

Universitat de Lleida

Assessment of extracellular vesicles and their applicability in diseases of veterinary importance

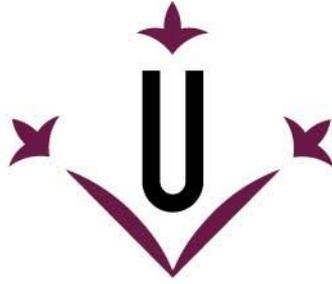
Sergio Roberto Montaner Tarbes

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Universitat de Lleida

PhD Thesis Research

Assessment of extracellular vesicles and their applicability in diseases of veterinary importance

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Thesis submitted for the degree of Doctor in Agricultural and Food Science and Technology

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“If knowledge can create problems,
it is not through ignorance that we can solve them.”

— Isaac Asimov

*A mis padres,
Adriana y Sergio por su apoyo y su motivación
Son unicos, son mi orgullo
Este es nuestro logro.*

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ABSTRACT

Vaccination continues to be the most important strategy for control and eradication of infectious diseases in public and animal health. Porcine Reproductive and Respiratory Syndrome virus (PRRSV) is one of the most important swine diseases in the world, causing an enormous economic burden due to reproductive failure in sows and a complex respiratory syndrome in pigs of all ages, in which available vaccines induce limited immune responses and protection. Extracellular vesicles (EVs) are now in the scope as important source of antigens and interactors in several pathogen-host relations. In the present research work, extracellular vesicles were evaluated as suitable antigen source for vaccination in this important swine disease. First, EVs derived from swine sera were assessed using different methodologies for isolation, characterization and concentration finding that contained specific and immunogenic viral proteins. Moreover, when PRRSV-sera derived EVs were tested in a targeted-pig vaccination trial using a prime-boost strategy, EVs demonstrated to be safe causing no infection neither secondary effects and were able to induce specific antibody and cell mediated IFN- γ secretion. In addition, EVs vaccinated animals showed less viral load and best clinical outcome when compared to those vaccinated with a classical approach of viral peptides in which adverse effects (increased viral load) were observed. To test the applicability of this approach in other viruses, African Swine fever, another important swine disease was selected and EVs content evaluated, finding similar results to those obtain in PRRSV, where specific viral proteins were associated to EVs enriched fractions. In conclusion, extracellular vesicles represent an important source to identify immunogenic antigens that could be used as a novel vaccination strategy to induce protection for porcine viral diseases such as PRRSV and can be extrapolated to other swine viral diseases

RESUMEN

La vacunación continúa siendo una de las estrategias más importantes para el control y la erradicación de enfermedades infecciosas en salud pública y salud animal. El Síndrome Reproductivo y Respiratorio Porcino (PRRSV) es una de las enfermedades porcinas más importantes en el mundo, causante de grandes pérdidas económicas debido a las fallas en cerdas reproductoras, un síndrome respiratorio complejo en cerdos de todas las edades y en el cual las vacunas disponibles inducen una respuesta inmune y protectora limitada. Las vesículas extracelulares (EVs) son actualmente consideradas como importantes fuentes de antígenos en múltiples relaciones patógeno-hospedador. En el presente estudio, se evaluaron las vesículas extracelulares como una fuente de antígenos adecuados para vacunación en esta enfermedad de importancia veterinaria. En primer lugar, se valoraron las EVs utilizando distintas metodologías para su aislamiento, caracterización y concentración, mediante las cuales fue posible detectar proteínas virales específicas e inmunogénicas en su contenido. Además, cuando las EVs derivadas de suero de animales infectados con PRRSV se probaron en un ensayo de vacunación en cerdos utilizando una estrategia de vacunación Prime-Boost, estas demostraron ser seguras al no causar infección ni efectos secundarios, y fueron capaces de inducir una respuesta de anticuerpos y secreción de IFN- γ específica en los animales vacunados. Conjuntamente, los animales vacunados con EVs mostraron una menor carga viral y un mejor desarrollo clínico cuando se comparó su estado con animales vacunados con un enfoque clásico de vacunas de péptidos en el cual se observó un aumento de la carga viral y otros síntomas clínicos. Para probar la aplicabilidad de esta metodología en otros virus, se seleccionó la Peste Porcina Africana (PPA), otra enfermedad porcina de gran importancia como modelo para evaluar el contenido de las EVs en esta enfermedad, encontrando resultados similares a los obtenidos con PRRSV en donde se detectaron proteínas virales específicas en las fracciones enriquecidas con EVs. En conclusión, las vesículas extracelulares representan una importante fuente para la identificación de antígenos inmunogénicos que pueden ser utilizados como una nueva estrategia de vacunación para inducir protección en enfermedades porcinas virales como PRRSV y además pudiendo extrapolarse a otras enfermedades virales en el cerdo.

RESUM

Les vacunes continuen sent l'estratègia més important pel control i l'erradicació de malalties infeccioses en salut pública i animal. El virus del Síndrome Respiratori i Reproductiu Porcí (SRRP) és una de les malalties porcines més importants en el món. Causa una enorme afectació econòmica degut als problemes reproductius en les femelles i al síndrome respiratori en porcs de totes les edats, en les quals les vacunes existents indueixen una resposta immune i protectora limitada. Les vesícules extracel·lulars estan al punt de mira com a font important d'antígens i interactors entre patògens i hostes. En aquest estudi, hem caracteritzat vesícules extracel·lulars per a cercar antigens per la vacunació d'aquesta malaltia porcina. En primer lloc, les vesícules extracel·lulars derivades de sèrum porcí han estat avaluades utilitzant diferents metodologies d'aïllament, caracterització i concentració, trobant proteïnes virals específiques i immunogèniques. A més a més, quan les vesícules derivades del sèrum de porcs infectats amb SRRP han estat testades en un assaig clínic de vacunació usant una estratègia prime-boost, les vesícules han demostrat ser segures, sense causar infecció ni efectes adversos i induïnt una resposta específica d'anticossos i secreció de IFN- γ . D'altra banda, els animals vacunats amb vesícules extracel·lulars van mostrar una càrrega viral inferior i uns millors resultats clínics comparats amb aquells vacunats amb el mètode clàssic de vacunes peptídiques, en els quals es van observar majors efectes adversos (major càrrega viral). Per provar la utilitat d'aquesta metodologia en altres virus una altra malaltia porcina important va ser seleccionada, el virus de la Febre Africana Porcina, i es va evaluar el contingut de vesícules extracel·lulars, trobant proteïnes virals específiques similars a aquelles obtingudes en SRRP. En conclusió, les vesícules extracel·lulars representen una font important per a la identificació d'antígens immunogènics que podrien ser usats com a una nova estratègia de vacunació per induïr protecció contra malalties virals porcines tals com SRRP i podent ser extrapolades a altres malalties porcines virals.

CHAPTER I. INTRODUCTION. Key gaps in the knowledge of
PRRSV.

Key gaps in the knowledge of the Porcine Respiratory Reproductive Syndrome Virus (PRRSV)

Running title: Key gaps in the knowledge of PRRSV

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Key words: Porcine Reproductive and Respiratory Syndrome Virus, PRRSV, virus biology, immunology, vaccinology, extracellular vesicles.

Abstract.

The porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important swine diseases in the world. It is causing an enormous economic burden due to reproductive failure in sows and a complex respiratory syndrome in pigs of all ages, with mortality varying from 2 to 100% in the most extreme cases of emergent highly pathogenic strains. PRRSV displays complex interactions with the immune system and a high mutation rate, making the development and implementation of control strategies a major challenge. In this review, the biology of the virus will be addressed focusing on newly discovered functions of non-structural proteins and novel dissemination mechanisms. Secondly, the role of different cell types and viral proteins will be reviewed in natural and vaccine-induced immune response together with the role of different immune evasion mechanisms focusing on those gaps of knowledge that are critical to generate more efficacious vaccines. Finally, novel strategies for antigen discovery and vaccine development will be discussed, in particular the use of exosomes (extracellular vesicles of endocytic origin). As nanocarriers of lipids, proteins and nucleic acids, exosomes have potential effects on cell activation, modulation of immune responses and antigen presentation. Thus, representing a novel vaccination approach against this devastating disease.

Economic Impact

PRRSV is responsible for respiratory disease in weaned and growing pigs, as well as reproductive failures in sows. It is considered one of the most important swine diseases worldwide, with an economic impact estimated at \$664 million in losses every year to U.S. producers, representing an increase of 18.5% in the last eight years (Holtkamp et al. 2013; Neumann et al. 2005). In Europe, the situation is similar and economic disease models have been carried out to determine the economic burden in the best and worst case scenario combining reproductive failure and respiratory disease, estimating annual losses from a median of €75.724, if the farm was slightly affected during nursing and fattening, to a median of €650.090 if a farm of 1000 sows is severely affected in all productive phases (Nathues et al. 2017). Nevertheless, there is scarce of information about the economic impact of this disease as a consequence of multiple factors (vaccination, treatment, respiratory symptoms, reproductive failure and other PRRSV-related diseases) making a difficult task to quantify exactly this parameter under field conditions. Thus, the exact economic impact of PRRSV remains a key gap in the knowledge for this disease.

Biology of PRRSV

The porcine reproductive and respiratory syndrome virus (PRRSV) was first isolated in the early 1990s in Europe and North America (Wensvoort et al. 1991; McCullough, Gorcyca, and Chladek 1992). It is an enveloped single-stranded positive-sense RNA virus of the family Arteriviridae, Genus Porarterivirus according to the International Committee of Taxonomy of Viruses (Adams et al. 2016). Presently, there are four distinct species included in this Genus (Porarterivirus), PRRSV-1 and PRRSV-2 (with 30-45% variation in nucleotide sequences), along with other two viruses that do not affect pigs (Lactate dehydrogenase-elevating virus and Rat Arterivirus 1) (Lunney et al. 2016). The genome size of PRRSV is about 15 kb with 10 open reading frames (ORFs), with replicase genes located at the 5'-end followed by the genes encoding structural proteins towards the 3'-end (Snijder and Meulenber 1998). The majority of the genome (approximately 60-70%) encodes non-structural proteins involved in replication (ORF1a and ORF1ab), whereas ORFs 2-7 encodes structural proteins (N, M, GP2-GP5, E) (Figure 1A and 1B) (Dokland 2010). Using ORF5 in molecular epidemiological studies, an enormous genetic variability has been described (Nguyen et al. 2014). Yet, data on whole genome sequencing is scarce and constitute another important gap in the knowledge of this virus and its evolution (box 1).

Box 1. Gaps in knowledge in PRRSV.

Biology	<ul style="list-style-type: none">• Deeper understanding of the function/structure of PRRSV proteins.• Whole genome sequences from different geographical origins to further study evolution and mutations.• Role of EVs in virus-host interaction.
Innate immune responses	<ul style="list-style-type: none">• Role of host genetics in innate immune responses• Effect of structural and non-structural proteins in innate immune response• Effect of PRRSV on antigen presenting cells.• Identification of macrophage cell lines capable of sustaining infections with widely different PRRSV strains.• Limited knowledge of other cell populations interacting with PRRSV.
Acquired immune responses	<ul style="list-style-type: none">• Cell immune protective mechanisms not fully elucidated.• No correlates of protection.• Role of neutralizing antibodies is controversial, as appear late in infection.• Role non-neutralizing antibodies is not defined: antibody-related mechanisms such as ADCC, CDC or ADCV.• The role of Tregs in cellular immune response.
Vaccines	<ul style="list-style-type: none">• Killed and subunit vaccines do not confer full protection.• Partial protection by MLVs and problems due to revert to virulent strain.• New vaccination strategies that are universal, virus free, immunogenic, DIVA and protective are desperately needed.

