA) and in 1/40 dilution of Alexa Fluor® 594 phalloidin (a high-affinity F-actin probe conjugated) (Invitrogen, Burlington, ON, Canada) during 30 minutes at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) as recommended by the manufacturer. PAM stained cells were visualized by confocal laser scanning microscopy (Olympus FV1000 IX81, Markham, ON, Canada). MARC-145 and SJPL cells were visualized using a DMI 4000B reverse fluorescence microscope. Images of these cells were taking with a DFC 490 digital camera and were analyzed using the Leica Application Suite Software, version 2.4.0 (Leica Microsystems Inc., Richmond Hill, Canada).

Cytochalasin D PRRSV replication inhibition

A total of $2x10^5$ PAM, $1x10^4$ MARC-145 and SJPL cells were infected using both protocols described above, where the *App* cell culture supernatant was replaced by 3µM of cytochalasin D (28) (Sigma-Aldrich, St. Louis, MO, USA). At 52 hours pi, PRRSV was tittered by the Kärber method (9, 24). Briefly, samples infected with PRRSV were subjected to three cycles of freeze-thaw and cellular suspensions were then clarified by low speed centrifugation at 1200g for 10 minutes. Serial 10-fold dilutions of supernatants were used to infect 96-well tissue culture plate of MARC-145 cells. Then, plates were incubated for 96 hours. Virus titers were expressed in tissue culture infectious dose 50 % per ml ($TCID_{50}$/mL). All experiments were repeated three times in duplicate.

App cell culture supernatant antiviral activity against other PRRSV strains

The PRRSV viruses used in this experiment were the PRRSV genotype I reference strain Lelystad virus (LV) and two PRRSV genotype II strains, NVSL strain and FMV09-11SS278. $1x10^4$ MARC-145 cells were infected with each virus using the protocol #1 described above. The infectious dose of each virus was calculated as described above. All experiments were repeated three times in duplicate.

Statistical analyses

A One-way ANOVA model, followed by Tukey's Multiple Comparison Test (GraphPad Prism Version 5.03 software) was used to establish if statistical significant differences existed between PRRSV infected and uninfected cells treated or not with the *App* cell culture supernatant in the cell viability and mortality tests. Moreover, the same test was employed to determine if PRRSV titers and β-actin mRNA relative expression in the presence or absence of cytochalasin D or/and *App* cell culture supernatant, respectively, were statistically significant. Two-way ANOVA model, followed by Bonferroni post-hoc tests (GraphPad Prism Version 5.03 software) was performed to determine if statistical significant differences exist between PRRSV infected and uninfected cells untreated or treated with *App* cell culture supernatant in the virus replication/transcription kinetic assays. Moreover, t test statistical analyses (unpaired t tests) were also performed in the PRRSV replication/transcription kinetic assay to compared *App* treated and untreated cells at all-time points with 4 hours pi. The same test was also used to determine if the type I and type II IFN mRNA relative expressions in PRRSV infected or uninfected cells treated or not with the *App* cell culture supernatant, were statistically different. Differences between experimental groups were considered statistically significant with a $P<0.05$.

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SUPPORTING INFORMATION

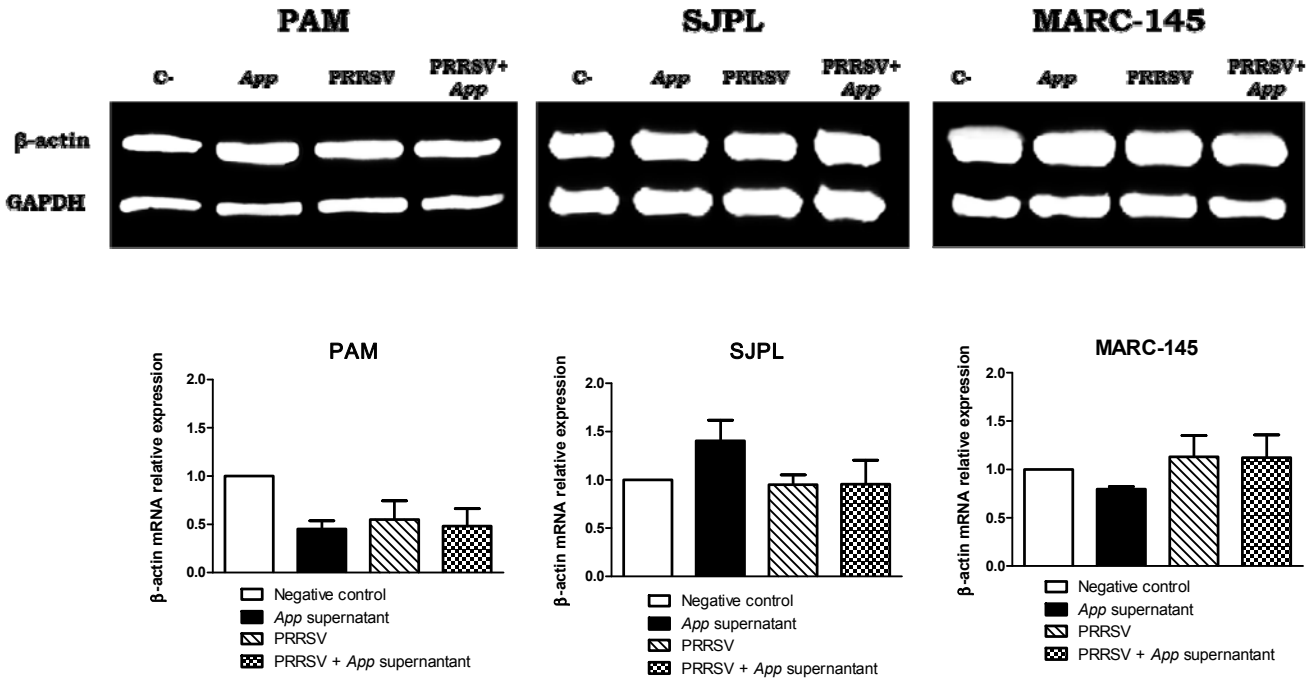

Figure S1: β-actin protein and mRNA expression in the presence of *App* cell culture supernatant.

At 52 hours pi cells were harvested or total RNAs were extracted to perform western blot and qRT-PCR assays, respectively. The β-actin protein expression level and mRNA relative expression were determined in PAM, SJPL and MARC-145 infected or uninfected cells treated or untreated with *App* cell culture supernatant.

GENERAL DISCUSSION

For more than two decades porcine reproductive and respiratory syndrom virus (PRRSV) has been the major concern of the swine industry, causing in United States an annual loss of approximately $560 million (1). This data clearly shows that current strategies to control PRRS are no longer efficient. To date, managent strategies are mainly: surveillance, whole herd depopulation and repopulation, herd closure and vaccination (25). There are two types of commercially available vaccines, the modified-live virus (MLV) vaccines and the killed-virus vaccines (24, 26), which cannot prevent PRRSV infection. Other studies have focused in to find anti-PRRSV specific drugs (27-29, 31, 33) without success, since there are no commercially available antiviral drugs to control or eradicate PRRS.

To the best of our knowledge, Lévesque and colleagues recently discovered which constitutes the first bacterial culture supernatant with antiviral properties against PRRSV (37). This antiviral activity is present in the cell culture supernatant of the *Actinobacillus pleuropneumoniae* (*App*) mutant (MBHPP147) of the S4074 serotype 1 reference strain, which produces non-active ApxI and ApxII toxins (*AppΔapxICΔapxIIC)*. The bacterial supernatant has a strong antiviral effect against PRRSV infection in the newly discovered SJPL permissive cell line, from monkey origin, but its effect is insignificant in MARC-145 infected cells. Following these findings, the goals of this project were 1) to demonstrate the *App* antiviral effect in the primary target cells of PRRSV: porcine alveolar macrophages (PAM) and 2) to identify the possible mechanisms used by *App* to inhibit PRRSV infection in PAM.

The results concerning the first objective of this project have been recently published ((37), Annexe I: Figures 3 and 4). In this study, it was clearly demonstrated that *App* cell culture supernatant inhibits PRRSV infection in PAM, which represents an important finding since PAM are the PRRSV natural host target cells.

Once demonstrated the *App* antiviral effect against PRRSV in PAM, the next step was to determine the specific mechanisms involved in this antiviral effect. For this purpose, firstly it was determined if *App* cell culture supernatant PAM treated cells were suitable for virus replication. Cell viability and mortality tests showed that PAM integrity or metabolism are not affected in the presence of *App* cell culture supernatant and that the *App* antiviral has no impact on cell death, which suggest that the bacterial antiviral mechanism is not via the

induction of cell cytotoxicity. Lévesque and collaborators also demonstrated, that in SJPL cells, the cell culture supernatant from the *App* mutant strain is not toxic to cells and that the SJPL cells were metabolically active and able to support other viral infections in the presence of *App* supernatant (37).

Types I and II interferon (IFN) are known as potent antiviral molecules against PRRSV (208, 229, 230). Therefore, in this study it was critical to determine if the bacterial supernatant was able to induce their expressions in PAM, even though it is well known that PRRSV has developed strategies to evade their antiviral effects in order to prolong its survival in the host (124, 129, 139, 211, 212, 221, 243). Results showed that mRNA relative expression of type I and II IFN in the presence of *App* cell culture supernatant is similar to that observed in untreated cells. Thus, these results suggest that *App* antiviral effect is not via the induction of IFN-α, IFN-β or IFN-γ. Lévesque and collaborators also observed that the bacterial supernatant does not induce the type I IFN expressions in the SJPL cell line (37). However an induction of type II IFN was detected, which suggest that *App* antiviral effect in SJPL cell line might be via the induction of IFN-γ (37). These results also suggest that the *App* antiviral action mechanism, in PAM and SJPL cells, might be different.

All stages during PRRSV replication cycle are dependent on host machinery. For instance, PRRSV entry is via receptor-mediated endocytosis and this process was demonstrated to be microfilament-dependent (13, 23); PRRSV uncoating is known to be dependent on acidic pH of the early endosomes and are also involved cellular proteases (13, 181, 182); PRRSV genome replication/transcription is believed to be produced in autophagosome-like double-membrane vesicles (184, 185). Furthermore, in order to elucidate the possible *App* supernatant antiviral mechanism it was developed a strategy to determine if the bacterial supernatant inhibits PRRSV replication cycle before or after viral genome replication / transcription in PAM, SJPL and MARC-145 cells. SJPL and MARC-145 cells were included in this study because 1) the *App* antiviral mechanism in these two cell lines is also unknown and 2) to compare their results with those obtained in PAM cells will allow to better understand the mechanism of action of the bacterial supernatant. Results clearly showed that the bacterial antiviral inhibits PRRSV infection in PAM and in SJPL, prior the first cycle of PRRSV

genome replication/transcription, which suggests that *App* antiviral activity possibly occurs during PRRSV entry, uncoating or genome replication/transcription stages.

