Université de Montréal

# Decoding protein networks during porcine reproductive and respiratory syndrome virus infection through proteomics

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*Ce mémoire intitulé*

# Decoding protein networks during porcine reproductive and respiratory syndrome virus infection through proteomics

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# RÉSUMÉ

Le virus du syndrome reproducteur et respiratoire porcin (VSRRP) est un pathogène de grande importance dans l'industrie porcine car il peut entraîner des pertes économiques. L'une des lignées cellulaires couramment utilisée pour la recherche et la production de vaccins est MARC-145 (cellules rénales de singe vert africain). Les interactions moléculaires entre les cellules hôtes et le virus sont essentielles pour comprendre le mécanisme par lequel le virus utilise la machinerie cellulaire pour se répliquer et infecter les cellules voisines. Notre objectif était d'analyser les changements protéomiques produits lors de l'infection par le VSRRP chez les cellules MARC-145, y compris la composition des virions et des exosomes produits par les cellules infectées. Les surnageants des cellules infectées et non infectées ont été purifiés pour obtenir les virions et exosomes des cellules hôtes. Par la suite, les protéines extraites ont été analysées par spectrométrie de masse à haute résolution quadripolaire-hybride-Orbitrap, et classées selon la fonction moléculaire et la localisation subcellulaire. Le besoin d'obtenir des données protéomiques fiables a conduit au prochain objectif : optimiser l'infection des cellules MARC-145 par le VSRRP. Pour évaluer l'efficacité de l'infection, nous avons synchronisé l'infection en utilisant un virus marqué avec une protéine fluorescente verte améliorée (eGFP) et en ajoutant des polycations à différentes concentrations pour stimuler la liaison des particules virales à la cellule. Pour vérifier le pourcentage de cellules infectées, nous avons utilisé la microscopie à fluorescence et la cytométrie en flux. Nos résultats suggèrent que les protéines cellulaires affectées au cours de l'infection par le VSRRP pourraient jouer un rôle important dans la réponse immunitaire de l'hôte et / ou le cycle de vie viral. L'efficacité de l'utilisation de polycations pour augmenter l'infection par le VSRRP a été démontrée.

**Mots clés:**

Virus du syndrome reproducteur et respiratoire porcin (VSRRP), interactions hôte-virus, exosomes, polycations, spinoculation, protéines, spectrométrie de masse.

# ABSTRACT

Porcine reproductive and respiratory virus (PRRSV) is a pathogen of high importance in the porcine industry because it can lead to significant economic losses. One of the cell lines routinely used for research and vaccine production is MARC-145 (African green monkey kidney cells). Molecular interactions between the host cells and the virus are essential to understand how the virus uses the cell machinery to replicate and infect neighbouring cells. Our goal was to analyze the proteomic changes involved during the PRRSV infection in MARC-145 cells, including the composition of the infected cells' virions and exosomes. The infected and non-infected cells' supernatants were purified to obtain the host cells' virions and exosomes. Extracted proteins were further analyzed by High-Resolution-Quadrupole-Hybrid-Orbitrap mass spectrometry and classified according to molecular function and subcellular localization. The need for obtaining reliable proteomics data led to the next goal of optimizing the infection of MARC-145 cells with PRRSV. To assess the efficiency of the infection, we synchronized the infection, used a virus tagged with an enhanced green fluorescent protein (EGFP) and added polycations at different concentrations to stimulate the binding of the viral particles to the cell. To verify the percentage of infected cells, we used fluorescence microscopy and flow cytometry. Our findings suggest the cellular proteins affected during the PRRSV infection could play important roles in host immune response and/or the viral life cycle. The efficiency of the use of polycations was demonstrated to be effective in increasing PRRSV infection.

**Keywords:**

Porcine reproductive and respiratory syndrome virus (PRRSV), host-virus interactions, exosomes, polycations, proteins, mass spectrometry.

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# LIST OF ABBREVIATIONS AND SIGLES

ATCC/VR-2332: American type culture collection/PRRSV reference strain for genotype II

C: Cytoplasm

CD151: Cluster of differentiation 151 (PRRSV cellular receptor)

CD163: Cluster of differentiation 163 (PRRSV cellular receptor)

CD4+/CD8+/ CD4+CD8+: T cells CD4+/CD8+/CD4+-CD8+ double positives

Ce: Centrosome

CL2621: African green monkey kidney cell line derived from MA-104

CM: Cellular membrane

CPE: Cytopathic effect

Cr: Chromosome

CS: Cell surface

Cy: Cytoskeleton

DMV: Double membrane vesicle

EMEM: Eagle's minimum essential medium

d.p.i.: Days post-infection

dsRNA: Double-stranded ribonucleic acid

EE: Exosome

EM: Extracellular Matrix

EMS: Endomembrane System

ER: Endoplasmic reticulum

ERM: Endoplasmic reticulum membrane

FBS: Foetal bovine serum

GM: Golgi membrane

GP: Glycoprotein

gRNA: Genomic RNA

HBC: Hemoglobin complex

IC: Intracellular

ICM: Integral Component of membrane

IFN α/β/γ: Interferon-alpha/beta/gamma

IgG/IgA: Immunoglobulin G and A

IL: Interleukin

IRC: Intracellular Ribonucleic Complex

ISG15: interferon-stimulated gene 15

JAK-STAT: Janus kinase- signal transducer and activator of transcription (Type I IFN signalling pathway)

LDH: Lactate dehydrogenase

LE: Late endosome

LV: Lelystad virus

Ly: Lysosome

M PRRSV: membrane viral protein of PRRSV

M: Membrane

MARC-145: African green monkey kidney cell line derived from MA-104

MHC: Major histocompatibility complex class

MI: Mitochondria

MIIM: Mitochondrial Inner Membrane

ML: Melanosome

MOI: Multiply of infection

N PRRSV: nucleocapsid viral protein of PRRSV

N: Nucleus

NFκB: nuclear factor-κB kappa-light-chain-enhancer of activated B cells

NL: Nucleolus

NPTr: Newborn pig trachea cells

NS: Nucleosome

Nsps: Non-structural proteins

OM: Organelle membrane

ORFs: Open reading frames

PAM: Porcine alveolar macrophages

PBS: Phosphate buffer saline

PFA: Paraformaldehyde

PM: Plasma membrane

Poly I:C: Polyinosinic–polycytidylic acid potassium salt

PRRS: Porcine reproductive and respiratory syndrome

PRRSV: Porcine reproductive and respiratory syndrome virus

R: Ribosome

RdRp: RNA-dependent RNA polymerase

RTC: Replication-transcription complex

SD: Standard deviation

sg mRNA: Sub-genomic messenger RNAs

ssRNA: Single-stranded ribonucleic acid

TNF-α: Tumor necrosis factor-alpha

V: Vesicles

VE: Viral envelope

# DEDICATORY

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# INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically significant diseases in the porcine industry worldwide. It was first described in the United States in the late 1980s and then in Europe in the early 1990s (1-3). The etiological agent is the porcine reproductive and respiratory syndrome virus (PRRSV), which belongs to the order *Nidovirales*, family *Arteriviridae,* and genus *Betaarterivirus* (4). The size of the viral particle is between 50-60 nm, and the length of the genome is about 15 kb, which comprises at least 10 ORFs (5).

PRRSV infects endothelial and germ cells and can affect the host respiratory tract, lungs, and reproductive organs (1, 6, 7). The primary natural host cells used for *in vivo* studies are the porcine alveolar macrophages (PAM). The development of permissive cells such as newborn pig trachea epithelial (NPTr) cells expressing CD163 (a receptor that mediates the PRRSV entry), and St-Jude porcine lung cells (SJPL, from monkey origin) (8, 9), allowed the research of the pathogenesis of PRRSV *in vitro*. Other cell lines routinely used for research, virus production, vaccines, and viral production are the MA-104, MARC-145, and CL2621 epithelial kidney cell lines from African green monkey (10).

The replication cycle of PRRSV starts with the viral particle's entry through the endocytosis process mediated by receptors such as CD163 (11). The endosome is the cellular vesicle that transports and releases the viral genome into the cell. The expression of replicase polyproteins pp1a and pp1ab begins following the viral release. These proteins are cleaved into 16 non-structural proteins and further assembled to form the replication and transcription complex (RTC) associated with intracellular membranes (12). The replication process produces sets of sub-genomic RNA (sgRNAs), which are transcribed to express viral structural proteins. The viral particles assemble by budding of nucleocapsids into the lumen of the endoplasmic reticulum or Golgi apparatus; these particles are released from cells via exocytosis (13, 14).

The complex interaction network of the host with PRRSV proteins during the infection is not well studied. Nonetheless, a research group demonstrated that there are changes in the

proteomic profiles of the PRRSV infected cells (15). Proteomic analyses of the supernatants from the PRRSV infected PAM cells showed that the proteins involved in inflammatory response pathways were the most affected. Furthermore, cell-to-cell communication pathways are affected, which could be related to protein secretion (16). Exosomes are cellular vesicles released to the extracellular space; these vesicles transport different components such as lipids, proteins and RNAs, playing an essential role in cell-to-cell communication (17). Infected and non-infected cells can release exosomes; these vesicles' content reveals information about the intracellular processes and the cellular state (18, 19).

The delivery of exosomes to neighbouring cells allows the communication between the cells to activate an early immune response against pathogens (19, 20). Viruses have evolved to evade cellular immune machinery; they have taken advantage of the exosomal pathway to transport its components (proteins, RNA) from infected to non-infected cells. Consequently, the virus spreads at a favourable rate in terms of infection and can hijack the immune response of receiving cells (21-24). Evidence showed that infected MARC-145 and porcine kidney cells (PK-15) produced exosomes containing viral proteins and viral RNA, may suggest an evasion of cellular immune response using the exosomal pathway (25).

The polycations correspond to a group of polymers that possess a positive charge. They can interact with cellular components such as anionic charges in the cells' surface and hydrophobic interaction with lipid bilayers membranes (26, 27). These molecules are one alternative for gene therapy because they act as vehicles to deliver nucleic acids to the cell (27-29). There is virological evidence that the addition of polycations, such as DEAE-dextran, enhances the transfection of lentiviral vectors in different cell lines (30). Additionally, the infectivity of viruses such as avian sarcoma virus, encephalomyocarditis virus, rotavirus and rabies virus showed enhancement when polycations were present at the early stages of infection (31-34).

Despite some progress in recent years, our knowledge of PRRSV and host cells' complex and dynamic interactions remains limited. Thus, many questions remain regarding PRRSV-

host cell interactions. Nidoviruses should be a subject of continuous study. Understanding the molecular mechanisms underlying the interaction between the virus and host cells will help overcome different limits, such as identifying potential pro- or antiviral host targets designing new vaccines and antiviral strategies. State of the art previously described motivated the present work, hypothesizing that during PRRSV infection, the intracellular levels of host proteins would be affected, and that could modulate various cellular pathways. This study's first objective was to identify host cellular proteins associated with PRRSV virions and exosomes produced by infected cells.

As an additional contribution to PRRSV's study, we proposed using polycations to improve PRRSV’s infectivity. The goal was to synchronize the infection and to obtain a significant number of infected cells by PRRSV. To this end, we performed the treatment with two polycations (polybrene and DEAE-dextran). The use of polycations increased transduction efficiency in lentiviral vectors; also increased infectivity of viruses such as HIV and rabies virus, among others (30, 35).

# CHAPTER I: LITERATURE REVIEW

## PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

### 1. Origin and clinical manifestation

The porcine reproductive and respiratory syndrome (PRRS), previously referred to as "mystery swine disease and mystery reproductive syndrome," emerged in North America and Europe in the late '80s and early '90s. The etiological agent is the PRRS virus (PRRSV), first described in the Netherlands in 1991 and designated as the Lelystad virus. Primary cell cultures of porcine alveolar lung macrophages were necessary for viral isolation (1, 3, 36). Later, in the United States, PRRSV was isolated from infected piglets and recovered from tissues following its isolation using continuous CL2126 cells (monkey kidney origin). The North American strain of PRRSV registered as ATCC VR-2332 (1, 2).

Recently, the strains of PRRSV previously identified as genotype I (Lelystad) and genotype II (ATCC VR-2332) are now *Betaarterivirus suid 1* and *Betaarterivirus suid 2,* respectively (2-4). Both genotypes of PRRSV have dramatically affected porcine industries in many countries, including Canada, Germany, the United Kingdom, Korea, Japan, and China. In 2007, a simultaneous outbreak of PRRSV in Vietnam and China led to identifying a highly pathogenic strain (HP-PRRSV). Further studies suggested that the HP-PRRSV strain is a variant of the genotype II (37, 38).

The PRRSV-susceptible animals are newborn piglets and growing pigs, which typically present mild to severe respiratory disease, and pregnant sows, where infection causes reproductive failure (10). The severity of the infection is determined by the immune status of the host and the strain pathogenicity. Secondary infection or opportunistic pathogens may predispose animals to more severe development of the disease (5).

## 2. Etiology

### 2.1. Taxonomy

PRRSV belongs to the order *Nidovirales*, family *Arteriviridae,* family *Variartevirinae*. After 2018 the classification changed and classified PRRSV in the genus *Betaarterivirus*, the genotype I in a subgenus named *Eurpoarterivirus* and species *Betaarterivirus 1*; the genotype II classified in the subgenus *Amproarterivirus*, and the species is *Betaarterivirus suid 2* (4, 39).

### 2.2. Morphology

PRRSV virion is spherical, with a diameter between 45-60 nm. It possesses a lipid-containing envelope that surrounds the helicoidal core; the size of this last is about 20-30 nm in diameter (40). The genomic RNA is linear, positive sense and single-stranded, about 15Kb in length. The genome is enclosed in the nucleocapsid and composed of the N (nucleocapsid) protein (5).

### 2.3. Genomic organization

The viral RNA of PRRSV is composed of at least ten open reading frames (ORF), which carry out the expression of different important proteins involved in the virus's replication and structure (5, 41). The ORFs 1a and 1b encode a replicase, which plays an essential role during viral replication (42). These ORFs encode two polyproteins (pp), pp1a and pp1ab, that are translated through programmed ribosomal frameshifting (RFS). The pp1a and pp1ab are later processed to different nonstructural proteins (nsps) (Figure 2), which are going to lead the genome replication of PRRSV (5, 43).

During the replication, subgenomic (sg) RNAs are synthesized from minus-strand RNAs (these are the product of a discontinuous synthesis), better known as the nested transcription, which is characteristic of the replication of viruses found within the *Nidovirales* order (44). The translation of the sgRNAs lead to the expression of two major structural proteins, Glycoproteins (GP) 5 and membrane (M) protein, minor structural GP2a, GP2b, GP3, GP4 proteins, hydrophobic

proteins E and ORF5a and phosphorylated structural protein N. These proteins are all encoded by the ORFs 2-7 (36, 42, 45-47).

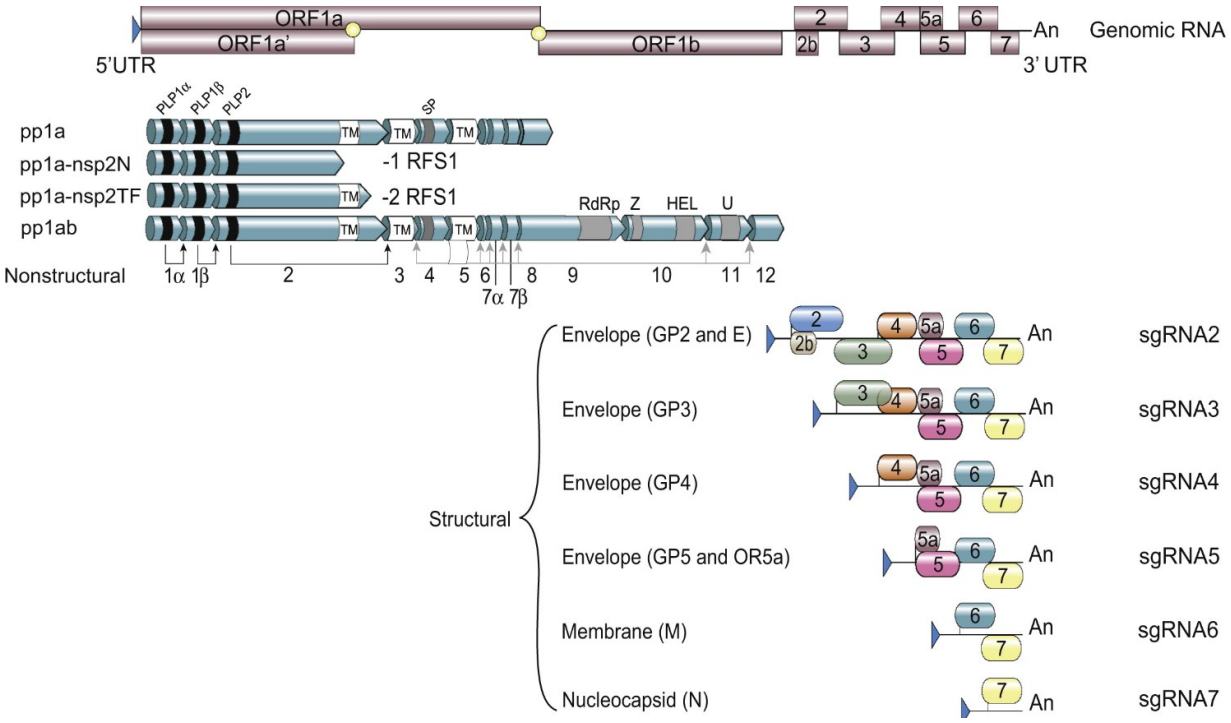



Figure 1. Representation of the genomic organization of PRRSV.

Adapted from Kappes and Faaberg (43). From the top, the untranslated regions (UTRs), then the ORFs starting by the ORF1a and 1a', followed by the ORF1b. The two yellow dots show where the ribosomal frameshift (RFS) takes place and enhances the polyproteins' (pp) translation. Then, the proteolytic cleavage of polyproteins and the production of nonstructural proteins (nsp) begin. Immediately following this event, the synthesis of subgenomic RNAs 2-7 (sgRNA) and the corresponding structural proteins begins. These components are represented in the figure as glycoproteins 2-5 (GP), OR5a, Membrane (M), and nucleocapsid (N).

## 2.4. Viral proteins and their function

The viral proteins are divided into non-structural and structural proteins; simultaneously, they are classified into major and minor proteins (42). The known functions of these proteins are elucidated in the following Table 1:

Table 1. PRRSV viral genes and related proteins (non-structural and structural), including their functions. Modified from Lunney et al. (5)

| ***Genes*** | ***Proteins*** | ***Functions*** |
|---|---|---|
| ***ORF1a*** | **nsp1α** | Regulation of sgRNA synthesis by the zinc finger protein (1), comprises the papain-like cysteine protease α (PLPα), inhibits the interferon 1 (INF) activity (41, 42, 48-53). |
| | **nsp1β** | Comprises PLPβ (5, 54), inhibits type I INF activity (1). |
| | **nsp2** | Comprises PLP2 domain, a negative regulator of innate immunity, replication, disruption of type I INF signalling (5, 54, 55) |
| ***ORF1a'-TF*** | **nsp2TF** | Comprises PLP2 domain, innate immune antagonist, replication, disruption of type I INF signalling (5, 54, 55) |
| | **nsp2N** | Comprises PLP2 domain, innate immune antagonist, replication, disruption of type I INF signalling (5, 54, 55) |
| ***ORF1a*** | **nsp3** | Autophagic induction (56), which interacts with membrane proteins, involve in replication processes (5), contains proteases (1). |
| | **nsp4** | Induces apoptosis, inhibits IFN expression (1, 5, 57). |

| | | |
|---|---|---|
| | **nsp5** | Autophagic induction (56) degrades the signal transducer and activator of transcription 3 (STAT3) (58). |
| | **nsp6** | The function is not described yet. |
| | **nsp7α** | RNA synthesis and replication (59). |
| | **nsp7β** | The function is not described yet. |
| | **nsp8** | The function is not described yet. |
| ***ORF1b*** | **nsp9** | Replication and transcription protein, viral RNA-dependent RNA polymerase (60, 61), NTPase (1). |
| | **nsp10** | Induce apoptosis (62). helicase (1). related to sgRNAs synthesis (60). |
| | **nsp11** | Endoribonuclease (5, 60). inhibition of type I INF (1), interaction with the RNA-silencing complex (63). |
| | **nsp12** | Synthetizise viral sgRNAs (13). |
| ***ORF2a*** | **GP2a** | Involved in viral attachment and infectivity is a minor envelope structural protein (5), interacts with the PRRSV receptor CD163 (64, 65). |
| ***ORF2b*** | **E** | It is a minor structural and non-glycosylated protein that can act as a viroporin and possesses ion-like channel properties (1, 65, 66). |

| | | |
|---|---|---|
| ***ORF3*** | **GP3** | It is a minor envelope structural protein, forms a heterodimer complex with GP2 and GP4 (64, 65), important for viral entry due to the interaction with PRRSV CD163 viral receptor (67). |
| ***ORF4*** | **GP4** | It is a minor envelope structural protein involved in viral attachment, interacts with the CD163 viral receptor (64, 65), is crucial for glycoproteins interaction (65), has a highly variable epitope (68). |
| ***ORF5*** | **GP5** | It is a major glycoprotein present in the viral envelope in significant quantities (64, 65). interacts with host cell receptors such as heparan sulphate and sialoadhesin (63). forms a complex with the M protein, is involved in budding and assembly of the viral particle (69). Induces the synthesis of neutralizing antibodies in PRRSV infected hosts (70, 71). |
| ***ORF5a*** | **GP5a** | (69)(69)Essential for viral infectivity (72). |
| ***ORF6*** | **M** | Viral entry; interacts with the heparan sulphate receptor (63), is a major non-glycosylated envelope protein (1), is involved in the budding and assembly of the viral particle (69), is the most conserved protein (49, 73). |
| ***ORF7*** | **N** | It is a non-glycosylated major structural protein (1) involved in RNA binding; it is inducing high titers of specific antibodies due to its high immunogenicity in infected host(1, 74). |

## 3. Cellular tropism

The tropism of PRRSV is very specific both *in vitro* and *in vivo* (75). The virus' natural host is swine and tends to infect cells of the monocytic lineage, such as the porcine alveolar macrophages (PAM) located in the lungs (9). However, the macrophages are just one of the different cell types that this virus infects, and it can infect the cells from other organs such as the tonsils, lymph nodes, and placenta (76).

The PAM cell line is one of the most preferred models for studying PRRSV; however, due to the high genetic variation in primary cells, the results' reproducibility could vary. Some researchers have recently developed a cell line of immortalized porcine macrophages to overcome this variability, better known as ZMAC cells for *in vitro* studies (77-79).

Historically, different host cell lines have been used for *in vitro* experiments. One of these cell lines, pig kidney cells (PK-15), was transfected with the receptor CD163, necessary for PRRSV's entry into the cells. This genetic modification allowed the efficient production of the PRRSV in these cells (80, 81). Another example of a cell line used to study PRRSV successfully is the epithelial cell line from the newborn porcine trachea (NPTr), which was also modified to express the receptor CD163. For example, these cells have been used to study PRRSV and influenza A virus co-infection, and the results showed that they are permissive for the PRRSV replication (8, 82).

Another cell line widely used to study PRRSV pathogenicity, viral replication, and host response is the MARC-145 cell line, derived from an African green monkey kidney. These cells are highly permissive, allow the production of PRRSV and the reproducibility of data; for these reasons, this cell line is suitable as *in vitro* model for vaccine production, molecular and proteomic studies (9, 77, 83).

Monocyte-derived dendritic cells have been used as well for *in vitro* studies. Proteomic profiles of infected dendritic cells showed to be affected during the PRRSV infection (84-86).

## 4. Virus life cycle in cells

The life cycle of PRRSV can be divided into different phases that will be described below. Figure 3 shows the stages of the viral cycle in target cells (87).

### 4.1. Virus entry

The cell entry of PRRSV is mediated by receptors, which can vary depending on the cell line. Cellular receptors described for PRRSV are heparan sulphate, the cluster of differentiation 163 (CD163, a scavenger receptor), tetraspanin CD151, sialoadhesin (CD169) and vimentin (80, 87).

The current PRRSV endocytosis model into PAM cells suggests that the attachment mediates this process to the heparan sulphate receptor. This last interacts with the sialoadhesin receptor, known as the M/GP5 heterodimer complex's binding site to the sialic-acid binding domain (88, 89). After the attachment, the internalization starts by transporting the virus into the endosome. Next, the receptor CD163 will interact with glycoproteins 2 and 4 of the virus, resulting in releasing the viral genome to the cytoplasm (89).

The PRRSV entry into the MARC-145 cells differs from the viral entry into the PAM cells due to the receptor sialoadhesin lacking (80). However, the MARC-145 cells express the receptor CD163, CD151, vimentin and heparan sulphate (65, 80). Other cellular elements that play an essential role in the PRRSV entry into MARC-145 cells are the lipid rafts (90). It was demonstrated that these molecules interact with GP3 and GP4 (91). In addition to the lipid rafts, various studies suggested that cholesterol is important for the PRRSV entry into the cell (90, 92, 93). The interaction between the lipid rafts and the receptor occurs with the co-localization of glycoproteins and the receptor CD163 in the cell membrane's lipid rafts (94).