PRRSV replicase genes consist of two ORFs, ORF1a and ORF1b, which occupy the 5' proximal three-quarters of the genome (Figure 1A). Both are expressed from the viral genome, with expression of ORF1b depending on a conserved ribosomal frameshifting mechanism. Subsequently, extensive proteolytic processing of the resulting pp1a and pp1ab polyproteins yields at least 14 functional non-structural proteins (nsps), specifically nsp1 to nsp12, with both the nsp1 and nsp7 parts being subject to internal cleavage (giving origin to nsp1 α and nsp1 β , and nsp7 α and nsp7 β , respectively), most of which assemble into a membrane-associated replication and transcription complex (Yanhua Li et al. 2012). Recently, a programmed ribosomal frameshift encoding an alternative ORF that generates two extra proteins, nsp2TF and nsp2N, was discovered in PRRSV and other Arteriviruses (Fang et al. 2012; Y. Li et al. 2018). These nsps, described for PRRSV, have proven to be necessary and sufficient for the induction of membrane modifications resembling those found in infected cells (van der Hoeven et al. 2016). Most importantly, all positive RNA viruses seem to induce one of two basic morphotypes of membrane modifications: invaginations or double-membrane vesicles.

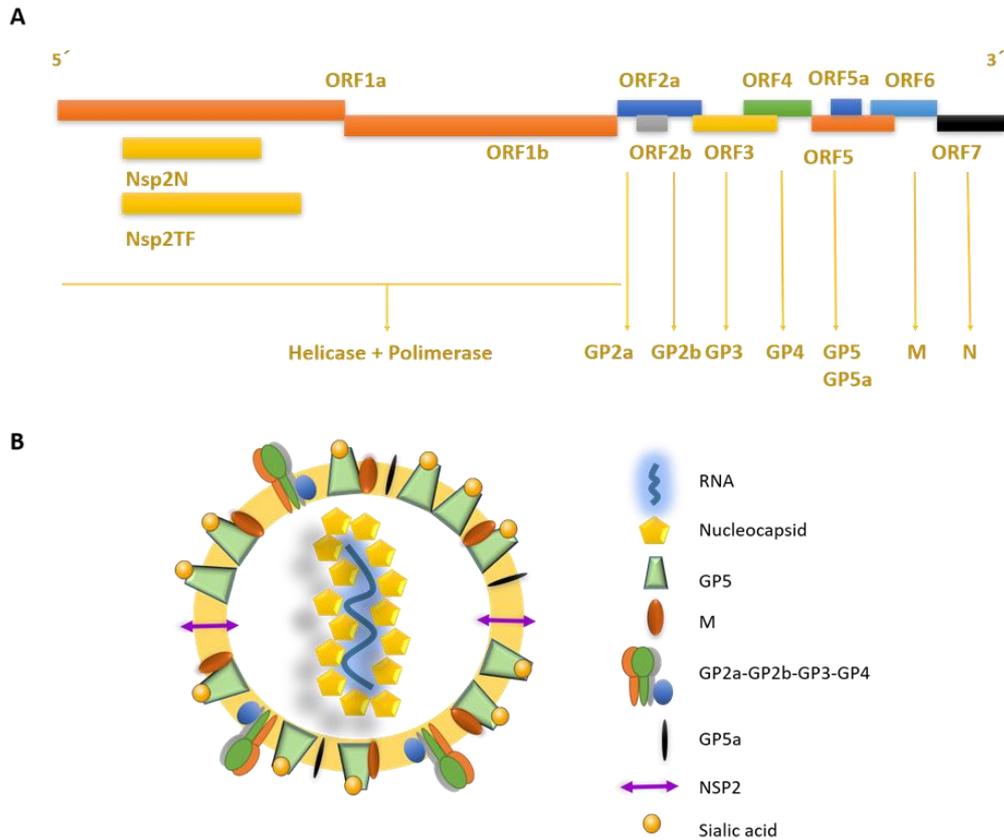


Figure 1. Genome structure and mature viral particle of PRRSV virus. (A) Non-structural proteins are located in the 5' end of the genome, codifying for two different polyproteins pp1a and pp1ab that are cleaved into at least 14 nsps (nsp1 to nsp12 and nsp1 α and nsp1 β , and nsp7 α and nsp7 β). Structural proteins located near the 3' end, are associated to the viral envelope and RNA packaging. (B) PRRSV mature viral particle, composed of a lipid bilayer envelop with viral receptor glycoproteins involved on infection and cell internalization. Single stranded positive RNA is associated with nucleocapsid protein in the internal layer of the virus.

PRRSV also has a set of 8 structural proteins, including a small non-glycosylated protein and a set of glycosylated ones: GP2a-b, GP3, GP4, GP5 and GP5a, M and N proteins (Huang et al., 2015). However, nsp2, traditionally classified as a non-structural protein, has been found to be incorporated in multiple isoforms within the viral envelope (Ovarian tumour domain protease region, hypervariable region and C-terminal region) (M. A. Kappes, Miller, and Faaberg 2013), giving new insights into the structure of this virus (Figure 1B). First, the nucleocapsid protein (N), as one of the most important parts of the mature viral particle, has been deeply characterized on PRRSV, finding important features shared in most non-segmented RNA viruses. The N protein consists of 123 amino acids for genotype 2 and 128 amino acids for genotype 1. The viral envelope glycoproteins (GP2 to GP5) are the first interactors with host cell receptors to initiate infection and are exposed to the immune system when viral particles are in blood and lymphoid tissue circulation (Figure 2). There is also another protein that contribute to virion structure, M protein, that is required during viral entry to interact with heparan sulphate cell receptor on macrophages. Later, GP5 is thought to bind to sialoadhesin and virus internalization and uncoating is triggered by a formation of a viral heterotrimer

(GP2a, GP3 and GP4) with scavenger receptor CD163 (Figure 2) (C. Shi et al. 2015; Veit et al. 2014). GP5 is the most abundant glycoprotein. First, it interacts with two cell entry mediators, heparan sulphate glycosaminoglycans and sialoadhesin/CD169 (Veit et al. 2014; C. Shi et al. 2015) to favour viral entry and then possibly with the N protein and its MHC-like domain to carry N-Viral RNA complex to the budding site (Figure 2). GP2, GP3 and GP4 are protected with glycan shields, like most PRRSV membrane proteins, to avoid antibody recognition and neutralization. GP2 has two glycosylation sites, GP3 have seven and GP4 have four, three of which are directly related to virus survival, causing lethal damage in virus production when more than two of these sites are mutated (Wei et al. 2012) (Figure 2).

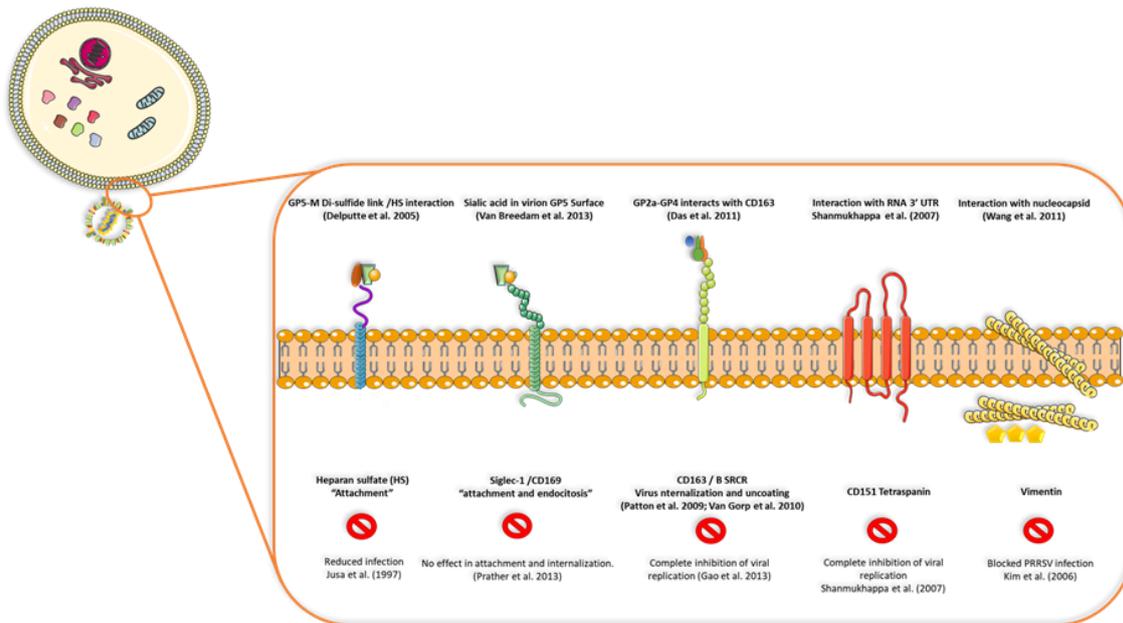


Figure 2. Interactions between viral proteins and cell receptors for virus attachment, entry, uncoating and release of genetic ssRNA to cell cytoplasm. Blocking CD163, CD151 tetraspanin or vimentin seems to inhibit viral replication or infection in the host cell, but reduced replication or no effect is seen when receptors such as heparan-sulphate or siglec-1 are blocked, demonstrating that some viral proteins and cell receptors are indispensable in terms of production of infectious viral progeny and dissemination in the host.

Virus replication and entry mechanisms in host cells.

Viral replication starts by interaction of viral glycoproteins with different cellular receptors (Figure 2) (C. Shi et al. 2015). CD163 and CD169 play a main role during infection, uncoating of the viral particle, activation of clathrin-mediated endocytosis and release of viral genome in the cytoplasm (Yun and Lee 2013). CD163 has been defined as the main receptor for viral infection by evaluating the effect of PRRSV on CD163 knockout pigs, where there is complete resistance to infection (Yang et al. 2018). Cysteine-rich domain 5 in this receptor seems to be necessary to establish interactions with PRRSV-1 species, since its deletion by CRISPR/Cas9 system (exon 7 of the gene encoding this region) implies protection for a large panel of these viruses demonstrated by in vitro challenge of edited-pig macrophages and in vivo experiments with Δ SRCR5 animals (Burkard et al. 2017; Wells et al. 2017; Burkard et al. 2018). More important, edited pigs show no side effects when kept under standard husbandry conditions

and CD163 seems to maintain its biological function (haemoglobin-haptoglobin scavenger) regardless the lacking cysteine-rich 5 domain, nevertheless, other unknown functions could be impaired by this modification. In conclusion, gene-edited pigs lacking SRCR5 region of CD163 could be an important asset to confront PRRSV epidemics with the final goal of eradication.

CD169 seems to be related only to co-interactions with sialic acid in the virion surface, however, knockout pigs for either exon 1, 2 or 3 of CD169 were not protected from infection and viral load as well as antibody responses were similar to heterozygous (CD169 +/-) or wild type pigs (CD169 +/+) (Prather et al. 2013). The former experiments suggested that other unknown mechanisms could be involved in PRRSV infection such as other receptors, new unknown susceptible cell types different from macrophages or possible leaking of CD169 expression in the knockout model.

Other molecules are also involved in viral entry, such as CD151 (Shanmukhappa, Kim, and Kapil 2007) and vimentin (W. W. Wang et al. 2011); blocking of any of these four molecules (CD163, CD169, CD151 and vimentin) had an effect on viral infection, either on internalization or complete inhibition of viral replication (C. Shi et al. 2015). After cell entry, PRRSV causes a series of intracellular modifications to complete its replication cycle, which includes rearrangements of intracellular membrane organelles to generate the replication complex. These include the formation of perinuclear double membrane vesicles apparently derived from endoplasmic reticulum, synthesis of genomic RNA (gRNA), transcription of segmented RNA (sgRNA) and expression of viral proteins (M. a. Kappes and Faaberg 2015; Yun and Lee 2013). At late stages of replication, the mature virions accumulate in the intracellular membrane compartments and they are then released into the extracellular space through exocytosis (Thanawongnuwech, Thacker, and Halbur 1997).