It was demonstrated by Garcia-Cuellar and colleagues that a 24 kDa cloned Zn-metalloprotease which is secreted by *App* is able to degrade actin protein *in vitro* (84). On the other hand, different studies have revealed the important role of actin cytoskeleton on PRRSV infection (13, 14, 23, 164). Following these findings, it was hypothesized that *App* actin cytoskeleton modulation maybe is responsible for the antiviral effect against PRRSV. A proteomic study was performed using the KINEX™ Microarray, which revealed that cofilin, a protein implied in actin signalling pathway, was modulated, which suggested a possible involvement of actin filaments (F-actin) in *App* antiviral mechanism.

Cofilin has two statuses: unphosphorylated and phosphorylated, where only the active (unphosphorylated) cofilin is able to bind F-actin and promote polymerization and depolymerisation (243). Western blot analyses revealed that in PAM infected cells treated with *App* cell culture supernatant there was more active cofilin than in the others experimental groups. However, the western blot test showed an increase of inactive cofilin in MARC-145 infected cells in the presence of *App* supernatant. Since results obtained with MARC-145 and PAM cells are opposite and it was well demonstrated that in MARC-145 cells the *App* antiviral effect is insignificant compared to PAM cells (37), the data obtained highly suggest that cofilin it is probably involved in *App* antiviral effect and that a modulation in F-actin exists.

The immunofluorescence assay (IFA) revealed a marked F-actin fluorescence intensity diminution in *App* cell culture supernatant treated cells, but was markedly reduced in PAM infected cells treated with the bacterial antiviral. A previous study suggested that a negative correlation between the F-actin expression level and PRRSV infection exists (23), indicating that probably PRRSV decreases F-actin to favour its infection. Therefore, it is possible to conclude that PRRSV establishes the F-actin quantities needed for its infection, but when certain low and high thresholds are exceeded PRRSV infection is inhibited. For that reason, it is believed that when PRRSV PAM infected cells are treated with *App* cell culture supernatant there is a detectable increase in active cofilin, thus a considerable F-actin depolymerisation

causing PRRSV infection inhibition. Interestingly, similar F-actin fluorescence intensity was obtained between the *App* supernatant treated and untreated MARC-145 cells, which suggest that F-actin depolymerisation phenomenon is involved in *App* antiviral effect.

Cytochalasin D, a drug that destabilizes actin filaments, has been extensively used to determine the role of actin cytoskeleton in many cellular processes and in viral pathogenesis (13, 23, 245-247). In order to confirm if actin cytoskeleton is implied in *App* supernatant antiviral effect, the viral titer was determined in the presence of the drug and it was compared to those obtained in the presence of the bacterial supernatant. Results showed that the drug inhibits PRRSV infection similarly to *App* cell culture supernatant in PAM, SJPL and MARC-145 cells ((37) and Annexe I: Figure 4), which confirmed that the observed F-actin depolymerisation phenomenon is involved in *App* cell culture supernatant antiviral effect. Interestingly, in MARC-145 cells no PRRSV infection inhibition was detected with the drug, as happens with the bacterial culture supernatant. This result is contradictory to those reported in the literature (23), where it was demonstrated that smaller drug doses (1-2 μM) than the dose used in this study (3 μM) were able to inhibits PRRSV infection in MARC-145 cells. Thus, it is believed that the Canadian PRRSV strain used in this study probably does not need the actin cytoskeleton to successful infect this cell line, which can explains why *App* cell culture supernatant has almost no effect on PRRSV infection. Overall, these results highly suggest that PRRSV inhibition in PAM is probably during the entry by endocytosis because before genome replication and transcription, the entry is the only stage, according to the literature (13, 23), that is dependent on actin cytoskeleton.

In conclusion, our results suggest that *App* cell culture supernatant antiviral activity probably increases F-actin depolymerisation by the activation of cofilin in PAM infected cells and subsequently this phenomenon can affects PRRSV entry by endocytosis. Further studies need to be conducted in order to discover the molecular bases of the *App* antiviral effect in PAM and to find the molecule (s) present in *App* cell culture supernatant responsible for this antiviral activity.

GENERAL CONCLUSION

Because PRRSV has become the major concern of pig producing countries, the discovery of the strong *App* cell culture supernatant antiviral effect against PRRSV in the main *in vivo* target cells of PRRSV, PAM, represents a new hope for the porcine industry worldwide. Moreover, the uncovered *App* antiviral mechanism yielded some interesting findings about PRRSV cellular infection mechanism in PAM and MARC-145 cells. In addition, the *App* antiviral effect in MARC-145 cells was showed to be PRRSV strain dependant, while in SJPL cell line was observed to be RNA viruses dependent, which demonstrated that *App* cell culture supernatant is a promising antiviral that can lead to the development of prophylactic or therapeutic specific drugs against others viral diseases.

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ANNEXES

ANNEXE I: ***Actinobacillus pleuropneumoniae*** **possesses an antiviral activity against porcine reproductive and respiratory syndrome virus.**

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AUTHOR CONTRIBUTIONS

This article includes the results related to the first objective of my project that is to demonstrate the *App* cell culture supernatant antiviral effect against PRRSV in porcine alveolar macrophages (PAM) (Figures 3 and 4).

Then, my contribution to this study was in performing the PAM collecting technique from lungs, PRRSV antigen detection in PAM by immunofluorescence assay and PRRSV titer determination in PAM by the Kärber method.

ABSTRACT

Pigs are often colonized by more than one bacterial and/or viral species during respiratory tract infections. This phenomenon is known as the porcine respiratory disease complex (PRDC). *Actinobacillus pleuropneumoniae* (*App*) and porcine reproductive and respiratory syndrome virus (PRRSV) are pathogens that are frequently involved in PRDC. The main objective of this project was to study the *in vitro* interactions between these two pathogens and the host cells in the context of mixed infections. To fulfill this objective, PRRSV permissive cell lines such as MARC-145, SJPL, and porcine alveolar macrophages (PAM) were used. A pre-infection with PRRSV was performed at 0.5 multiplicity of infection (MOI) followed by an infection with *App* at 10 MOI. Bacterial adherence and cell death were compared. Results showed that PRRSV pre-infection did not affect bacterial adherence to the cells. PRRSV and *App* co-infection produced an additive cytotoxicity effect. Interestingly, a pre-infection of SJPL and PAM cells with *App* blocked completely PRRSV infection. Incubation of SJPL and PAM cells with an *App* cell-free culture supernatant is also sufficient to significantly block PRRSV infection. This antiviral activity is not due to LPS but rather by small molecular weight, heat-resistant *App* metabolites (< 1 kDa). The antiviral activity was also observed in SJPL cells infected with swine influenza virus but to a much lower extent compared to PRRSV. More importantly, the PRRSV antiviral activity of *App* was also seen with PAM, the cells targeted by the virus *in vivo* during infection in pigs. The antiviral activity might be due, at least in part, to the production of interferon γ. The use of *in vitro* experimental models to study viral and bacterial co-infections will lead to a better understanding of the interactions between pathogens and their host cells, and could allow the development of novel prophylactic and therapeutic tools.

INTRODUCTION

Respiratory disease in pigs is common in modern pork production worldwide and is often referred to as porcine respiratory disease complex (PRDC) [1]. PRDC is polymicrobial in nature, and occurs following infections with various combinations of primary and secondary respiratory pathogens. There are a variety of viral and bacterial pathogens commonly associated with PRDC including porcine reproductive and respiratory syndrome virus (PRRSV) and *Actinobacillus pleuropneumoniae* (*App*) [1]. Both are considered pathogens of major importance or relevance for the pig industry [1]. Furthermore, bacterial-viral co-infections can exacerbate the pathogenicity of respiratory pig diseases [1]. For example, co-infections with *Mycoplasma hyopneumoniae* and swine influenza virus (SIV) exhibited more severe clinical disease [2], PRRSV and *Streptococcus suis* co-infection experiments confirmed that PRRSV predisposes pigs to *S. suis* infection and bacteremia [3] and increases the virulence of PRRSV in pigs [4], *M. hyopneumoniae* infection increases effectiveness of PRRSV infection and lesions [5], and PRRSV infection was able to accelerate *Haemophilus parasuis* infection and loads [6]. Those studies on co-infections principally looked at the macroscopic lesions and at the clinical signs. Only a few recent studies are investigating more closely the direct interactions and mechanisms involved between the pathogens. As an example, Qiao and collaborators showed that PRRSV and bacterial endotoxin (LPS) act in synergy to amplify the inflammatory response of infected macrophages [7]. Thus, it is crucial to develop new *in vitro* models to investigate in more details the mechanistic and the interactions involved in polymicrobial infections.

Porcine reproductive and respiratory syndrome (PRRS) is the most economically devastating viral disease affecting the swine industry worldwide [8]. The etiological agent, PRRSV, possesses a RNA viral genome with ten open reading frames [8-10]. PRRSV virulence is multigenic and resides in both the non-structural and structural viral proteins. The molecular characteristics, biological and immunological functions of the PRRSV structural and non-structural proteins and their involvement in the virus pathogenesis were recently reviewed [8]. The disease induced by PRRSV 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 stillborn, mummified and weak-born pigs) [11,12] and respiratory

problems in pigs of all ages associated with a non-specific lymphomononuclear interstitial pneumonitis [11-13].

App is the causative agent of porcine pleuropneumonia, a severe and highly contagious respiratory disease responsible for major economic losses in the swine industry worldwide [14]. The disease, transmitted by aerosol or by direct contact with infected pigs, may result in rapid death or in severe pathology characterized by hemorrhagic, fibrinous, and necrotic lung lesions. Exposure to the organism may lead to chronic infection such that animals fail to thrive; alternatively, they survive as asymptomatic carriers that transmit the disease to healthy herds. Many virulence factors of this microorganism have been well characterized [14-16]. To date, fifteen serotypes of *App* based on capsular antigens have been described [17,18]. The prevalence of specific serotypes varies with geographic region [17].