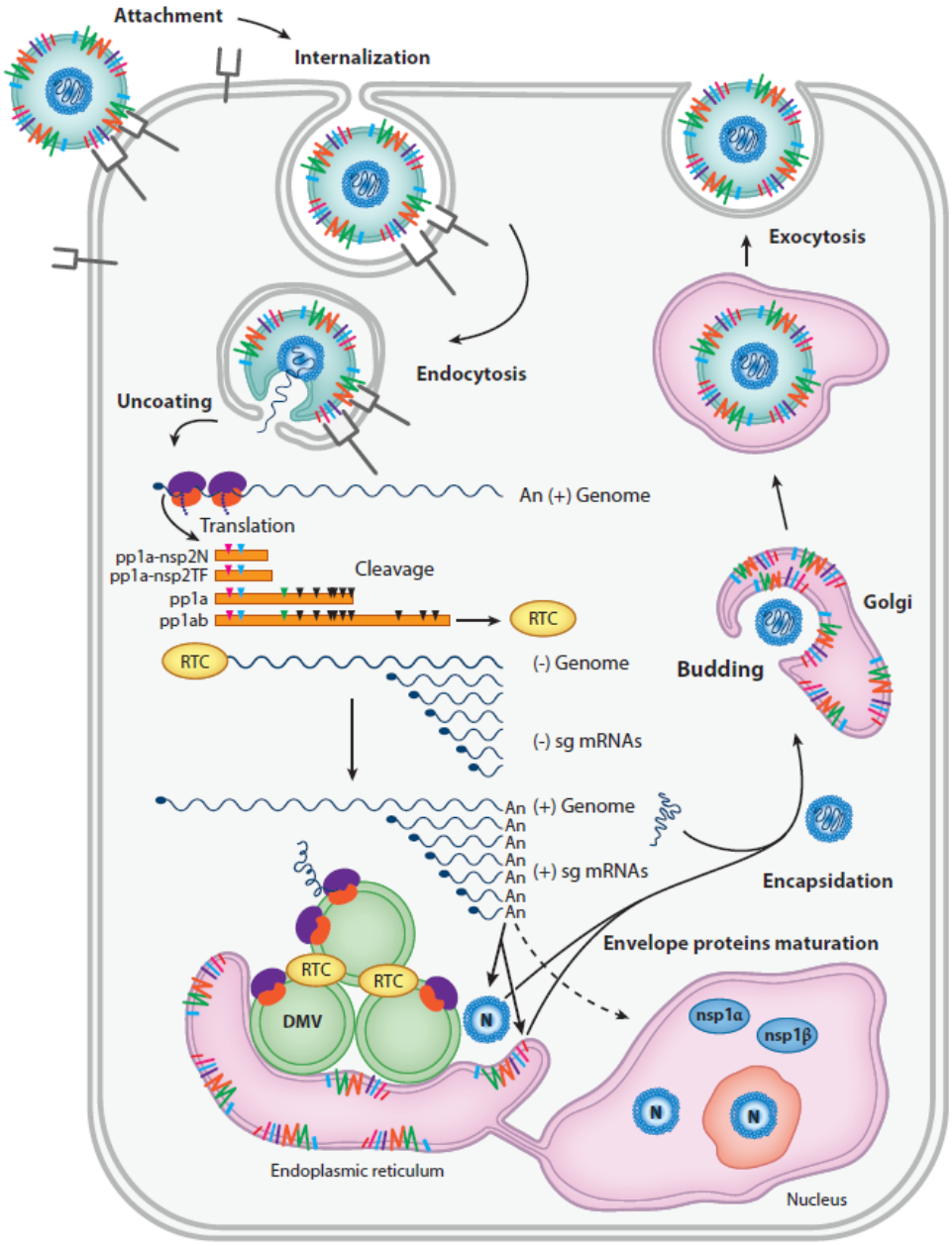



Figure 2 Schematic representation of the PRRSV life cycle.

Adapted from Luney et al. (5). The infectious cycle of PRRSV begins upon attachment of the virion to the cell surface and receptor-mediated endocytosis, followed by an uncoating process that will release the viral genome to the cytoplasm. Next, the translation starts, yielding the replicase polyproteins pp1a-nsp2TF, pp1a-nsp2N, pp1a, and pp1ab. Later, upon proteolytic cleavage, at least 14 non-structural proteins are generated that will proceed to the assembly of a

replication and transcription complex (RTC). The RTC joins the synthesis of minus-strand RNA and will produce subgenomic (sg) minus-strand RNAs, which later will serve as templates to synthesize plus-strand sg mRNAs to allow the expression of structural protein genes. The new generation of viral RNA genomes is packed into nucleocapsid, and this process involves the double-membrane vesicles (DMVs). Then, new nucleocapsid complex buds (N) formed by the smooth endoplasmic reticulum and the Golgi vesicles acquire the envelope. Right after maturation, the new virions are released from the cell through the exocytic pathway (5).

## 4.2. Virion uncoating

The viral life cycle continues with the viral particle's internalization and release of the viral genome to the cytoplasm. The process of internalization is carried out by early endosomes (5, 88, 95). The uncoating occurs when the pH of the endosome drops. Consequently, the envelope fuses with the endosomal membrane and leads to the release of nucleocapsid into the cytoplasm (89, 96-98).

It has been demonstrated that during the uncoating, the protease cathepsin E plays an important role. The cathepsin E is localized in the endosomes and other non-lysosomal organelles in the cell (95). Another key molecule for this process might be the tetraspanin CD151, which is involved in signalling pathways. It was shown that CD151 binds to the 3'-untranslated region (UTR) RNA of PRRSV and confers susceptibility of BHK-21 cells to PRRSV infection (1, 88, 99).

In addition to the role of this cathepsin E and CD151, it was suggested that as the envelope viral protein (E) has a function of viroporin (table 1), this protein will allow the exchange of ions inside the endosome, and therefore the uncoating can be done (66, 97).

## 4.3. PRRSVgenome replication

After the viral particle's entry and uncoating, the PRRSV uses the host cellular translational machinery to instruct the host to make virus components. This process begins with translating the ORF1a and ORF1b into the replicase polyproteins (pp) pp1a and pp1ab. The expression of the -1 ribosomal frameshift signal (100) will result in the processing of at least 10 non-structural proteins (nsps) (60, 63) and the synthesis of an additional protein by the -2 ribosomal frameshift. These proteins are assembled to form a replication and transcription complex (RTC) associated with the formation of double-membrane vesicles (DMVs). This association suggests that transcription and translation of the viral genome have been carried out (1, 101-103).

The RTC is implied in replication and translation processes, where the replication is comprised by the synthesis of negative full-length mRNA (Figure 4). This negative full-length mRNA will serve as a template for the continuous generation of subgenomic mRNA (sg mRNA); the RNA-dependent RNA polymerase (RdRp) is involved in the synthesis of these new RNAs; this enzyme is synthesized by the ORF1b (see table 1) (43, 45, 102). The transcription initiates with a change from continuous production of sg RNAs to discontinuous production of sgRNAs, followed by the translation to structural proteins (104, 105). The discontinuous synthesis of these sgRNAs represents the nested transcription, a characteristic of the viruses included in the order *Nidovirales* and is synthesized from PRRSV's genome (Figure 4) (101, 105, 106).

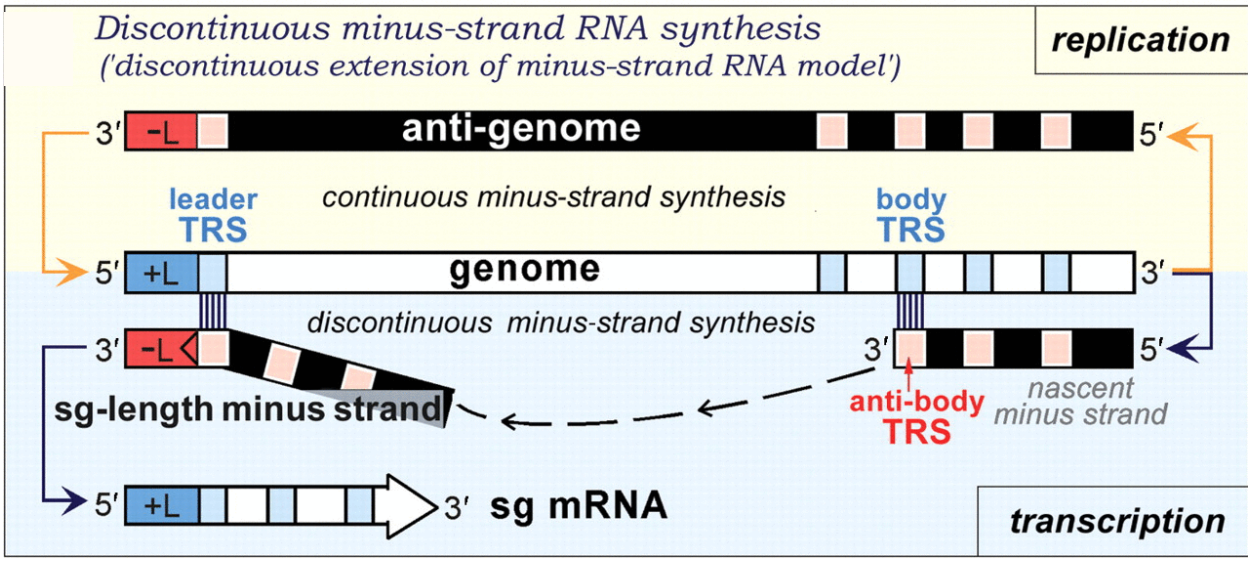



Figure 3 Schematic representation of the discontinuous (-) sgRNA synthesis.

Based on the model proposed by Sawicki and Sawicki (106). Figure 4 shows on top the first full-length minus-strand sgRNA that serves as a template for the continuous production of other minus strands sgRNAs. At the same time, these sgRNAs are going to serve as templates to produce discontinuous sg mRNAs. Transcription-regulatory sequence (TRS) can be presented as body TRS; its function is to act as a signal attenuator for (-) RNA synthesis. The new (-) strand produced will have an antibody TRS, later recognized by the genome's 5'-proximal region. This recognition is due to the interaction between the base-pairing and the leader TRS. The addition of the anti-leader (-L) to the new (-) strands will provide the capacity to the (-) sgRNAs to be templates for transcription. Adapted from Pasternak et al. (44).

### 4.4. Encapsidation

Next, the translated proteins are assembled to form new virions; viral N protein is involved in this process (107). There is evidence that suggests that N protein interacts with the nsp9, which corresponds to the RdRp. The N protein will further assemble the nucleocapsid, and the PRRSV genome is encapsidated simultaneously (61, 108, 109).

### 4.5. Budding

In the next step, the newly produced nucleocapsids acquire a viral envelope by budding into the endoplasmic reticulum (ER) or Golgi region, taking the smooth intracellular membranes of these organelles (5, 107). It is suggested that the glycoprotein 5 (GP5) and the membrane protein (M) are involved in this step, forming the GP5/M complex and signalling the transportation to the budding site (110).

### 4.6. Exocytosis

After the budding, the viral life cycle's last step is releasing new viral particles from the host cell. The newly assembled intermediate viral particles accumulate in the lumen of the ER. They are then transported by vesicular transport to the cell surface, where they are released via exocytosis (53, 111). Another way for PRRSV to exit the cell is by cell lysis; this happens when the immune system of the cell recognizes proteins of the virus (112), the virus can induce or apoptosis, nsp4 and nsp10 are responsible in the induction of this process, and this occurs in late infection of PRRSV (62).

## 5. PRRSV transmission and pathogenesis

The natural host of PRRSV is swine. This virus affects growing pigs, newborns and older piglets, causing mild to severe respiratory disease. In sows, the virus is responsible for impairing the reproductive functions (5, 113). This last accelerates the farrowing of stillborn and mummified fetuses, leading to economic losses due to decreased production in the porcine industry (114-116). In 2013 financial losses were estimated from C$116 to C$219 per year in Canada (117). The transmission of PRRSV could be by bioaerosol, ingestion, insemination or sexual intercourse and direct contact. In vertical transmission, the most critical period is the last trimester of gestation (114).

PRRSV presence in different biological fluids such as saliva, semen, nasal secretions, urine, saliva, feces and mammary gland secretions is a consequence of the viral shedding

during the viremia. After this last, a massive spread of the virus between pigs occurs. Additionally, the spaces where the pigs are confined make them in direct contact. The production of aerosols by the pigs can become critical transmission points, as they breathe, sneeze or cough, and share the sources of water and food (114, 118-121).

After the exposure of the natural host to PRRSV, the replication process starts in the macrophages. Allowing the virus's entry and spread into lymphoid tissues and through the body via the lymph-hematic route (114). A study in gnotobiotic pigs showed that the viremia started after 12 h post-infection (122). The viremia period may vary from less than four weeks to 3 months; the viremia onset can be linked to the pigs' age, being the oldest group, which develops it most rapidly (114, 123, 124).

During PRRSV infection, the most affected organs are the heart, thymus, spleen, hepatic sinusoids, lungs, Peyer's patches and tonsils (1, 114). The lungs are reported to be the organ where the viral load is high from day one post-infection to day 28, but the virus can still be detected at day 49 (125, 126). After the viremia, the viral replication rate decreases and the virus confines in the lymphoid tissues, suggesting that the probabilities of transmission are high at this stage, but the contagiousness is low (113, 114).

PRRSV's vertical transmission is done via transplacental from viremic dams to fetuses and can cause abortions, the birth of weak or normal appearance pigs. PRRSV can replicate in fetuses after 14 days of gestation. Still, this infection is uncommon at an early stage of pregnancy. The third trimester is the most critical due to an effective cross of PRRSV through the placenta (119).

## 5.1. Cell damage

PRRSV infection leads to lesions and clinical disease due to different mechanisms such as apoptosis; this mechanism affects infected and proximate non-infected cells (the major cause of cell death). The induction of inflammatory cytokines results in the immune response's activation, causing pulmonary edema and bronchial constriction. The induction of polyclonal B cell activator impairs serum immunoglobulins, lymphoid hyperplasia, circulating immune complexes, immune complexes associated with cell

inflammation, and induction of auto-antibodies to Golgi antigens. Reduction in bacterial phagocytosis and killing by macrophages increases septicemia susceptibility (113, 119, 127, 128).

# 6. Immune responses

## 6.1. Innate immunity

The innate immunity is the first host defence system from pathogens. In the case of PRRSV, it suppresses the host's immune response. Consequently, the antiviral response in the different infected cells, such as macrophages (primary infected cells by PRRSV) or plasmacytoid dendritic cell, is suppressed (5, 119, 129).

### 6.1.1. Macrophages

The macrophages are the first cells infected by PRRSV *in vivo*, as these cells are the first defence of the lungs; therefore, the lungs are a suitable organ for viral replication (129-131). It is suggested that early viral replication in macrophages inhibits apoptosis (which will be further explained), and the pro-apoptotic pathway is activated when the infection is at the late stage (132).

### 6.1.2. Natural killer cells (NK)

The natural killer cells are part of the innate immune response, and they are lymphocytes that could be in lymphoid and non-lymphoid tissues; these cells are distributed throughout the whole body (133). Evidence about PRRSV infection in PAMs demonstrates that these NK cells are suppressed; thus, the susceptibility of PAMs to the cytotoxicity of the NK cells was low and could indicate an attempt of the virus to evade the innate immune response (134).

### 6.1.3. PRRSV modulation of TLR pathways and inhibition of type I interferons

The production of cytokines, such as the type I interferons (IFN), constitutes innate immunity mechanisms to defend the host against viral infections. In the case of the porcine,

there are at least 39 subtypes of type I IFNs described (17 IFN-α subtypes, 11 IFN-δ subtypes, 7 IFN-ω subtypes, and single-subtype subclasses of IFN-αω, IFN-β, IFN-ε, and IFN-κ) (135). The host pattern recognition receptors (PRRs) play an essential role in the induction of cytokines; for RNA viruses, these receptors include the toll-like receptors and the retinoic acid-inducible gene (RIG) -I-like receptors (RLRs) (136, 137). Double-stranded RNA (dsRNA) viruses are recognized by TLR3 and RLR (137). The toll-like receptors activated by PRRSV include TLR2, TLR3, TLR4, TLR 7, and TRL8 (138, 139).

The process of induction of inflammatory cytokines and type I IFN starts with the activation of TLRs and RLRs, followed by the activation of cellular factors such as interferon regulatory factor 3 (IRF3), IRF7, and transcriptional nuclear factor-*k*B (NF-*k*B) (137). It has been demonstrated that PRRSV infection in PAMs downregulates the production of type I INF-α, impeding the ligation to the TLR3, consequently increasing the replication rate of PRRSV (138, 140). The effect of PRRSV infection in the TLR3 expression in PAMs and porcine dendritic cells (DCs) was demonstrated by stimulating the cells with polyinosinic-polycytidylic acid [(poly I: C) ); this last mimics viral RNA and is used as a ligand of TLR3]. The collected data showed that PRRSV inhibited the expression of TLR3 and TLR7 in macrophages and DCs, while poly I:C down-regulated the expression of TLR7 and TLR8 in macrophages (141).

PRRSV proteins such as the N, nsp1, nsp2, and nsp11 proteins have been reported as modulators of host innate immune response (142). The N and nsp1 proteins have been found in the nucleus and cytoplasm of the cells. The innate immune response is modulated by nsp1, inhibiting the induction of IFN. The inhibition of IFN by nsp1is performed through the degradation of the CREB-binding protein (CBP); it is suggested that the nsp1α located in the cytoplasm blocks the activation of NF- κB (plays an important role in apoptosis). The IFN activity inhibition delays the clearance of the virus from the host (143, 144).

The cell cycle and ribosome biogenesis are affected by the N protein; these cellular processes' hijacking improves viral production (143). The nsp2 protein allows PRRSV to evade innate immunity by deconjugating ubiquitin and Papain-like from signalling

molecules; this evasion is done because the IFN and inflammatory response are silenced (143).

### 6.1.4. Cytokines expression during PRRSV infection

There are cytokines such as interleukin 10 (IL-10), IL-1β, IL-8, IL-12, IL-4, tumour necrosis factor α (TNF-α), and IFN-γ that are affected during PRRSV infection (143). These cytokines are produced by macrophages, B cells, T cells, endothelial cells, and epithelial cells (145). The role of the IL-10 is to act as an immunosuppressive cytokine, production of INF-γ works as a protective mechanism for the host, and TNF-α has a synergic activity with type I INFs to protect the host (146-148).

Blood samples of inoculated pigs with PRRSV viral proteins nsp1 and GP5 showed a high level of production of IL-10 compared to non-inoculated pigs (149); the expression of IL-10 has reduced the expression of other cytokines such as TNF-α and INF-γ; this reduction occurs in the onset of the infection (148-150). There is evidence that one of the viral proteins responsible for the upregulation of IL-10 is the N protein (143).

Porcine alveolar macrophages and peripheral blood mononuclear cells (PBMC) showed an increase in the production of IL-6 (which is a pro-inflammatory cytokine) when pigs were infected by PRRSV (151). The increased production of different cytokines suggests that the host is trying to fight the infection. Still, the expression of a single cytokine during the infection is not enough for PRRSV's clearance (152).

The IL-1β and IL-8 levels were measured to determine if they could act as an indicator of PRRSV infection persistence in lymphoid tissues. Both cytokines were up-regulated at early infection, suggesting that both cytokines play an important role in PRRSV immunity (153). High transcription levels of IL-8 at the early stage of infection, while IL-12 and IFN-γ mRNAs were reported with high transcription levels at a late stage of infection in PBMCs. Other cytokines such as TNF-α, IL-12, and INF-α were present in high levels in the serum of infected pigs, suggesting that the cytokines' expression could give hints to target immune responses to develop potential vaccines (152).

An important pathway for signalling at the intracellular level is the Janus kinase signal transducer and activator of transcription (JAK-STAT) and is affected during PRRSV infection (58, 145, 154). The production of host cytokines significantly affects this pathway; the importance of the JAK-STAT pathway is linked to its function of signalling the host immune response (145, 154). The suggested mechanism of PRRSV to dysregulate the JAK-STAT pathway is related to the inhibition of the interferon-stimulated gene factor 3 (ISGF3) (154), which plays an essential role in the activation of interferon-stimulated genes (ISG) responsible for the host immune response (145).

There is additional evidence that shows the nsp1β protein acting to suppress the JAK-STAT pathway in different cell lines such as MARC-145 and PAMs, the interaction between nsp1 and the nuclear protein PIAS1 suggest that nsp1 modulates the immune response (155). All this information helps better understand the role or importance of the cytokines when PRRSV's viral infection occurs because it can interfere with cellular pathways.

## 6.2. Adaptive immunity against PRRSV

Adaptative immunity is an essential barrier to preventing the development of infectious diseases; this immunity is activated when the host has been in contact with an infectious agent. The innate immunity cannot completely clear the pathogen from the host (156). The activation of adaptive immunity is based on the immune memory (previous exposure to an antigen) of the host and is thus more specific (157); the machinery of this immunity includes the antibody (humoral) and cellular mediated responses (158).

### 6.2.1. Antibody response

Antibodies are produced when an antigen stimulates the cell; the antibody binds to the pathogen antigen to label it to recognize the phagocytic cells to destroy the pathogen further. The neutralizing antibodies are important to mediate the immune protection against viral agents, and these antibodies take action at a late stage of infection (158, 159). The presence of antibodies (neutralizing and non-neutralizing) at an early stage of infection has not been shown to contribute to an immune response against PRRSV (160).

The antibody response induced by PRRSV starts to be evident after 7-9 days post-infection (d.p.i) (5); the targets are the viral proteins E, N, M, and GP5. The antibodies appear at different times of the infection; the chronologic order is 7 d.p.i for the E protein, and proteins M, N and GP5, the detection is after 2 weeks post-infection (161). The N protein is used as an antigen to detect antibodies. Other non-structural proteins (nsp) from PRRSV have shown a high immunogenic reactivity; most precisely, the nsp1 nsp2, and nsp7 (162).

In early infection, the antibodies do not necessarily clear the virus from the host. Instead, the antibody-dependent enhancement (ADE) occurs, referring to non-neutralizing antibodies transferred from infected to non-infected macrophages and enhancing viral infectivity (129, 160, 163). This mechanism has been demonstrated *in vitro* in infected macrophages, but *in vivo* remains unclear (164, 165).

The presence of neutralizing antibodies induced by PRRSV was confirmed when pregnant sows were gradually challenged using immunoglobulin (Ig) from hyperimmune animals. A passive transfer of antibodies to these sows was developed, preventing abortions at a rate of 95%. The newborns and dams did not present any viremia sign, confirming that the PRRSV-Igs can confer immunity against PRRSV (166). The viral neutralizing antibodies are detected in the serum after three weeks post-infection, and they remain for a long time and at low concentrations (119). Gnotobiotic pigs infected with PRRSV showed the presence of neutralizing antibodies at 32 and 52 d.p.i., suggesting that the presence of neutralizing antibodies aids PRRSV's clearance from the lungs (167).

### 6.2.2. The B and plasma cells response

The activation of B cells caused by a persistent infection with PRRSV leads to specific antibodies' production. Plasma cells are important too because, along with B cells, they determined antibodies levels in serum from infected pigs in lymphoid tissues (168). PRSSV causes a humoral immune response in infected pigs; immunoglobulins M and G production are detected after 5 and 7 d.p.i, respectively (116). The presence of B cells has been confirmed in lymphoid organs of infected pigs, tonsils. The IgG of plasma cells was detected in infected pigs' lymph nodes and spleen (168, 169).

### 6.2.3. T cell response

The swine T cells are characterized by the cluster differentiation (CD) markers such as CD3, CD4, and CD8 and the secretion of IFN-γ. The population of CD4/CD8$^{++}$ significantly increased during viral infection with PRRSV, meaning that memory T cells play an important role in memory responses (129, 170, 171). Some epitopes of non-structural proteins such as nsp2, nsp9, and nsp10 were identified to trigger the secretion of IFN-γ during PRRSV infection. The induction of T helper cells (Th1/Th2) during PRRSV infection is related to the production of IL-10 and the upregulation of Th1-specification factor TBX-21 (T-bet) in CD4$^{+}$ (129, 143)

### 6.2.4. Apoptosis

Apoptosis, also known as programmed cell death, is a mechanism affected during PRRSV infection. The viral proteins reported to affect this mechanism are GP2, GP5, nsp4, and nsp10; these proteins' role is to induce apoptosis in cells (62, 172). To better understand the activation of the apoptotic process, there are two described pathways. The intrinsic, which is activated when a cellular stressor is present. The stressors could be DNA damage, toxic drugs, or unscheduled DNA replication. During intrinsic apoptosis, the mitochondrial outer membrane permeabilizes (MOMP), releasing cytochrome c and leading to the apoptosome's formation and activation of the initiation caspase-9. The second pathway is the extrinsic pathway, where death receptors are expressed; specifically, this refers to the formation of the death-inducing signalling complex (DISC), leading to activation of caspases-8 and -10 (173-176).

The cells can suffer changes in their morphology and physiology due to apoptosis; these changes include cell shrinkage, membrane blebbing, condensation of nuclear chromatin, activation of endogenous endonucleases, and DNA fragmentation (176-178). This last is known to be enclosed in apoptotic bodies that are further phagocytized by a phagosome. Apoptotic bodies have been observed in histological lesions in PAMs infected with PRRSV, suggesting that PRRSV is involved in apoptosis (1, 172, 179).

The induction of apoptosis by PRRSV is believed to happen through a cross-talk of the intrinsic and extrinsic pathways (62, 180). Viral proteins such as nsp4 and nsp10 have shown to have a tole as pro-apoptotic proteins, nsp4 induces degradation of the anti-apoptotic cellular protein (BcI-XL), and nsp10 activates procaspases-8 and -9 (62). Additionally, it has been suggested that viral E protein also has a pro-apoptotic role by activating caspase-3 (181). Further studies are still needed to understand better how apoptosis and PRRSV infection interact (176).