A non-classical spread pathway has been detected in several viruses including PRRSV where virus dissemination is mediated by cell to cell nanotubules (Guo et al. 2016). It was reported that almost all PRRSV proteins interact with myosin and actin (especially F-actin and Myosin IIA) where nanotubules connected cells allowing the movement of structural proteins and RNA, infecting naïve cells in a non-classical way even in the presence of neutralizing antibodies in the cell media. In addition, this non-classical pathway demonstrated that PRRSV cell entry receptors were not necessary to establish infection, as non-permissive cells became infected when were contacted by infected cells via nanotubes. This spreading strategy has been proposed as a mechanism to facilitate infection either by surfing of viral particles between adjacent cell membranes or as a receptor-independent mechanism for infection (Alemu et al. 2013); Importantly, has been reported for other viruses such as HIV-1 where nanotube number on macrophages increases after infection (Eugenin, Gaskill, and Berman 2009) and Herpesvirus transmission between bovine fibroblasts (Panasiuk et al. 2018). Interestingly, although several viral proteins were detected in nanotubules (nsp1 β , nsp2, nsp2TF, nsp4, nsp7, and nsp8, GP5 and N), GP4 was detected in only a few nanotubes. In particular, the role of GP4 in this non-classical spread pathway is not fully understood and it will be interesting to further evaluate GP4 interaction with other cellular components to elucidate the reason why GP4 is not transported to new recipient naïve cells. Altogether these data indicate that PRRSV has evolved different pathways to spread even though, *in vivo*, the virus shows narrow cell

tropism for monocytes and macrophages (Loving et al. 2015; Snijder, Kikkert, and Fang 2013) (box 1).

Immunology of PRRSV and mechanisms involved in immune evasion.

Innate immune response

The innate immune response is the first system any given pathogen encounters, specially to prevent viral replication and invasion into mucosal tissues (respiratory tract in the case of PRRSV) and, importantly, to initiate the strong adaptive immune response to fight against intracellular infectious agents (Lunney et al. 2016). Type I interferons (IFN α/β) comprise one of the most potent mechanisms against invading viruses in the first stages of infection, triggering an array of IFN-stimulated genes (ISG) (Schneider, Chevillotte, and Rice 2014). Generally speaking, all nucleated cells have the ability to produce IFN α/β but plasmacytoid DC (pDC) are the most potent producers of this family of cytokines (Asselin-Paturel and Trinchieri 2005). PRRSV has evolved a set of mechanisms for suppressing IFN α/β in vivo, maintaining low expression levels of this cytokines on infected pigs (Albina et al. 1998) during almost all time-course of infection shortly after transient elevation in the lungs (Van Reeth et al. 1999). Suppression of IFN α/β also takes place in vitro in PRRSV infected MARC-145 and porcine alveolar macrophages (Albina et al. 1998; Buddaert, Van Reeth, and Pensaert 1998; Miller et al. 2004). Further studies have shown that IFN type I suppression is a major strategy of PRRSV to modulate host antiviral defence. In fact, several viral proteins have been identified as IFN antagonists (nsp1 α , nsp1 β , nsp2, nsp4, nsp11 and N) (Han and Yoo 2014; Lunney et al. 2016; Y. Sun et al. 2012; Yoo et al. 2010). As an example, for N protein, upon dsRNA stimulation, IFN- β production was shown to decrease proportionally with increasing levels of N expression and additionally it was found to downregulate IFN-dependent gene production by dsRNA interfering with dsRNA-induced phosphorylation and nuclear translocation of IRF3 (Sagong and Lee 2011).

Among PRRSV non-structural proteins with type I IFN modulation capacity, nsp1 has been considered as the strongest antagonist of IFN- β production by acting on interferon regulatory factor 3 (IRF3) phosphorylation and nuclear translocation. Almost all nsps, excepting nsp1, have been related to the perinuclear region, associated with intracellular membranes, supposedly derived from the endoplasmic reticulum (ER), which are modified into vesicular double-membrane structures with which the viral replication and transcription complex (RTC) is thought to be associated with (Fang and Snijder 2010; Pedersen et al. 1999; van der Hoeven et al. 2016). Nsp1 translocate to the nucleus during the first hours of infection, where it is capable of inhibiting IRF3 association with CREB-binding protein (CBP), promoting CBP degradation by a proteasome-dependent mechanism, without which the transcription enhanceosome may not assemble the transcription machinery for the interferon expression (Fang and Snijder 2010; Huang, Zhang, and Feng 2015). Recently, post-transcription protein expression of IFN β was shown to be regulated by PRRSV by means of upregulating cellular miRNA in porcine alveolar macrophages (L. Wang et al. 2017)

Nsp2 is the largest (mature) PRRSV protein and contains at least four distinct domains: The N-terminal CP/OTU domain, a central hypervariable region, a putative transmembrane domain, and a C-terminal region of unknown function that is rich in conserved cysteine residues. This protein is unique in the context of PRRSV due to its genetic heterogeneity, its participation in diverse roles supporting the viral replication cycle, and its packaging within the PRRSV virion (M. A. Kappes, Miller, and Faaberg 2015, 2013). Previous studies suggest that nsp2 has different roles related to immune evasion mechanisms. It has been determined that nsp2 OTU domain (thiol-dependent deubiquitinating domain) inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) by interfering with the polyubiquitination process of I κ B α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor) and, subsequently, preventing the degradation of the I κ B α protein (Z. Sun et al. 2010). Moreover, viable deletion mutants in nsp2, when infecting cells, caused a downregulation of cytokines (IL-1 β and TNF- α) mRNA expression, in comparison with that of parental virus, suggesting that certain regions of nsp2 might contribute to the induction of a virus-specific host immune response and that deletion of such a region could produce a more virulent virus (Z. Chen et al. 2010).

There are several isoforms of nsp2, sharing a consistent core set between viral strains, which are integrated into mature virion at the final stage of replication (Figure 1B), although some of them could be strain-specific. Inclusion of nsp2 within the PRRSV virion suggests that it may function in previously unknown roles related to extracellular function, entry, or immediate-early viral replication events (M. A. Kappes, Miller, and Faaberg 2013). Truncated forms of nsp2 have also been identified, named nsp2TF and nsp2N, with apparent roles in modulation of immune evasion. When deletion mutants for those forms were used to infect cells, there was a significant change in gene expression, a strong activation of those involved in cytokine-cytokine receptor interaction, TNF signalling, toll-like receptor signaling, NOD-like receptor signalling, NF- κ B signaling, RIG-I-like receptor signalling, chemokine signaling, JAK-STAT signalling, cytosolic DNA-sensing, and NK cell mediated cytotoxicity (Y. Li et al. 2018), suggesting that an active role (direct or indirect) is played by these truncated forms in modulating host cells innate immune response, making PRRSV infectious cycle more complicated than it was initially thought.

Nsp11, is a Nidovirus conserved endoribonuclease with an uridylyate-specific endonuclease (NendoU). It has been demonstrated in vitro that overexpression of nsp11 enhanced viral titer (X. Shi et al. 2016). Moreover, nsp11 antagonizes type I IFN, specifically IFN β production, activated by the retinoic acid inducible gene 1 like receptor, showing substrate specificity towards Mitochondrial Antiviral Signaling proteins (MAVS) and RIG-I (transcripts and proteins), and demonstrating that this activity was associated to the endoribonuclease activity of this protein in which transfection mutant viruses were unable to degrade MAVS mRNA and impair IFN β production (Y. Sun et al. 2016). Another mechanism whereby this protein limits antiviral response is related to inflammasome and synthesis of IL-1 β , due to its important role in both the innate and adaptive immune response and in pathological mechanisms. It has been shown that PRRSV could activate NLRP3 inflammasome in early stages of infection but induce host's immunosuppression later as measured by determining the levels of pro-IL-1 β and procaspase-1 mRNA and the mature IL-1 β protein in porcine alveolar macrophages (PAM) (C. Wang et al. 2015). It is not surprising that nsp11 also interacts with the RNA-silencing complex (RISC), as it has been demonstrated in vitro in a MARC-145 cell line that this protein and

nsp1 α are responsible for inhibiting RISC and downregulating argonaute-2 protein expression increasing viral titer significantly, which demonstrates a direct relationship between this silencing complex and viral replication at least in vitro (J. Chen et al. 2015).

Other non-structural proteins have been studied but there is an important gap on information about in vivo and in vitro functions and interaction in signalling pathways. Additionally, the enormous variation among strains makes it difficult to characterize all protein variants and interactions with cell systems (macrophages, Dendritic cells “DCs”, monocytes and others) (box 1).

Recently, a body of evidence associates host genetics with different outcomes following PRRSV infection in the respiratory and reproductive form of the disease (Hess et al. 2016; Reiner 2016; Rashidi et al. 2014; Serão et al. 2014; Harding et al. 2017). Although pathways and mechanisms involved in specific disease-resistance traits have not yet been fully characterized, it is clear that the genetic variation in disease resilience is polygenic, regulating aspects of both innate resistance and acquired immunity (Harding et al. 2017). In connection with innate response, the average daily gain (ADG) after PRRSV infection was associated with a single genomic region in chromosome 4 (SSC4) which is best represented by the SNP tag marker WUR, located in the 3' non-coding region of the interferon-inducible guanylate-binding protein 1 (GBP1) gene (Boddicker et al. 2014). The pig genetic resistance to PRRSV infection has been historically overlooked in PRRSV research probably generating a confounding factor in immune response studies. A key gap in the knowledge of PRRSV is linked the pig genetic variability after PRRSV infection with the enormous variability of the virus itself (box 1).

In pigs, PRRSV replicates in cells belonging to the innate immune system. PAMs are the primary cells to be infected in the lungs as well as other cells of the monocyte/macrophage lineage, which later could disseminate the virus to other tissues or support replication to release viral particles into the bloodstream (C. Shi et al. 2015) (Figure 2). Moreover, PRRSV is thought to be able to infect professional antigen presenting cells such as DCs and monocyte derived dendritic cells, (MoDC) impairing their normal antigen presentation ability by inducing apoptosis, down-regulating the expression of IFN- α , MHC class I, MHC class II, CD11b/c and CD14, upregulating the expression of IL-10 and inducing minimal Th1 cytokine secretion (Flores-Mendoza et al. 2008; Loving, Brockmeier, and Sacco 2007; Calzada-Nova et al. 2011; Gimeno et al. 2011). Nevertheless, new evidence suggest by in vivo and in vitro experiments that specifically lung cDC1, cDC2 and MoDCs are not infected by PRRSV-1 viruses from subtypes 1 and 3 and one possible explanation is the lower expression of CD163 and CD169 in those 3 DC subtypes, associating previous results of infection in DCs to culture conditions of monocytes in vitro that could cause a sensibilization to infection by certain strains as Lena (Bordet, Blanc, et al. 2018). In addition, these findings were also tested in tonsil cDC and tracheal cDC1 and cDC2 observing that those cell populations are not infected by PRRSV virus (Reséndiz, Valenzuela, and Hernández 2018; Puebla-Clark et al. 2019).

Moreover, a new type of PAM has been characterized and named porcine intravascular macrophages (PIM) due to its association to endothelial lung capillaries and not to the alveoli, presenting strong capacity to phagocytised blood-related particles (Bordet, Maisonnasse, et al. 2018). Importantly, when infected PIM cells gave similar results of viral load to those derived from infected PAM, but significantly upregulates of TNF α and non-significantly IL-6 and IL-

8 expression after infection when compared to normal alveolar macrophages, indicating that these cells have an important pro-inflammatory role during PRRSV infection in the lungs (Bordet, Maisonnasse, et al. 2018). New interactions between cells and the virus need to be further explored to unravel possible immunological features that leads to correlates of protection.

Recently, it has been shown that a domain within Nsp1 α is able to stimulate the secretion of CD83, which in turn inhibits MoDC function *in vitro*, impairing the ability of MoDC to stimulate T cell proliferation (X. Chen et al. 2018). Production of IFN α/β and the mechanisms for cell activation by pDC are severely suppressed during PRRSV infection, although these cells are not permissive to PRRSV infection (Baumann et al. 2013; Calzada-Nova et al. 2010). However, this phenomenon is strain dependent, as other PRRSV strains are able to stimulate pDC for IFN α/β production in large quantities (García-Nicolás et al. 2016). Again, there is an enormous variability between PRRSV strains in relation with their effect on antigen presenting cells which prevent scientists from finding common mechanisms. It might be of interest to link this key gap of knowledge for PRRSV with host genetics (box 1). Moreover, in PRRSV-infected cells, N is abundantly expressed benefiting from the discontinuous transcription mechanism (Ke and Yoo 2017). This protein is also distributed in the nucleus, induced by two nuclear localization signals called cryptic NLS or NLS-1 and functional NLS or NLS-2 (positions 10 to 13 and 41 to 47 respectively) (Music and Gagnon 2010). The effect of N protein has been examined in PAMs and MoDCs using transfection, finding a significant upregulation of IL-10 gene expression.