Recent advances in pathogen detection methods allow better understanding of interactions between pathogens, improve characterization of their mechanisms in disease potentiation and demonstrate the importance of polymicrobial disease [1]. In the present study, the *in vitro* interactions between PRRSV and *App* in PRRSV permissive cell models were investigated. Thus, MARC-145 cells, SJPL cell line and pulmonary alveolar macrophages (PAM) were used in this study since they have been shown previously to be permissive to PRRSV infection and replication [8,19]. Results indicate that *App* possesses a strong antiviral activity against PRRSV *in vitro.*

RESULTS

PRRSV infection effect on *App* bacterial adherence

Bacterial adherence of *Appwt* and *AppΔapxIΔapxIIC* to PRRSV-infected and non-infected SJPL and MARC-145 cells was compared (Figure 1). Prior infection of both cell types with PRRSV did not significantly affect the adhesion of neither *Appwt* nor *AppΔapxIΔapxIIC* strain.

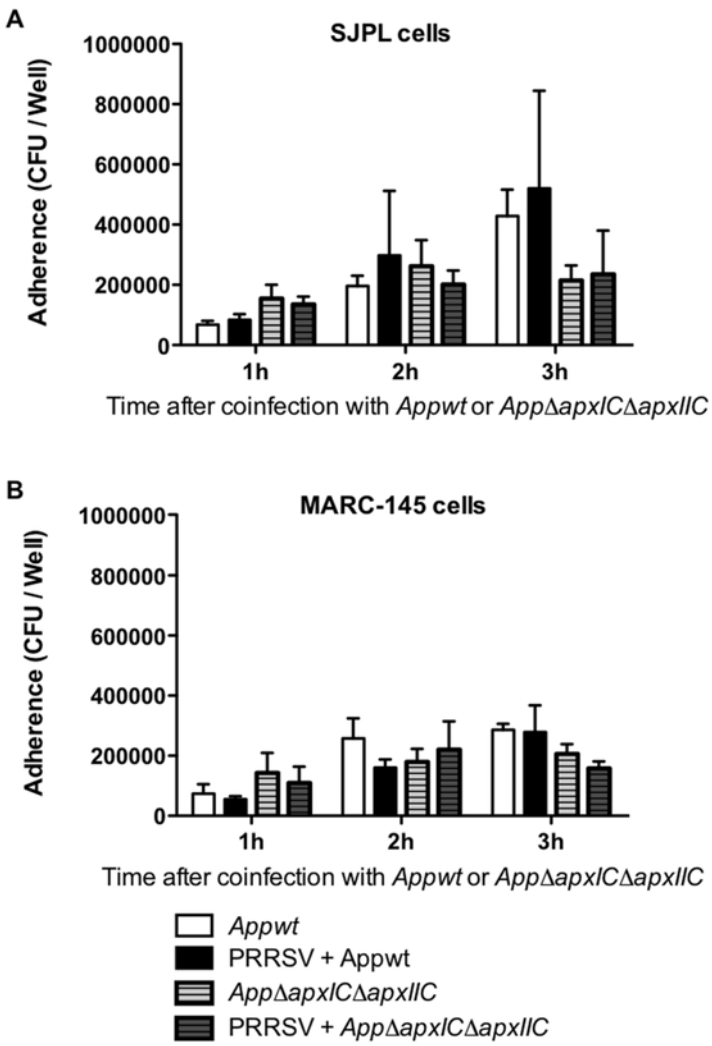

Figure 1: Bacterial adherence over time of *Appwt* or *AppΔapxIΔapxIIC* in PRRSV co-infected SJPL and MARC-145 cells.

SJPL (A) and MARC-145 (B) cells were infected with or without PRRSV at an MOI of 0.5 during 72 hours, and then cells were co-infected with *Appwt* or *AppΔapxIΔapxIIC* at an MOI of 10. Bacterial adherence was measured in CFU per well after 1, 2 and 3 hours post bacterial infection as described in Auger *et al*., 2009 [20]. Values are presented as ± Standard Deviation (SD). No statistical significance was obtained following two-away ANOVA analysis. All experiments were repeated 3 times.

Impact of *App* and PRRSV co-infection on cell cytotoxicity

Auger *et al*. 2009 [20] have previously published that SJPL cell death induced by *App* occurs through necrosis and not apoptosis. Consequently, based on this previous report, only a cytotoxicity experiment was performed in order to verify if PRRSV infection increases the cytotoxicity of *App*. Moreover, this assay was done to confirm that inactivation of the toxins ApxI and ApxII in the mutant *AppΔapxIΔapxIIC* reduces cell death seen with *Appwt* strain. Thus, LDH cytotoxicity assays to detect cell death were performed on cells infected with PRRSV for 72 hours and then co-infected with *Appwt* strain or *AppΔapxIΔapxIIC*. As shown in Figure 2, the cytotoxic activity of *Appwt* was higher in both cell lines after 2 hours of incubation, around 36% in SJPL cells (Figure 2A) and around 14% in MARC-145 cells (Figure 2C) compared to the one of *AppΔapxIΔapxIIC* mutant after 6 hours of incubation, which was less than 15% in SJPL cells (Figure 2B) and around 7% in MARC-145 cells (Figure 2D). As expected, the *AppΔapxIΔapxIIC* mutant is markedly less cytotoxic than the parental strain *Appwt*. Thus, *AppΔapxIΔapxIIC* mutant allows much longer incubation periods with cells and facilitate *in vitro* observation. Furthermore, co-infection with PRRSV and *AppΔapxIΔapxIIC* increased SJPL and MARC-145 cells death compared to *App* single infection (Figure 2B and D, respectively), showing an additive cytotoxicity effect of PRRSV and *AppΔapxIΔapxIIC*. Because of its markedly reduced cytotoxicity, the *AppΔapxIΔapxIIC* was used for all the subsequent experiments.

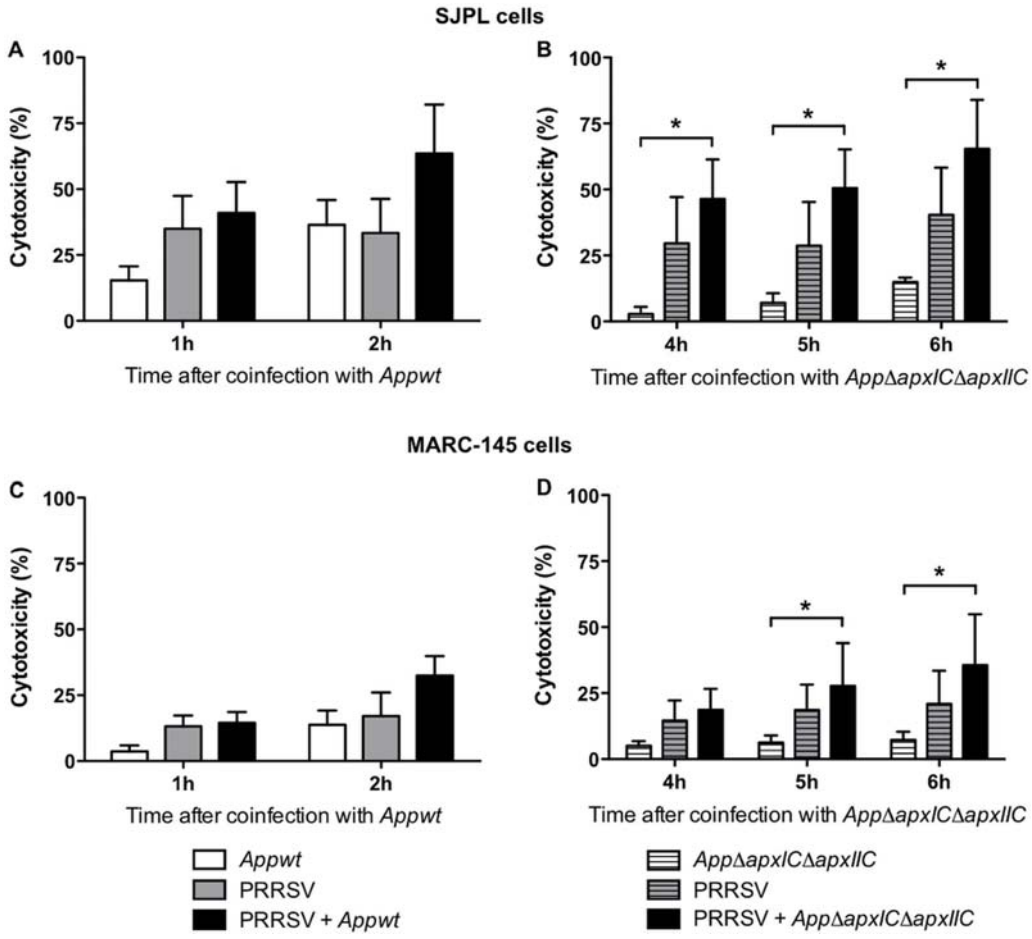

Figure 2: Cytotoxicity over time of *Appwt* or *AppΔapxIΔapxIIC* in PRRSV co-infected SJPL and MARC-145 cells.

SJPL (A and B) and MARC-145 cells (C and D) were infected with or without PRRSV at an MOI of 0.5 during 72 hours, and then cells were co-infected with *App* (for 1 or 2 hours) (A and C, respectively) or with *AppΔapxIΔapxIIC* (for 4, 5 and 6 hours) (B and D, respectively) at an MOI of 10. Cytotoxicity was measured in % using lactate dehydrogenase (LDH) CytoTox assay [20]. Values are presented as ± Standard Deviation (SD). Two-away ANOVA analysis was used to obtain statistical data. * $P<0.05$. All experiments were performed 3 times.