### 6.2.5. Cell-to-cell communication and microvesicles

Cell-to-cell communication has been reported to occur during PRRSV infection by using the intercellular nanotubes mechanism. The viral materials transported could be viral RNA, certain replicases and structural proteins, and these materials are transported to the neighbouring cell's cytosol (182). PRRSV infection has been shown to hijack the mechanism of microvesicles, such as exosomes. These vesicles are responsible for transporting the information between infected and non-infected cells; most specifically, they encapsulate different proteins from the host such as vimentin, heat shock proteins, among others (25).

# CURRENT CONTRIBUTIONS OF PROTEOMIC INTERACTIONS BETWEEN THE HOST CELLS AND PRRSV

## 1. Proteomic changes in permissive cells

Recently proteomics has become a useful tool in virology. The study of viral and host proteins has led to a better understanding of the interaction during the infection process, the composition of viral particles, and the hijacking of cell machinery and pathways by viruses (183). Studies in human immunodeficiency virus (HIV), dengue virus, influenza virus, hepatitis C and B viruses, rabies and others, incorporated proteomics to understand the

mechanism by which the viruses can enter into the cell, composition of vesicles secreted during the infection and the viral progeny (184-191).

In PRRSV studies, some have elucidated the proteomic changes at the cellular level. MARC-145 cells are an immortalized cell line from the African green monkey kidney and allow PRRSV infection and production. The genetic stability of these cells compared to the PAMs, makes them suitable for *in vitro* research (192, 193). Proteomic studies in infected MARC-145 and PAMs cells have given tools to understand better how PRRSV enters the cell and affect different cellular pathways; this part of the document summarizes some of these studies and, in some cases, enounce the technologies used.

PRRSV infection causes imbalances in cells; the secretome carries information about the events occurring inside the cells. One of the technologies used for proteomic studies in PRRSV infection is stable isotope labelling (SILAC). This last is considered a promising technology to minimize quantification errors. SILAC allowed the identification of 204 host proteins from the secretome of infected MARC-145 cells and determined that these proteins relate to the cell-to-cell communication pathway (194). Another technique that aims to precipitate the proteins is immunoprecipitation (IP). Based on the use of antibodies that bind to specific proteins, IP permitted the analysis of PRRSV-infected MARC-145 cells lysates, finding that PRRSV may modulate cellular trafficking (182).

Additionally, the interaction between host proteins and PRRSV proteins has been studied using proteomics as well. The nsp2 of PRRSV was responsible for impairing different biological processes and molecular functions in MARC-145 cells. The cellular proteins affected belonged to the reproduction, cellular component organization or biogenesis, signalling, and immune system. The functions affected were transporter activity, structural molecule activity, binding, and catalytic activity. Mass spectrometry, coupled with liquid chromatography, was the method that allowed the identification of the cellular proteins interacting with nsp2 (195).

Another viral protein that has been shown to interact with the MARC-145 proteins is the M protein of PRRSV, affecting biological, cellular and metabolic processes. Among the

molecular functions, the most affected were binding, catalytic activity, and structural molecule activity. The cellular components most affected were macromolecular complex, cell, membrane, organelle, and membrane-enclosed lumen (73).

## 2. Proteomic changes in the natural host cells

Proteomic changes in PAMs during PRRSV infection were analyzed using the 2DE analysis coupled with MALDI-TOF MS/MS. Leading to the identification of 27 proteins and confirmed using western blot. Identifying proteins such as HSP70, aconitase, HSP27, and cathepsin D protein showed that the viral infection modulates host cells' molecular pathways (196). Mass spectrometry (MS) has been widely used and has demonstrated to be versatile because it can couple to other techniques. Isobaric tags for relative and absolute quantification (iTRAQ) coupled with liquid MS (LC-MS/MS) facilitated identifying 160 proteins from infected PAMs. These proteins affected metabolic functions and biological processes of infected PAMs (197).

The insertion of viral proteins into the cell can be a tool to study cellular proteins' expression and interaction. Comparing the proteomic profiles of PAMs and N protein-expressed PAMs was done to evaluate the cellular responses of PAMs to PRRSV N protein. N protein-expressed PAMs were clones generated by constructing a PRRSV N protein plasmid inserted into the cell line using gene transfer. Comparing the two cellular models showed that 15 proteins were affected in the cells expressing the N protein, 11 of them down-regulated, and four up-regulated. The processes affected by these proteins included cell division, metabolism, inflammation response, stress response, protein folding; the researchers highlighted the upregulation of HSP27 protein, which could be involved in the virus replication (198).

The strain of the virus can determine the expression of cellular proteins. There is evidence of PAMs infected with a highly pathogenic and attenuated strain. From the highly pathogenic strain, the proteins HSPB1, pyruvate kinase M2 (PKM2), and proteasome subunit alpha type 6 (PSMA6) were up-regulated, while the attenuated strain showed down-regulation in of the same proteins. Suggesting that these proteins' up-regulation can

cause alteration in different cell pathways such as inhibition of apoptosis, enhancing the viral replication and the evasion of the immune response (199).

The secretome of PAMs infected cells revealed 96 proteins affected. Showing up-regulation of 49 proteins and down-regulation of 46 proteins. The pathways affected included immune and inflammatory responses, signalling pathways, biological processes. Cells' secretome gives vital information about what is occurring inside the infected and non-infected cells (16).

## 3. Comparison of proteomic changes MARC-145 cells and PAM cells

MARC-145 and PAMs cells are continually being used to study PRRSV pathogenesis; there is a remarkable difference in these cells' origin. Thus, it is essential to elucidate the proteomic profiles of both cell lines. There is evidence about a common expression of proteins such as HSP27 and galectin-1 in MARC-145 and PAMs cells. Increased expression of HSP27 can indicate apoptosis modulation, and in the case of galectin-1, it can relate to infection or replication (200).

The interference of PRRSV-infected MARC-145 cells and PAMs showed the role of nsp4 and nsp10 in the pro-apoptotic activity of both cell lines. The activation of caspase-12 occurs in MARC-145 cells at 24 hours post-infection (h.p.i), and caspase-8 is activated in PAMs at 12 h.p.i, inducing apoptosis in both cell types. In the MARC-145 cells expressing nsp4 and nsp10, the affected pathways were death receptor pathway, mitochondrial-dependent pathway, and ER stress-dependent pathway. In PAMs, the cells expressing the nsp4 activated the ER stress-dependent pathway rather than the nsp10 (62).

The role of viral proteins such as nsp5 in PAM and MARC-145 cells is to induce the degradation of STAT3. One of the signalling pathways affected during PRRSV infection is the STAT3 pathway, composed of cytokines. The downregulation of this pathway by PRRSV infection in MARC-145 cells and PAMs is related to the interaction with nsp5. This non-structural protein induces the degradation of STAT3 by ubiquitination.

Suggesting that this protein may play a role in the perturbation of the host innate and adaptive immune responses (145).

All the proteomic studies during PRRSV infection done up to date have contributed to understanding the interaction between the host cell and the virus. However, the technologies used in each approach are different, and this could influence the outcome. Additional contributions are necessary to build up a solid knowledge about the influence of the viral proteins on the host machinery.

# CHAPTER II: MATERIAL AND METHODS

## 1. Cell culture and virus

MARC-145 cells (from African green monkey kidney origin) were cultured and maintained in T-175 flasks (Eppendorf, 0030712013, Canada). The maintenance medium was Eagle's Minimum Essential Medium (EMEM) (Wisent, 320-005-CL, Canada). The supplements for the medium were 10% Fetal bovine serum (FBS) (Wisent, 080-550, Canada), 1 % HEPES (1M) (Wisent, 330-050-CL, Canada), 1% penicillin/streptomycin (Wisent, 450-201-EL, Canada) and 1% amphotericin B (Wisent, 450-105-QL, Canada) (this recipe corresponds to the complete growth medium).

Cell incubation was at 37°C and 5% $CO_2$. Cells were passed once the cells reached 80% of confluency (every week). To this purpose, cell wash with PBS was necessary, then added 0.05% trypsin+EDTA (Gibco, 15400054, Canada), and cells incubation was at 37°C for 10 minutes. Determined the viability of the detached cells by trypan blue exclusion assay (201). Next, seeded $1x10^6$ cells in the flask and changed the growth medium once a week. For the cells used for viral production, $4x10^6$ cells were seeded and incubated at 37°C for 24 hours.

The strain used was the PRRSV Quebec reference strain IAF-Klop (*Betaarterivirus suid 2*) for viral production and infection experiments (9). For the optimization of PRRSV infectivity (experiments with the polycation-treatments), the HP-PRRSV strain has been used (kindly provided by Dr. Wang Chengbao). This strain can express the enhanced green fluorescent protein (EGFP), which was inserted between the N protein and the 3'-UTR of the virus's cDNA as previously described (48, 202).

### 1.1.Cells infection

MARC-145 cells at 80% of confluency were washed once with PBS and then infected with PRRSV. The multiplicity of infection (M.O.I) used was 0.1. The infections required a modified complete growth medium (without FBS). The viral attachment was performed by incubating the cells with the virus for 2 h at 37°C. After incubation, the cell culture

supernatant was removed (contained non-attached viruses). The infected cells were washed twice with PBS. Next, fresh medium containing 2% FBS, HEPES and antibiotics, was added, and then the flasks were incubated for 48 h at 37°C.

### 1.2.Infection of cells with PRRSV-EGFP and viral production

MARC-145 cells at 80% of confluency, grown in T25 flasks, were infected with PRRSV-EGFP ($10^{7.25}$ viruses/ml). Before the infection, the cells were washed once with PBS. Then for the infection, added the viral inoculum to the cells using EMEM 2% FBS+ antibiotics and HEPES, followed by incubation at 37°C, 5% $CO_2$. The infection was monitored for three days, and the cytopathic effect (CPE) was checked during this time. Once the cytopathic effect reached 45% of the infected cells, as observed under the fluorescence microscope (after the three days of post-infection), the cells were lysed through two cycles of freeze and thaw. The supernatants were centrifuged at 15000 xg, 4°C for 15 minutes. The pellet was discarded, and the supernatant containing viral particles was aliquoted in microtubes. The virus titer was determined using the Spearman-Karber method. The results were expressed in tissue culture infectious dose 50% per mL (TCID50/mL) (203).

## 2. Purification of viral particles

For viral particles obtention, the cells were lysed by repeated freezing and thawing cycles. The supernatants were purified. Briefly, two cycles of freezing (at -80°C) and thawing were performed. The cell culture media were then centrifuged at 4°C, 15000x*g* for 30 minutes to remove the cell debris (pre-clearing step). Thereafter, the supernatants were collected and centrifuged through a 30% sucrose (Millipore, SX1075-1, Canada) cushion [prepared in the TNE buffer, which contains 50 mMTris HCl (pH 7.5) (Fisher, BP152-5, Canada), 100 mM NaCl (Sigma, RES20908-A704X, Canada), and 1 mM EDTA (Millipore, 324503100GM, Canada)]. The centrifugation was performed at 4°C, 112 000 x *g* for 4 h (Optima L-1000XP, Beckman Coulter, Mississauga, ON, CA), using the rotor SW28 (Beckman Coulter rotor).

The resulting pellet was resuspended in the TNE buffer and centrifuged through a continuous gradient of 5% - 45% cesium chloride (prepared in the TNE buffer) at 4°C, 107

000 x *g*, overnight (Optima L-1000XP, Beckman Coulter, Mississauga, ON, CA), using rotor SW 41Ti (Beckman Coulter rotor). Next, ten fractions were collected. Two virus-enriched fractions were carefully selected (fractions containing viral particles equivalent to at least 50 µg of proteins were selected)—this step allowed to obtain purified viral supernatants. Aliquots from those two fractions were treated with subtilisin, an enzyme that digests microvesicles. Briefly, to the virus-enriched fractions, an equal volume of subtilisin (Sigma, P5380-100MG, USA) (dissolved in 2x Digestion Buffer to a final concentration 2mg/ml) was added. For the mock samples preparation, an equal volume of 2x Digestion Buffer (Tris HCl 40mM, $CaCl_2$ 2mM, pH 8.0) but without subtilisin was added. Next, all ultra-pure and corresponding control samples were incubated for 18 hrs at 37°C.

After the enzymatic digestion by the subtilisin, a protease-inhibiting cocktail (cOmplete, Mini, EDTA-free, Roche Catalog No. 4693159001; 1 tablet dissolved in 7 ml of TNE buffer) was added at a 1:1 ratio and incubated at room temperature for 15 minutes (to inhibit the subtilisin's activity). The samples were purified through a 30% sucrose cushion, at 4°C for 4 hours, 107 000 x *g* (Optima L-1000XP, Beckman Coulter, Mississauga, ON, CA), using the rotor SW 41Ti, as described before (54, 204, 205). After the purification, the viruses were resuspended in TNE sterile.

## 3. Viruses titration (or Virus quantification)

The cytopathic effect (CPE) in the cell culture system was used to determine the viral titers. The titer of the infectious viral particles present in each supernatant, purification steps and gradient fractions (after each centrifugation step) was calculated using the 10-fold dilution Spearman-Karber method (9, 14, 203, 206). The plates were incubated at 37°C for 96 h. The CPE was checked every day; the infected wells (wells with a visible CPE) were labelled as positive and viral titers have been calculated according to the Spearman Kärber formula. The results were expressed in tissue culture infectious dose 50% per mL (TCID50/mL) (203).

## 4. Viral RNA purification

The presence of viral RNA in the virus-containing samples (different purification steps and gradient fractions) were confirmed by quantitative real-time PCR method. Briefly, the viral RNAs were extracted following the manufacturer's spin protocol of the QIAamp viral RNA mini kit (Qiagen, 52906). Briefly, 140 µl of the sample were taken and mixed with 560 uL of Buffer AVL- Carrier RNA by vortexing for 15 seconds; then, the mixture was incubated for 10 minutes at room temperature and briefly centrifuged. Next, 560 uL of ethanol was added and mixed by vortexing for 15 seconds, followed by brief centrifugation. The 630 uL from this solution was transferred to a QIAamp Mini column and centrifuged at 6 000 x *g* for 1 min; the liquid was discarded (this step was repeated with the remaining solution). Then, 500 uL of the Buffer AW1 was added to the column and centrifuged for 1 min at 6 000 x *g*; the liquid was discarded. The sample was washed with 500 uL of the Buffer AW2 by centrifugation at 20 000 x g for 3 min, followed by second centrifugation at 20000 x g for 1 min. Finally, the sample was eluted with 60uL of Buffer AVE and centrifuged at 6 000 x *g* for 1 minute. The final elution was collected and subjected to an RT-qPCR assay (see below).

## 5. PRRSV RT-qPCR assay

The viral RNA extracted was used in the master mix for RT-qPCR; the enzyme used was Taqman Fast virus 1-step 4x (Thermo Fisher, Walthman, MA, USA). A positive sample of PRRSV (IAF-Klop) RNA purified was used. The target for PRRSV detection was the ORF6, and the sequences were:

PRRSV-F: GCTTTCATCCGATTGCGGCAARTGA;

PRRSV-R: CACCAATGTGCCGTTRACCGTAGT,

and PRRSV-Probe: [6~FAM]TAACCACGCATTTGTCGTCCGGCGT[BHQ1a~Q] (Eurofins).

The samples were poured into a 96-well plate (BioRad, MSB1001) and sealed by using plate film (BioRad, HSP9655). Then, the plate was centrifuged at 1 320 x *g* for 1 min to bring all the liquid to the bottom. Next, the plate was read by the Bio-Rad CFX-96 sequence detector. The amplification steps used for the PCR started with the retrotranscription (RT) at 50°C for 5 min, then the first denaturation at 95°C for 20 seconds (these conditions were done just in one cycle). After the RT step, a denaturation step was performed at 98°C for 2 seconds, and the annealing was done for 5 seconds at 57°C; the denaturation and annealing steps were repeated during 40 cycles.

## 6. Proteins concentration

The samples' protein concentrations (for each step of viral purification and in the gradient fractions) were determined with the colorimetric Quick Start Bradford Protein Assay (BioRad, 5000201) following the Microassay protocol of the company. Briefly, a 96-well plate was used for this assay. The content of protein was determined by measuring absorbance at 595nm in the spectrophotometer plate reader. The unknown samples' protein concentration was calculated using a created standard curve using BSA (Wisent00-095-AG, Canada) of the BioRad assay, as suggested by the manufacturer. All reactions were performed in triplicates for each experimental sample and BSA protein-based standard curve.

## 7. High-Resolution-Hybrid-Quadrupole-Orbitrap MS and proteomic analysis

A mass spectrometry approach was used to identify the proteins present in the exosomes or associated with the viral particles. The samples (the viral purification and virus-enriched fractions of the gradients) were subjected to digestion with trypsin to obtain the tryptic peptides. The proteomic profiles were assessed using these tryptic peptides. The peptides were fractionated by passing through the ion source to be classified by charge; the quadrupole mass filter provided the mass's wide-pass to sort the peptides by their weight

(*m/z*) (207-210). The proteomic information from the tryptic peptide was identified using the website Prospector (http://prospector.ucsf.edu/prospector/mshome.htm). These peptides' scores were plotted using the Graphpad Prism software (version 7.00, GraphPad Prism Software Inc., San Diego, CA). The statistical analysis was done by calculating One Way ANOVA with Turkey multiple comparison tests, using the peptides from the infected MARC-145 cells compared to the negative control (non-infected cells). The $p < 0.05$ was considered statistically significant.

## 8. Polycations

Polycations such as polybrene (Sigma Aldrich, H9268-5G, USA) and DEAE-dextran (Sigma Aldrich, D9885, USA), previously used for lentiviral vectors transduction studies (30), were used in our study. We evaluated their potential to enhance the attachment of the viruses to the cells. Different concentrations of polybrene and DEAE-dextran were tested to evaluate the capacity of these polycations to affect the infectivity of PRRSV on the MARC-145 cells. For the polybrene, we used 2, 4, 8, 12 and 16 µg/ml, while for the DEAE-dextran concentration at 3.75, 7.5, 15, 30 and 60 µg/ml were chosen based on literature (30, 31, 211, 212). The selected concentrations were prepared from the stocks of the polybrene (4 mg/ml, stored at 4°C) and DEAE-dextran (20 mg/ml, stored at -20°C). the solutions were filtrated through a 0.22 µm filter to ensure the stock's sterility; then, the stocks were aliquoted in 1.5 ml tubes and stored to ensure the stability of the polycations (the stocks were prepared in MiliQ water). For polycation-treatment experiments, the dilutions of the polycations were done by using fresh EMEM medium containing 2%FBS, antibiotics and HEPES (30, 35, 211, 213).

### 8.1. Viability and cytotoxic assay

The cytotoxicity of polybrene and DEAE-dextran were evaluated in non-infected MARC-145 monolayer cells. To this end, the 20 0000 cells per well were seeded onto 6-well plates (Corning, Canada); the cells were treated by adding 1 ml of fresh medium containing selected serial concentrations: 0, 2, 4, 8, 12 and 16 µg/mL of polybrene and 0, 3.75, 7.5, 15, 30 and 60 of DEAE-dextran to each well by triplicates, and the control was performed

using just fresh medium without polycations. The plates were incubated at room temperature for 2 hours. After the incubation time, the medium with polycations was removed from the wells and washed twice using PBS. After that, 3 ml of fresh medium without polycations were added to each well. Finally, the plates were incubated at 37°C for 24 and 48 hours.

After the polycation-treatment, the medium containing the polycations was removed, and the wells were washed with PBS. Next, 1 ml of trypsin-EDTA 0.05% was added to the cells, and cells were incubated for 10 minutes. Then, the detached cells were collected in 15 ml tubes, and the cells remaining in the wells were collected by an additional 2 ml of PBS-washing. The cells were resuspended in the PBS, and viable cells were counted using microscopy and trypan blue dye exclusion test.

Additionally, the kit Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, G1780) was used to determine the cytotoxicity of the polycations to the cells. This kit uses the LDH method to measure cytotoxicity. Briefly, 30 000 cells per well were plated in 24-well plates and incubated for 24 h at 37°C. The same concentrations used above were tested and the same procedure, but with some modifications. After adding the polycations to the cells and 2h incubation at room temperature, the polycations were removed, and the wells were washed 3 times with PBS. Then, the complete growth medium was added, and the cells were incubated for 18 h and 48 h at 37°C.

Next, the cell culture supernatants were removed and diluted with fresh medium (1:1 for the18 h and 1:3 for the 48 h post-treatment incubation samples). 50uL from each sample was mixed with 50uL of the kit substrate in a 96-well plate. The plate was covered and incubated at room temperature for 30 minutes in the dark (the substrate is light sensitive). Then, 50 uL of Stop Solution was added, and the plates were read at 490nm. We used different controls for this cytotoxicity assay. The first one was the No-Cell control, where the wells did not contain the cells (had only complete growth medium). These samples were used to measure the background levels of LDH of the culture medium. The second control was the Only Cells control, where the LDH levels (i.e. cytotoxicity) of the untreated cells were measured.

Additionally, the Maximum LDH Release was evaluated by adding 10µL of 10x Lysis Solution to the control wells (untreated cells) growing in 100uL of complete medium for 45 minutes. This procedure allowed to lyse all the cells in the control wells and measure the Maximum LDH Release by adding the CytoTox 96® substrate. This last control allowed the calculation of the percentage of cytotoxicity in the samples compared to the Maximum LDH Release control.

## 8.2. Optimization of PRRSV infection using polycations

MARC-145 cells were grown in 6-well plates and incubated for 24 h at 37°C. Then, cells were infected with PRRSV-EGFP at an M.O.I. of 0.1 using EMEM with antibiotics, HEPES and without FBS. The medium containing different concentrations of the polycations (0, 2, 4 and 8 ug/ml) was added to the cells to evaluate the effect of polycation on PRRSV-EGFP infectivity. Then cells were incubated for 2 h at room temperature, and washed twice with PBS. Finally, a complete growth medium pre-warmed to 37°C was added to the cells and cells were incubated for 28 h, prior to flow cytometry assay (Accuri C6 flow cytometer BD Biosciences).

## 8.3. The multiplicity of infection of PRRSV-EGFP

Different concentrations (0, 0.02, 0.1 and 0.5 MOI) of PRRSV-EGFP viral inoculum were tested to select the multiplicity of infection (M.O.I), which would be used for further experiments. Additionally, the effect of the washing step to remove unbound virus was evaluated by washing off unbound viral particles with cold-PBS at different times of post-infection (20 h, 24 h and 28 h). 6-well plates containing MARC-145 cells at 80% of confluency were used, and all experiments were done in triplicate. The infected cells and the cytopathic effect were assessed using fluorescence microscopy (DMI 4000B reverse fluorescence microscope image) analyzed using the Leica Application Suite software, version 2.4.0 Leica Microsystems Inc., Richmond Hill, Canada) and the NIH software ImageJ. The expression of the EGFP in cells was used as an indicator of the PRRSV infection.

## 9. PRRSV infectivity assessed by flow cytometry

The PRRSV-EGFP infected MARC-145 cells were subjected to flow cytometry. To this end, the medium was removed, and the cells were washed with cold PBS. The cells were detached from the cell culture plate by adding 1 ml of trypsin + EDTA 0.05% and incubating them for 10 min at room temperature. Next, the detached cells were transferred into 15 ml tube; then, 1 ml of fresh complete cell growth medium was added to the cells to inhibit the trypsin and EDTA activities; the well was washed twice with 2 ml of PBS to collect the remaining cells. The harvested cells were centrifuged at 400 x *g*, and the supernatant was discarded. The cells containing the pellet were resuspended in 1 ml of PBS. Next, the cells were transferred into a 1.5 ml tube and spun down at 400 xg for 1 minute. The cells were resuspended and fixed in 0.5 mL of 2% paraformaldehyde (PFA) (Sigma Aldrich, 158127, USA) on ice for 30 minutes. PFA-fixed cells were washed twice with ice-cold PBS, resuspended in 1ml of cold PBS, and the GFP-positive cells were counted using the flow cytometer.

## 10.Statistical analysis

One way ANOVA and Turkey's multiple comparison test (GraphPad Prism Version 8 software) was performed to analyze the statistical difference among semipurified and the purified fractions of PRRSV. Two way ANOVA and Turkey's multiple comparison test (GraphPad Prism Version 8 software) was used to analyze the statistical difference in different parameters measured such as polycations concentrations, polycations concentrations with PRRSV, and viral titers of PRRSV infections and polycations. The statistical significance was determined using a $P<0.05$ for all the analyzed data.