Natural killer (NK) cells constitute another powerful arm of the innate immune system against PRRSV, particularly when considering the high percentage of circulating NK cells in pigs (Denyer et al. 2006). The cytotoxic function of NK cells is reduced in PRRSV infected pigs from day 2 after infection up to three to four weeks (Albina et al. 1998; Dwivedi et al. 2011; G. J. Renukaradhya et al. 2010). Initial studies using *in vitro* systems demonstrated that the stimulation of porcine NK cells with proinflammatory cytokines (IL-2 and IL-15) was capable of activating NK cells and inducing them to express high levels of IFN- γ and perforins to cause lysis of infected cells, but a different scenario appears if cells are evaluated post-infection, indicating that a virus such as PRRSV is capable of impairing NK cell cytotoxicity (Shekhar and Yang 2015). *In vitro*, the NK cytotoxicity against PRRSV-infected PAMs was decreased and degranulation of NK cells inhibited (Cao et al. 2013). *In vivo*, the immune response is the same as that observed *in vitro*, with some studies reporting that approximately half of viremic pigs had a reduction greater than 50% in NK cell-mediated cytotoxicity and enhanced secretion of IL-4, IL-12 and IL-10 and reduced frequency of cytotoxic T-cells (CD4-CD8 $^+$ T) and double positive T cells (CD4+CD8 $^+$ T) and upregulated frequency regulatory T- cells (Tregs) (Dwivedi, Manickam, Binjawadagi, Linhares, et al. 2012).

Acquired immune responses

Innate immune responses against PRRSV are obstructed by different mechanisms as are adaptive responses. The modest and delayed B cell mediated neutralizing antibody response is one of the main characteristics associated to PRRSV acquired immune responses. Even though PRRSV specific antibodies appear early at 7-9 days post-infection, the efficacy of those antibodies remains unclear. Neutralizing antibodies take longer, appearing nearly 1 month after

infection (Loving et al. 2015). However, passive transfer of these neutralizing antibodies conferred almost full protection in a PRRSV reproductive model (95% of offspring alive after challenging pregnant sows with high neutralizing antibody titer). Nevertheless, in another experiment using the reproductive model, when the presence of PRRSV was examined after the transfer of neutralizing antibodies, lungs, tonsils, buffy coat cells, and peripheral lymph nodes contained replicating PRRSV similar to infected controls, although pigs were apparently protected against infection. In summary, passive transfer of high neutralizing antibody titer conferred protection to gilts and offspring (not detectable viremia) but did not eliminate the presence of viral particles in peripheral tissues nor transmission to animals they were in contact with (Osorio et al. 2002; Lopez et al. 2007; Lopez and Osorio 2004). Curiously, the role of neutralizing antibodies in the protection against the respiratory form of the disease is a key gap of knowledge for PRRSV. This point is critical to define precisely targets for improved vaccines based on the humoral immune response against this virus (box 1).

N protein is involved in several mechanisms for immune evasion and is also one of the most immunogenic structural proteins (Music and Gagnon 2010). Antibodies against N appear early during acute infection, together with those against M and GP5 proteins, but are non-neutralizing and could be involved in antibody dependent enhancement (Mateu and Diaz 2008; Murtaugh, Xiao, and Zuckermann 2002).

There are other “antibody-related mechanisms” that do not necessarily involve neutralizing activity. Antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent complement-mediated cytotoxicity (CDC) and antibody-dependent complement mediated virolysis (ADCV) have been examined in the context of PRRSV, although none of these mechanisms were evident during infection or have not been deeply investigated on in vitro and in vivo models of this virus (M. C. Rahe and Murtaugh 2017). It is important to note that neutralizing antibodies appear late in PRRSV infection and other immune mechanisms (cellular or antibody mediated immune response) might be acting to suppress viral replication in blood, causing the virus to be isolated in lymphoid tissues and maintaining suboptimal replication that will finally end in viral clearance. For type PRRSV-2 it has been demonstrated that immunization of pigs with ectodomain peptides from GP5/M complex did not induce neutralizing antibodies (J. Li and Murtaugh 2012) although those ectodomain-specific antibodies generated were capable of binding virus.

An important feature that makes difficult to validate the location of neutralizing epitopes is the number of glycosylations in or around it. For PRRSV-1 strains, up to 3 glycosylations may be found in, or flanking the GP5 neutralizing epitope that is located between amino acids 37–45 (Faaberg et al. 2006), whereas for PRRSV-2 strains there are four potential glycosylation sites (Darwich, Díaz, and Mateu 2010). When tested, PRRSV with mutations in GP5 glycosylation sites (either at N44 or in the hypervariable region, upstream the neutralizing epitope) enhanced immunogenicity with increased concentration of antibodies directed to this epitope 5 to 10-fold higher compared with those induced by the wild type strains (Faaberg et al. 2006). Same results were obtained when administering another deglycosylation mutant (double deglycosylation in the putative glycosylation moieties on GP5) twice, which conferred better protection against homologous challenge (J.-A. Lee et al. 2014). In addition, when this protein is expressed early during infection, it stimulates production of early neutralizing antibodies and IFN- β , two main antiviral mechanisms, demonstrating its role in induction of self-protection mechanisms from the host (Gao et al. 2014). Available data about neutralizing antibodies induced by this protein

are controversial, which may be due to the high variation among PRRSV strains (Butler et al. 2014) and, as previously commented, the host genetics. ORF5 is also complemented by a small frameshift of the subgenomic mRNA called ORF5a, encoding a type I membrane protein consisting primarily of alpha helix with a membrane-spanning domain (called GP5a) that is incorporated into virions as a very minor component, playing a role in viral replication, as mutation in the initiation codon or premature termination related to expression for this protein leads to non-efficient viral replication and lower titer (Firth et al. 2011; Johnson et al. 2011). This protein is capable of eliciting specific antibody immune response in natural infections and after immunizations, although those are not neutralizing neither protective in a challenge trial after infection, making difficult to define the role of this particular small protein in the whole immune response and viral clearance of PRRSV infection (Robinson et al. 2013). In summary, the role of humoral immunity remains elusive in PRRSV infection (neutralizing and non-neutralizing antibodies) and a better characterization will be required to overcome this relevant gap of knowledge (box 1).

Treg typically increase in number in chronic viral diseases to prevent a persistent inflammatory response and pathological damage associated to viral infections. Conversely, Tregs are described as key contributors in modulating the host immune response to viral infection. This cell population is an important component in regulating the magnitude of the immune response to infection (in viruses such as HIV and HCV), thus preventing excessive inflammation and tissue damage. However, they can also be inappropriately induced by viruses to switch the balance of the immune response in favour of maintaining viral replication (Belkaid 2007). In PRRSV, the role of Tregs remains unclear and appears to be a consequence of IL-10 induction of some strains as early as 2 days post infection (Dwivedi, Manickam, Binjawadagi, Linhares, et al. 2012). In some experiments, in vitro infected DCs with PRRSV-1 exhibited an unbalanced ability to stimulate T cell immune responses in a strain-dependent manner, but no Tregs were detected, at least in vitro, as measured by expression of CD25 and FoxP3 markers (Silva-Campa et al. 2010). When using PRRSV-2 strains, the case seems to be different, as the virus was capable of stimulating IL-10 production with concomitant generation of Tregs (Wongyanin et al. 2012) which was associated to nucleocapsid protein expression in the in vitro system. This group also suggested that IL-10 production and Treg could be related to impaired gamma interferon (IFN- γ) production and altered development of protective T-cell response by inhibiting T-cell proliferation as seen in the early stage of infection with viruses such as HCV. Vaccine strains currently in use in the United States do not provide adequate heterologous protection, one possible explanation could lay on their inability to induce an adequate IFN- γ response due to their ability to stimulate Tregs, at least in vitro (Cecere, Todd, and LeRoith 2012). Structural conformation, but not nuclear localization, of the expressed N protein was suggested as essential for the ability to induce IL-10 that, in consequence, causes induction of Tregs as measured by markers CD4+CD25+Foxp3+ (Wongyanin et al. 2012). It should be noted that when the role of the nuclear localization signal was evaluated using deletion mutants, results suggested that NLS-2 was not essential for virus survival, although pigs developed a significantly shorter duration of viremia and higher neutralizing antibodies than those of wild-type PRRSV-infected pigs (C. Lee et al., 2006). The role of Tregs cells in the immune response against PRRSV is a key gap of knowledge in order to develop more efficacious PRRSV vaccines (box 1).

Moreover, reports have highlighted the impact of PRRSV infection on thymic cellularity mainly as a loss of CD4+/CD8+ cells in the thymus of PRRSV-infected pigs. Acute lymphopenia, thymic atrophy, and lymphadenopathy associated with the presence of PRRSV antigen in the thymus are some of the mechanisms whereby PRRSV suppresses the immune response. In addition, presence of PRRSV antigens in the thymus could also induce tolerance and presents a mechanism that could explain the presence of Tregs during PRRSV infection (Butler et al. 2014). Nevertheless, the picture is not complete and basic knowledge about the effect of PRRSV on cell development in the thymus would be of great interest to understand the effect of this viruses in the host.

PRRSV immunology thus remains an unsolved puzzle due to complex interactions between different viral strains and the host. Similar immune responses could be the key feature of this virus, such as persistence viremia, a strong inhibition of innate cytokines (IFN- α/β , TNF- α , IL-1 β , IFN- γ), dysregulation of NK cell function (cytotoxicity and degranulation), rapid induction of non-neutralizing antibodies, delayed appearance of neutralizing antibody, late and low CD8+ T-cell response, and induction of regulatory T cells (Tregs) (Du et al. 2017). As a whole, neutralizing antibodies and PRRSV-specific IFN- γ secreting cells do not fully depict the immune effector functions related to protective immunity, as the viral targets related to them are unknown. As a consequence, correlates of protection remain elusive for this infection due to the laborious work in vitro and in vivo and the enormous genetic diversity that causes confusion and makes it difficult to predict how immune responses against one isolate or strain could be applied to another in a cross-protective immune prediction model (Murtaugh and Genzow 2011; Amadori and Razzuoli 2014). Without any doubt, the most important gap of knowledge for PRRSV is the lack of correlates of protection that makes extremely difficult to have robust models to check vaccines efficacy against this disease (box 1).

Vaccination strategies in PRRSV. Classical and novel vaccines.

Since the beginning of PRRSV outbreaks in Europe and the USA, the development of efficacious PRRSV vaccines has been a challenge. Classical approaches are not working properly for several reasons: viral mutation can lead to more pathogenic strains, there is a lack of knowledge on how the porcine immune system interacts with all PRRSV proteins, and most importantly, there is no robust parameter (surrogate marker) that can be unequivocally linked with viral clearance. Thus, there is no relationship between complete homologous or heterologous protection and classic immunological parameters such as an increase/decrease in particular cell population (Sang, Rowland, and Blecha 2014), IFN- γ production, neutralizing antibodies (Martínez-Lobo et al. 2011), non-neutralizing antibodies and clinical outcome (M. Rahe and Murtaugh 2017). In addition, highly divergent strains make it more difficult to develop a universal vaccine for this virus (M. a. Kappes and Faaberg 2015).

Several different vaccines against PRRSV have reached the market and have been reviewed recently (Nan et al. 2017). Most of these vaccines rely upon modified live virus (Porcilis PRRS from Merck, Ingelvac PRRSFLEX EU from Boehringer Ingelheim, Amervac-PRRS from Hypra, Pysrvac-183 from Syva) against PRRSV-1, as well as some to control PRRSV-2 (Fostera PRRS from Zoetis, Ingelvac PRRS MLV / Ingelvac PRRSATP from Boehringer Ingelheim). There is also evidence that most MLV vaccines of both PRRSV-1 and PRRSV-2 species elicit specific humoral and cell-mediated immune (CMI) responses, as they confer

protection to homologous parental strains and partial protection to heterologous strains. Although it is possible to control some PRRSV outbreaks by use of MLV in combination with good practices, there are major safety issues such as a high mutation rate leading to reversion to virulence and recombination among vaccine and wild type strains. Cases have been reported in which new viruses have been introduced as a consequence of MLV vaccines. For example, nucleotide sequence identities of atypical Danish isolates were between 99.2-99.5% with the vaccine virus RespPRRS and 99.0-99.3% with VR2332, which is the parental virus to the vaccine virus, supporting the conclusion that the introduction of PRRSV-2 in Denmark was due to the spread of vaccine virus (Madsen et al. 1998). In China a recombination event was reported in which a PRRSV variant with nucleotide deletions and insertions in the non-structural protein 2 (nsp2) gene also showed a possible recombination event between a MLV strain and a prototype Chinese field strain (Wenhui et al. 2012).