App effects on PRRSV infection

In SJPL cells, co-infection with *AppΔapxIΔapxIIC* and PRRSV shows absence of PRRSV N viral protein detection by IFA compared to control where SJPL cells were infected with PRRSV alone (Figure 3A) suggesting an inhibition of PRRSV infection and/or replication

(Figure 3B). MARC-145 cell line was used to compare results obtained with SJPL cell line since MARC-145 cells are the most common cells used during *in vitro* PRRSV studies. Interestingly, results were different between the two cell lines. In PRRSV infected MARC-145 cells, only a small reduction of cells expressing the PRRSV N protein was observed following a co-infection with *AppΔapxIΔapxIIC* (Figure 3G). Thus, SJPL cells were qualitatively more responsive to the *App* antiviral affect than MARC-145 cells. Moreover, since SJPL cells were recently shown to be from monkey origin [21] and not from swine as first described [22], evaluation of the antiviral activity of *App* was tested in a porcine relevant cell model, the PAM cells. Co-infection with *AppΔapxIΔapxIIC* and PRRSV in PAM cells also presented total absence of PRRSV N protein detection (Figure 3L), as in SJPL cells (Figure 3B), suggesting that *AppΔapxIΔapxIIC* can also inhibits PRRSV in PRRSV's *in vivo* porcine target cells, the porcine alveolar macrophages. Incubation with UV-inactivated *AppΔapxIΔapxIIC* bacteria after PRRSV infection allowed the detection of N proteins of PRRSV by IFA in all cell types (Figure 3C, 3H and 3M) showing that UV-inactivated bacteria were not able to block PRRSV infection. Interestingly, the bacteria-free culture supernatant of *AppΔapxIΔapxIIC* also effectively blocked PRRSV infection in SJPL and PAM cells (Figure 3D and 3N, respectively). A weak inhibition was observed in MARC-145 cells (Figure 3I). pH did not vary between all the tested conditions, being stable at around 7.3 ± 0.1. The active metabolites present in the culture supernatant did not seem to be *App* LPS (Figure 3E, 3J and 3O) nor peptidoglycan fragments (assayed with NOD1 or NOD2 ligands) (Figure S1D and S1F, respectively). Dilutions of *AppΔapxIΔapxIIC* supernatant showed a dose-dependent effect on PRRSV's detection by IFA. A 1:2 dilution resulted in twice as much PRRSV N protein when observed with IFA (data not shown). The loss of antiviral activity of *AppΔapxIΔapxIIC* supernatant was observed with 1:10, 1:20 and 1:40 dilutions.

PRRSV titers were measured to confirm IFA observations and to quantify the inhibitory effect of *AppΔapxIΔapxIIC* on PRRSV infection. SJPL, MARC-145 and PAM cells were infected or treated as described previously. In SJPL cells after 72 hours post PRRSV infection, viral titer obtained was 6.25 log10 $TCID_{50}$/ml (Figure 4A), in MARC-145 cells, was 7.6 log10 $TCID_{50}$/ml (Figure 4B) and in PAM cells, 6.0 log10 $TCID_{50}$/ml (Figure 4C). Co-infection with *AppΔapxIΔapxIIC* or treatment with its culture supernatant blocked completely PRRSV

replication (P<0.01) in SJPL cells (Figure 4A). But in MARC-145 cells, their antiviral effect on PRRSV replication was markedly less efficient. More specifically, in MARC-145 cells, PRRSV titers were 4.9 log 10 $TCID_{50}$/ml (which correspond to a 751 fold decrease compared to PRRSV non-treated infected cell) and 6.5 log10 $TCID_{50}$/ml (which correspond to a 19 fold decrease compared to PRRSV non-treated infected cell) for *AppΔapxIΔapxIIC* (P<0.01) and its cell-free culture supernatant (P<0.05) treated cells, respectively (Figure 4B). In PAM cells, results obtained with PRRSV's titration showed that live *AppΔapxIΔapxIIC* completely blocked PRRSV replication (P<0.001) and that its culture supernatant significantly inhibits PRRSV infection in PAM, reducing its amount of infectious virions to 2.9 log10 $TCID_{50}$/ml (P<0.001 compared to PRRSV infection at 10^6 TCID50/mL) which correspond to a 1250 fold decrease (Figure 4C). Stimulation of the cells with *App* purified LPS or co-infection with UV inactivated bacteria did not have any effect on PRRSV titer in all cell types (Figure 4A, 4B and 4C). Those results confirm the IFA data obtained previously. In addition, it is important to note that inhibition in PAM is total with live *AppΔapxIΔapxIIC* as observed previously in SJPL cells and below PRRSV inoculum when treated with *AppΔapxIΔapxIIC* cell culture supernatant. Thus, those results indicate that *AppΔapxIΔapxIIC* antiviral effect against PRRSV can be observed not only in SJPL cells but also in porcine alveolar macrophages.

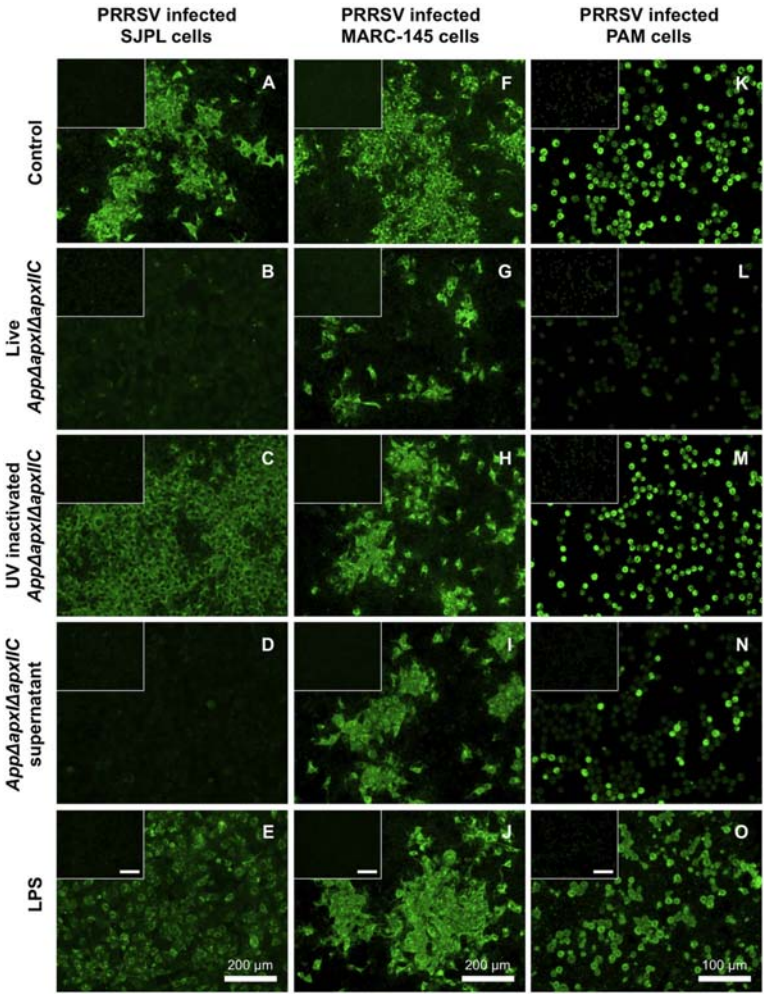

Figure 3: PRRSV antigen detection in SJPL, MARC-145 and PAM cells co-infected with *AppΔapxIΔapxIIC*.

PRRSV N protein revealed by IFA in SJPL (A-E), MARC-145 (F-J) and PAM cells (K-O) were infected with PRRSV at an MOI of 0.5 for 4 hours (A, F and K) then co-infected with live *AppΔapxIΔapxIIC* at an MOI of 10 (B, G and L), or with UV inactivated *AppΔapxIΔapxIIC* at an MOI of 10 (C, H and M), or with *AppΔapxIΔapxIIC* supernatant (D, I and N) or treated with LPS 4µg/ml (E, J and O) for 48 hours. Inserts are negative control where cells were not infected with PRRSV. White scale bar represents 200 µm for SJPL and MARC-145 cells, and 100 µm for PAM cells. Pictures were taken at 100X magnification for SJPL and MARC-145 cells, and 200X for PAM cells.

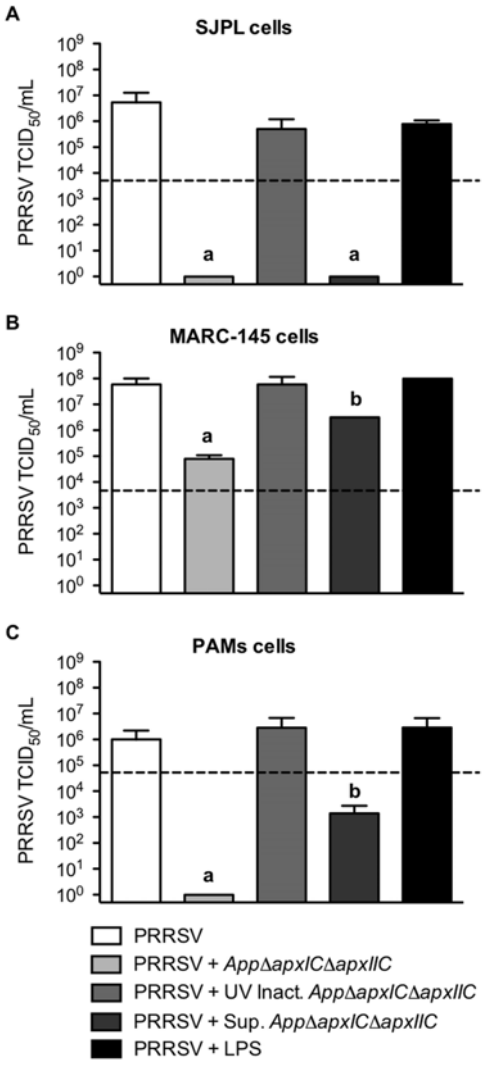

Figure 4: PRRSV titer in *App* treated SJPL, MARC-145 and PAM cells.

SJPL (A), MARC-145 (B) and PAM (C) cells were infected with PRRSV MOI of 0.5 for 4 hours and then co-infected with *AppΔapxIΔapxIIC* MOI of 10, or with UV inactivated *AppΔapxIΔapxIIC* MOI of 10, or treated with LPS (4 μg/ml) or culture supernatant of *AppΔapxIΔapxIIC* for 48 hours. PRRSV titer was determined on MARC-145 cells by the Kärber method. Values are presented as ± Standard Deviation (SD). One-away ANOVA analysis was used to obtain statistical data. When bars within a cell type are labeled with superscripts letters, it indicates that these sets of data are statistically different from the other bars ($P < 0.05$).

Fractionation of cell culture supernatant of *AppΔapxIΔapxIIC*

Fractionation of the cell culture supernatant of *AppΔapxIΔapxIIC* indicated that the ihnibitory effect on PRRSV infection is mediated by small *App* metabolite(s) weighting < 1 kDa (Figure 5C). The same results were obtained with all small fractions tested, < 3 (Figure S2D), 10 (data not shown) and 50 kDa (Figure S2F). Additionally, treatment at 56°C for 30 min of these low molecular weight *App* metabolite(s) did not inactivate their ihnibitory effect on PRRSV infection and/or replication in SJPL cells, showing that those *App* antiviral metabolites are heat-resistant (data not shown).