# CHAPTER III: RESULTS

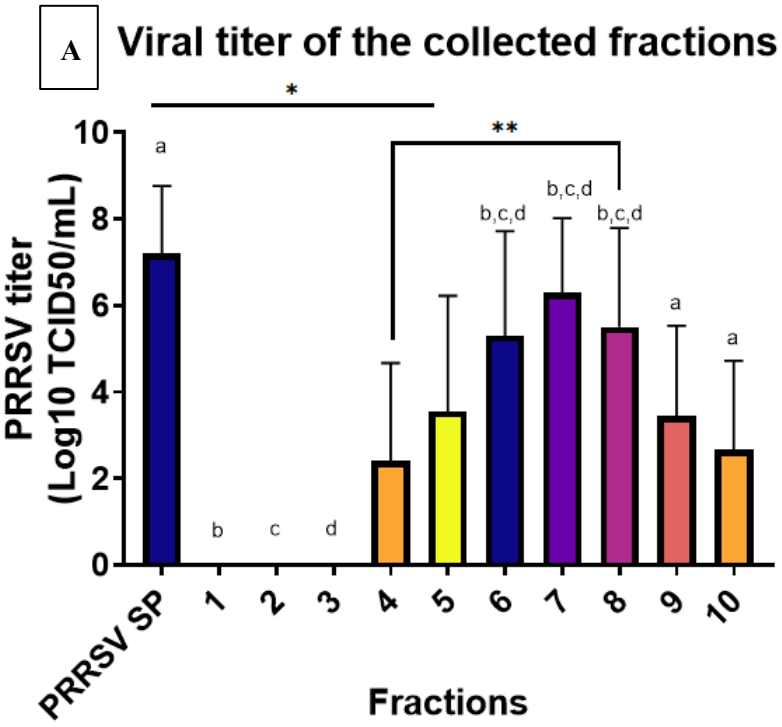
## 1. Proteomic analyses of the infection of PRRSV in MARC-145 cells

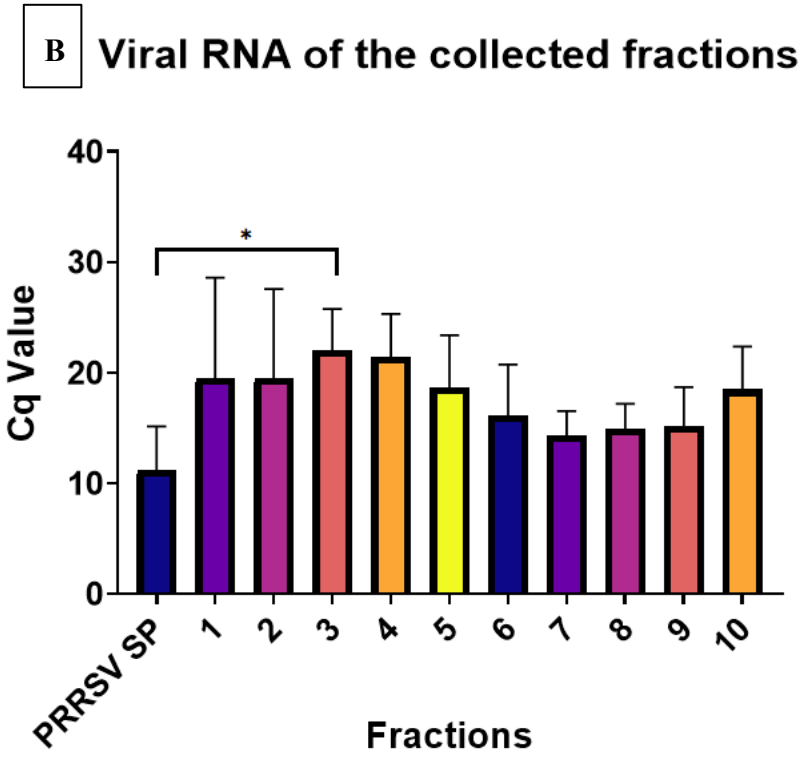
### 1.1. Purification of viral particles

The viral particles were purified from the infected MARC-145 cells; negative control with mock-infected cells was performed. This experiment was repeated 5 times and performed a validation after different purification steps. Once the purification step with 5-45% CsCl ended, ten fractions were collected. It was evident to see a difference between the different samples when parameters were validated (figures 1A-1C).

The semipurified samples contained a higher viral titer, a low quantity of cycles (Cq) and a high concentration of proteins; all these parameters compared to the purified fractions. The possible explanation for this difference could be that the semipurified samples contained cellular, microvesicles, and viral proteins.

The purified samples allowed to determine whether the viral particles were concentrated, the parameters evaluated to determine this are shown in figures 1A to 1C. The viral titers of fractions 7 and 8 were $10^7$ and $10^6$ $TCID_{50}$/mL, and these fractions contained the lowest quantity of cycles (Cq), and the higher amount of proteins compared to the other fractions. After the selection of the fractions containing the virus, the enzyme subtilisin was used to purify the viral particles.


A
Viral titer of the collected fractions
PRRSV titer (Log10 TCID50/mL)
Fractions
PRRSV SP
1
2
3
4
5
6
7
8
9
10
a
b
c
d
b,c,d
b,c,d
b,c,d
a
a
*
**


B
Viral RNA of the collected fractions
Cq Value
Fractions
PRRSV SP
1
2
3
4
5
6
7
8
9
10
*

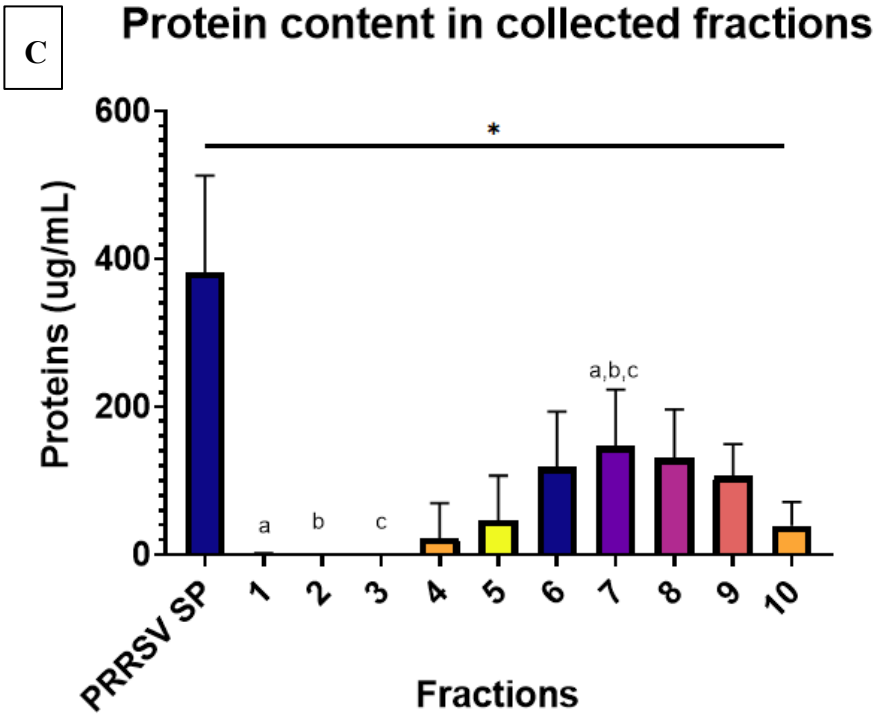



Figure 1. Validation of the viral purification supernatants of PRRSV from infected MARC-145 cells after 48 hours of post-infection.

The purification steps are semipurified (SP, 30% sucrose cushion) and purified (CsCl 5-45%), divided into ten fractions. (A) represents the titration of the semipurified and different fractions expressed in $TCID_{50}$/mL. (B) corresponds to the quantification cycle (Cq) values of the different samples evaluated, the target gene was ORF6 of PRRSV. (C) shows the total amount of proteins contained in each sample. Results are presented as the mean ± SEM (standard error of the mean) from five independent experiments. Bars labelled with letters and asterisk (*) indicate that the data of these groups is significantly different ($p<0.05$).

### 1.2.Proteomic identification and analysis

The proteomic identification and analysis of two semipurified samples and the selected fractions were carried out using a high-resolution-hybrid-quadrupole-orbitrap mass spectrometer. Tryptic peptides were extracted after trypsinization of the different samples. The change in the expression of the proteins was determined by comparing the scores of peptides of infected and non-infected cells (negative control).

In figure 2, the horizontal dotted line represents the significance threshold (1.3), meaning that the peptides located on top of this threshold are statistically significant (p-value <0.05), as it could be seen most of these peptides decrease their abundance in PRRSV infected cells.

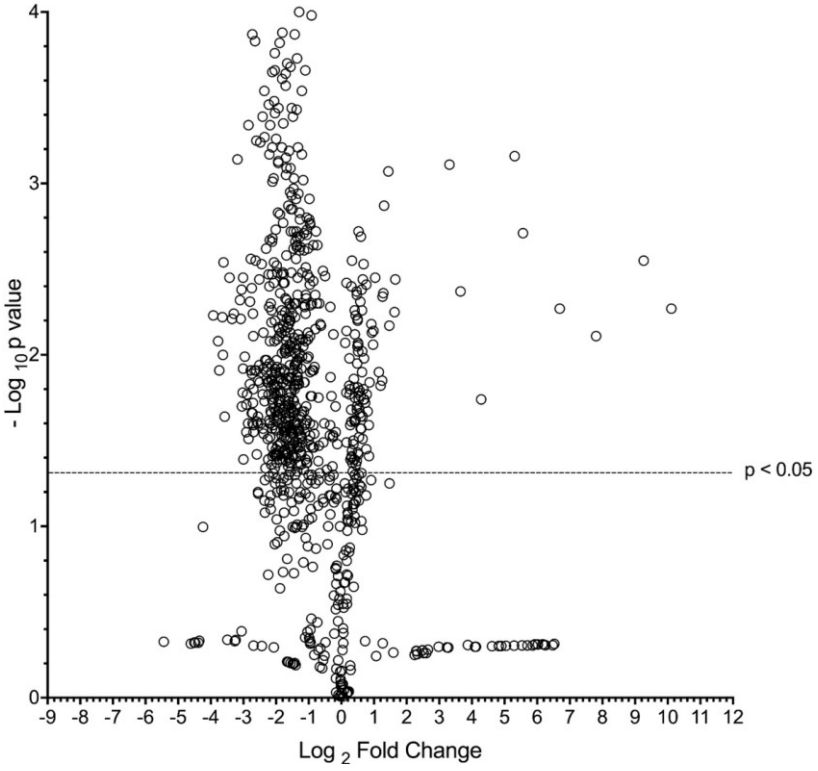
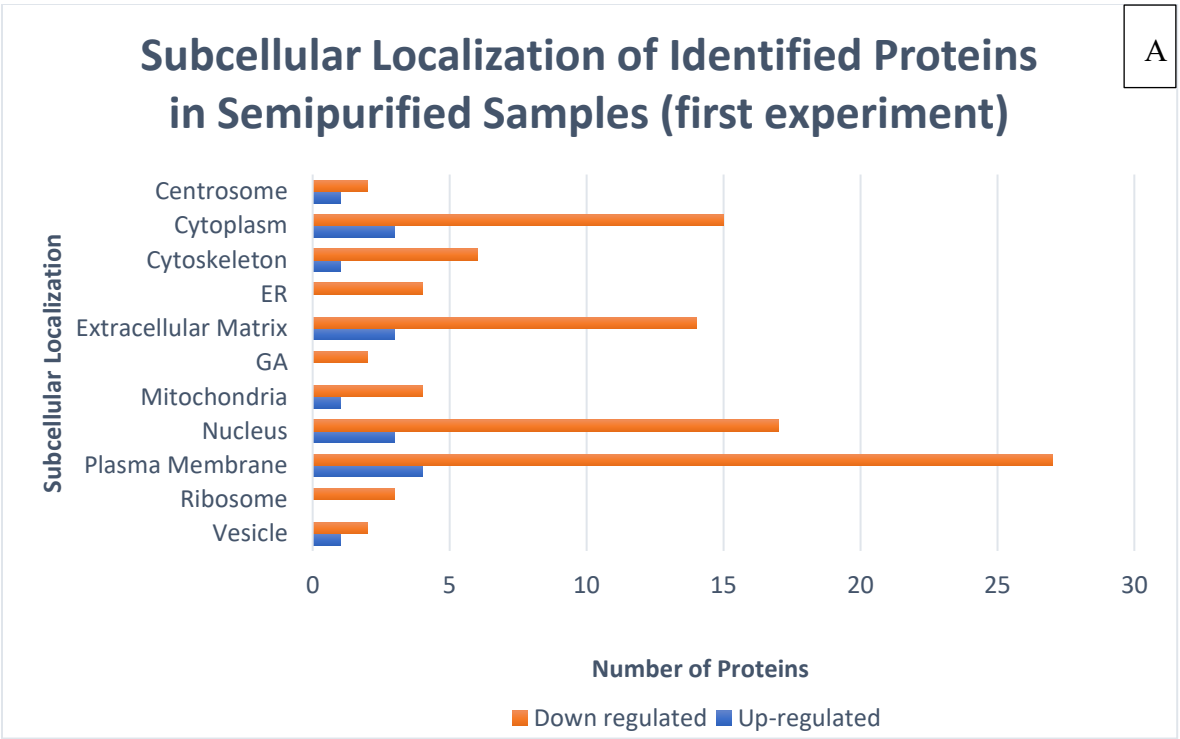


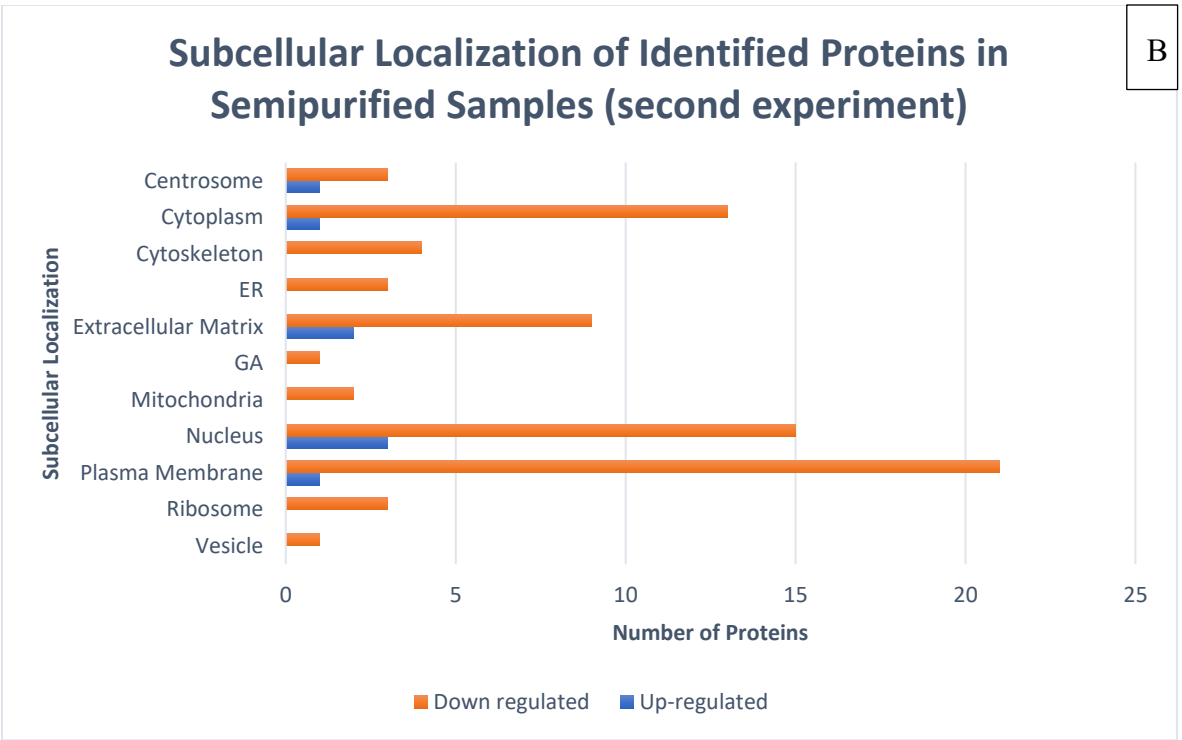

Figure 2. Tryptic peptides of the proteins contained in the semipurified samples of MARC-145 cells infected by PRRSV after 48 hours of post-infection.

The *X*-axis represents the fold change of tryptic peptides associated with the identified proteins on a $\log_2$ scale compared with the negative control. The *Y*-axis the $-\log_{10}$ *p*-value ($p < 0.05$).

The obtained proteins were blasted against the UniProt *Chlorocebus aethiops* database (downloaded on September 9, 2016). Afterwards, the proteins' classification was according to subcellular localization (annex 1. Tables 1 and 2) and molecular functions (Fig. 3 and 4). As most of the proteins were down-regulated, figures 3A and B showed most of the affected proteins frequently localized in the plasma membrane, nucleus and cytoplasm. In the functional classification, figures 4A, B, C and D showed that most of the affected pathways were nucleic acid binding, metabolic processes, immune response and cell cycle.

We could identify membrane cofactor protein and latent transforming growth factor-beta binding protein 3 (annex 1. Tables 1 and 2) as proteins associated with cellular vesicles in semipurified and purified samples. In figure 3A and B, the classification by subcellular localization shows that PRRSV's viral infection is affecting the proteins related to vesicles. Also, Figures 4A and B show the functional classification of the proteins. One of the affected functions is transportation. These two classifications could be linked, as the microvesicles' function is the cell to cell communication.


A
Subcellular Localization of Identified Proteins in Semipurified Samples (first experiment)
Subcellular Localization
Centrosome
Cytoplasm
Cytoskeleton
ER
Extracellular Matrix
GA
Mitochondria
Nucleus
Plasma Membrane
Ribosome
Vesicle
0
5
10
15
20
25
30
Number of Proteins
Down regulated
Up-regulated



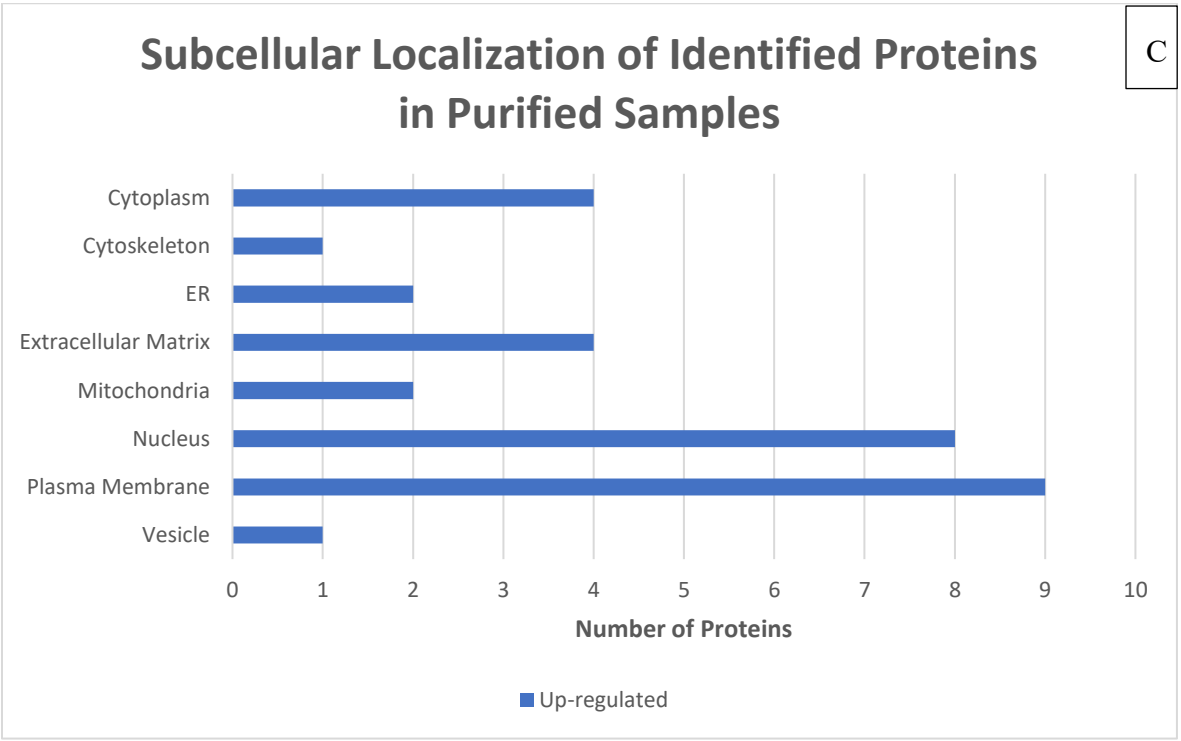
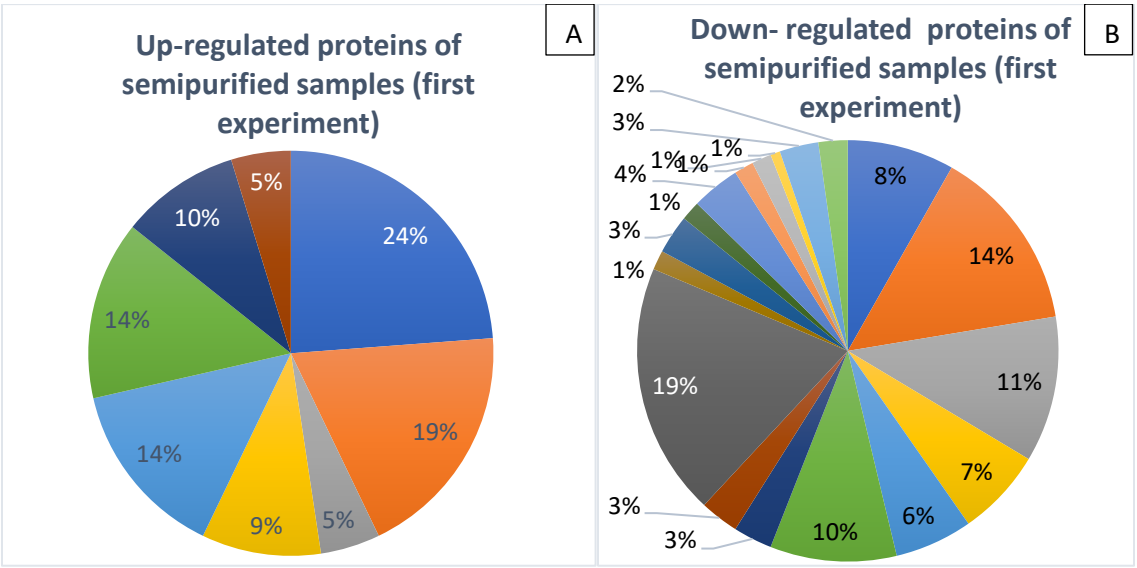



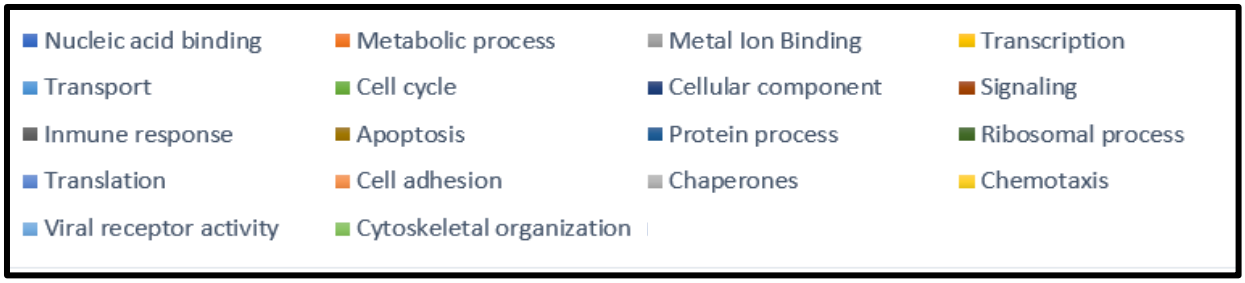

Figure 3. The number of proteins affected during PRRSV infection in the analyzed samples after 48 hours of post-infection.

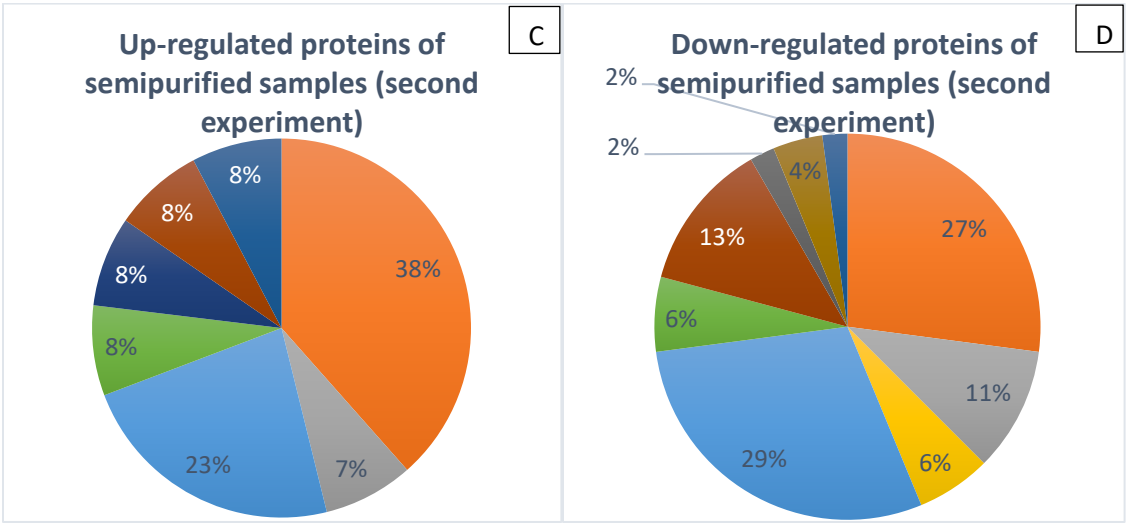
A. corresponds to the first experiment, B.corresponds to the second experiment, and C. corresponds to the purified proteins obtained during the first experiment after the semipurified samples' purification.