Current inactivated vaccine approaches are not highly effective since elicited immune responses are not enough to prevent spreading of the virus. However, this type of vaccine can augment anamnestic virus neutralizing antibodies and virus-specific IFN- γ responses following a wild-type virus infection or PRRSV-MLV vaccination which can contribute to viral clearance (Piras et al. 2005; Scotti et al. 2007). Thus, the combination of modified live vaccines with inactivated ones can be a reasonable approach to control the disease under field conditions (Díaz et al. 2013) but unfortunately, there is no robust data comparing this approach with other options available on the market. On the other hand, most inactivated vaccines are not approved for use in the United States due to the poor efficacy showed in challenge trials (Charentantanakul 2012) as measured by production of PRRSV specific neutralizing and non-neutralizing antibodies and low cellular immune responses leading to their failure in the porcine market. According to the Centre for Food Security and Public Health of Iowa State University, only “BIOSUIS PRRS Inact EU+Am” is approved to be used in the US. However, new strategies are being evaluated to overcome these problems (G. J. Renukaradhya et al. 2015), including nanoparticle entrapped antigens (Dwivedi et al. 2013; Dwivedi, Manickam, Binjawadagi, Joyappa, et al. 2012; G. Renukaradhya et al. 2014; Mokhtar et al. 2017), plant-based approaches (Uribe-Campero et al. 2015) or vectored vaccines (Cruz et al. 2010).

Several attempts have been made to use structural proteins to develop vaccines against PRRSV because they are specific targets of neutralizing antibodies. For this reason, one may hypothesize that antibodies against those proteins could be the main key to inhibit viral replication and spread as it is common for many viruses. Approaches such as VLPs combining different structural proteins have been tested (Binjawadagi et al. 2016; Eck et al. 2016; García Durán et al. 2016), finding that anamnestic response is possible (boosted IgG and IFN- γ producing cells) in previously vaccinated or infected pigs but not in the pre-challenge period. These structural proteins are able to prime the immune system, but no reduction of viremia was observed after challenge (Eck et al. 2016). Those results suggest that other viral proteins may be targeted to induce a protective response in pigs. A plausible explanation for this finding may be based on the presence of few neutralizing epitopes in their sequences, most of which are located in variable regions of the proteins, to the phenomena of glycan shielding for epitopes and to the high variability observed between PRRSV virus strains. Again, a critical gap of knowledge for PRRSV is to precisely characterize common epitopes that are present in all PRRSV strains. Epitopes responsible for generating an efficient immune response eliciting cross-protective immunity remained elusive. Taken together, this evidence points to the need

for new vaccination approaches that comply with a pathogen free strategy, capable of eliciting effective cellular and antibody responses with mid to long term protection against homologous strains and preferable to heterologous challenge as well.

Extracellular vesicles as a new vaccination approach.

Extracellular vesicles (EVs) are gaining increased scientific attention as novel vaccines against infectious diseases, including animal diseases of veterinary importance by its capacity of self-antigen presentation, activation of host cell and antibody immune responses and more important, to induce protection in lethal challenge trials (Andre et al., 2004; del Cacho et al., 2016; Marcilla et al., 2014; Martin-Jaular et al., 2011; Montaner-Tarbes et al., 2016; Raposo et al., 1996; Schorey and Bhatnagar, 2008; Yáñez-Mó et al., 2015) (Box 2). In the case of PRRSV, artificial microRNAs (amiRNA) were initially synthesized to try suppressing expression of sialoadhesin (Sn) or CD163 by recombinant adenoviral vectors to be contained in exosomes, causing a subexpression of Sn and CD163 at mRNA and protein level, and reducing viral titer when porcine macrophages were pre-treated with amiRNA thus providing new evidence supporting the hypothesis that EVs can also serve as an efficient small RNA transfer vehicle for pig cells (Zhu et al. 2014). More recently, PRRSV viral proteins associated to extracellular vesicles (EVs) in the size range of exosomes, were reported (Montaner-Tarbes et al. 2016). Moreover, a targeted-pig trial using EVs from sera of infected pigs who had overcome the disease, demonstrated that EVs are capable of inducing specific IFN- γ secreting cells after a prime-boost strategy, are safe, free-of-virus and can differentiate infected from vaccinated animals (Montaner-Tarbes et al. 2018), moreover, it was demonstrated that those EVs contained antigenic viral proteins recognized by pig immune sera and not by the pre-immune one. Of interest, however, a recent article indicated that PRRSV derived EVs are capable of transmitting the virus from one cell to another (T. Wang et al. 2017). Whether these discrepancies are due to in vivo vs in vitro experimental work and methods applied to isolate EVs from serum samples or culture supernatant, remains to be determined.

EVs have also been explored as novel control strategies in other viral diseases. For example, in respiratory syncytial virus infection, EVs are released with a selected modified cargo when compared with uninfected epithelial cells. When analysed in detail, several viral proteins and diverse species of RNA were detected and capable of activating innate immune responses through induction of cytokine and chemokine release (H. S. Chahar et al. 2018). Similar scenarios of viral proteins exported in EVs have been observed and extensively reviewed for HIV/HCV/HTLV-1 (H. Chahar, Bao, and Casola 2015), EBV (Teow et al. 2017), and other viral diseases. Moreover, viral products of various origin and size including Ebola Virus VP24, VP40 and NP, Influenza Virus NP, Crimean-Congo Haemorrhagic Fever NP, West Nile Virus NS3, and Hepatitis C Virus NS3, when fused with Nef C-terminal domain through DNA vectors, were directed to the EVs membrane or packaged into them and remained stable after fusion. More importantly, when injected in mice, DNA vectors expressing the diverse fusion products elicited a well detectable antigen-specific CD8⁺ T cell response associating with a cytotoxic activity potent enough to kill peptide-loaded and/or antigen-expressing syngeneic cells, proving its promising results as a cytotoxic T lymphocyte vaccine (Anticoli et al. 2018).

Box 2. Exosomes and therapeutic applications in PRRSV.

Pan et al., 1983; Harding et al., 1983.	•Discovered in 1983. Known as a garbage-disposal mechanism in the maturation of reticulocytes to erythrocytes.
Raposo et al., 1996	•Exosomes released from B-lymphocytes are capable of antigen presentation as contain MHC-II in their surface and induce antigen-specific MCH-II restricted T-cell responses.
Zhu et al., 2014	•First use of exosomes in PRRSV. Application of exosome for delivering artificial microRNAs targeting sialoadhesin and CD163 receptors.
Montaner-Tarbes et al., 2016.	•Serum-derived exosomes from non-viremic animals previously exposed to the porcine respiratory and reproductive virus (PRRSV) contain antigenic viral proteins.
Lener et al., 2015	•Guidance of International Society of Extracellular Vesicles regarding the most important aspects of safety and regulatory requirements that must be considered for pharmaceutical manufacturing and clinical application of extracellular vesicles including strategies to promote the therapeutic application of EVs in future clinical studies.
Montaner-Tarbes et al. 2018.	• First targeted-pig trial using serum derived extracellular vesicles as a new vaccination platform for PRRSV virus.
FDA clinical trials for veterinary diseases	• To the best of our knowledge, no clinical trials in veterinary medicine have been approved by FDA

Concluding remarks

PRRSV is a complex disease and several gaps in the knowledge of its economic impact, biology and evolution, genetic polymorphism, mechanism of viral infections, elicitation of protective immune responses and novel control strategies, have been reviewed here (Box 1). Since the late 1980's, different approaches have permitted to examine more closely this virus allowing the discovery of new features of the complex replication cycle, the identification of proteins and nucleic acids playing a role together with extracellular vesicles and nanotubules in facilitating spreading, and a better understanding of immune evasion (non-neutralizing antibodies, glycan shielding, mutation, recombination events, among others) to further vaccine development. Presently available PRRSV vaccines have many limitations in terms of heterologous protection, but some efforts have been made by combining new adjuvant formulations with modified live viruses, DNA and peptide vaccines, as well as extracellular vesicles a new vaccination approach. Advancing in all these gaps in knowledge, will eventually accelerate eliminating and eventually eradicating this devastating veterinary disease of such huge economic importance.

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This review was discussed and written by all co-authors

Conflict of Interest

All authors declare no conflict of interest.

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CHAPTER II. OBJECTIVES

Main objective.

The main objective of this thesis is to expand the applicability of extracellular vesicles (EVs) in the treatment and diagnosis of diseases of veterinary interest with a particular focus on the development of a new vaccine for the control of Porcine Reproductive and Respiratory Syndrome Virus.

As a part of an Industrial doctoral program, this thesis particularly aimed:

Specific objectives

- 1. To isolate and determine the molecular composition of serum-derived extracellular vesicles enriched fractions obtained from naïve pigs, viremic animals and non-viremic animals previously infected with PRRSV.**
 - To isolate and characterize EVs from serum obtained from naïve pigs, viremic animals and non-viremic animals previously infected with PRRSV by flow cytometry, nanoparticle tracking analyses (NTA) and Cryo-Electron Microscopy and to validate EVs enriched fractions.
 - To analyse the proteomic composition of EVs enriched fractions by liquid chromatography/mass spectrometry in order to find molecules of immunologic interest in PRRSV diagnostics and treatment.
 - To define and standardize a Standard Operation Procedure for EVs' enrichment that must be reproducible and scalable.
 - To protect and patent the intellectual property derived from the proof-of-concept on EVs immunogenic capacity.

- 2. To evaluate safety and immunogenicity of EVs derived from PRRSV convalescent swine sera as a new vaccination platform on *in vivo* targeted-pig trials.**
 - To obtain EVs from PRRSV convalescent swine sera that must be homogeneous in terms of molecular markers and proteomic signatures for safety and immunogenicity trials.
 - To evaluate safety of EVs enriched fractions by *in vitro* assays.
 - To evaluate safety of EVs enriched fractions using an *in vivo* assay.
 - To evaluate cellular and humoral immune responses elicited by serum derived EVs in a heterologous prime-boost vaccination trial in comparison with a vaccination approach based on peptides.

- 3. To evaluate efficacy in an *in vivo* challenge trial using swine sera-derived EVs as a vaccination platform and compared with a classical peptide vaccination approach (CONFIDENTIAL by sponsors' request).**

- To evaluate *in vivo* the capacity of serum derived EVs enriched fractions to elicit protection in a challenge trial with a wild type PRRSV virus strain.
- To compare the protection degree achieved by two different vaccination schemes using either EVs enriched fractions or viral peptides.
- To determine the presence of viral particles in different tissues to evaluate dissemination and localization after challenge.

4. To isolate and determine the molecular composition of serum-derived extracellular vesicle enriched fractions obtained from infected animals with two African Swine fever virus strains (OURT88/3 and Benin Δ MGF)(CONFIDENTIAL / Manuscript In preparation).

- To evaluate viral protein content and EVs molecular markers on EVs enriched fractions at 7 days post infection using two different attenuated viral strains (OURT88/3 and Benin Δ MGF).
- To determine the viral and host proteins differentially expressed in serum derived EVs enriched fractions at 24 days post infection using two different attenuated viral strains (OURT88/3 and Benin Δ MGF).
- Compare host relative protein expression levels between controls and infected animals in order to determine the effect of viral strain on EVs enriched fractions content.