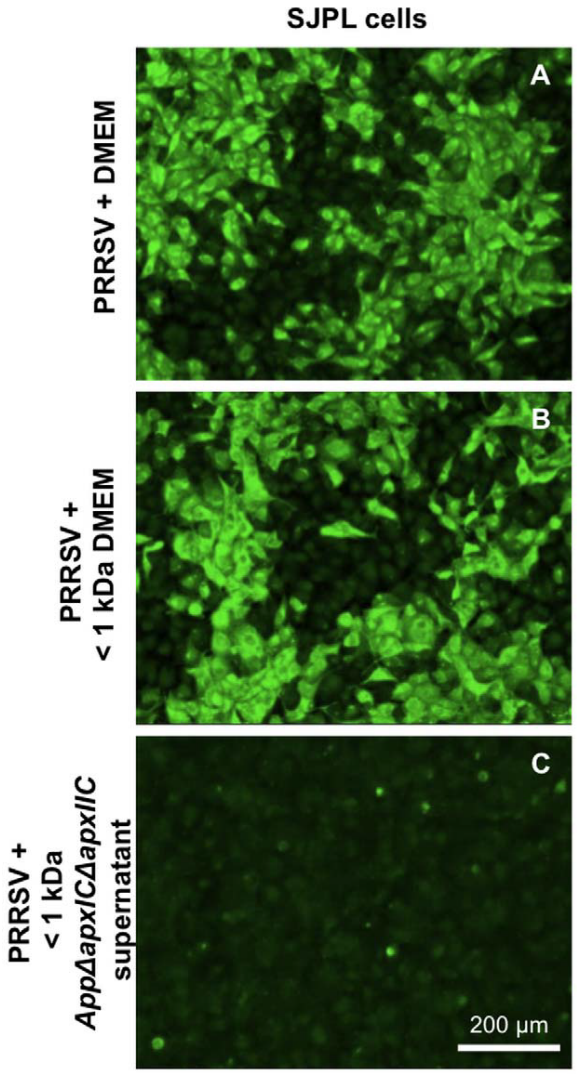

Figure 5: *AppΔapxIΔapxIIC* cell culture supernatant < 1 kDa fraction antiviral activity against PRRSV.

Detection of the N viral protein in PRRSV infected SJPL cells by immunofluorescence. SJPL cells were infected with 0.5 MOI of PRRSV for 4 hours then incubated with DMEM culture medium alone (DMEM) (A) or either a DMEM culture medium fraction of < 1 kDa (DMEM < 1 kDa) (B) or a *AppΔapxIΔapxIIC* cell culture supernatant < 1 kDa fraction (*App* < 1 kDa) (C) added to complete SJPL culture medium for 48 hours. White scale bar represents 200 µm. Pictures were taken at 100X magnification.

Antiviral efficacy of *AppΔapxIΔapxIIC* cell culture supernatant against several other viruses

Since *AppΔapxIΔapxIIC* cell culture supernatant inhibits PRRSV replication, other viruses were tested in order to verify if this inhibition is virus specific or if it is a general antiviral effect. First, the SJPL cells permissivity was tested in regards to different DNA genome viruses such as: BAV3, BHV-1, BHV-4, CPV, EHV-1, and PCV2; as well as RNA genome viruses such as: BVDV-1, Influenza H1N1, and Influenza H3N2. BAV3, BHV-1, EHV-1, BVDV-1, Influenza H1N1, and Influenza H3N2 viruses were able to infect and replicate in SJPL cells (Table 1). Thus, treatment with *AppΔapxIΔapxIIC* culture supernatant was performed after infection with those viruses in SJPL cells, to verify its spectrum of antiviral activity. Overall, 50% of the viruses tested that are able to replicate in SJPL cells (excluding

PRRSV) were inhibited by *AppΔapxIΔapxIIC* cell culture supernatant. Those inhibited viruses were: EHV-1, Influenza H1N1 and H3N2. However, it is important to note that the inhibition of PRRSV replication observed following treatment with *AppΔapxICΔapxIIC* supernatant was significantly higher compared to than the inhibition observed against EHV-1, Influenza H1N1 and H3N2 (Table 1). These results are important because they indicate that SJPL cells were still able to allow the replication of several viruses in the presence of *AppΔapxIΔapxIIC* cell culture supernatant, indicating that the SJPL cells are still metabolically active and fit for viruses' replication.

Table 1: Antiviral activity of *AppΔapxIΔapxIIC* supernatant against several animal DNA and RNA viruses in SJPL infected cells.

Table 1. Antiviral activity of *AppΔapxIΔapxIIC* supernatant against several animal DNA and RNA viruses in SJPL infected cells.

Viruses	Virus titer		Relative virus replication inhibition
	Without *AppΔapxIΔapxIIC*	With *AppΔapxIΔapxIIC*	
	($TCID_{50}$ log10 ± SD)		
DNA genome			
BHV-4	Neg	-	-
CPV	Neg	-	-
PCV2	Neg	-	-
BAV3	2.75±0.35	2.88±0.18	0.74±2.45
BHV-1	4.54±0.48	4.42±0.59	1.32±5.75
EHV-1	5.00±0.71	3.75±0.35$^{\mathcal{F}}$	17.78±6.17
RNA genome			
BVDV-1	4.38±0.18	4.25±0.35	1.35±2.45
H1N1	5.40±0.57	4.23±0.50*	14.8±5.75
H3N2	4.85±0.50	3.82±0.45**	10.72±4.68
PRRSV	5.44±0.56	1.61±0.59***	6760.83±6.46^{a}

All experiences were performed at least 2 times.
Statistical *P* value compared to *AppΔapxIΔapxIIC* untreated cells: $^{\mathcal{F}}P = 0.15$.
Statistically significative compared to *AppΔapxIΔapxIIC* untreated cells: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.
Statistically significative compared to other viruses: $^{a}P<0.01$.
doi:10.1371/journal.pone.0098434.t001

Effect of *AppΔapxIΔapxIIC* cell culture supernatant on the mRNA level of type I and type II IFNs

Since the levels of mRNA expression of type I (IFNα and IFNβ) and type II (IFNγ) interferons are known to be implicated in the cellular antiviral effect against PRRSV [23-26], mRNA levels of those cytokines were measured by qRT-PCR (Figure 6). No modulation of IFNα was observed in any of the tested conditions, including the Poly I:C control. This observation was

also previously made by Provost *et al*., 2012 [19]. PRRSV infection in SJPL cells significantly increased IFNβ levels compared to mock infected cells, as previously described in Provost *et al*., 2012 [19]. Treatment with *AppΔapxICΔapxIIC* supernantant alone induced a significant increase of IFNβ mRNA compared to mock infected cells, but co-treated cells did not showed a significant increase compared to mock infected cells. PRRSV infection in SJPL cells did not modulate IFNγ mRNA levels. However, treatment with *AppΔapxICΔapxIIC* supernantant alone or as co-treatment significantly increased IFNγ mRNA compared to mock infected SJPL cells.

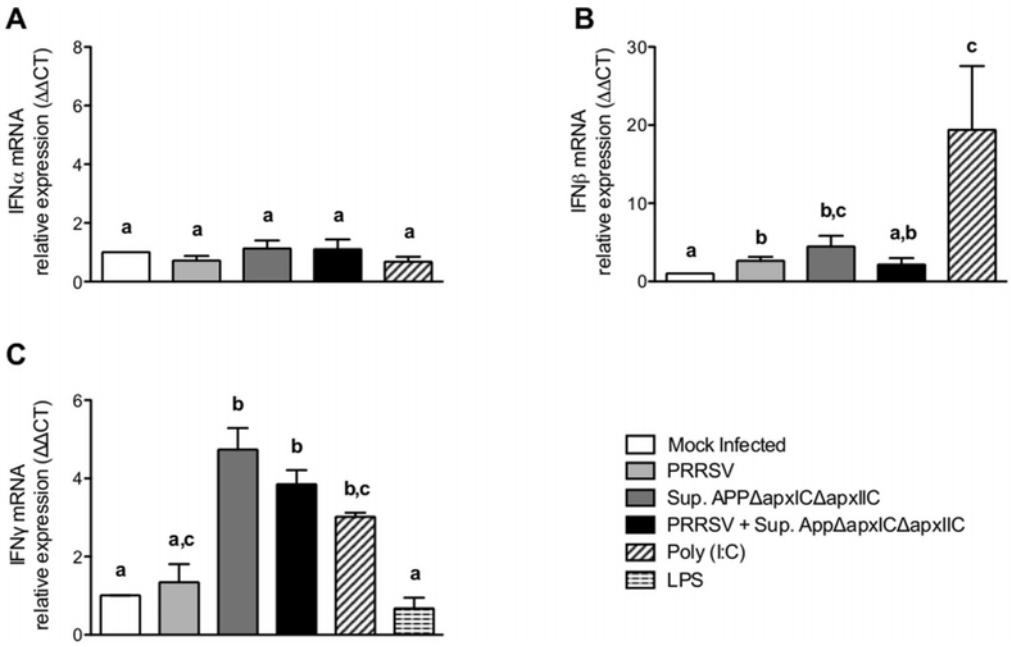

Figure 6: *AppΔapxIΔapxIIC* cell culture supernatant and PRRSV effects on mRNA quantification of type I (IFNα, IFNβ) and type II (IFNγ) interferons.

qRT-PCR results expressed in relative expression (ΔΔCT) for IFNα (A), IFNβ (B) and IFNγ (C) in SJPL cells. The cells were mock infected or infected with 0.5 MOI of PRRSV for 4 hours then treated without or with *AppΔapxICΔapxIIC* cell culture supernatant for 48 hours. Poly (I:C) and LPS were used as positive controls. Data labeled with superscripts of different letters indicates that these sets of data are statistically different ($P < 0.05$).

DISCUSSION

Many studies have previously shown that respiratory viral infections can increase bacterial adherence to cells. For example, influenza A infection increases adherence of *Streptococcus*

pyogenes to MDCK cells [27], rhinovirus infection increases adherence of *Streptococcus pneumoniae* to cultured human airway epithelial cells [28], and respiratory syncytial virus (RSV), human parainfluenza virus 3 (HPIV-3), and influenza virus increase the adherence of *Haemophilus influenzae* and *S. pneumoniae* to respiratory epithelial cells [29]. However, in the present study, no modulation of *App* adherence was observed when cells were infected with PRRSV.