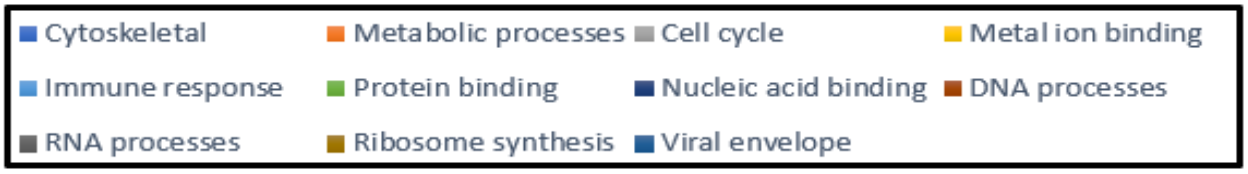
The proteins from the purification through CsCl in the first experiment were up-regulated. In figures 3 C and 4 E, the subcellular localization showed that most of these proteins are distributed in the nucleus and plasma membrane. Additionally, pathways such as immune response, cell cycle, metal ion binding and metabolic processes were the most affected during the infection of PRRSV (Fig. 4A-E).

The presence of proteins related to vesicles (Fig. 3C), and transportation function (Fig. 4E) in the purified samples, suggest that the last purification using subtilisin will lyse the external proteins of the viral particle. This lysis will reveal proteins of the host that are contained in the progeny after the budding and exocytosis of new viral progeny.




Nucleic acid binding
Metabolic process
Metal Ion Binding
Transcription
Transport
Cell cycle
Cellular component
Signaling
Inmune response
Apoptosis
Protein process
Ribosomal process
Translation
Cell adhesion
Chaperones
Chemotaxis
Viral receptor activity
Cytoskeletal organization


C
Up-regulated proteins of semipurified samples (second experiment)
38%
7%
23%
8%
8%
8%
D
Down-regulated proteins of semipurified samples (second experiment)
27%
11%
6%
29%
6%
13%
4%
2%
2%


Cytoskeletal
Metabolic processes
Cell cycle
Metal ion binding
Immune response
Protein binding
Nucleic acid binding
DNA processes
RNA processes
Ribosome synthesis
Viral envelope

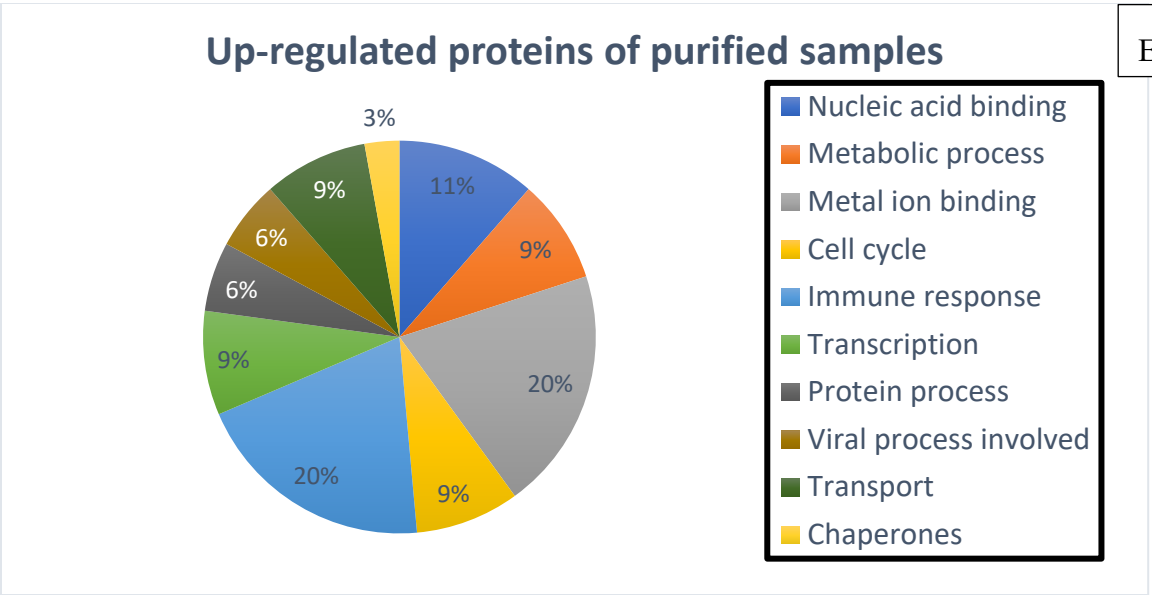



Figure 4. Classification by the molecular function of the identified proteins after PRRSV infection after 48 hours of post-infection (n corresponds to the number of proteins).

A and B correspond to experiment 1; C (n=8) and D (n=110) to experiment 2; E (n=21) corresponds to the purification of the identified proteins in experiment 1.

### 1.3.Protein to protein interaction

The interactome of figure 5 represents the interaction of the identified proteins from the host during PRRSV infection (annex 1., Tables 1 and 2). The protein-protein interaction network was constructed using the database String.

We could observe the interaction edges of co-expression (meaning that the proteins are expressed at the same time and represented by black lines) and text mining (yellow lines).

Proteins such as ninein (NIN), centromere protein J (CENPJ), microcephalin 1 (MCPH1) and topoisomerase (DNA) II binding protein 1 (TOPBP1), are important for processes of protein organization and protein binding (UniProt).

Other co-expressed proteins are Bone marrow stromal cell antigen 2 (BST2), Tripartite motif-containing 22 (TRIM22), Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), BST2, and APOBEC3G are associated to innate immune response, defence response to viral infection (replication), TRIM22 has a positive regulation of autophagy. APOBEC3G has an interaction with Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 4 (putative) (APOBEC4), which is also related to the innate immune response to viral infection, more specifically to retrovirus (UniProt).

Another co-expression presented in the interactome happens between DnaJ (Hsp40) homolog, subfamily C, member 1 (DNAJC1), Ribosomal protein S6; Belongs to the eukaryotic ribosomal protein eS6 family (RPS6) and 40S ribosomal protein SA (RPSA), these group of proteins are involved in the translation process, and RPSA can act as a receptor for viruses (UniProt).

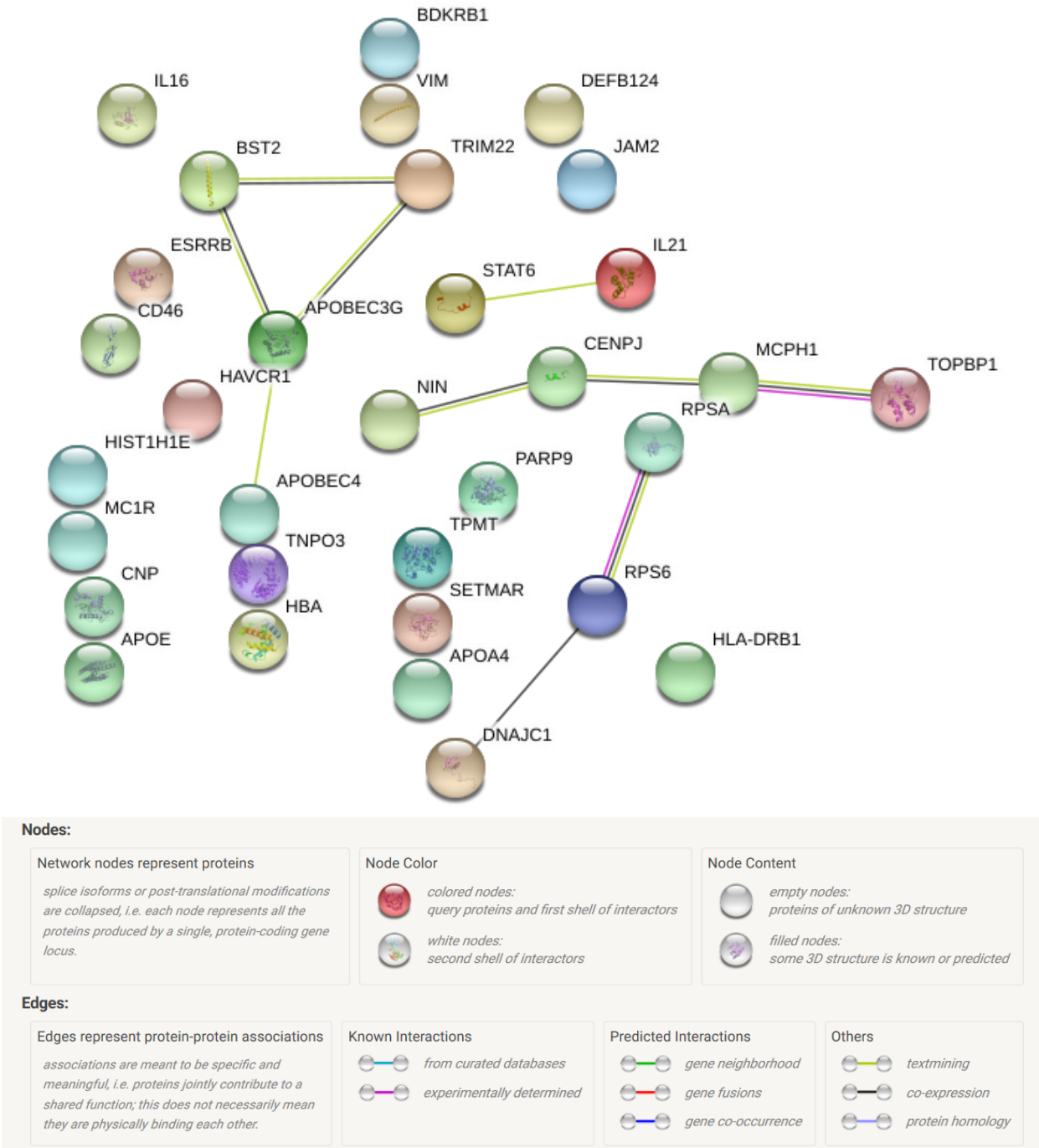



Figure 5. Interactome of the identified proteins during PRRSV infection in MARC-145 cells after 48 hours of post-infection.

It was hypothesized that the number of proteins obtained after the purification with 30% sucrose and 5-45% CsCl could increase. Also, it is important to know at what point of the

viral life cycle the proteins have their peak of production; in this way, we could get more proteins and compare if the expressed proteins are the same during the viral cycle. Some studies suggest that PRRSV's viral proteins were interacting with host cell proteins after 48h post-infection at high levels of expression; the affected proteins from the host were related to immune response, most exactly cytokines such as L-1β, IL-6, IL-8, and TNF-α (78, 214). It was necessary to modify the infection protocol to optimize the infection and get as many infected cells as possible and, therefore, a higher amount of proteins.

# 2. PRRSV infection optimization in MARC-145 cells using polycations

Two positively charged molecules (polycations) were necessary to increase the viral attachment to the cell surface. This strategy was based on previous evidence of the high efficiency of transfection when polybrene and DEAE-dextran, when added to viral infections (30, 35, 213).

## 2.1.Polycations cytotoxicity of MARC-145 cells

Two parameters evaluated to know if using polycations were suitable for optimizing the infection in MARC-145 cells were the viability of cells after the exposure to polycations and the cytotoxicity of polybrene DEAE-dextran. The treatments lasted 1 hour, and then the cells with the fresh medium were incubated for 48h. The results showed a low impact on the viability of cells after 48 h post-treatment. The polycations in cells were compared by performing negative controls using non-treated cells (Fig. 6).

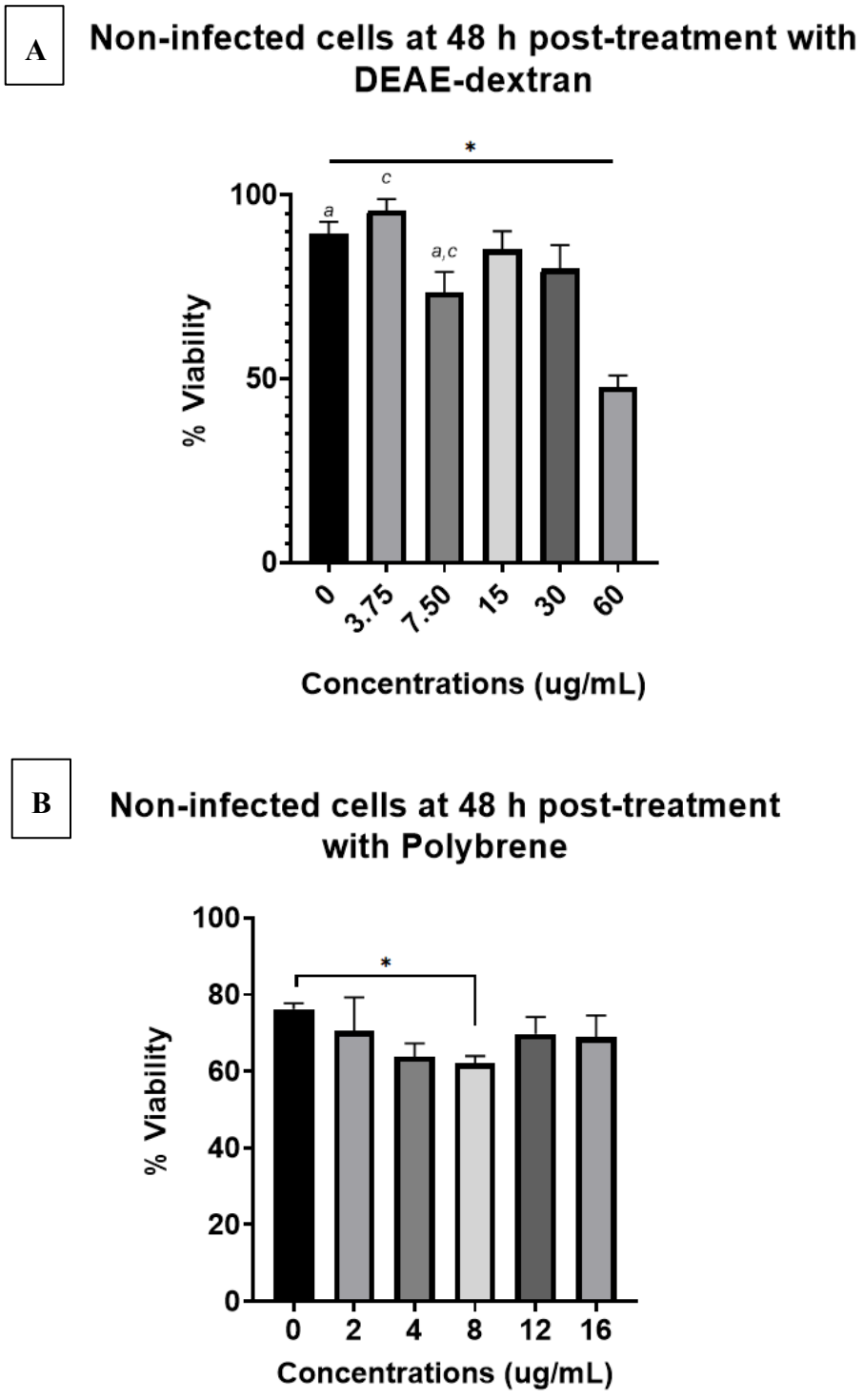
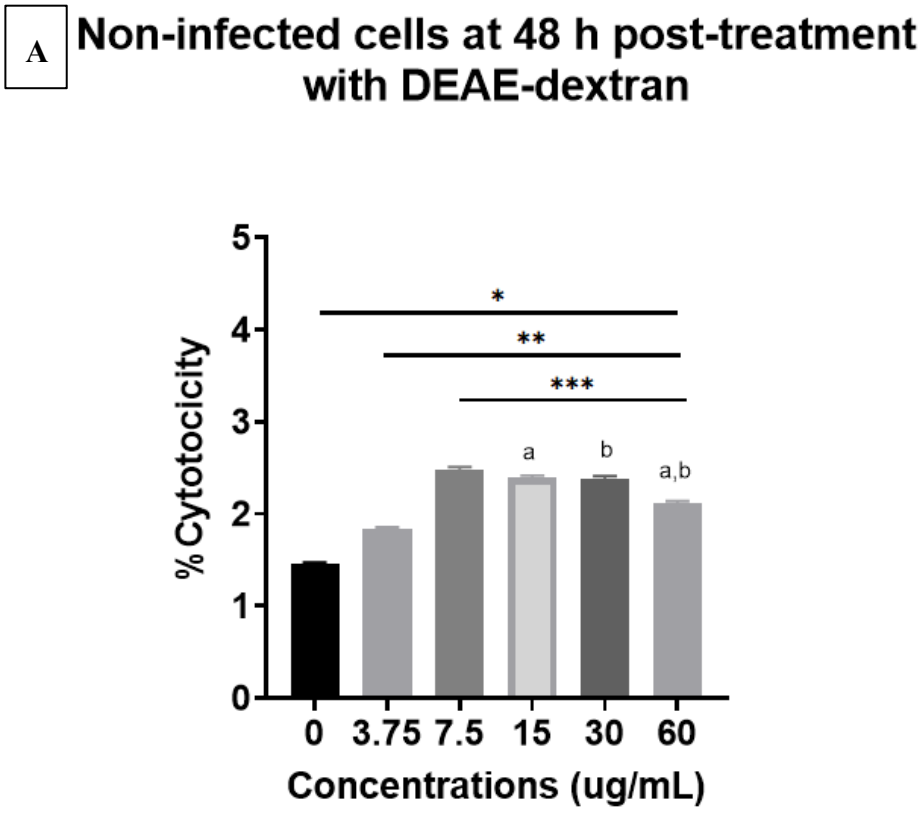



Figure 6. Effect of polycations at different concentrations in the viability of non-infected MARC-145 cells after 48 hours of treatment.

MARC-145 cells were treated with different polycations concentrations for 2 h; then, the treatments were removed. A. shows the impact of DEAE-dextran in non-infected cells. B. shows the effect of polybrene in non-infected cells. Results are presented as the ± SD (standard deviation) values of the different concentration tested of polycations from three replicates. The bars identified with letters or asterisks (*) indicate that their data is significantly different in the tested concentrations of each treatment ($p>0.05$).

Knowing that the MARC-145 cells remained viable after the exposure to polycations, it was crucial to know the cytotoxic effect of these molecules in the cells as well. This parameter was measured by the production of the enzyme lactate dehydrogenase (LDH) of treated cells. The percentage of cytotoxicity caused by DEAE-dextran and polybrene is represented in figure 7.



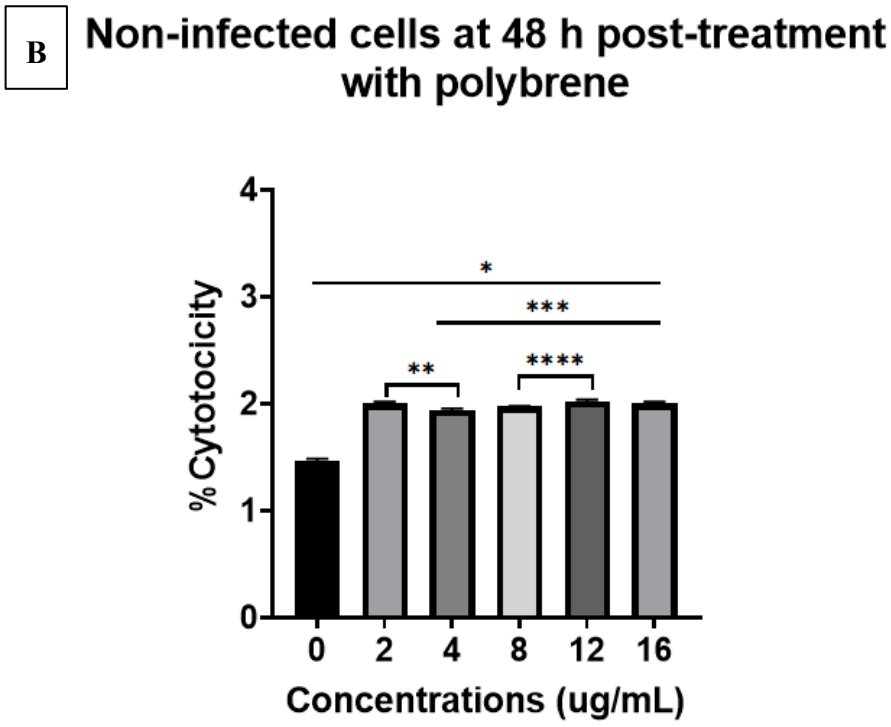



Figure 7. Percentage of cytotoxicity by polycations treatments in non-infected MARC-145 cells.

MARC-145 cells were treated with different polycations concentrations for 2 h; then, the treatments were removed. Cells with the fresh medium were incubated for 48 h, at 37°C. A. represents the percentage of cytotoxicity of DEAE-dextran in MARC-145 cells after the treatment. B. represents the cytotoxic effect of polybrene in MARC-145 cells after the treatment. Results are presented as the ± SD (standard deviation) values of the different concentration tested polycations from three replicates. The bars identified with asterisks (*) indicate that their data is significantly different in the tested concentrations of each treatment ($p>0.05$).

After the validation of the effect of polycations in non-infected MARC-145 cells, it was evident that these molecules do not have a toxic effect on the cells (Figs.6 and 7). The percent of cytotoxicity for both polycations showed values lower than 5%. The cells' viability did not decrease more than 50% after the exposure of cells to these two molecules. The polycations DEAE-dextran and polybrene are safe to use with MARC-145 cells, and that let us continue to the next step to optimize PRRSV infection.

### 2.2.Infection of MARC-145 cells by PRRSV-EGFP strain

In parallel to the cytotoxicity and viability tests, the infection of MARC-145 cells with PRRSV was adjusted by selecting the more suitable multiplicity of infection (M.O.I) of the strain of PRRSV-EGFP (Dr. Wang Chengbao kindly provided this strain) (48). In addition to the selection of the M.O.I, factors such as time and synchronization of the infection were evaluated.

The time was one of the parameters to determine the time when the virus finishes its first cycle of infection in the cells, the hours proposed in the experiments were based on the results obtained by Provost et al. (9) and results observed during preliminary tests performed in the laboratory (data not showed). The synchronization was proposed to evidence how the infection behaved if the plates containing cells with the virus were washed and non-washed. The washing step in the plates means that the infection was synchronized.

Infected cells were observed using fluorescence microscopy (Figs. 9 and 10), the cytopathic effect (CPE) was first observed in the phase-contrast, and then the cells infected by PRRSV-GFP were observed using the fluorescence filter. The software ImageJ was used to count the number of infected cells in each experiment. The final count of infected cells was expressed as a percentage of infected cells. Figure 8 shows that the highest percentage of infected cells was obtained at M.O.I. of 0.5 for the washed after 28 hours of infection, and for non-washed plates, the M.O.I. was 0.5 after 20 hours of infection. It is also evident that the tendency for the non-washed plates is not regular. Washed plates showed a better tendency for an increase in the percentage of infected cells during the time of the infection and the M.O.I. tested.

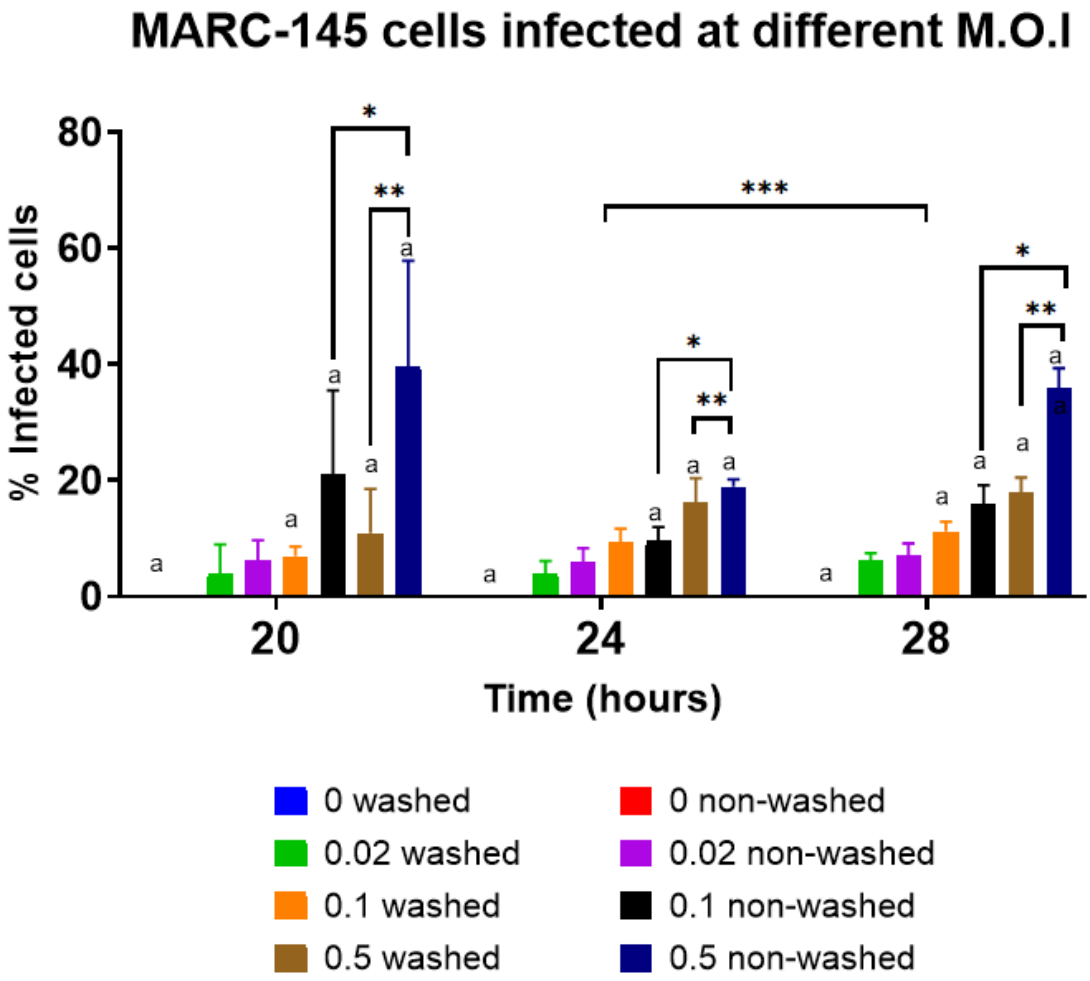



Figure 8. Percentage of infected cells by PRRSV at different M.O.I. and time of infection. MARC-145 cells were infected at different M.O.I. and the time of infection was variable.