CHAPTER III. RESULTS

**CHAPTER 3.1. Serum-derived exosomes from non-viremic animals
previously exposed to the Porcine Respiratory and Reproductive Virus
contain antigenic viral proteins**

Serum-derived exosomes from non-viremic animals previously exposed to the Porcine Respiratory and Reproductive Virus contain antigenic viral proteins

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ABSTRACT

PRRSV is the etiological agent of one of the most important swine diseases with a significant economic burden worldwide and limitations in vaccinology. Exosomes are 30–100 nm vesicles of endocytic origin. Remarkably, immunizations with exosomes containing antigens from tumors or pathogens are capable of eliciting protective immune responses, albeit variably, in cancer and infectious diseases. Here we describe the isolation, molecular composition and immunogenicity of serum-derived exosomes from naïve animals, from PRRSV viremic animals and from animals previously PRRSV infected but already free of viruses (non viremic). Exosomes were isolated through size exclusion chromatography and characterized by different methodologies. Exosome-enriched fractions from naïve and natural infected animals contained classical tetraspanin exosomal markers (CD63 and CD81) and high concentrations of particles in the size-range of exosomes as detected by nanoparticle tracking analysis and cryo-TEM. NanoLC-MS/MS was used to identify viral antigens associated to exosomes. PRRSV-proteins were detected in serum samples from only viremic animals and from animals previously infected already free of viruses (non-viremic), but not in controls. Moreover, immune sera from pigs previously exposed to PRRSV specifically reacted against exosomes purified from non-viremic pig sera in a dose-dependent manner, a reactivity not detected when naïve sera was used in the assay. To facilitate future studies, a scaling-up process was implemented. To the best of our knowledge, this is the first molecular characterization of serum-derived exosomes from naïve pigs and pigs actively or previously infected with PRRSV. The presence of antigenic viral proteins in serum-derived exosomes free of virus, suggest their use as a novel vaccine approach against PRRSV.

Keywords

Size Exclusion Chromatography, Nucleocapsid Protein, Extracellular Vesicle, Nanoparticle Tracking Analysis, PRRSV Infection.

Introduction

Porcine reproductive and respiratory virus (PRRSV) is the etiological agent of one of the most important swine diseases with a significant economic burden worldwide. Only in the US, it is estimated that \$560 million yearly losses are directly related to this disease [1]. Current vaccines against PRRSV have focused on methods using modified live or attenuated virus [2], peptides [3], vectored vaccines [4], inactivated virus and subunit vaccines [5, 6, 7]. Available vaccines, however, have limitations such as little protective immunity [8], possible reversion to virulence [9], and incapability of eliciting long lasting and heterologous protection among European and American genotypes [10]. In addition, PRRSV strains have high antigenic variability and genetic polymorphisms [11, 12] and the highest mutation rate of RNA viruses [5]. All together, these limitations indicate that new alternatives to conventional vaccines are desperately needed aiming to control and eventually eradicating PRRSV.

Exosomes are 30–100 nm vesicles of endocytic origin originally described as a “garbage-disposable” mechanism of reticulocytes in their terminal differentiation to erythrocytes [13, 14]. This cellular origin and function were shown not to be unique as 10 years later, B-cells were also described to secrete exosomes with antigen presentation capacity and with the ability of generating specific T-cell responses [15]. Since these seminal observations, exosomes have been shown to be secreted by all immune cells and explored as novel vaccination approaches [16]. In fact, proof-of-principle Phase I clinical trials using dendritic cell-derived exosomes coupled to tumor-associated antigens have shown their safety and immunogenicity in cancer and Phase II trials are presently being conducted [17]. Of interest, antigens from infectious diseases associated with exosomes also demonstrated their capacity for eliciting specific and protective immune responses in preclinical mouse models [18, 19, 20]. For instance, vaccination with extracellular vesicles and exosomes can induce a strong immune response and increase survival in *Mycobacterium tuberculosis*, *Eimeria tenella*, *Toxoplasma gondii* [18, 19] and full protection against a lethal challenge in *Plasmodium yoelii* experimental infections [21]. Moreover, outer membrane vesicles (OMVs) derived from *Bordetella pertussis* used as vaccine in mice ameliorated infection following challenge with several strains [20]. For virus, exosomes play an important role not only involved in pathogenesis and virus spreading [22] but also in cell communication and protection against infection [23]. All together, these data strongly suggest the value of exosomes as a new vaccination approach in human health. Yet, no reports have shown their potential value for vaccination in animal health.

In this work, we describe the isolation and molecular composition of serum-derived exosomes obtained from naïve pigs, from viremic animals and from non-viremic animals previously infected with PRRSV. Our results unequivocally identified viral antigens associated to exosomes in viremic and non-viremic pigs. Moreover, viral proteins contained in serum-derived exosomes from non-viremic animals exhibit antigenic potential as judged by ELISA assays. A scaling-up protocol for obtaining serum-derived exosomes was also developed. Thus, opening the possibility of exploring these non-viremic nanovesicles as a novel vaccination approach against PRRSV.

Materials and methods.

Samples

Sera were obtained from large white X Landrace pigs of approximately seventeen weeks of age that had suffered a PRRSV natural outbreak in two conventional farms and from animals of one PRRSV negative farm (naive pigs). The two PRRSV positive farms belong to the same

integration company but from different sow origin. The PRRSV negative farm pertains to a different integration Company; thus, avoiding any confounding with samples. Viral as well as serological status of animals against PRRSV antigens were analyzed, respectively, by RT-PCR TaqMan® NA/EU PRRSV Reagents (Applied Biosystems) and IDEXX PRRS X3 Antibody Test (IDEXX). An independent diagnostics laboratory for porcine diseases in Lleida [24] confirmed these analyses following their own standard operational procedures.

Sera from all animals were classified as non-viremic (NV, PRRSV negative by RT-PCR) or viremic (V, PRRSV positive by RT-PCR), being both groups serologically positive to PRRSV using an IDEXX PRRS X3 Antibody Test. On the other hand, sera from naive control animals (CN) were PRRSV negative and free from antibodies against PRRSV. Details of sera used in this study are included in Additional file 1. All studies were approved by the ethical committee of the University of Lleida, Spain, and performed under their guidelines for animal care (DAAM7684).

Exosome isolation: size-exclusion chromatography

Isolation of serum-derived exosomes by size exclusion chromatography (SEC) were performed as previously described [25]. Briefly, Sepharose CL-2B (Sigma-Aldrich, St. Louis, MO, USA) was packed in 10 mL syringes to a final volume of 10 mL and equilibrated with PBS-Citrate 0.32% (w/v). Frozen serum samples were thawed on ice, centrifuged at 500×g for 10 min at room temperature to remove cellular debris, and 2 mL aliquots were applied to each column. Collection of 20 fractions of 0.5 mL each started immediately using PBS-citrate as the elution buffer. Protein content of each fraction was analyzed using Bradford protein quantification assay according to manufacturer's instructions (Bradford reagent, Sigma-Aldrich). To determine protein profiles, samples were loaded into 10% polyacrylamide BIORAD precast gels, separated at 120 V for 45 min and stained using SilverQuest™ Staining kit (Invitrogen).

Flow cytometry analysis of molecular markers associated with extracellular vesicles

A bead-based assay for detection of two classical exosome markers, CD63 and CD81 was used to phenotypically identify SEC fractions containing exosomes [26]. Briefly, exosomes were coupled with Aldehyde/Sulfate Latex Beads, 4% w/v, 4 µm (Invitrogen) and then blocked with PBS 1X/BSA 0.1% (Sigma-Aldrich) /NaN₃ 0.01% (Sigma-Aldrich). Fractions were incubated in microtest conical bottom 96-well plates for 30 min at 4 °C with anti-CD63 and anti-CD81 antibodies (culture supernatant monoclonal antibodies) at 1:10 dilution. After washing, a 1/100 dilution of secondary antibody FITC (Southern Biotech) was incubated for 30 min at 4 °C. After removal of unbound secondary antibodies by centrifugation, beads were suspended in PBS and analyzed by flow cytometry using a BD FACSVerser (BD Biosciences) equipment. Median Fluorescence Intensity (MFI) and beads count data were obtained by FlowJo analysis Software of every sample-reading file.

Nanoparticle tracking analysis (NTA)

Diameter size and concentration of vesicle population was determined using NanoSight LM10 equipment (Malvern). Fractions were evaluated using different dilutions in sterile-filtered PBS 1X (1/10 to 1/50) and the following parameters: camera at 30 frames per second (FPS), camera level at 16, temperature between 21–25 °C and video recording time 60 s in order to estimate the concentration and size distribution of vesicle population through light scattering and Brownian motion. Nanosight NTA Software analyzed raw data videos by triplicate and results

were obtained in PDF datasheet with all selected values (Mean size, Median size, Mode size and concentration).

Cryo-electron microscopy (Cryo-TEM)

Ten microliter aliquots from individual SEC fractions containing exosomes were directly laid on Formvar-Carbon EM grids and frozen in ethanol. Samples were analyzed on a Jeol JEM 2011 transmission electron microscope at an accelerating voltage of 200 kV. Samples and the 626 Gatan cryoholder were maintained at $-182\text{ }^{\circ}\text{C}$ during the whole process. To minimize electron beam radiation, images were recorded on a Gatan Ultrascan cooled CCD camera under low electron dose conditions. Vesicle size was determined using the ImageJ software (NIH) and setting calibration was performed pixels/nanometer.

Mass spectrometry

Liquid Chromatography (nanoLCULTRA-EKSIGENT) followed by mass spectrometry (nanoLC-MS/MS) was performed on an LTQ Orbitrap Velos (Thermo Fisher). Briefly, samples were reduced with 10 mM DTT (Dithiothreitol), alkylated with 55 mM iodoacetamide and precipitated by 10% TCA. After washing with acetone, 2 μL of 8 M urea were added and samples brought to a final concentration of 1.6 M urea. One microgram of trypsin (*Sus scrofa*) was added and digestions were carried overnight at $37\text{ }^{\circ}\text{C}$. The reaction was stopped with 1% formic acid. The amount of sample submitted to mass spectrometry analyses was based on nanoparticle tracking analysis (see below) and ranged from 9.8×10^7 to 3.9×10^8 particles/mL among all samples analyzed. MS/MS was performed in the LTQ using data dependent dynamic exclusion of the top 20 most intense peptides using repeat count = 1, repeat duration = 30 s, exclusion list size of 500 and exclusion list duration = 30 s as parameters. The top 20 most intense peptides were isolated and fragmented by low energy CID, 35% collision energy.

Database search and protein identification

Raw spectral data from Xcalibur™ (Thermo Scientific, v2.1) was searched against a custom database compiled from [27] in FASTA format for uploading it into Andromeda Search Engine 1.4. The database contained complete and partial sequences of PRRSV (22 976 sequences) and *Sus scrofa* (59 898 sequences). The sequence for trypsin from *Sus scrofa* (Accession P00761 from [28]) and default contaminant database were also included in the search carried out with Maxquant 1.5/Andromeda 1.4 software. Contaminants and proteins identified only by site modification were filtered out from the list. Proteins found in all groups were scored positive if they had at least two unique peptides and 1% False Discovery Rate (FDR) for protein and peptide identification. After filtering, proteins of each group were compared in a Venn diagram using Venny 2.0 software [29] to determine which proteins were unique and shared among samples.

Gene ontology (GO) analyses by PANTHER overrepresentation test

Porcine proteins identified by Maxquant 1.5/Andromeda 1.4 Software were filtered by elimination of “contaminants” and “Only identified by site proteins”. Then, the most common proteins with highest score were submitted to UniprotKb “retrieve/ ID mapping [28] to convert GI numbers (Maxquant results) to UniprotKB ID number and eliminating redundant hits. Then, the final protein list (184) (.tab format) was submitted to PANTHER Overrepresentation Test (release 20150430) [30] using Annotation Version: PANTHER version 10.0 Released 2015-05-15, Reference List: *Sus scrofa* (all genes in database) and perform all three GO-Slim

analysis available (Biological process, Cellular component and Molecular function) [31]. Also, exosomal proteins of *Sus scrofa* were compared against exosome proteins of different farm animals (*Bos taurus*, *Equus caballus*, *Gallus gallus* and *Rattus norvegicus*) using Funrich analysis software [32].