Appwt induced, as expected, a high percentage of cytotoxicity in SJPL cells (Figure 2). Its derivative, *AppΔapxIΔapxIIC*, that is expressing the non-activated toxins ApxI and ApxII, showed a much lower cytotoxicity in SJPL cells. Furthermore, as previously described in Provost et al. 2012, PRRSV infection in SJPL cells induced a significant increase of cell death [19]. However, co-infection with PRRSV and *AppΔapxIΔapxIIC* did not result in a significant increase of cell death when compared to PRRSV infection alone, supporting that *AppΔapxIΔapxIIC* is less (if not) toxic to eukaryotic cells and that cytotoxicity is mainly caused by PRRSV in co-infected cells. Interestingly, this less toxic *App* mutant enables longer exposure in *in vitro* experiments and allowed us to observe *App*'s antiviral effect on PRRSV.

The antiviral effect of *AppΔapxIΔapxIIC* was first observed on SJPL cells co-infected with PRRSV (Figure 3). Subsequently, other results showed that the antiviral activity was also present in the bacterial supernatant and was not due to *App* purified LPS, nor NOD ligands, but probably to low molecular weight metabolites of < 1 kDa. Inhibition of PRRSV replication by *AppΔapxIΔapxIIC* is not generated by contact between bacterial and eukaryotic host cell, since it was also observed with *App* cell culture supernatant; thus without the presence of *App* bacterial cells. Furthermore, this antiviral effect is not only observed in SJPL cells but also in the PRRSV natural host target cells, i.e. PAM. This suggests that the antiviral action of *AppΔapxIΔapxIIC* can be efficient in different cell species and types. Viral inhibition in PAM cells was complete in presence of the bacteria *AppΔapxIΔapxIIC* and was partial when treated with its cell culture supernatant. Other combinations of treatments have been tested. Data obtained gave some information about the mechanism of the antiviral activity of *AppΔapxIΔapxIIC* supernatant. Overall, they suggested that *AppΔapxIΔapxIIC* supernatant's antiviral activity is not interfering with PRRSV attachment and entry. Other experiments are

currently in progress to further investigate by which mechanisms the *AppΔapxIΔapxIIC* supernatant is inhibiting PRRSV replication.

Despite the fact that MARC-145 and SJPL are of monkey origin, they are phenotypically distinct as demonstrated by our group in Provost *et al.* (2012) [19]. In this previous report, we demonstrated that SJPL and MARC-145 cells do not have the same division rate and that the development of the cytopathic effect (CPE) induced by PRRSV in SJPL cells was delayed compared to MARC-145 cells. Furthermore, the cytokine profiles after PRRSV infection were different between the two cell lines. These results suggested that PRRSV infection could be different in each. Thus, the difference in PRRSV infection between both cell lines could explain the difference observed for the *AppΔapxIΔapxIIC* supernatant antiviral activity.

Type I IFNs, produced by many cell types, are part of the innate immunity response [30]. Moreover, it is well known in the literature that type I IFNs are often part of the cellular response against viral infections, including PRRSV infections [23,25]. Results of this study showed that there is no modulation of IFNα mRNA levels. IFNβ mRNA levels were increased in PRRSV and in *AppΔapxIΔapxIIC* supernatant alone but no significant increase was observed in the PRRSV + *AppΔapxIΔapxIIC* supernatant condition when compared to mock infected cells. Thus, the impaired IFNβexpression following co-treatment might be due to PRRSV replication which might block IFN production induced by *AppΔapxIΔapxIIC* supernatant. Additionally, those results demonstrate that since PRRSV can inhibit type I IFN induction and signalling [31-34], antiviral activity induced by *AppΔapxIΔapxIIC* supernatant may not rely on its ability to induce IFNβ. However, this does not mean that IFNβ is not part of the antiviral activity of *AppΔapxIΔapxIIC* supernatant, since most viruses are still sensitive to type I IFNs.

Type II IFNγ, mainly produced by activated T cells and Natural Killer cells, is mostly responsible for adaptive Th1 response, which is part of cell-mediated immunity [35]. Furthermore, its implication in antiviral response against PRRSV was also demonstrated [24,26]. Nonetheless, IFNγ mRNA levels in SJPL cells were significantly increased by *AppΔapxIΔapxIIC* supernatant alone and in PRRSV + *AppΔapxIΔapxIIC* supernatant condition. This observation might give a clue by which cellular response *AppΔapxIΔapxIIC*

supernatant induces its antiviral effect; i.e. via the increased of IFNγ mRNA levels by the cell. However, it is important to mention that it is not known if SJPL cells possess IFNγ receptors, which are necessary for IFNγ mediated signalling. Further investigations are needed to confirm this hypothesis.

PRRSV can lead to persistent infections [36,37] and current PRRSV vaccines are not yet optimal, since they lack the ability to induce a strong immune response and since they do not provide complete immunity against homologous PRRSV infections (for review see [38,39]). Moreover, most PRRSV vaccines are live attenuated virus and thus present a safety issue; some vaccinated pigs were shown to produce shedding of virulent PRRSV particles [40]. Thus, it is important to further investigate new possible ways to control PRRSV infections. In that regards, an antiviral molecule or metabolite might be a good alternative to the currently used vaccines. Recently published studies showed few compounds that can inhibits PRRSV as glycosides, terpenoids, coumarins, isoflavones, peptolides, alkaloids, flavones, macrolides [41], N-acetylpenicillamine [42], cyclosporine A [43], sodium tanshinone IIA sulfonate [44], flavaspidic acid AB [45], Ribavirin [46], and morpholino oligomer [47], or compounds derived from plant as a polysaccharide isolated from *Achyranthes bidentata* [48] or a mushroom extract from *Cryptoporus volvatus* [49]. However, there is no commercially available antiviral drug against PRRSV on the market.

In conclusion, to the best of our knowledge, this is the first description of an *App* antiviral activity. This study might lead to the development of a new treatment against PRRSV derived from *App* cell culture supernatant. However, more investigations are needed to identify and/or purify the target metabolite(s) secreted by *App* before generating a possible new antiviral molecule against PRRSV. Moreover, since we have demonstrated that the antiviral effect of the metabolite(s) secreted from *App* is not only specific to PRRSV, but also effective against other RNA viruses, this antiviral activity might as well lead to a new antiviral treatment. For example, molecules such as Ribavirin, which is currently used against human respiratory syncytial virus (RSV) [50,51] and hepatitis C infection [52], was initially demonstrated to have a broad antiviral activity against animal viruses [53]. This study might therefore allow the development of a new antiviral molecule against PRRSV, but also against other viruses such as influenza.

MATERIALS AND METHODS

Cells

All cells products were ordered from Invitrogen Corporation GibcoBRL (Burlington, ON, CA) unless specified. MARC-145 cells, a subclone of African green monkey kidney MA104 cells, were grown in minimum essential medium (MEM) supplemented with 10% of foetal bovine serum (FBS) (Wisent Inc, St-Bruno, QC, Canada), 0.1 mM HEPES, 2 mM L-glutamine, 10 U/mL of penicillin, 10 µg/mL of streptomycin and 250 g/L antibiotic-antimitotic solution [54]. The SJPL cell line (St. Jude porcine lung epithelial cell) was provided by Dr. R.G. Webster (St. Jude Children's Hospital, Memphis, TN, USA) [22] and later was demonstrated to be from monkey origin [21]. This cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Wisent Inc), 1 mM sodium pyruvate, 2 mM L-glutamine, 1 µM MEM nonessential amino acid, 10 U/mL of penicillin, 10 µg/mL of streptomycin and 250 g/L antibiotic-antimitotic solution and, 100 mg/L gentamicin. Porcine alveolar macrophages (PAM) were harvested from lungs of 2 to 14 weeks old pigs as described previously [19]. Pigs were sacrificed following ethic protocol 12-Rech-1640 approved by our institutional ethic committee (Comité d'éthique de l'utilisation des animaux – CÉUA) following the guidelines of the Canadian Council on Animal Care. Briefly, an instillation of the lungs with PBS containing 10 units/mL penicillin, 10 µg/mL streptomycin and 100 mg/L gentamicin was realized. Then, phosphate buffer saline solution (PBS) was collected and PAM removed following low speed centrifugation. Cells were washed with DMEM medium complemented with 2 mM L-glutamine, 0,1 mM HEPES, 1 µM non-essential amino acids, 250 g/L amphotericin B (Wisent Inc), 10 units/mL penicillin, 10 µg/mL streptomycin and 100 mg/L gentamicin. Cells were then collected following low speed centrifugation and were resuspended in freezing medium (same as wash medium plus 20% foetal bovine serum and 10 % DMSO (Sigma-Aldrich, St-Louis, MO, USA)) and slowly frozen, than stored in liquid nitrogen until further utilization. PAM cells were cultured for 24 hours in complete DMEM prior to assay. All cells were cultured and infected at 37°C in 5% CO_2 atmosphere.

Bacterial and viral strains

The *App* strains used in this study were the S4074 serotype 1 reference wild type strain (*App*wt) and a mutant of this strain (MBHPP147) producing non-active ApxI and ApxII toxins (*AppΔapxICΔapxIIC)*, kindly provided by Ruud P.A.M. Segers (MSD Animal Health, Boxmeer, The Netherlands). *App* strains were cultured on brain heart infusion (BHI) broth and/or agar (Gibco) supplemented with 15 µg/ml nicotinamide adenine dinucleotide (NAD) at 37°C in 5% CO_2. The PRRSV strain used in this study was the Canadian genotype II reference strain IAF-Klop [55].

Adherence assay

For the adherence assay, 10^5 epithelial cells/well were seeded into 24 well-tissue culture plates (Sarstedt, Numbrecht, Germany) and incubated overnight (O/N). Cells were infected with PRRSV at 0.5 multiplicity of infection (MOI; virus particles or bacterial cells per cell). *App*wt and *AppΔapxIΔapxIIC* from an overnight culture grown at an OD_{600nm} of 0.6 were resuspended in complete cell culture medium to a concentration of 10^6 CFU/ml. One ml of either suspension was added to each well at an MOI of 10 after 72 hours PRRSV infection, and plates were incubated for 1, 2 or 3 hours. Non-adherent bacteria were removed by washing four times with Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco). Cells with adherent bacteria were released from the wells by adding 100 µl of 1X trypsin-EDTA (Gibco) and resuspended in 900 µl DPBS buffer. Serial dilutions were performed and poured on agar plates to determine the number of bacteria that adhered to the epithelial cells. Bacteria colonies were counted as colonies forming unit per well (CFU/well) as described by Auger *et al*., 2009 [20].