Additionally, the plates containing the cells with the virus were split into two groups, washed and non-washed. The infected cells were observed using the fluorescence microscope, followed by counting them using the ImageJ software (four fields of each well replicate were considered for counting the total and the infected amount of cells). Negative controls containing mock-infected cells were performed. Results are presented as the ± SD (standard deviation) values of three replicates. The bars identified with letters and asterisks (*) indicate that their data is significantly different in the tested M.O.I. of each treatment, and the three asterisks

correspond to the significant difference among the data between 24 and 28 hours of post-infection ($p>0.05$).

Figure 9. Detection of infected cells at different times of post-infection, using different M.O.I. in washed plates.

MARC-145 cells were infected with PRRSV-EGFP at different concentrations of virus (M.O.I.) during different hours. Mock infected cells were used as a negative control. The visualization of the cells was in light-phase contrast (observing the CPE) and the UV (fluorescence lamp, observing the infected cells); the pictures were merged using a phase-fluorescence lamp

| M.O.I | Time (h) | CPE | Fluorescence | Merge |
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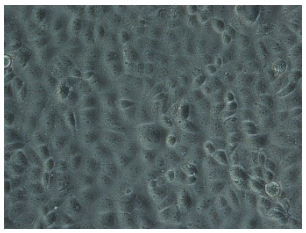
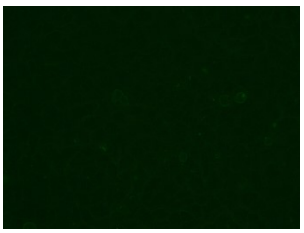
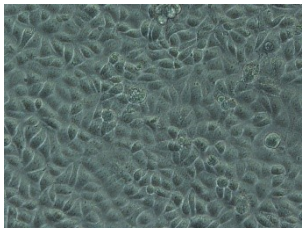
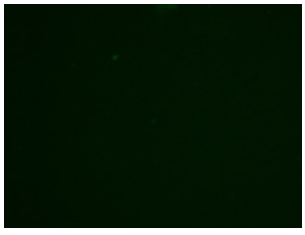
| 0 | 20 |  |  |  |
| | 24 |  |  |  |

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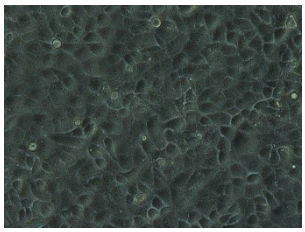
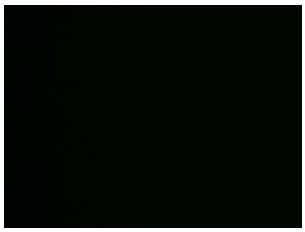
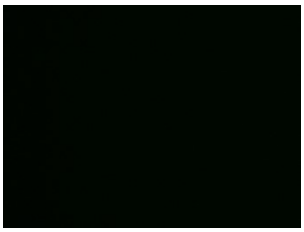
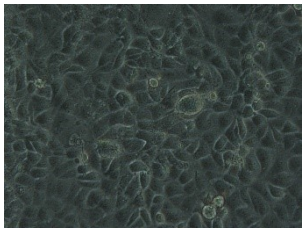
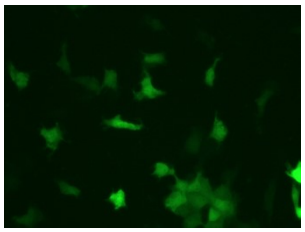
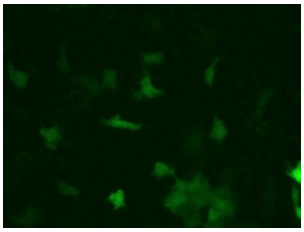
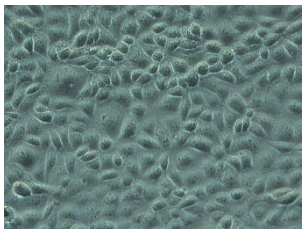
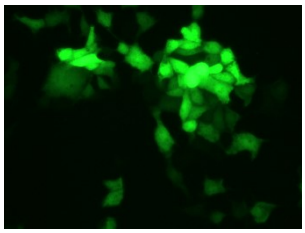
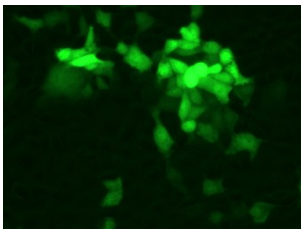
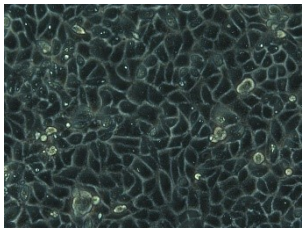
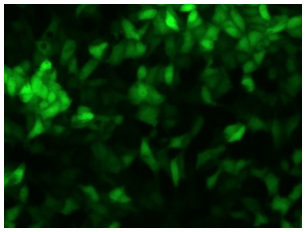
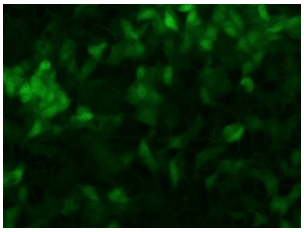
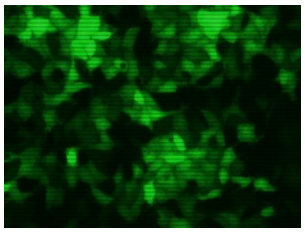
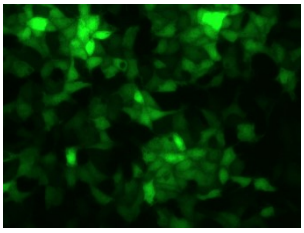
| | 28 |  |  |  |
| | 20 |  |  |  |
| **0.02** | 24 |  |  |  |
| | 28 |  |  |  |
| **0.1** | 20 |  |  |  |

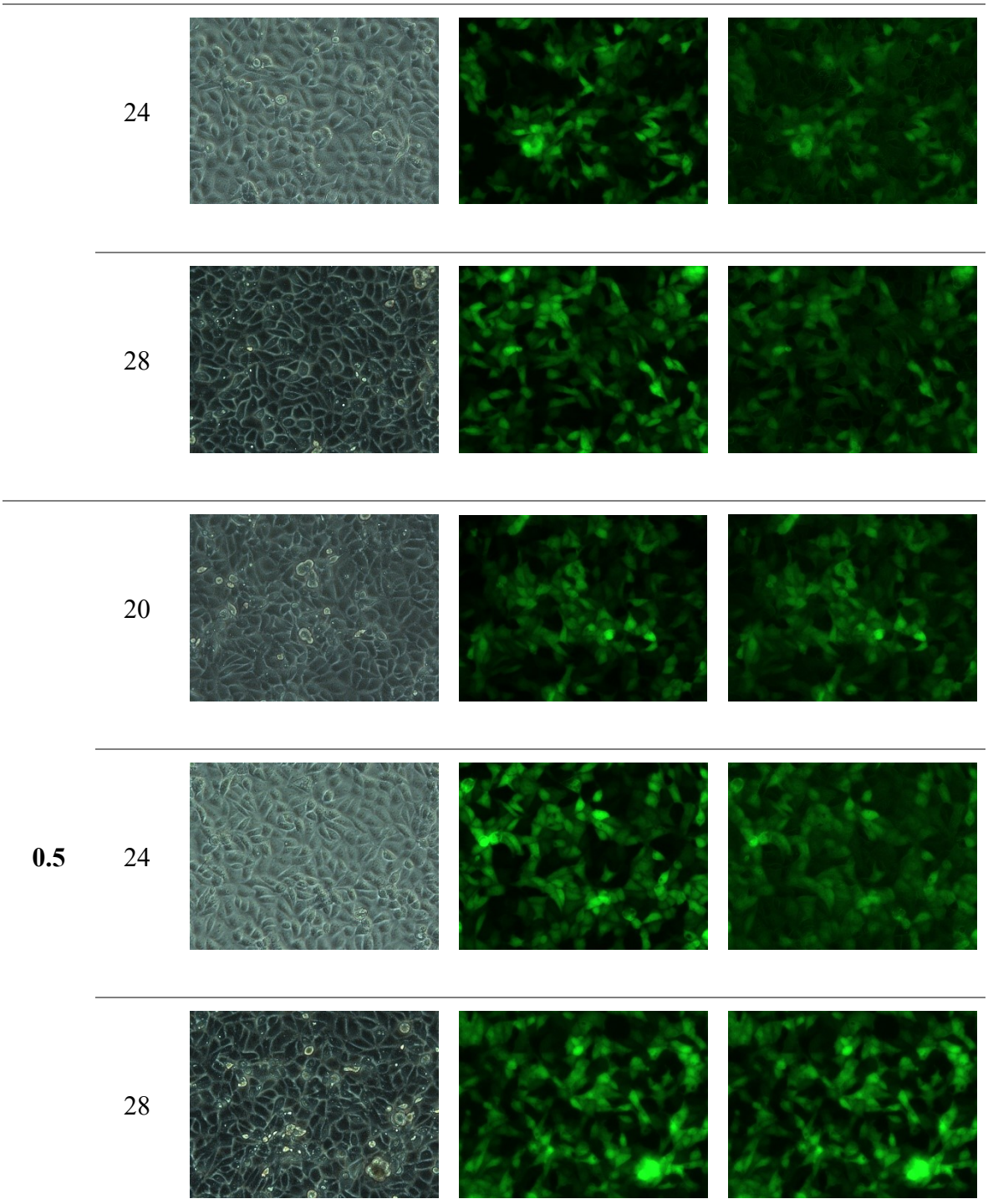



Figure 10. Detection of infected cells at different times of post-infection, using different M.O.I. in non-washed plates.

MARC-145 cells were infected with PRRSV-EGFP at different concentrations of virus (M.O.I.) during different hours. Mock infected cells were used as a negative control. The visualization of the cells was in light-phase contrast (observing the CPE) and the UV (fluorescence lamp, observing the infected cells); the pictures were merged using a phase-fluorescence lamp.

| **M.O.I** | **Time (h)** | **CPE** | **Fluorescence** | **Merge** |
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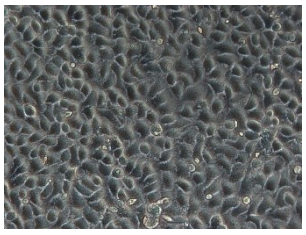
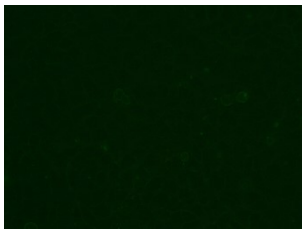
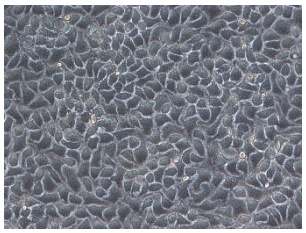
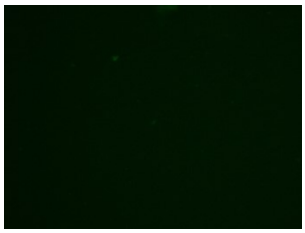
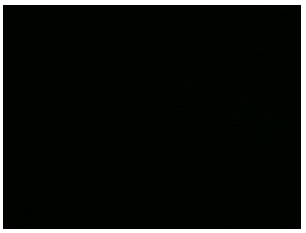
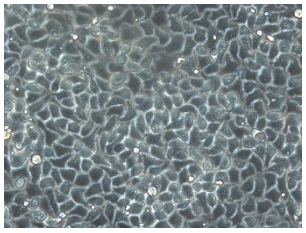
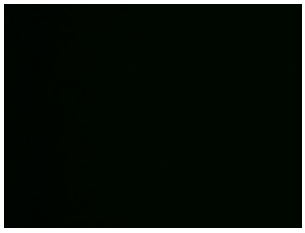
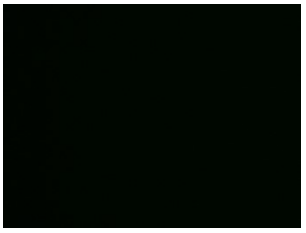
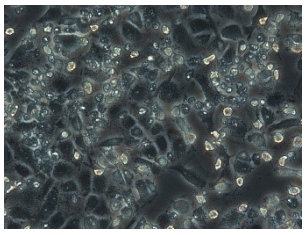
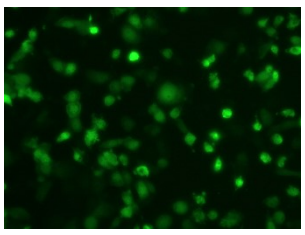
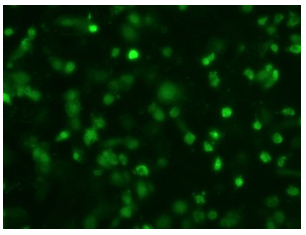
| **0** | 20 |  |  |  |
| | 24 |  |  |  |
| | 28 |  |  |  |
| **0.02** | 20 |  |  |  |

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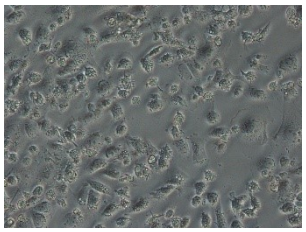
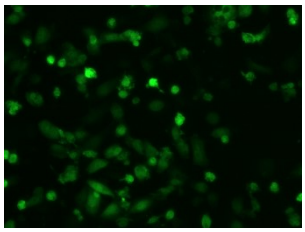
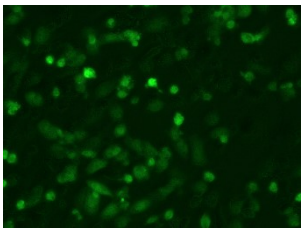
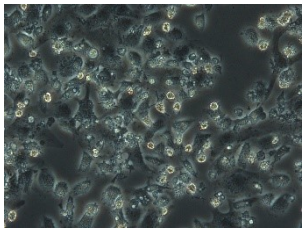
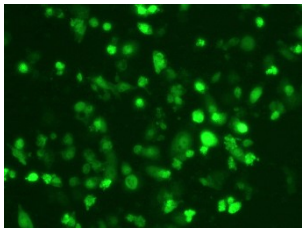
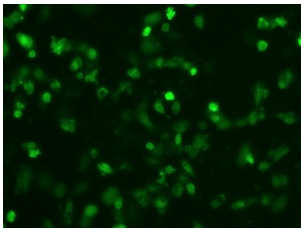
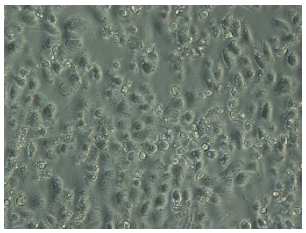
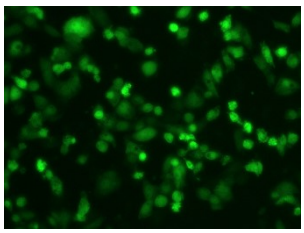
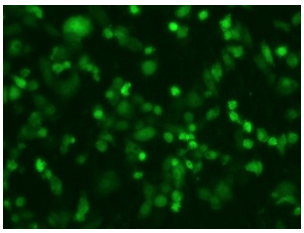
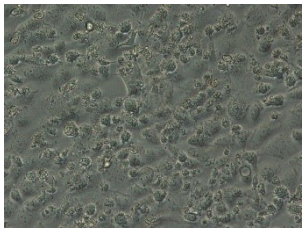
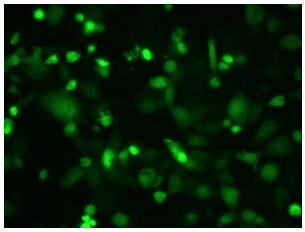
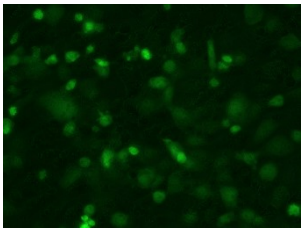
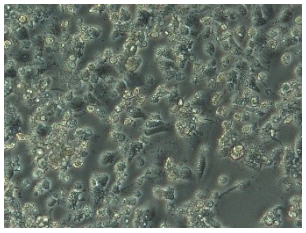
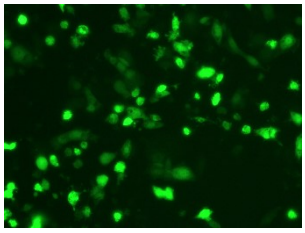
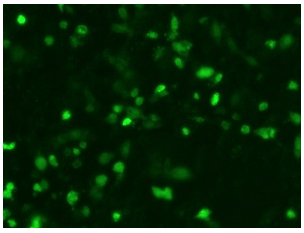
| | 24 |  |  |  |
| | 28 |  |  |  |
| | 20 |  |  |  |
| **0.1** | 24 |  |  |  |
| | 28 |  |  |  |

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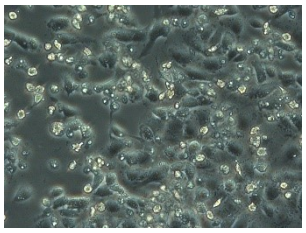
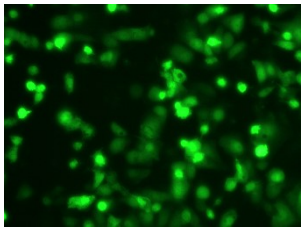
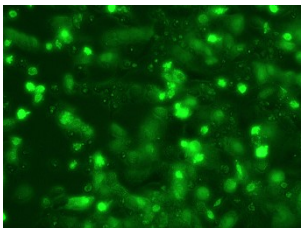
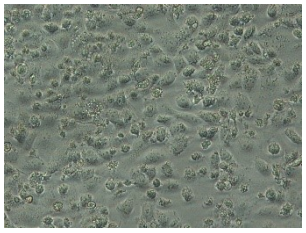
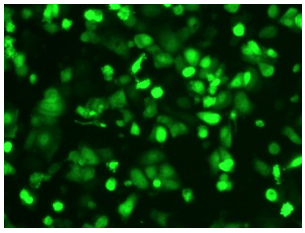
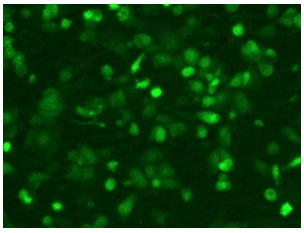
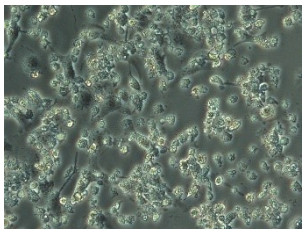
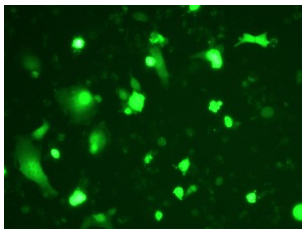
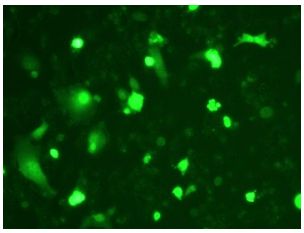
| | 20 |  |  |  |
| **0.5** | 24 |  |  |  |
| | 28 |  |  |  |

### 2.3. Effect of the polycations during the infection of MARC-145 cells with PRRSV-EGFP

The parameters evaluated above allow to determine that the infections were suitable when the synchronization (washed step) was done, the M.O.I. was 0.1 and the time 28 hours of infection, with this information the next step was to evaluate different concentrations of the polycations independently when the MARC-145 cells were being infected. Additionally, as the concentrations tested of polycations were not toxic for the cells (Figs. 6-7) and based on literature evidence (31, 33, 215), it was decided to use low concentrations of these molecules to see the effect in the infection with PRRSV.

To quantify the effect that the polycations had on the infection, the approach used was flow cytometry. The outputs were given by measuring the eGFP mean of the infected cells (Fig 11) and the percentage of infected cells (fig 12). Finally, as a confirmation using a routinely approach from the laboratory the titration of the infected cells with the polycations was done as well.

The number of infected cells increased when polycations were added; we could observe that the percentage of fluorescence cells are almost double or more compared to the treatment without the polycations. The suitable concentration to increase the infectivity of PRRSV seems to be 4 μg/mL in the case of DEAE-Dextran and 8μg/mL for polybrene (Figs 11 and 12).

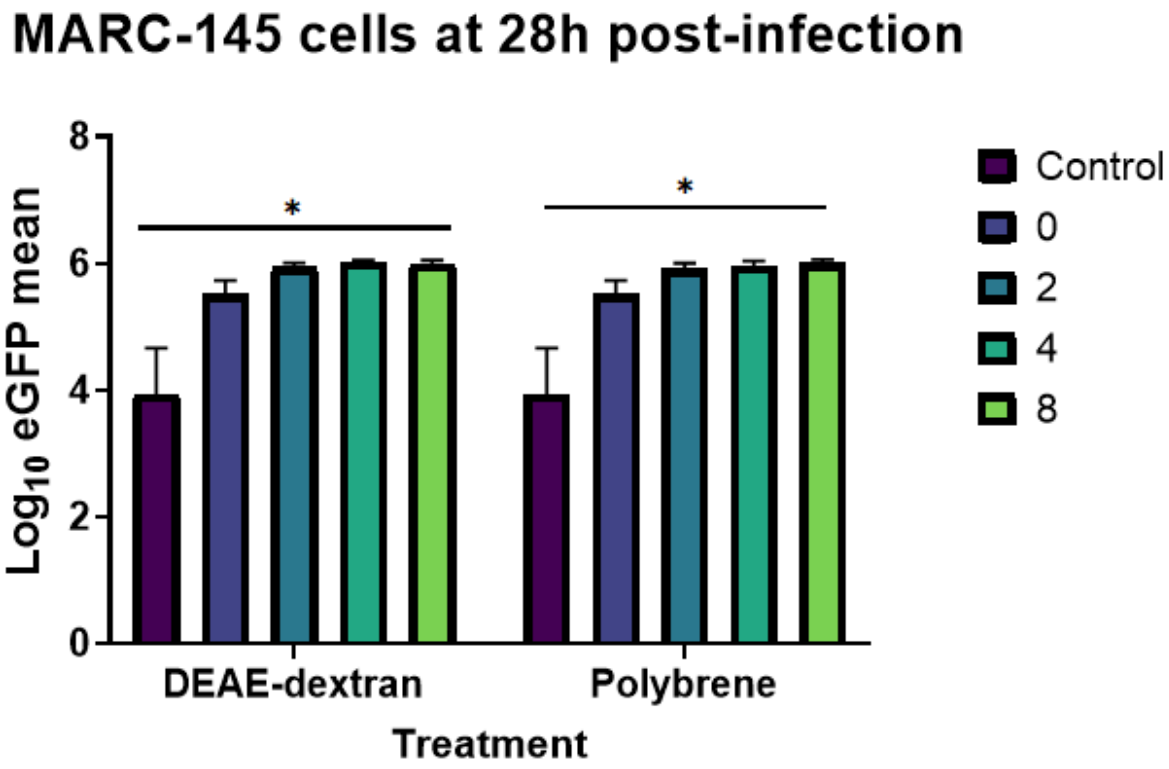



Figure 11. Mean values of measuring the intensity of eGFP protein during PRRSV-EGFP infection in MARC-145 cells after 28 hours of post-infection, adding different concentrations of polycations.

In the graph, each bar represents the concentration of polycations added; the units of these concentrations are given in μg/mL (a legend on the right). Results are presented as the ± SD (standard deviation) values of the concentrations of the polycations in the infection from three replicates. The bars identified with asterisks (*) indicate that their data is significantly different among the concentrations tested for each treatment ($p>0.05$).

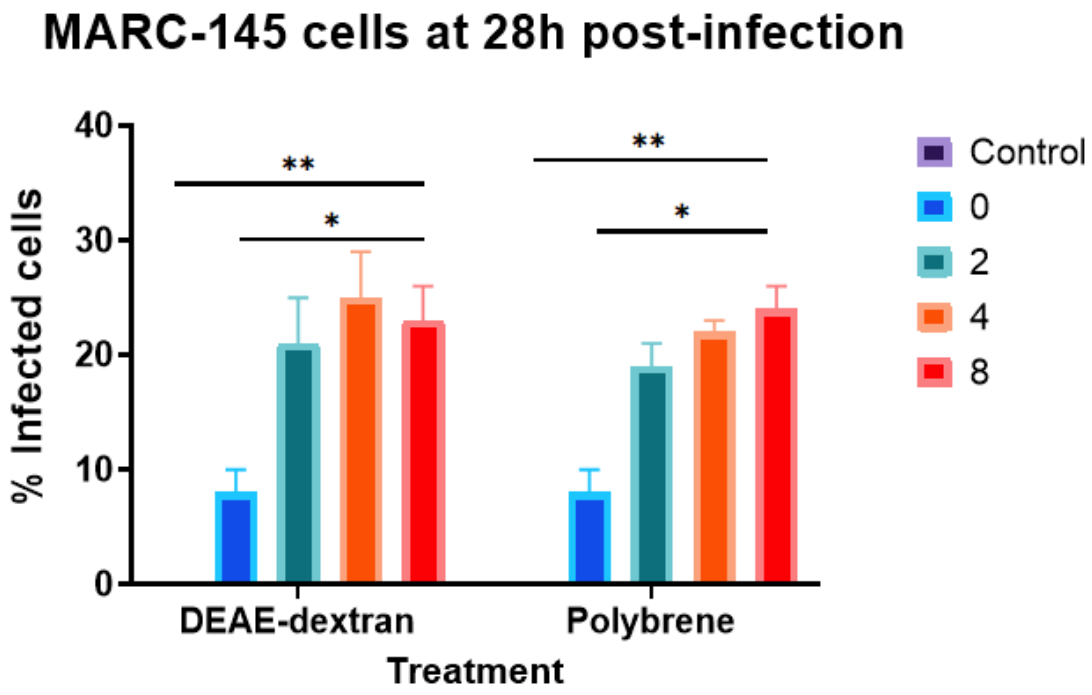



Figure 12. Percentage of infected cells during PRRSV-EGFP infection in MARC-145 cells after 28 hours of post-infection, adding different concentrations of polycations.