ELISA assays

An indirect ELISA protocol was initially performed (dilution chessboard) for titration of sera coming from NV, V and CN pigs using a secondary antibody Goat anti-Pig IgG (Fc): HRP (AbSerotec AAI41P) and Porcilis PRRSV vaccine as coating antigen (Intervet Lot. A200ED03) (Additional file 2). Using a range of sera dilutions previously titrated, circulating IgG antibodies from NV and CN pigs were tested in a double ELISA test against homologous NV serum-derived exosomes (sandwich ELISA) and against whole viral vaccine (Porcilis PRRS Vaccine “intervet” lot. A200ED03) as previously described. For sandwich ELISAs, plates were first coated with anti-CD63 antibodies and after washing and blocking, SEC fractions (100 μ L per well) containing exosomes were incubated 90 min at 37 °C. Sera samples were afterwards incubated for 1 h at room temperature, washed and incubated with secondary antibody Goat anti-Pig IgG (Fc): HRP (AbSerotec AAI41P) at 1:10 000 dilution and optical density was measured at 450 nm using Varioskan equipment (Thermo Scientific).

Scaling-up process for vesicle enrichment and isolation

The process of polyethylene-glycol (PEG) precipitation was based on scale-up process for retrovirus stock in order to maintain structure and functionality of extracellular vesicles [33, 34]. Thus, two adult healthy animals (80–100 kg) were anesthetized and approximately 500 mLs of peripheral blood from each animal collected by venous puncture. Blood was collected into 50 mL Falcon tubes to facilitate collection of sera and minimize hemolysis. Sera were precipitated overnight at room temperature by adding PEG at 8.5% w/v ratio. Pellets were resuspended in 20 mLs of PBS and loaded into PuriFlash Dry Load Columns 80G (Interchim) filled with 100 mL of sepharose CL-2B (separation matrix) and 5 mLs fractions collected for further analysis.

Results

Characterization of serum-derived exosomes after purification by size exclusion chromatography

SEC was used to isolate exosomes from sera of naïve animals (CN) pertaining to a PRRSV negative farm, and sera from viremic animals (V) or non-viremic (NV) animals from two PRRSV positive farms where different PRRS viruses were detected. Preliminary studies on sequence polymorphisms from PRRSV viruses isolated in these farms revealed 85% homology among them (data not shown). Twenty 0.5 mL aliquot fractions were collected from each serum and individually analysed for (i) for their protein content and (ii) for the presence of two “classical” exosome markers, CD63 and CD81. In all samples analysed, exosome markers were identified in fractions 7–10, whereas total protein content increased in later fractions (Figure 1A). In addition, NTA revealed that preparations from all animals were highly homogeneous in terms of particle size (100–200 nm with a medium size of 127 nm) and concentration (10^9 – 10^{10} particles per mL) (Figure 1B; Additional file 3). Furthermore, electrophoresis of the protein content of SEC fractions from different samples revealed a similar profile in early fractions and, as expected, an enrichment of plasma proteins in late fractions (Figure 1C). Vesicle size and structure were also analysed by cryo-TEM. Similar to

NTA, vesicles from 80–200 nm in diameter were predominantly observed whereas electro-dense bodies typically associated with viral particles, were not (Figure 1D).

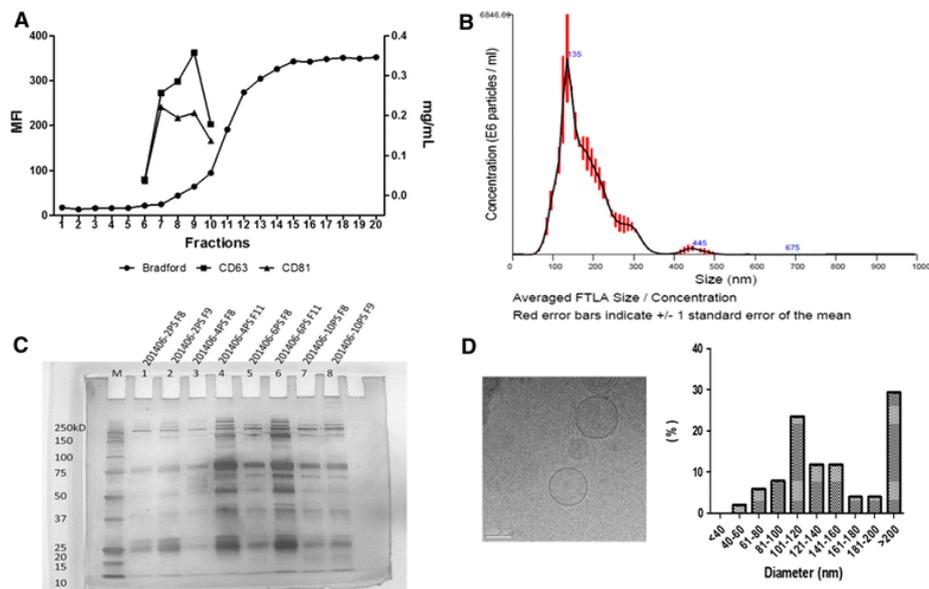


Figure 1. **Characterization of porcine serum-derived exosomes sera by different methodologies.** Bradford and flow cytometry analyses (A), nanoparticle tracking analysis (B), cryo-electron microscopy (C) and SDS-PAGE/Silver Staining (D) are represented. MFI: Median fluorescence unit, mg/mL: Bradford measure unit, M: Molecular weight marker, F6-F9: Fraction number from SEC and percentage (%) size distribution (nm).

Proteomic analysis

To characterize the exosome protein composition from different groups of animals, liquid chromatography and mass spectrometry were applied for protein sequencing and identification from samples of one NV animal and two V animals (Figure 2). Of importance, peptides pertaining to viral proteins were identified in serum-derived exosomes from all animals whereas others were uniquely identified. Thus, peptides from major envelope glycoprotein GP5-Tm:pFc (a fusion protein of GP5 with no transmembrane domain and pig fragment crystallizable portion), from envelope glycoprotein GP3, NSP2 and partial ORF2b were detected in exosomes from all (NV and V) animals. Other peptides from nucleocapsid protein, envelope glycoprotein GP3 protein, major envelope glycoprotein GP5 and replicase polyprotein 1ab, were only identified in exosomes from V animals whereas peptides from envelope glycoprotein 3 were identified in exosomes from one V and one NV animal. Interestingly, peptides from RNA-dependent RNA polymerase and nucleocapsid protein N, were detected only in exosomes from NV animals.

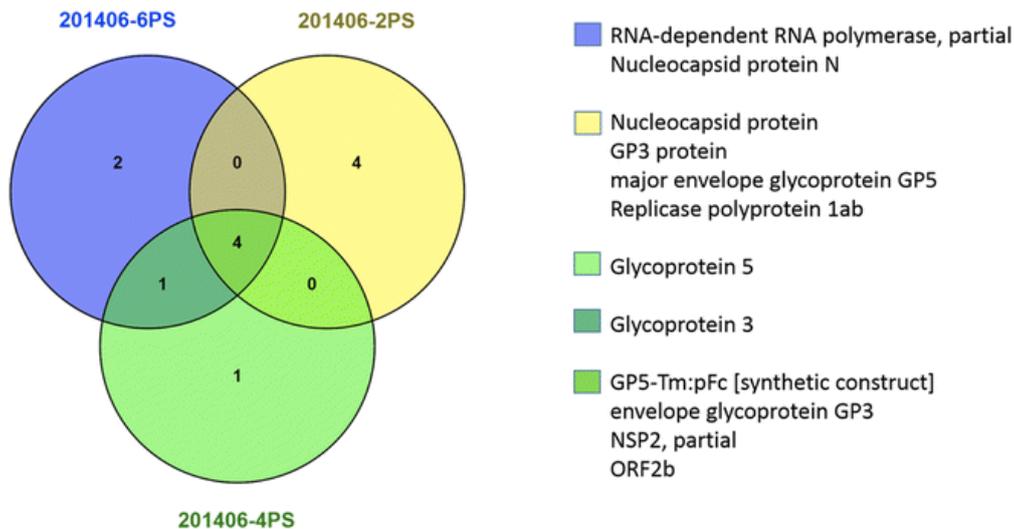


Figure 2. **Venn diagram showing overlap and unique peptides of viral proteins detected in different sample groups: non-viremic (201406-6PS) and viremic (201406-2PS and 201406-4PS).** The overlapping and unique peptides identified for proteins in these sample groups are shown.

To confirm the presence of unique viral proteins in exosomes from NV animals, three additional samples were also analysed by liquid chromatography and mass spectrometry and confirmed the presence of RNA-dependent RNA polymerase, partial and nucleocapsid protein N. None of these proteins were found in serum-derived exosomes from naïve animals (CN) further confirming the specificity of these results (not shown).

The proteomics analysis also identified more than 400 porcine proteins contained within exosomes (Additional file 4). Of interest, GO analysis showed an important enrichment of lipid transport, response to external stimulus, proteolysis, enzymatic activities and extracellular space proteins, all related to exosomes composition and function (Table 1). Besides, when comparing exosome porcine proteins in our database with exosomes from other farm animals using the Funrich software [32], 48 proteins were shared among *B. taurus* and *S. scrofa* (approximately 2.9% of total proteins), 5 with *E. caballus*, 6 with *G. gallus* and 3 with *R. norvegicus* as outlier in the evolutionary line (Additional file 5). Interestingly, even though the pig protein database is smaller than others in these analyses, there is a coincidence of 3% with *Bos taurus*, and at least 4 of these proteins are classical exosomal markers (CD5, CD9, CD81 and CD63) (Additional file 5).

Table 1. Gene Ontology analysis of *Sus scrofa* proteins detected in exosomal enriched fractions

PANTHER GO-Slim Biological Process	REF LIST. 21483	Exp. List (91)	expected	over/under	fold Enrichment	P-value
lipid transport	305	10	1.29	+	> 5	1.67E-04
response to external stimulus	347	10	1.47	+	> 5	5.24E-04
proteolysis	690	12	2.92	+	4.11	7.98E-03
response to stress	659	11	2.79	+	3.94	2.48E-02
immune system process	1399	17	5.93	+	2.87	1.65E-02
localization	2610	28	11.06	+	2.53	4.69E-04
transport	2484	26	10.52	+	2.47	1.93E-03
response to stimulus	2364	23	10.01	+	2.3	2.31E-02
Unclassified	9376	24	39.72	-	0.6	0.00E+00
PANTHER GO-Slim Molecular Function	REF LIST. 21483	Exp. List (91)	expected	over/under	fold Enrichment	P-value
lipid transporter activity	106	8	0.45	+	> 5	3.51E-06
peptidase inhibitor activity	227	10	0.96	+	> 5	8.65E-06
serine-type peptidase activity	293	11	1.24	+	> 5	8.91E-06
enzyme inhibitor activity	337	10	1.43	+	> 5	3.08E-04
peptidase activity	605	16	2.56	+	> 5	9.26E-07
receptor binding	947	18	4.01	+	4.49	1.42E-05
hydrolase activity	2181	23	9.24	+	2.49	5.06E-03
protein binding	2729	28	11.56	+	2.42	8.53E-04
Unclassified	10794	35	45.72	-	0.77	0.00E+00
PANTHER GO-Slim Cellular Component	REF LIST. 21483	Exp. List (91)	expected	over/under	fold Enrichment	P-value
extracellular space	6	3	0.03	+	> 5	1.22E-04
extracellular region	624	18	2.64	+	> 5	6.31E-09
Unclassified	17295	62	73.26	-	0.85	0.00E+00

Specific immune recognition of PRRSV-proteins in serum-derived exosomes

To determine whether serum-derived exosomes from NV animals contained antigenic viral proteins, swine sera was tested by indirect and sandwich ELISA. First, sera was titrated using Porcilis PRRSV vaccine as coating antigen and showed maximum differences between CN and NV animals at 1/50–1/100 dilutions and 1/10 000 dilution of the secondary antibody without being at saturation values (Additional file 2). As shown in Figure 3, statistically significant differences ($p < 0.05$) between sera from NV and CN animals using three individual NV exosome preparations (1PS, 2PS and 3PS) at 1/50 and 1/100 dilutions, were observed. Moreover, similar reactivity and statistical significance were observed when sera from CN and NV animals were tested against a commercially available vaccine (Porcilis PRRSV vaccine, Intervet) as the coating antigen. In addition, evaluation of antigenicity was done in concentrated exosome samples obtained through PEG/SEC isolation. Non-viremic sera but not naïve was able to recognize exosomes derived from non-viremic animals in a dose dependent manner (Figure 4).