Cytotoxicity detection assay

For the cytotoxicity detection assay, 10^5 epithelial cells/wells were seeded into 24 well-tissue culture plates (Sarstedt) and incubated O/N. Cells were infected with PRRSV at 0.5 MOI. *Appwt* and *AppΔapxIΔapxIIC* from an overnight culture grown at an OD_{600nm} of 0.6 were resuspended in complete cell culture medium to a concentration of 10^6 CFU/ml. One ml of either suspension was added to each well at an MOI of 10 after 72 hours PRRSV infection,

and plates were incubated for 1 or 2 hours with *Appwt* or for 4, 5 and 6 hours with *AppΔapxIΔapxIIC*. The cellular cytotoxicity was determined using the lactate dehydrogenase (LDH)-measuring CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI) as described by the manufacturer. Noninfected cells were used as a negative control, while total cell lysate was used for the 100%-cytotoxicity positive control, since all LDH is released when cells are mechanically lysed. Optical densities were measured at 490 nm with a Power Wave X340 (Biotek Instruments Inc, Winooski, VT) microplate reader and used to calculate the percentage of cytotoxicity [55].

Immunofluorescence assay

The presence of PRRSV antigens in infected cells was determined by an immunofluorescence assay (IFA). Cells were infected or treated as described below. Following treatment and/or infections, cells were fixed with a 4% paraformaldehyde (PFA) solution prepared as previously described [19]. Mock-infected or non-treated cells were used as negative controls. After an incubation period of 20 minutes at room temperature, the PFA solution was removed and cells were washed three times with Phosphate buffer solution (PBS). Then, cells were incubated during 10 minutes at room temperature with a PBS solution containing 0.1% Triton X-100 for cell membrane permeabilization. After removing the Triton X-100 solution, cells were washed three times with a PBS-Tween 20 solution (PBS containing 0.02% Tween 20). Thereafter, cells were incubated 30 minutes with PBS containing 0.02% Tween 20 and 1% foetal bovine serum albumin. Then, the α7 rabbit monospecific antisera (anti-N PRRSV protein) [55] was diluted 1/100 in the blocking solution and added to the cells and incubated at 37°C for 90 minutes. Cells were then washed and incubated for 60 minutes with the blocking solution containing a 1/160 dilution of anti-rabbit specific antiserum FITC conjugated (Sigma). Finally, cells were visualized using a DMI 4000B reverse fluorescence microscope, image of the cells were taken with a DFC 490 digital camera and the images were analyzed using the Leica Application Suite Software, version 2.4.0 (Leica Microsystems Inc., Richmond Hill, Canada).

Antiviral activity of *AppΔapxIΔapxIIC* against PRRSV

Cells were infected with 0.5 MOI of PRRSV and incubated in DMEM without serum or other additives for 4 hours, then all non-attached virus were removed from the medium with soft washing step using PBS. Thereafter fresh medium was added. *AppΔapxIΔapxIIC* from an overnight culture grown at an OD_{600nm} of 0.6 were resuspended at an MOI of 10 in complete cell culture medium to a concentration of 10^6 CFU/ml. To obtain *AppΔapxIΔapxIIC* UV inactivated, resuspended *AppΔapxIΔapxIIC* at an MOI 100:1 were inactivated for 2 hours under UV light (315 nm) in a rocking petri dish and their inactivation was confirmed by plating on BHI-NAD. To obtain *AppΔapxIΔapxIIC* supernatant, resuspended *AppΔapxIΔapxIIC* at an MOI of 10 were centrifuged at 500 g for 15 minutes and harvested supernatants were passed through a 0.22 μm filter to remove all residual bacteria. Bacterial culture supernatants were further fractionated through ultrafiltration membranes with cut-off of 50, 10, 3 (Amicon Ultra-15, Millipore, Billerica, MA) or 1 kDa (Macrosep 1K, Pall Life Sciences, Port Washington, NY) to obtain *AppΔapxIΔapxIIC* cell culture supernatant fractions. *AppΔapxIΔapxIIC* supernatant was also diluted 1:2, 1:10, 1:20, 1:40. One ml of the suspensions was added to each well 4 hours after PRRSV infection, and plates were incubated for 48 hours. pH measurements were performed directly in the wells of treated SJPL cells using an Accumet basic AB15 pH meter (Fisher Scientific, Ottawa, ON). The presence of PRRSV N antigen was determined by IFA. The infectious dose of the virus was determined from serial dilutions and calculated by the Kärber method [56]. Briefly, samples infected by PRRSV were subjected to three cycles of freeze-thaw and cellular suspensions were then clarified by low speed centrifugation at 1200g for 10 minutes. Supernatants were serially diluted then used to infect MARC-145 cells in a 96-well tissue culture plate. The plate was incubated for 96 hours. Virus titers were expressed in tissue culture infectious dose 50 per ml ($TCID_{50}$/ml). Presence of PRRSV was also evaluated by qRT-PCR using a commercial kit (Tetracore Inc., Rockville, MD, USA) as previously described [57].

Treatment with LPS and NOD ligands

Cells were infected with PRRSV at 0.5 MOI of in DMEM without serum and other additives and incubated for 4 hours. Then infected cells were washed and fresh medium was added.

Cells were treated with 4µg/ml of LPS purified from *Appwt* [58], or 100 to 1,000 ng/ml of C12-iE-DAP (a NOD1 ligand, InvivoGen, San Diego CA), or 100 to 1,000 ng/ml of L18-MDP (a NOD2 ligand, InvivoGen) for 48 hours. The presence of PRRSV N protein was determined by IFA. The virus titer was determined as described above.

***App* cell culture supernatant antiviral activity against other DNA and RNA viruses**

The DNA genome viruses used in this experiment were: bovine herpes virus type 4 (BHV-4) of strain FMV09-1180503; porcine circovirus 2 (PCV2b) of strain FMV05-6302 and bovine adenovirus 3 (BAV3); bovine herpes virus type 1 (BHV-1); canine parvovirus (CPV); equine herpes virus type 1 (EHV-1). The RNA genome viruses used in this experiment were: bovine viral diarrhea virus type 1 (BVDV1) of strain NADL (ATCC VR-534); swine influenza H1N1 of strain A/Swine/Saint-Hyacinthe/148/90 [59]; and Swine Influenza H3N2 of strain A/Swine/Quebec/4001/05 [60]. Cells were infected with each virus at different dilutions (1/10; 1/100; 1/1000; 1/10000; 1/1000000; 1/10000000) for 4 hours in DMEM as described for PRRSV and than treated with *AppΔapxIΔapxIIC* culture supernatant for 48 hours as described above. The infectious dose of each virus was calculated as described above for PRRSV using SJPL cells.

Analysis of cytokine mRNAs expression by real time reverse transcriptase-quantitative PCR

SJPL cells and PAMs were treated and infected as described above or transfected with Polyinosinic–polycytidylic acid potassium salt (Poly (I:C)) [50 µg/mL] (Sigma-Aldrich Inc., St-Louis, USA) as a positive control for innate immunity induction, using polyethylenimine (PEI) [1µg/µL] (Sigma) for 48 hours or treated with 1µg/ml of lipopolysaccharide (LPS) from *E. coli* (Sigma) for 20 hours, as an IFNγ inducer. Total cellular RNA was extracted from cells using Trizol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. Quantification of RNA was performed with a Nanodrop (NanoDrop Technologies, Inc., Wilmington, Delaware, USA). 1 µg of total RNA was reverse-transcribed using the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada). The cDNA was amplified using the SsoFast EvaGreen Supermix kit (Bio-rad, Hercules, CA, USA). The PCR amplification program for all cDNA consisted of an enzyme activation step of 3 min at 98°C,

followed by 40 cycles of a denaturing step for 2 sec at 98°C and an annealing/extension step for 5 sec at 57°C. The primers used for amplification of the different target cDNA were previously described in Provost *et al*., 2012 [19]. All primers were tested to achieve amplification efficiency between 90% and 110%. The primer sequences were all designed from the NCBI GenBank mRNA sequences using web-based software primerquest from Integrated DNA technologies. The Bio-Rad CFX-96 sequence detector apparatus was used for the cDNA amplification. The quantification of differences between the different groups was calculated using the $2^{-\Delta\Delta Ct}$ method. Beta-2 microglobulin (B2M) was used as the normalizing gene to compensate for potential differences in cDNA amounts. The non-infected PAMs and SJPL cells were used as the calibrator reference in the analysis.

Statistical analyses

A two-way ANOVA model, followed by Bonferroni post-hoc tests (Graphpad PRISM Version 5.03 software) were used to determine if a statistically significant difference exists between infections performed in adherence and cytotoxicity assays. One-way ANOVA model, followed by Tukey's Multiple Comparison Test (Graphpad PRISM) were used to determine if a statistically significant difference exists between PRRSV titer ($TCID_{50}$) obtained in MARC-145, SJPL and PAM cells. Unpaired t tests were used for the qRT-PCR statistical analysis. Differences were considered statistically significant with a $P<0.05$.