Results are presented as the ± SD (standard deviation) values of the concentrations of the polycations in the infection from three replicates. In the graph, each bar represents the concentration of polycations added; the units of these concentrations are given in µg/mL (a legend in the right). The bars identified with asterisks (*) are indicating a significant difference among the concentrations tested for each treatment ($p>0.05$).

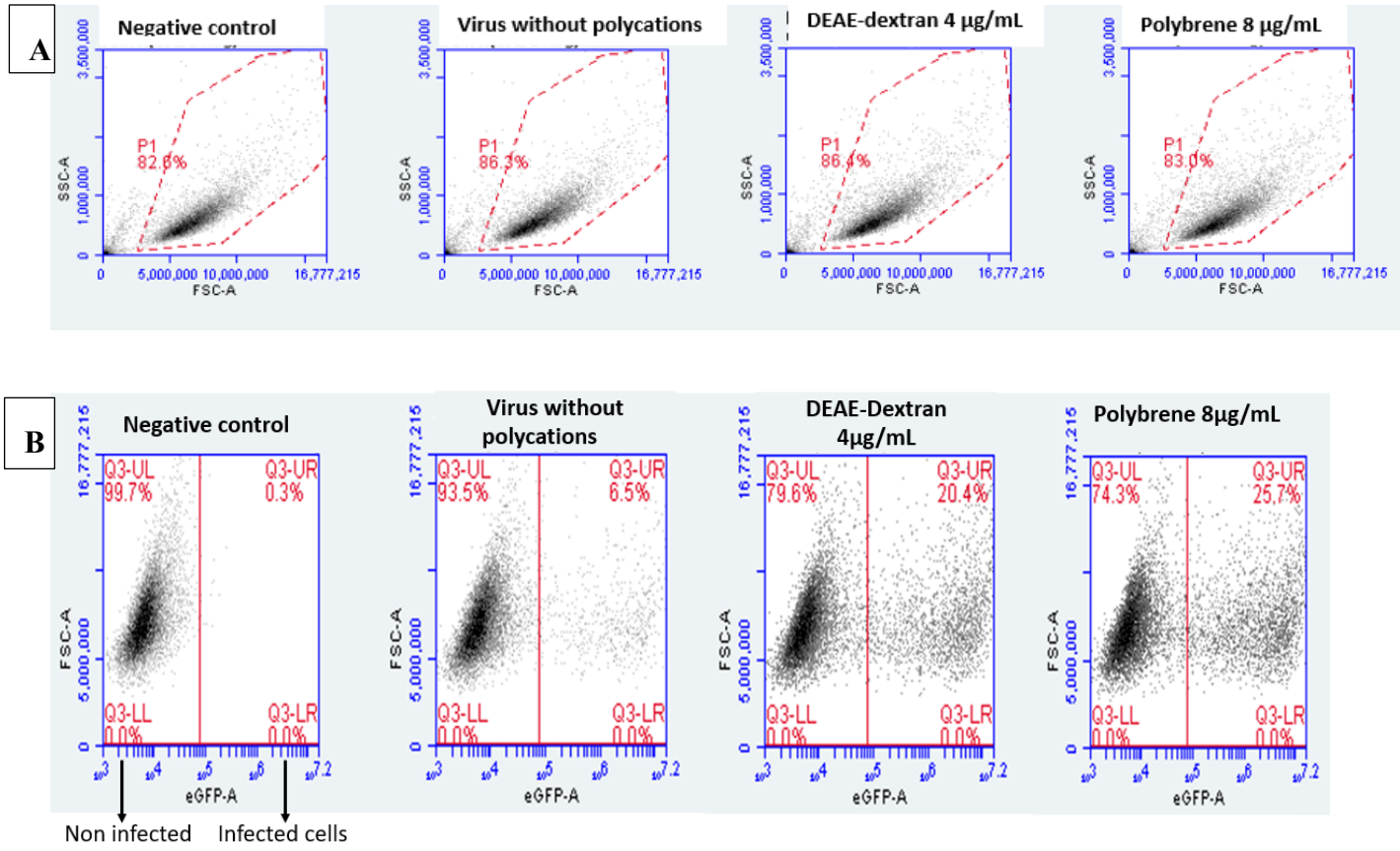



Figure 13. Populations of cells at the different variables tested after 28 hours of post-infection. The flow cytometry analysis performed for these samples allowed to evidence the number of infected cells based on the quantification of eGFP.

The dot plots showed for this figure were selected representatively to appreciate in A. the gating of the population of cells analyzed for each sample. The samples' order from left to right starts with the negative control (mock-infected cells), positive control (virus without polycations), followed by the concentrations used to test the polycations. In B. the distribution of the population of infected and non-infected cells, the samples are organized as in A.

The quantification of the infectious viral particles confirmed that polybrene enhances the number of viruses produced compared to the non-treated cells (0ug/mL). These results confirm the flow cytometer readout where the suitable concentration for the infection of MARC-145 cells with PRRSV is 8 ug/mL for polybrene (Fig 14).

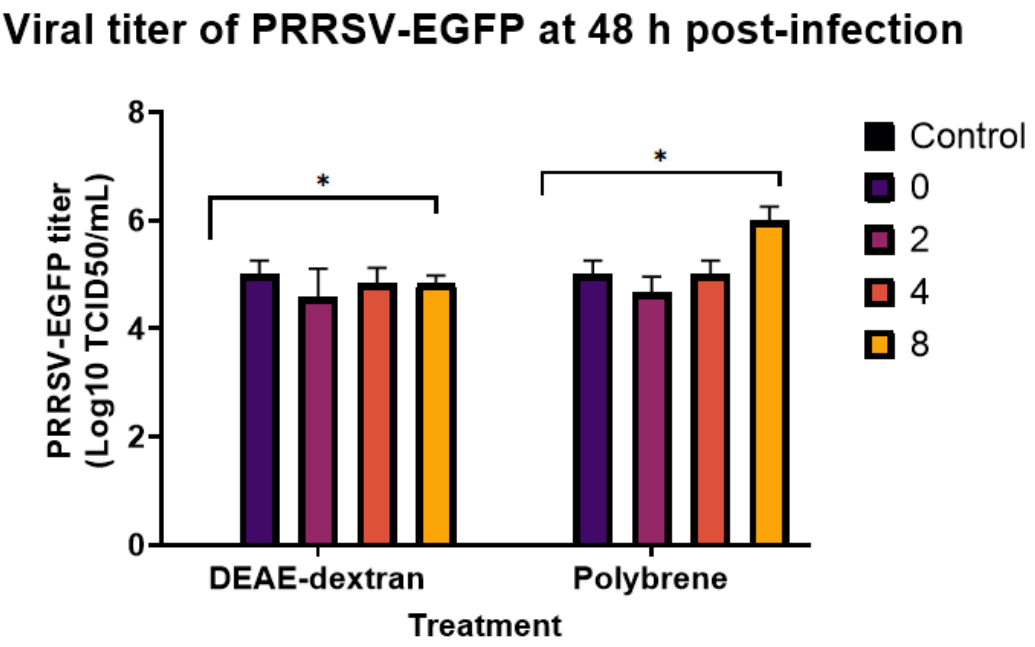



Figure 14 Viral titers obtained during PRRSV-EGFP infection after 48 hours of post-infection in MARC-145 cells, adding different concentrations of polycations.

Results are presented as the ± SD (standard deviation) values of the concentrations of the polycations in the infection from three replicates. The units of these concentrations are given in µg/mL (a legend in the right). The bars identified with asterisks (*) are indicating a significant difference among the concentration of 8 µg/mL and the negative control ($p>0.05$).

# CHAPTER IV: GENERAL DISCUSSION

PRRSV is responsible for causing one of the most devastating diseases in the pig industry (1); therefore, it is essential to understand molecular interactions between the host cell and the virus during the infection. We aimed here to identify host cellular proteins associated with PRRSV virions and exosomes produced by infected cells. The experiments' outcomes will contribute to the current knowledge of the host protein interactions during PRRSV infection.

## 1. Proteomic analysis of PRRSV infection in MARC-145 cells infected with PRRSV

The study of proteins is considered a promising tool to understand the role of proteins in virology. Mass spectrometry analysis has been useful for achieving the recent progress in viral proteomics; this last allowed the description of the proteomic composition of virions and has helped in the elucidation of host-virus interactions (183).

The purification of viral particles is important for studying the virus of interest, as this step can contribute to the characterization of the viral particle (216). In this study, we proposed to purify the lysates of MARC-145 cells infected with PRRSV to analyze the proteins contained in each step of purification and verify if there was a difference between the stages (purification).

Initially, the results showed a high abundance in the number of proteins in the semipurified samples (30% sucrose cushion) compared to the purified samples (5-45% CsCl). First, the validation showed in figure 0-1 let us determine where the viral particles were present. The results correlated the parameters such as protein content, viral titer, and viral RNA; additionally, PRRSV infection caused downregulation in most of the identified host proteins.

### 1.1. Proteins of semipurified samples

The semipurified samples led to the identification of 96 proteins (figure 1 and annex 1). Compared to a previous PRRSV secretome study, we obtained fewer proteins; this could be

due to the technic used (194). One of the proteins we could identify was vimentin (annex 1, table 2), which is part of the cell cytoskeleton and function as a receptor in MARC-145 and PAM cells and is suggested to form a complex with N and nsp2 of PRRSV to allow viral entry (65, 217). A previous study confirmed the presence of vimentin in the exosomes coming from the serum of PRRSV infected animals (218). There is a correlation to our results because we expected to have proteins coming from exosomes in the semipurified samples. Additionally, a study of the secretome of PRRSV-infected PAMs showed an upregulation in vimentin expression (200). Contrary to our results, the infection of PRRSV in MARC-145 cells caused the downregulation of vimentin. This information can suggest that PRRSV modulates protein expression depending on the cell type.

In other viruses such as the African swine fever virus, vimentin expression causes a rearrangement of the protein and interfering in the cytoskeleton structure (219). This evidence suggests that PRRSV infection can regulate the expression of some cytoskeleton proteins to spread its progeny. The last statement can be supported by additional cytoskeletal proteins identified in the present thesis, as is the case of actin cytoplasmic 1. This protein is important in metabolic processes and is a nanotubes component that allows cell-to-cell communication (85, 182, 220). A study reported that PRRSV infection in monocyte-derived dendritic cells (MoDCs) showed a change in actin protein expression, suggesting that these changes affect the cytoskeleton (85). Another study confirms the modulation of actin and myosin's expression during PRRSV infection in MARC-145 cells, indicating that PRRSV can use MARC-145 cells' nanotubes to transport viral components (182). Additionally, cellular actin expression during PRRSV infection may have an essential role in transmitting the virus from cell-to-cell (221).

Another important protein identified was the junctional adhesion molecule; its functions include maintaining the cytoskeletal organization, regulate cell adhesion (222). To date, there are no reports or studies about the role of this protein in MARC-145 cells and PRRSV infection. However, the Vero cells (African green monkey kidney origin) have expressed the junctional adhesion molecule and seemed to have an essential role in viral entry (223). One group of viruses that use the junctional adhesion molecule as a receptor for viral entry is the genus *Orthoreovirus*; this protein interacts with the viral protein σ1s involved in cell

cycle arrest and apoptosis (224, 225). Apoptosis by HIV infection showed to affect the junctional molecules (226). Finally, similar to our data, porcine circovirus (PCV) decreased expression in infected endothelial cells, suggesting that this impacts the inflammatory responses to viral infection (227). Our data indicate that the downregulation of cytoskeletal proteins may contribute to PRRSV infection. Some of these proteins can play roles in viral entry and cell-to-cell communication.

We organized the identified molecules in subcellular localization shown in figure 3; the cellular structures with a significant amount of proteins were plasma membrane, cytoplasm and nucleus. One of the proteins identified in the cytoplasm and nucleus was Heat shock 70 KDa protein 1 (HSP70A1); identifying this protein is relevant because it coincides with recent studies that suggest that this protein may play an important role in PRRSV pathogenesis in MARC-145 cells (83). It is believed that the nsp12 of PRRSV interacts with HSP70, resulting in the improvement of viral replication (228). The inhibition of the HSP70 in PRRSV infection resulted in a reduction of viral replication (229). However, it is suggested that the upregulation of HSP70 protects the infected cell against apoptosis (83). The downregulation of the HSP70A1 in infected MARC-145 cells from our experiments can indicate that the infection is modulating this protein. The downregulation of the HSP70A1 in infected MARC-145 cells from our experiments can indicate that the infection modulates this protein; either could be a cellular mechanism of defence against infection or could be the virus inducing apoptosis.

Another protein found in the cytoplasm and nucleus was the CCCH-type zinc finger antiviral protein (ZAP); the information to date about this protein's role during PRRSV infection is limited. Nevertheless, a study reports the interaction between the nsp9 of PRRSV with ZAP, resulting in the suppression of the viral infection (230). The mechanism of action to suppress viral infection occurs when ZAP binds to viral mRNA and degrades it, avoiding a viral mRNA accumulation in the cytoplasm (231, 232). Our results could indicate that the infection modulates the expression of this protein to stimulate viral replication.

According to our results, PRRSV infection affected proteins involved in molecular pathways such as immune response and cell cycle (figure 4). In this group of proteins, we

could identify different cytokines. Most of them were down-regulated, as it was the case of IL15 and IL-16. IL-15 is essential for NK cell activation; this cytokine interacts with the N protein of PRRSV (233). IL-16 was down-regulated in PAMs infected with HP-PRRSV, indicating that PRRS can induce the expression of pro-inflammatory cytokines (234).

We could also identify the cytokine transforming growth factor beta-1 (TGF β-1) important for PRRSV pathogenesis (235). Previous studies enounced that the inhibition of this protein can inhibit PRRSV replication and induce an immune response after the infection (236). The upregulation of this protein *in vivo* showed to influence the host immune response negatively (237). All this evidence indicates that the downregulation of TGF β-1 may relate to MARC-145 immune response against PRRSV infection.

The co-expression network represented in figure 5 gave us an approach to what is happening in PRRSV-infected MARC-145. We could identify the TRIM22 protein that shows to affect PRRSV replication; it is suggested that the N protein interacts with TRIM22 (238). A study showed that this protein was present in PAMs after a co-infection with PRRSV and the porcine epidemic diarrhea virus (a member of the *Nidovirales*), finishing in the stimulation of INF-β production (239). The deletion of TRIM22 seemed to improve PRRSV replication in a previous study (238); this could explain the downregulation observed in our infections. The interaction of TRIM22 with APOBEC3G and BST2 is not described yet. However, BST2 and APOBEC3G both have antiviral functions (240, 241), suggesting that the co-expression of these proteins during PRRSV infection in MARC-145 is related to immune response.

Among the 27 up-regulated proteins found in the semipurified samples, we could identify the signal transducer and activator of transcription (STAT2). This protein mediates the IFN-activated signalling(58, 145, 242). There is evidence in PRRSV infection that the nsp11 interacts with STAT2 of MARC-145 cells and degrades it, antagonizing the INF signalling pathway (240, 243). Contrary to our findings, because STAT2 was up-regulated by PRRSV infection in MARC-145 cells, it may suggest that the cell activates the INF signalling to protect the cell against the virus.

According to the classification proposed in this thesis for the subcellular localization, one of the organelles that bore up-regulated proteins was the nucleus (figure 3 A and B). The

zinc finger protein 80 (ZNF80) is one of the identified proteins found in the nucleus. Despite there is not enough information about this protein, it is believed that it is essential for transcription regulation (244). There are no reports to date that could explain PRRSV's interaction with ZNF80; but, there is evidence showing the nsp1α to have a zinc finger motif essential for PRRSV transcription subgenomic mRNAs (245). Additionally, the deletion of this zinc finger domain inhibits PRRSV infection (246). Based on this information, the up-regulation of the ZNF80 could suggest that the process of viral transcription may be occurring in the cell.

There is evidence that PRRSV infection activates host immune response and interferes with the cell-to-cell communication pathway (194). We could identify proteins related to microvesicles, such as the membrane cofactor protein (CD46); this protein contributes to exosomes' internalization (247). This protein has shown to play an important role as a receptor of adenoviruses (248, 249). A previous study showed that PRRSV-infected PAMs caused down-regulation in the expression of CD46 (250); this correlates to our results, but this protein's specific role during PRRSV infection in cell-to-cell communication remains still unknown.

The infection with PRRSV was responsible for the change of expression of the decay cofactor protein (CD55). This protein is a regulator that contributes to the inhibition of the complement lysis in human cells (251). Infected PAMs showed repression of this CD55, which can correlate with our results because it was down-regulated by PRRSV infection (252). The role of CD55 in MARC-145 cells is still unknown. However, in human cells, CD55 and CD46 protect the cell by inactivating C3-cleaving enzymes (C3 convertases); these last are essential in the complement lysis pathway (253). Our results showed that CD55 and CD46 were down-regulated; this could indicate that the virus modulates these proteins to avoid cell lysis. Additionally, CD55 was present in exosomes of human cells (251), which can suggest that cell-to-cell communication is happening during PRRSV infection.

We obtained a significant number of proteins in the semipurified samples; the results indicate that most of them interfered with the cell balance. Most of the affected molecular functions included metabolic processes, immune response and cell cycle pathways. Our

results also demonstrated the possible presence of exosomes. Nevertheless, more studies are needed to confirm that PRRSV infection modulates the cell-to-cell communication pathway. Also, the protein-protein interaction is important to elucidate. We could identify some proteins' possible interactions (figure 5), but these proteins' roles remain unknown.

### 1.2. Proteins of purified samples

After the semipurification step, we performed a purification using a cesium chloride density gradient. We collected the fractions and chose the ones with more viral and protein content (figure 3). We aimed here to obtain samples with a significant number of viral particles and get rid of exosomes. Interestingly, we could observe the up-regulation of the proteins in these samples. A total of 21 proteins were identified and sorted in subcellular localization, finding that most of them were present in the nucleus and plasma membrane. Additionally, these proteins affected pathways such as immune response, metal ion binding and nucleic acid-binding

One noteworthy result identified protein was the major histocompatibility complex class II (MHC class II CEAE-DRB3); this protein is essential for immune response and antigen presentation and processing, located in the plasma membrane (UNIPROT). Pig monocyte-dendritic cells (Mo-DC) infected with PRRSV showed to down-regulate the MHC class II protein expression (254). Contrary to our results, we could observe the up-regulation of the MHC class II proteins. Suggesting a possible link with the cell type, but more information is needed to corroborate this.

Another protein identified was the SAM domain and HD domain-containing protein 1 (SAMHD1); this protein's identification suggests that PRRSV infection modulates this protein's expression to manipulate the host immune response (255). There is evidence that supports that PAMs and MARC-145 cells infected with HP-PRRSV up-regulated the expression of SAMHD1 and suggests that this up-regulation inhibited the replication of the virus (255). In our case, the up-regulation of SAMHD1 in the purified samples may indicate that the virus is packing this protein in its structure to avoid the immune mechanism; further studies are crucial to proving this.

It was interested to find in our results that the zinc finger protein 80 (ZNF80) was present in the purified samples. As mentioned above, there is still a lack of information about this protein's role during PRRSV infection. However, the little evidence about PRRSV transcription inhibition due to the zinc finger domain (237) led us to think about the possible hijacking or modulation in this protein to be encapsidated in the viral particle to improve the viral life cycle.

The purified samples allowed us to evidence the change in the protein content between the different purification steps. As expected, we obtained a significant decrease in protein content. Cellular proteins present in purified fractions may suggest that the virus is encapsidating these proteins to transcribe and replicate in the cell successfully. However, additional functional experiments and studies are needed to confirm this statement. Additionally, a final purification step with subtilisin is necessary to elucidate the proteins encapsidated into pure viral particles (this work is in progress).

## 2. Optimization of PRRSV infection in MARC-145 cells using polycations

Our proteomic results presented some limitations. One of the limitations was the low protein content in the purified fractions (Figure 1C); we realized it during data collection. Therefore, we addressed this limitation by proposing an optimization of PRRSV infection by adding polycations to the infection. To our knowledge, this is the first time that polycations are used in PRRSV infection to MARC-145 cells. Polycations have been effective in the increasing transduction of Lentiviral vectors (30). Thus, the evidence led us to hypothesize that the addition of polycations will increase the number of infected cells and consequently, the protein content will increase as well. The concentrations used in these optimizations worked well in lentiviral vectors and HIV transfections (30, 213, 256).

### 2.1. Cytotoxicity/viability

We evaluated the cytotoxic effect and MARC-145 cells' viability after the addition of polybrene and DEAE-dextran (Figures 6 and 7). Our results showed a low cytotoxic effect

in the cells (<5%), and the viability did not show to be affected except for the concentration of 60ug/mL of DEAE-dextran (Figure 7A). In contrast to our findings, polybrene showed a mild cytotoxic effect in keratocytes when the concentration used was 8ug/mL (256). Another study showed that mesenchymal cells and mice myoblasts did not have a cytotoxic effect after treatment with polybrene at 40 ug/mL (257). Besides, the cytotoxic effect of DEAE-dextran at high concentrations was confirmed in different cell lines (258). This last is similar to what we observed at the concentration of 60 ug/mL of DEAE-dextran, suggesting that at high concentrations, the use of this polycation is not suitable for MARC-145 cells. In comparison, polybrene seems to be less toxic for MARC-145 cells.

## 2.2. PRRSV-EGFP infection in MARC-145 cells

The PRRSV strain was modified between the N gene and 3-UTR by inserting the enhanced green fluorescent protein (EGFP) gene, as described before (202). The use of this virus tagged with EGFP allowed us to see the infected cells by the virus (figures 8-12), and in this way, we could further estimate the number of infected cells by flow cytometry. It was the first time using the virus PRRSV-EGFP in MARC-145 cells to optimize infections; therefore, the literature available to discuss this topic is limited. However, the virus replicated successfully in our cells, which correlates with previous studies (48, 202). Additionally, a report suggests that using PRRSV-EGFP to infect cells may be a useful tool for the viral study and may have further applications (193); this reinforced our hypothesis of combining polycations during PRRSV infection.

## 2.3. Effects of polycation in the viral infection

The addition of polycation during PRRSV infection increased the number of infected cells compared to non-treated cells (Figure 12). This correlates with previous studies on adenoviruses, where researchers demonstrated that the interaction of charges when using polycations facilitates viral internalization into the cells (212).

The effect of the polycations in viral infectivity was not significant in our results (Figure 14). However, polycations have increased the infectivity of viruses such as rabies virus (31, 34). Another virus that showed a substantial increase in its infectivity when adding

DEAE-dextran was HIV(30, 259). This evidence led us to think about the possibility of an increase in the concentration of the polycations used.

# CHAPTER V: GENERAL CONCLUSIONS

The proteins involved in nucleic acid binding, metabolic processes, immune response and cell cycle pathways were among the most affected by the PRRSV infection. The identified proteins are predominantly the host cytoplasmic, plasma membrane and nuclear proteins. Besides, some proteins are associated with the host cell organelles.

Proteins found in the purified samples were all up-regulated. The zinc finger protein 80 may be important for PRRSV infection in MARC-145 cells; this protein was identified in the semipurified and purified samples. The information about the interaction between proteins is still limited. Therefore, functional experiments are needed to corroborate the different proteins' roles during PRRSV infection in MARC-145 cells.

The use of polycations, such as DEAE-dextran and polybrene, showed an increase in the number of infected cells. While viral infectivity was not significantly affected. Polybrene was the most effective in allowing viral adsorption into the cell in terms of infectivity. DEAE-dextran was the most effective in terms of the high number of infected cells.

# CHAPTER VI: PERSPECTIVES

The characterization of the proteomic profile of infected cells at different time post-infection will help to identify host cellular pathways affected by PRRSV infection and the dynamics of that change.

Functional experiments will allow the study of the role of the identified proteins for PRRSV infection and pathogenesis (siRNA, co-immunoprecipitation, overexpression).

The proteomic analysis of the purified and ultra-purified fraction will allow us to determine the host proteins that are specifically encapsidated or associated with PRRSV virions.

It is important to determine the effect of polycations at high concentrations during PRRSV infection in MARC-145 cells; this will prove if polycations impact viral infectivity.

It is essential to observe the polycations' effect during PRRSV infection in natural host cells such as PAM or NPTr-CD163 is necessary.

Proteomic studies with PRRSV infected cells using polycations are necessary to determine the number of proteins obtained and corroborate the current study results.

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# Annex 1.

Table 0-1. identified proteins organized by subcellular localization and molecular function in the first experiment, from the semipurified samples.