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SUPPORTING INFORMATION

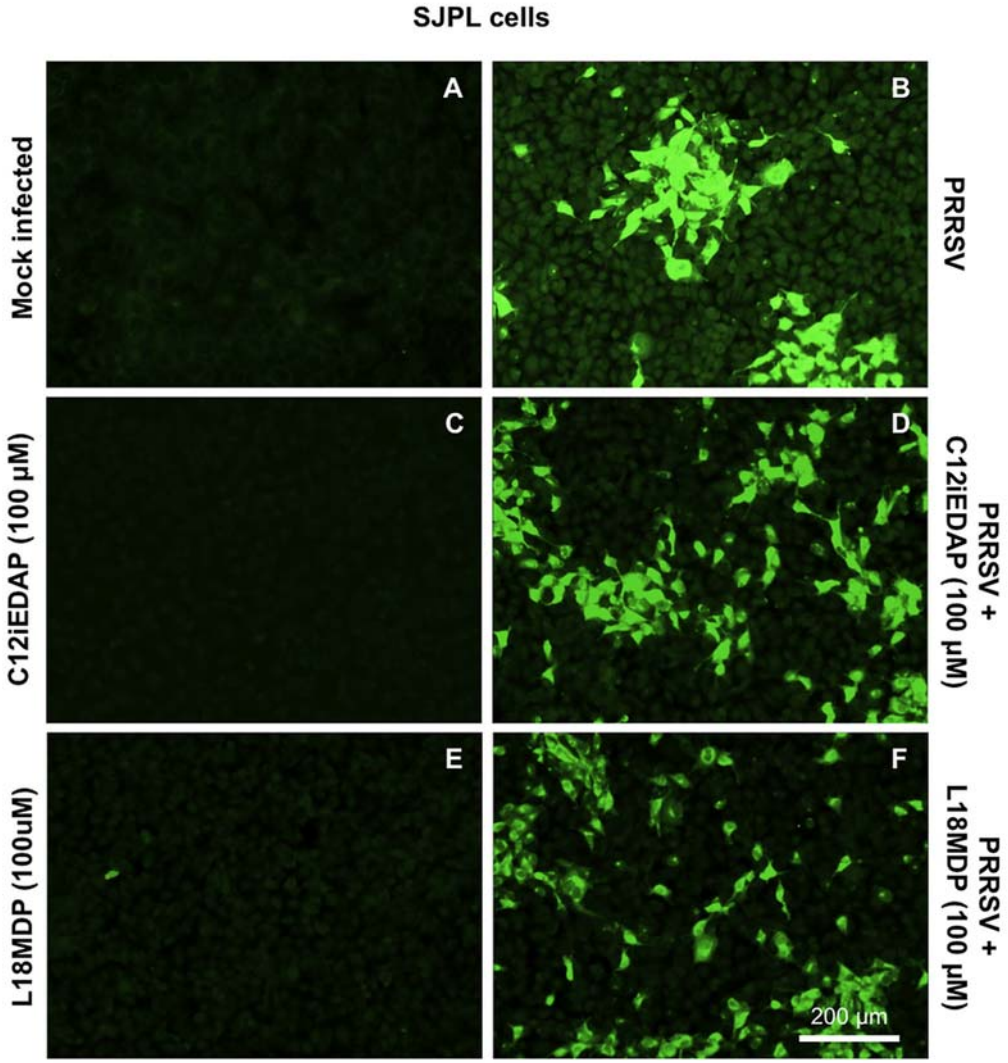

Figure S1: NOD1 and NOD2 inhibition effect on PRRSV replication.

Detection of the N viral protein in PRRSV infected SJPL cells by immunofluorescence. SJPL cells were infected with PRRSV MOI of 0.5 for 4 hours (B) and then treated with 100 µM of C12-iE-DAP (a NOD1 ligand) (D), or 100 µM of L18-MDP (a NOD2 ligand) (F) for 48 hours. Control are SJPL cells untreated (A) treated only with 100 µM of C12-iE-DAP (C), or 100 µM of L18-MDP (E) for 48 hours. White scale bar represents 200 µm. Pictures were taken at 100X magnification.

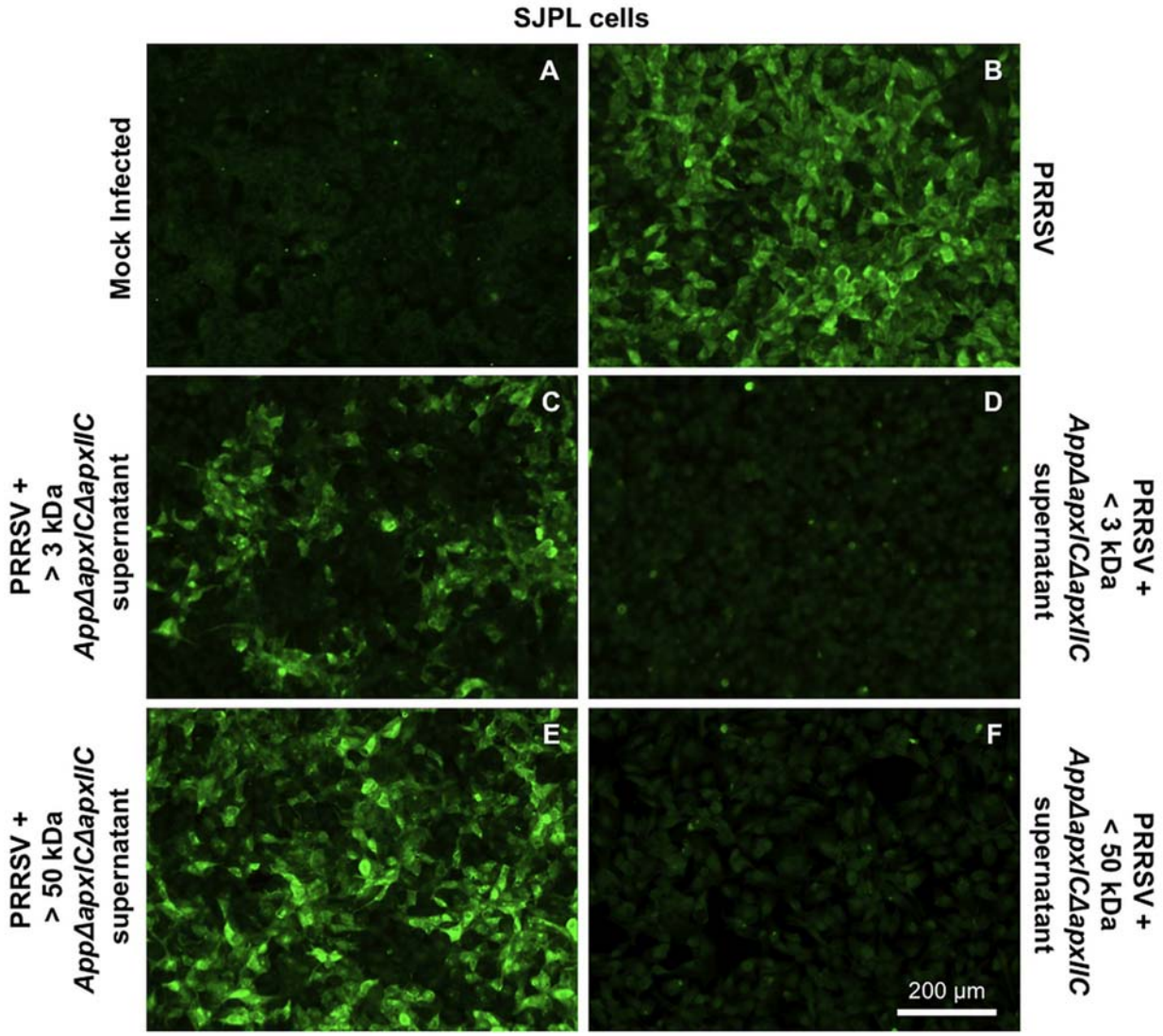

Figure S2: Antiviral activities of *AppΔapxIΔapxIIC* cell culture supernatant fractions against PRRSV.

Detection of the N viral protein in PRRSV infected SJPL cells by immunofluorescence. SJPL cells were untreated (A) or infected with 0.5 MOI of PRRSV for 4 hours (B) then incubated with > 3 kDa (C), or < 3 kDa (D), or > 50 kDa (E), or < 50 kDa (F) fraction of *AppΔapxIΔapxIIC* cell culture supernatant. White scale bar represents 200 µm. Pictures were taken at 100X magnification.

ANNEXE II: Curriculum vitae

Scholarships obtained

- Bourse de congrès du CRIPA. Centre de recherche en infectiologie porcine et avicole. Faculté de médicine vétérinaire. Université de Montréal. 2014
- Bourse d'études supérieures du Canada Alexander-Graham-Bell M (BESC M). Conseil de recherches en sciences naturelles et en génie du Canada (CRSNG). 2013.
- Bourse de congrès du CRIPA. Centre de recherche en infectiologie porcine et avicole. Faculté de médicine vétérinaire. Université de Montréal. 2012.

Publications and presentations

- **Y. Hernandez**, C. Provost, J. Ferreira-Barbosa, J. Labrie, M. Jacques, C.A. Gagnon. *Actinobacillus pleuropneumoniae* (*App*) blocks porcine reproductive and respiratory syndrome virus (PRRSV) replication prior its genome replication and transcription. IUMS, July 27-August 1, 2014. Poster presentation, VIR-PW2015.
- Provost C., Lévesque C., Labrie J., **Hernandez Reyes Y**., Burciaga Nava JA., Jacques M., Gagnon CA. Antiviral activity of *A. pleuropneumoniae* against PRRSV. 23rd IPVS congress, June 8-11 2014. Cancun, Mexico. Poster presentation P568: manuscrit volume 2;page 548.
- C. Lévesque, C. Provost, J. Labrie, **Y. Hernandez Reyes**, J. A. Burciaga Nava**,** C. A. Gagnon, M. Jacques. *Actinobacillus pleuropneumoniae* possesses an antiviral activity against Porcine Reproductive and Respiratory Syndrome Virus. PloS one. 2014;9(5):e98434.
- C. Provost, **Y. Hernandez Reyes**, C. Lévesque, J. Labrie, M. Jacques. C.A. Gagnon. Co-infection study between porcine reproductive and respiratory syndrome virus and bacteria led to the discovery of a possible new bacterial antiviral. International porcine

reproductive and respiratory syndrome (PRRS) symposium. Beijing, China, 2013. Poster presentation.

- **Hernandez, Y,** Provost, C., Ferreira-Barbosa, J., Labrie, J., Gagnon, C. A., Jacques, M. The antiviral activity of *Actinobacillus pleuropneumoniae* against porcine reproductive and respiratory syndrome virus in the porcine alveolar macrophages. Conference of Research Workers in Animal Diseases (CRWAD). Chicago, Illinois, États-Unis, 2012. Oral presentation.

- **Hernandez, Y*.,** Provost, C., Ferreira-Barbosa, J., Labrie, J., Gagnon, C. A., Jacques, M. Étude de l'effet antiviral d'*Actinobacillus pleuropneumoniae* (App) contre le virus du syndrome reproducteur et respiratoire porcin (VSRRP) dans les macrophages alvéolaires porcins. Journée de la recherche de la Faculté de Médicine Vétérinaire. Saint-Hyacinthe, 2012. Poster presentation.