**PRRSV semipurified Up-regulated**

| **Location** | | **Gene name** | **ID** | **Protein name** |
|---|---|---|---|---|
| | **Nucleic acid binding** | | | |
| | | | G8Z7M5_CHLAE | **PIWIL3** |
| N | | ZNF80 | P51502_CHLAE | **Zinc finger protein 80** |
| C, N | | STAT2 | Q4R4G0_CHLAE | **Signal transducer and activator of transcription** |
| | *Number of proteins* | | *2* | |
| | **Metabolic process** | | | |
| C, Cy | | ACTB | Q76N69_CHLAE | **Actin, cytoplasmic 1** |
| MM | | ND5 | Q50DM0_CHLAE | **NADH-ubiquinone oxidoreductase chain 5** |
| | *Number of proteins* | | *2* | |
| | **Metal ion biding** | | | |
| N | | ZNF80 | P51502_CHLAE | **Zinc finger protein 80** |
| | *Number of proteins* | | *1* | |

| | | | | |
|---|---|---|---|---|
| | **Transcriptional regulation** | | | |
| N | | ZNF80 | P51502_CHLAE | **Zinc finger protein 80** |
| C, N | | STAT2 | Q4R4G0_CHLAE | **Signal transducer and activator of transcription** |
| | *Number of proteins* | | *2* | |
| | **Transport** | | | |
| EM | | APOA5 | H2DJB6_CHLAE | **Apolipoprotein A-V** |
| EM | | APOA4 | H2DJB1_CHLAE | **Apolipoprotein A-IV** |
| PM | | Tfrc | H9BFK2_CHLAE | **Transferrin receptor 1** |
| | *Number of proteins* | | *3* | |
| | **Cell cycle** | | | |
| M | | CD4 | Q08338_CHLAE | **T-cell surface glycoprotein CD4** |
| | *Number of proteins* | | *1* | |
| | **Cellular component** | | | |
| Ce | | NIN | K7R0Z9_CHLAE | **NIN** |
| M, VE | | env | B6SEI5_CHLAE | **Envelope glycoprotein** |
| | *Number of proteins* | | *2* | |
| | **Sperm motility** | | | |
| EM | | SEMG1 | A4K2Y6_CHLAE | **Semenogelin I isoform a preproprotein** |

| | | | | |
|---|---|---|---|---|
| EM | | | A4K2Y7_CHLAE | **Semenogelin II** |
| | *Number of proteins* | | *2* | |
| | **Signaling** | | | |
| C, N | | STAT2 | Q4R4G0_CHLAE | **Signal transducer and activator of transcription** |
| | *Number of proteins* | | *1* | |

**PRRSV Semipurified Down-regulated**

| Location | Gene name | ID | Protein name |
|---|---|---|---|
| Cell Cycle | | | |
| EM, N, C, CS | TGFB1 | P09533_CHLAE | Transforming growth factor beta-1 |
| M, EMS, N | FIBP | O46431_CHLAE | Acidic fibroblast growth factor intracellular-binding protein |
| EM | LACRT | Q0Q2K9_CHLAE | Lacritin |
| Intracellular | MOS | P10650_CHLAE | Proto-oncogene serine/threonine-protein kinase mos |
| Cy | MCPH1 | F8S681_CHLAE | Microcephalin 1 |
| *Number of proteins* | | *5* | |
| Metabolic Process | | | |

| | | | |
|---|---|---|---|
| EM, N, C, CS | TGFB1 | P09533_CHLAE | Transforming growth factor beta-1 |
| HBC | HBA | P01926_CHLAE | Hemoglobin subunit alpha |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| ER, ML | PDIA3 | Q4VIT4_CHLAE | Protein disulfide-isomerase A3 |
| M, ML | | K4GWC8_CHLAE | 2',3'-cyclic-nucleotide 3'-phosphodiesterase |
| EM | WNT7A | Q1KYL3_CHLAE | Protein Wnt-7a |
| ICM, M | CYP3A7 | Q4U0S9_CHLAE | Cytochrome P450 3A7 |
| MIIM | Cytb | C0SW44_CHLAE | Cytochrome b |
| IC | MOS | P10650_CHLAE | Proto-oncogene serine/threonine-protein kinase mos |
| EM | MBL2 | Q66S37_CHLAE | Mannose-binding protein C |
| C, LE, Ly, N | Zc3hav1 | B0LB09_CHLAE | CCCH-type zinc finger antiviral protein |
| OM, ERM | CYP2C8 | Q4U0S8_CHLAE | Cytochrome P450 2C8 |
| CM | TSHR | Q8HY54_CHLAE | Thyrotropin receptor |
| *Number of proteins* | | *13* | |
| Immune Response | | | |
| EM, N, C, CS | TGFB1 | P09533_CHLAE | Transforming growth factor beta-1 |

| | | | |
|---|---|---|---|
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| EM | IL15 | P40221_CHLAE | Interleukin-15 |
| ICM | | V5W4N9_CHLAE | MHC class IB antigen |
| ICM | | V5W5X5_CHLAE | MHC class IA antigen |
| EM | MBL2 | Q66S37_CHLAE | Mannose-binding protein C |
| CM, PM | BDKRB1 | Q95L01_CHLAE | B1 bradykinin receptor |
| MHC | Chsa-B | F6KY63_CHLAE | MHC class I antigen |
| MHC | | V5W5Y9_CHLAE | MHC class IB antigen |
| EM | DEFB106 | Q5IAB1_CHLAE | Beta-defensin |
| M, Mi | MAVS | M1QCQ2_CHLAE | Mitochondrial antiviral signaling protein |
| Cy, N, PM, NL | IFI16 | T2F9S9_CHLAE | Gamma-interferon-inducible protein 16 |
| *Number of proteins* | | *12* | |
| Apoptosis | | | |
| EM, N, C, CS | TGFB1 | P09533_CHLAE | Transforming growth factor beta-1 |
| *Number of proteins* | | *1* | |
| Protein Process | | | |
| C, N | COMMD3 | A0A0A1GNW4_CHLAE | COMM domain containing 3 |

| | | | |
|---|---|---|---|
| | | | |
| Cy | AIP | O97628_CHLAE | AH receptor-interacting protein |
| *Number of proteins* | | *2* | |
| Transport | | | |
| HBC | HBA | P01926_CHLAE | Hemoglobin subunit alpha |
| EM | ApoL4 | C4N367_CHLAE | Apolipoprotein L4 |
| M | XPR1 | E2J867_CHLAE | Xenotropic and polytropic retrovirus receptor 1 |
| EM | | Q642Y7_CHLAE | Apolipoprotein E |
| EM | APOA4 | H2DJB1_CHLAE | Apolipoprotein A-IV |
| M | Slc10a1 | A0A0U3VGV8_CHLAE | Solute carrier family 10 member a1 |
| Cy, OM, NM | | K7X429_CHLAE | TNPO3 |
| *Number of proteins* | | *7* | |
| Metal Ion Binding | | | |
| HBC | HBA | P01926_CHLAE | Hemoglobin subunit alpha |
| N | | B6CK33_CHLAE | Estrogen receptor beta |
| N, NL | YPEL2 | Q65Z58_CHLAE | Protein yippee-like 2 |
| ICM, M, MI | CYP3A7 | Q4U0S9_CHLAE | Cytochrome P450 3A7 |

| | | | |
|---|---|---|---|
| MIIM | Cytb | C0SW44_CHLAE | Cytochrome b |
| C, LE, Ly, N | Zc3hav1 | B0LB09_CHLAE | CCCH-type zinc finger antiviral protein |
| EE, EM | | Q8MJK0_CHLAE | Latent transforming growth factor-beta binding protein 3 |
| OM, ERM | CYP2C8 | Q4U0S8_CHLAE | Cytochrome P450 2C8 |
| *Number of proteins* | | *8* | |
| Ribosomal process | | | |
| CM, R, PM, N | RPSA | Q2L9X0_CHLAE | 40S ribosomal protein SA |
| *Number of proteins* | | *1* | |
| Translation | | | |
| CM, R, PM, N | RPSA | Q2L9X0_CHLAE | 40S ribosomal protein SA |
| Cy | RPS4X | Q76N24_CHLAE | 40S ribosomal protein S4, X isoform |
| *Number of proteins* | | *2* | |
| Cell Adhesion | | | |
| CM, R, PM, N | RPSA | Q2L9X0_CHLAE | 40S ribosomal protein SA |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| *Number of proteins* | | *2* | |

| | | | |
|---|---|---|---|
| Chaperones | | | |
| C, IRC | HSPA1 | Q28222_CHLAE | Heat shock 70 kDa protein 1 |
| *Number of proteins* | | *1* | |
| Transcription | | | |
| C, N, EM | IL16 | O62674_CHLAE | Pro-interleukin-16 |
| N | | B6CK33_CHLAE | Estrogen receptor beta |
| N | | O97553_CHLAE | B42b |
| C, N | STAT1 beta | Q1T7F0_CHLAE | Signal transducer and activator of transcription |
| *Number of proteins* | | *4* | |
| Regulation of Transcription | | | |
| C, N, EM | IL16 | O62674_CHLAE | Pro-interleukin-16 |
| N | | B6CK33_CHLAE | Estrogen receptor beta |
| *Number of proteins* | | *2* | |
| Chemotaxis | | | |
| C, N, EM | IL16 | O62674_CHLAE | Pro-interleukin-16 |
| *Number of proteins* | | *1* | |
| Virus Receptor Activity | | | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule* |

| | | | |
|---|---|---|---|
| M | XPR1 | E2J867_CHLAE | Xenotropic and polytropic retrovirus receptor 1 |
| M, GM | | Q865L8_CHLAE | Decay-accelerating factor CD55 |
| *Number of proteins* | | *3* | |
| Signalling | | | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| N | | B6CK33_CHLAE | Estrogen receptor beta |
| EM | WNT7A | Q1KYL3_CHLAE | Protein Wnt-7a |
| *Number of proteins* | | *3* | |
| Aggregation | | | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| *Number of proteins* | | *1* | |
| Cytoskeleton Organization | | | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| *Number of proteins* | | *1* | |
| Nucleic Acid Binding | | | |
| M, ML | | K4GWC8_CHLAE | 2',3'-cyclic-nucleotide 3'-phosphodiesterase |
| N, NS, Cr | | A3R0T6_CHLAE | Histone H1e |

| | | | |
|---|---|---|---|
| C, N | STAT1 beta | Q1T7F0_CHLAE | Signal transducer and activator of transcription |
| ER | DNAJC1 | A0A0A1GK97_CHLAE | DnaJ (Hsp40) homolog, subfamily C, member 1 |
| N | | O97553_CHLAE | B42b |
| C, N | STAT1 beta | Q1T7F0_CHLAE | Signal transducer and activator of transcription |
| *Number of proteins* | | *6* | |
| Cell Integrity | | | |
| ER, ML | PDIA3 | Q4VIT4_CHLAE | Protein disulfide-isomerase A3 |
| *Number of proteins* | | *1* | |
| Female Pregnancy | | | |
| | | Q9N0P2_CHLAE | Pregnancy-specific glycoprotein x |
| *Number of proteins* | | *1* | |
| Cellular component | | | |
| M, VE | Env | B6SEI5_CHLAE | Envelope glycoprotein |
| Ce | NIN | K7R0Z9_CHLAE | NIN |
| *Number of proteins* | | *2* | |
| Post-translation modification | | | |

| | | | |
|---|---|---|---|
| IC | MOS | P10650_CHLAE | Proto-oncogene serine/threonine-protein kinase mos |
| *Number of proteins* | | *1* | |

Table 0-2. Identified proteins organized by subcellular localization and molecular function in the second experiment, from the semipurified samples.

**PRRSV semipurified Up-regulated**

| **Localization** | **Gene name** | **ID** | **Protein name** |
|---|---|---|---|
| | Nucleic acid-binding | | |
| N | TOP1 | TOP1_CHLAE | DNA topoisomerase 1 |
| C, N | STAT2 | Q4R4G0_CHLAE | Signal transducer and activator of transcription |
| | *Number of proteins* | | *2* |
| | Metabolic process | | |
| EM | APOA5 | H2DJB6_CHLAE | Apolipoprotein A-V |
| N | RAD17 | RAD17_CHLAE | Cell cycle checkpoint protein RAD17 |

| | | | |
|---|---|---|---|
| | *Number of proteins* | | *2* |
| | Transport | | |
| EM | APOA5 | H2DJB6_CHLAE | Apolipoprotein A-V |
| | *Number of proteins* | | *1* |
| | Cell cycle | | |
| EM, PM | HEBGF | HEBGF_CHLAE | Proheparin-binding EGF-like growth factor |
| N | RAD17 | RAD17_CHLAE | Cell cycle checkpoint protein RAD17 |
| | *Number of proteins* | | *2* |
| | Signaling | | |
| C, N | STAT2 | Q4R4G0_CHLAE | Signal transducer and activator of transcription |
| | *Number of proteins* | | *1* |

**PRRSV Semipurified Down-regulated**

| Location | Gene name | ID | Protein name |
|---|---|---|---|
| Cell Cycle | | | |
| EM, N, C, CS | TGFB1 | P09533_CHLAE | Transforming growth factor beta-1 |
| M, EMS, N | FIBP | O46431_CHLAE | Acidic fibroblast growth factor intracellular-binding protein |

| | | | |
|---|---|---|---|
| Cy | MCPH1 | F8S681_CHLAE | Microcephalin 1 |
| C | ASZ1 | ASZ1_CHLAE | Ankyrin repeat, SAM and basic leucine zipper domain-containing protein 1 |
| PM | HTR1A | X2CFA9_CHLAE | Serotonin receptor type 1A |
| | *Number of proteins* | | *5* |
| Metabolic Process | | | |
| EM, N, C, CS | TGFB1 | P09533_CHLAE | Transforming growth factor beta-1 |
| HBC | HBA | P01926_CHLAE | Hemoglobin subunit alpha |
| ICM | JAM-1f | Q2WGK1_CHLAE | Junctional adhesion molecule |
| ER, ML | PDIA3 | Q4VIT4_CHLAE | Protein disulfide-isomerase A3 |
| M, ML | | K4GWC8_CHLAE | 2',3'-cyclic-nucleotide 3'-phosphodiesterase |
| C, N, M, PM, Cy | PARP9 | A0A060III8_CHLAE | Poly ADP-ribose polymerase family member 9 |
| C, LE, Ly, N | Zc3hav1 | B0LB09_CHLAE | CCCH-type zinc finger antiviral protein |
| C, EM, V | CD46 | MCP_CHLAE | Membrane cofactor protein |
| | | | |
| | *Number of proteins* | | *13* |
| Immune Response | | | |

| | | | |
|---|---|---|---|
| EM, N, C, CS | TGFB1 | P09533_CHLAE | Transforming growth factor beta-1 |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| EM | IL15 | P40221_CHLAE | Interleukin-15 |
| ICM | | V5W4N9_CHLAE | MHC class IB antigen |
| ICM | | V5W5X5_CHLAE | MHC class IA antigen |
| CM, PM | BDKRB1 | Q95L01_CHLAE | B1 bradykinin receptor |
| MHC | Chsa-B | F6KY63_CHLAE | MHC class I antigen |
| MHC | | V5W5Y9_CHLAE | MHC class IB antigen |
| EM | DEFB106 | Q5IAB1_CHLAE | Beta-defensin 1 |
| Cy, N, PM, NL | IFI16 | T2F9S9_CHLAE | Gamma-interferon-inducible protein 16 |
| C, EM, V | CD46 | MCP_CHLAE | Membrane cofactor protein |
| EM | CSF2 | CSF2_CHLAE | Granulocyte-macrophage colony-stimulating factor |
| ICM | | C3W5K7_CHLAE | Tetherin |
| Cy | | A0A0F7DHA3_CHLAE | Cyclic GMP-AMP synthase |
| PM, GM, EMS, C | BST2 | D7RVC9_CHLAE | Bone marrow stromal cell antigen 2 |
| C | APOBEC3G | H2E0A5_CHLAE | Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G |

| | | | |
|---|---|---|---|
| | *Number of proteins* | | *14* |
| Apoptosis | | | |
| EM, N, C, CS | TGFB1 | P09533_CHLAE | Transforming growth factor beta-1 |
| | *Number of proteins* | | *1* |
| Protein Process | | | |
| GM, N | TRIM22 | B0F4P0_CHLAE | Tripartite motif containing 22 |
| Ce | NIN | K7R0Z9_CHLAE | Ninein |
| Ce, Cy, PM, N | CENPJ | F8S641_CHLAE | Centromere protein J |
| PM | MCPH1 | F8S681_CHLAE | Microcephalin 1 MCPH1 |
| N, Cy | TOPBP1 | TOP1_CHLAE | Topoisomerase (DNA) II binding protein 1 |
| | *Number of proteins* | | *5* |
| Transport | | | |
| HBC | HBA | P01926_CHLAE | Hemoglobin subunit alpha |
| EM | | Q642Y7_CHLAE | Apolipoprotein E |
| Cy, OM, NM | | K7X429_CHLAE | TNPO3 |
| | *Number of proteins* | | *7* |
| Metal Ion Binding | | | |

| | | | | |
|---|---|---|---|---|
| HBC | HBA | P01926_CHLAE | Hemoglobin subunit alpha | |
| N | | B6CK33_CHLAE | Estrogen receptor beta | |
| N | ZNF80 | ZNF80_CHLAE | Zinc finger protein 80 | |
| | *Number of proteins* | | | *8* |
| Ribosomal process | | | | |
| CM, R, PM, N | RPSA | Q2L9X0_CHLAE | 40S ribosomal protein SA | |
| R | rpS6 | Q9TSP8_CHLAE | Ribosomal protein S6 | |
| | *Number of proteins* | | | *1* |
| Translation | | | | |
| CM, R, PM, N | RPSA | Q2L9X0_CHLAE | 40S ribosomal protein SA | |
| R | rpS6 | Q9TSP8_CHLAE | Ribosomal protein S6 | |
| | *Number of proteins* | | | *2* |
| Cell Adhesion | | | | |
| CM, R, PM, N | RPSA | Q2L9X0_CHLAE | 40S ribosomal protein SA | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule | |
| | *Number of proteins* | | | *2* |
| Chaperones | | | | |

| | | | |
|---|---|---|---|
| C, IRC | HSPA1 | Q28222_CHLAE | Heat shock 70 kDa protein 1 |
| | *Number of proteins* | | *1* |
| Transcription | | | |
| C, N, EM | IL16 | O62674_CHLAE | Pro-interleukin-16 |
| N | | B6CK33_CHLAE | Estrogen receptor beta |
| | *Number of proteins* | | *4* |
| Regulation of Transcription | | | |
| C, N, EM | IL16 | O62674_CHLAE | Pro-interleukin-16 |
| N | | B6CK33_CHLAE | Estrogen receptor beta |
| N | ZNF80 | ZNF80_CHLAE | Zinc finger protein 80 |
| | | | |
| | *Number of proteins* | | *2* |
| Chemotaxis | | | |
| C, N, EM | IL16 | O62674_CHLAE | Pro-interleukin-16 |
| | *Number of proteins* | | *1* |
| Virus Receptor Activity | | | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule* |
| C, EM, V | CD46 | MCP_CHLAE | Membrane cofactor protein |

| | | | |
|---|---|---|---|
| | | | |
| | *Number of proteins* | | *3* |
| Signalling | | | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| N | | B6CK33_CHLAE | Estrogen receptor beta |
| | *Number of proteins* | | *3* |
| Aggregation | | | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| | *Number of proteins* | | *1* |
| Cytoskeleton Organization | | | |
| ICM | JAM-1 | Q2WGK1_CHLAE | Junctional adhesion molecule |
| | *Number of proteins* | | *1* |
| Nucleic Acid Binding | | | |
| M, ML | | K4GWC8_CHLAE | 2',3'-cyclic-nucleotide 3'-phosphodiesterase |
| N, NS, Cr | | A3R0T6_CHLAE | Histone H1e |
| ER | DNAJC1 | A0A0A1GK97_CHLAE | DnaJ (Hsp40) homolog, subfamily C, member 1 |
| N | ZNF80 | ZNF80_CHLAE | Zinc finger protein 80 |

| | | | |
|---|---|---|---|
| | *Number of proteins* | | *6* |
| Cell Integrity | | | |
| ER, ML | PDIA3 | Q4VIT4_CHLAE | Protein disulfide-isomerase A3 |
| | *Number of proteins* | | *1* |
| Female Pregnancy | | | |
| | | Q9N0P2_CHLAE | Pregnancy-specific glycoprotein x |
| | *Number of proteins* | | *1* |
| Cellular component | | | |
| Ce | NIN | K7R0Z9_CHLAE | NIN |
| C | VIM | VIME_CHLAE | Vimentin |
| | *Number of proteins* | | *2* |

# Annex 2.

Table 1. Comparison between proteins found in this study and others.

| **Proteins of this study** | **Proteins from exosomes of pigs serum (218)** | **Proteins of secretomes in PAM cells (16)** | **cellular proteins in virions (54)** | **Cellular proteins in MARC-145 cells (200)** | **Cellular proteins in PAM cells (200)** |
|---|---|---|---|---|---|
| **Apolipoprotein A-V** | ankyrin-1 | MHC class II antigen (HLA-DQB1) | Heat shock 70 kDa protein 8 isoform 1 | Heat shock 27 kDa protein 1 | Vimentin |
| **Apolipoprotein B mRNA editing enzyme catalytic polypeptide 4** | apolipoprotein B-100-like | C-C motif chemokine 23 (CCL23) | ribosomal protein P0 | | Heat shock protein beta-1 (HSPB1) |
| **Ankyrin repeat, SAM and basic leucine zipper domain-containing protein 1** | apolipoprotein B-100 | C-C motif chemokine 3-like 1 (CCL3L1) | Heat shock protein 27 | | |

| | | | | | |
|---|---|---|---|---|---|
| **40S ribosomal protein SA** | apolipoprotein A-II precursor | DnaJ homolog subfamily A member 1 (DNAJA1) | Heat shock 60 kDa protein 1 | | |
| **Acidic fibroblast growth factor intracellular-binding protein** | apolipoprotein B, partial | Histone H2A (H2AFX) | | | |
| **Beta-defensin 1** | heat shock 70kDa protein 8 | Histone H2A (H2AFX) | | | |
| **Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G** | Apolipoprotein E | Interleukin-1 receptor antagonist protein (IL1RN) | | | |
| **Apolipoprotein E** | C-X-C motif chemokine 4 | Interleukin-23 subunit alpha (IL23A) | | | |

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| **B1 bradykinin receptor** | heat shock 70kDa protein 6 (HSP70B) | Ribosomal protein L5 (RPL5) | | | |
| **2',3'-cyclic-nucleotide 3'-phosphodiesterase** | Apolipoprotein A-I | Serine/threonine-protein kinase 24 (STK24) | | | |
| **C-C chemokine receptor type 3** | heat shock 90kD protein 1, beta isoform X1 | | | | |
| **CCCH-type zinc finger antiviral protein** | Heat shock protein beta-1 | | | | |
| **Cell cycle checkpoint protein RAD17** | Heat shock protein HSP 90-alpha | | | | |
| **Centromere protein J** | histone H2B type 1-H-like | | | | |

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| **CXC chemokine receptor 4** | histone H2A type 1-F-like | | | | |
| **Cyclic GMP-AMP synthase** | histone H2A type 1-F-like | | | | |
| **DNA topoisomerase 1** | MHC class I antigen | | | | |
| **DNA topoisomerase 1** | MHC class I antigen, partial | | | | |
| **DnaJ (Hsp40) homolog, subfamily C, member 1** | poly [ADP-ribose] polymerase 6 isoform X15 | | | | |
| **Estrogen receptor beta** | serine/threonine-protein kinase ATR | | | | |
| **Gamma-interferon-inducible protein 16** | Transforming growth factor-beta-induced protein ig-h3 | | | | |

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| **Gonadotropin-releasing hormone II receptor** | vimentin isoform X2 | | | | |
| **Granulocyte-macrophage colony-stimulating factor** | zinc finger protein 518A isoform X18 | | | | |
| **Heat shock 70 kDa protein 1** | | | | | |
| **Hemoglobin subunit alpha** | | | | | |
| **Hepatitis A virus cellular receptor 1** | | | | | |
| **Histone H1e** | | | | | |
| **Interleukin-15** | | | | | |
| **Junctional adhesion molecule** | | | | | |
| **Melanocyte-stimulating** | | | | | |

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| **hormone receptor** | | | | | |
| **Membrane cofactor protein** | | | | | |
| **MHC Class I Antigen** | | | | | |
| **MHC class IB antigen** | | | | | |
| **MHC class I-related chain** | | | | | |
| **Microcephalin 1** | | | | | |
| **NIN** | | | | | |
| **Peptidyl-prolyl cis-trans isomerase FKBP5** | | | | | |
| **Poly ADP-ribose polymerase** | | | | | |

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| **family member 9** | | | | | |
| **Pregnancy-specific glycoprotein** | | | | | |
| **Proheparin-binding EGF-like growth factor** | | | | | |
| **Pro-interleukin-16** | | | | | |
| **Promyelocytic leukemia protein** | | | | | |
| **Protein disulfide-isomerase A3** | | | | | |
| **Protein ERGIC-53** | | | | | |
| **Protein sprouty homolog 2** | | | | | |

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| **Ribosomal protein S6** | | | | | |
| **Serine/threonine-protein kinase 4** | | | | | |
| **Serotonin receptor type 1A** | | | | | |
| **SETMAR** | | | | | |
| **Signal transducer and activator of transcription** | | | | | |
| **T-cell surface glycoprotein CD4** | | | | | |
| **Tetherin** | | | | | |
| **Thiopurine S-methyltransferase** | | | | | |
| **TNPO3** | | | | | |

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| **Transforming growth factor beta-1** | | | | | |
| **Transforming growth factor beta-2** | | | | | |
| **TRIM22** | | | | | |
| **Vimentin** | | | | | |
| **Zinc finger protein 80** | | | | | |