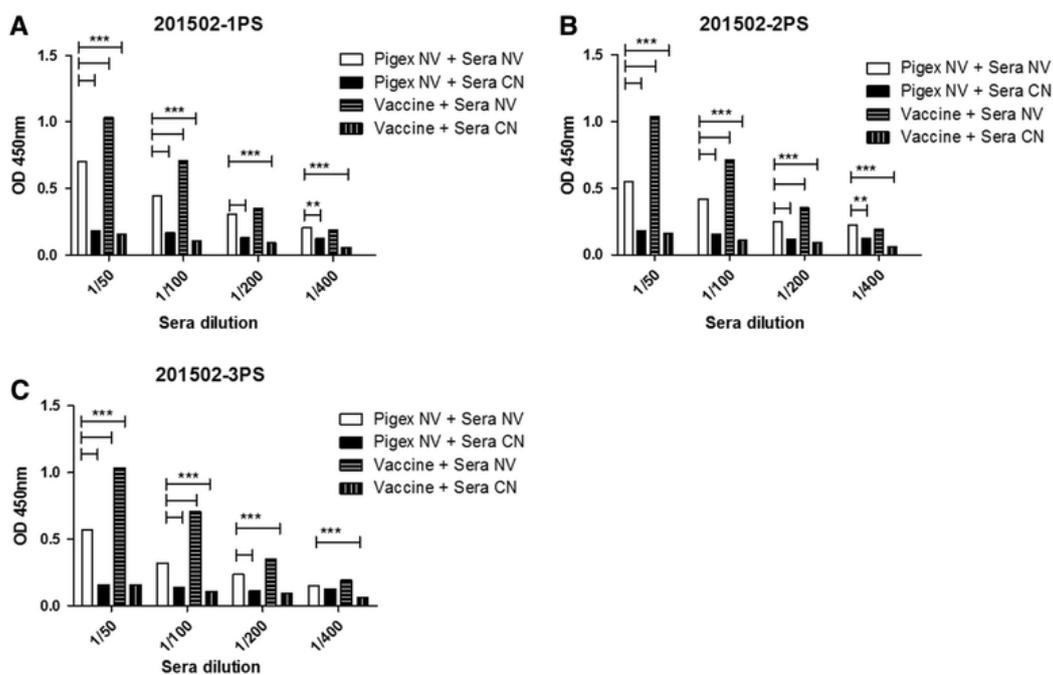


Figure 3. **ELISA assay for evaluation of NV and CN porcine sera immune recognition over inactivated viral particle PRRSV vaccine (Porcilis PRRS, Intervet) and exosomes derived from NV porcine sera of different origin.** Analyses of naïve (CN) and non-viremic (NV) sera against exosomes derived from sample 201502-1PS (A), 201502-2PS (B) and 201502-3PS (C). Optical density (OD) was measured at 450 nm and it is represented in the “Y” axis. “X” axis shows the dilution factor for sera samples (1/50 to 1/400). For each animal, exosomes were isolated and captured using anti-CD63 antibody and tested against both sera (** $p < 0.01$; *** $p < 0.0001$).

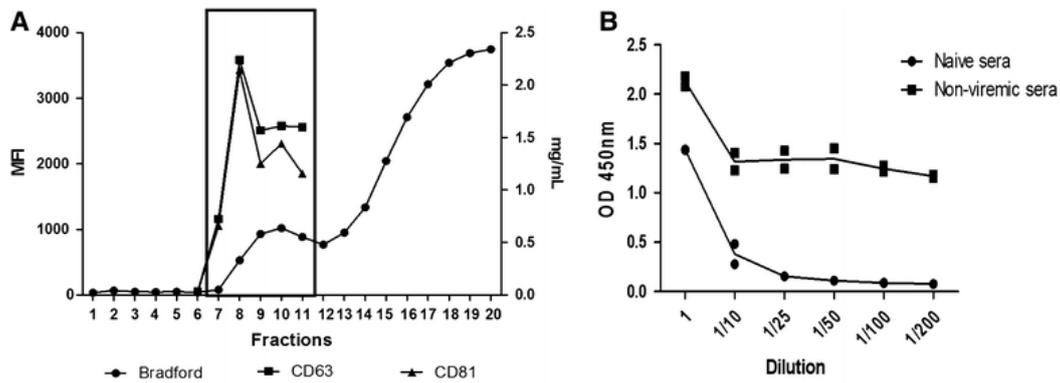


Figure 4. **Characterization of exosomes isolated using PEG/SEC methodology from porcine sera.** Bradford and flow cytometry analyses (A), Sandwich ELISA for exosomes derived from non-viremic animal (B), are represented. MFI: Median fluorescence unit, mg/mL: Bradford measure unit, CD63-CD81 are molecular markers for exosomes characterization. For FACS and Bradford analyses is evident that higher values are represented in comparison with non-concentrated samples (10-fold change). In addition, the indirect quantification through Bradford of protein associated with exosomes it is possible when sera is concentrated through PEG and separated using SEC, making this an important tool for further evaluation of immunogenic properties in vitro and in vivo. For the sandwich ELISA, exosomes derived from non-infected animal were tested against two types of porcine sera. Optical density (OD) was measured at 450 nm and represented in the “Y” axis. “X” axis shows the dilution factor for exosomes samples (1 to 1/200). Duplicated values are represented as squares and dots and mean as a line.

Scaling-up process for vesicle enrichment and isolation

Approximately half liter of peripheral blood was obtained from each of two anesthetized animals and their sera collected following standard procedures. Sera were precipitated using PEG, pellets suspended into 20 mL aliquots and passed through individual Sepharose CL-2B 100 mL columns. Results demonstrated that this escalation procedure yielded purified exosomes with the same NTA profile, SDS-PAGE and cryo-EM as those obtained from 2 mL aliquots of serum (Figure 4). Noticeably, the yield was significantly increased as proteins were detected by the Bradford assay in SEC fractions containing exosomes (Figure 4A) and NTA analysis revealed a twofold increase in the number of particles (10^{10-11} particles/mL) as opposed to those obtained from 2 mLs (10^{8-9} particles/mL). Last, PEG-precipitation did not affect the immunological properties of exosomes as immune sera similarly and specifically recognized them (Figure 4B).

Discussion

Here, for what we believe is the first time, we describe the isolation, characterization, antigenicity and scaling-up process of serum-derived exosomes from naïve pigs and from pigs actively or previously infected with PRRSV.

Firstly, size-exclusion chromatography was applied to analyze serum-derived exosomes from pigs in a small and scaling-up procedure. This single-standing methodology is presently considered a solid and reproducible method for isolation and characterization of extracellular vesicles in the size range of exosomes from different biological fluids such as plasma [25, 35], saliva [36] and urine [37]. In fact, it removes most contaminant abundant proteins and purifies 100–200 nm particles associated with classical exosomal markers. Accordingly, our results

showed that exosomes eluted in fractions 7 to 10, whereas more abundant serum proteins (such as albumin), as judged by the Bradford assay, eluted in later fractions. Moreover, two “classical” exosomal markers, CD63 and CD81 [38] showed maximum MFI values in these same fractions (Figures 1, 4), where high concentrations of particles in the size-range of exosomes were also detected by NTA and cryo-TEM analyses and low protein content was detected. Of interest, bicosome-like structures (vesicles within vesicles) were observed in agreement with similar structures found in structural studies from other fluids [39, 40, 41]. These results strongly suggest that porcine serum samples have similar and reproducible SEC elution profiles as described in human samples, reinforcing the use of SEC as a single-standing and easily implementing technology facilitating field studies of extracellular vesicles in animal diseases of veterinary importance.

To identify PRRSV viral proteins associated with serum-derived exosomes, we used nanoLC-MS/MS. Remarkably, RNA-dependent RNA polymerase, partial and nucleocapsid protein N, were detected only in exosomes from the non-viremic animals (Figure 2). The nucleocapsid protein (N) is one of the most abundant and immunodominant viral proteins during PRRS infection [42]. This protein interacts with different cellular factors of the host to facilitate virus infection and its role is crucial for mature viral particle formation within the cell, binding to the viral RNA genome and replication machinery including RNA-dependent RNA polymerase, and also interacts with itself to form the core capsid [43, 44]. Of interest, the N protein and three non-structural (Nsp) PRRSV proteins have been identified as playing an important role in type I IFN suppression and modulation of the NF- κ B pathway as it is translocated to the nucleus during early stages of infection [43, 45]. Late in infections, nucleocytoplasmic transport of the N protein increases the cytoplasmic concentration of this protein. It is tempting to speculate that an increase in virus N protein at cytoplasm during chronic infections might favor the release of the N-protein and RNA-dependent RNA polymerase in exosomes, which could at least partially explain the data from the proteomic analysis. In the absence of supporting data, this remains to be further demonstrated. Regardless, N proteins has been expressed in different models such as *Baculovirus* and *Escherichia coli* [42] and soya bean seeds [46]; interestingly, in all cases it was capable of inducing both cellular and humoral immune response (murine model) or being recognized by PRRSV immune porcine sera.

To test the antigenic properties of serum-derived exosomes from previously infected animals, we first captured exosomes isolated from three non-viremic (NV) animals that were free of detectable virus (RT-PCR negative) at the time of sera collection. Analysis of serum-derived exosomes from V animals was not performed in sandwich ELISA as PRRSV virus has the same density and size of exosomes; thus, confounding such analyses. Immune sera from pigs previously exposed to PRRSV, specifically reacted to these exosomes in a dose-dependent manner and similar, albeit at lower values, to antigens contained in the Porcilis attenuated vaccine; these results thus demonstrate that viral proteins contained in the exosome preparation from NV animals are antigenic. The immunogenic properties of exosomes containing pathogen-associated antigens have been tested in several preclinical models and diseases [18, 19, 47]. Yet, to the best of our knowledge, no reports are presently available on antigenic properties of serum-derived exosomes with no pathogen load detected in peripheral circulation. This observation may be of importance for future vaccine approaches.

As a *bonafide* aspect of these analyses, we present the first proteomics analysis of pig proteins contained in serum-derived exosomes (Additional file 2). More than 400 porcine proteins associated with lipid transport, response to external stimulus, response to stress, immune system processes, some enzymes and extracellular space proteins are enriched in our exosomal fractions indicating cargo-selection related to cell communication and metabolic processes.

These proteins thus represent a first baseline proteome of porcine serum-derived exosomes facilitating future studies between host and pathogens in PRRSV and other animal diseases.

The use of nanovesicles in vaccine approaches against PRRSV is bringing new and recent exciting data. It has been previously reported that nanoparticle entrapped antigens are more effective than conventional vaccine platforms [48, 49, 50] and demonstrated increasing titers of virus neutralizing antibodies in serum and lungs. Additionally, a different kind of artificial exosome was used to deliver microRNA into porcine alveolar macrophages (PAMs) to suppress expression of CD163 or Sialoadhesin receptors in cell surface making those less susceptible to PRRSV infection and replication [51]. Our results, including a scaling-up process, thus warrant further exploring serum-derived exosomes from PRRSV infections as a different vaccination approach. Regulatory aspects, similar to what has been recently positioned by the International Society of Extracellular Vesicles on human health [52], should not encounter major obstacles in future animal trials.

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CHAPTER 3.2. Targeted-pig trial on safety and immunogenicity of serum-derived extracellular vesicle enriched fractions obtained from Porcine Respiratory and Reproductive virus infections

Targeted-pig trial on safety and immunogenicity of serum-derived extracellular vesicle enriched fractions obtained from Porcine Respiratory and Reproductive virus infections

Running title: Safety and immunogenicity of serum-derived PRRSV extracellular vesicles

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Key words: extracellular vesicles, vaccines, targeted-pig trial, safety, immunogenicity, Porcine Reproductive and Respiratory Syndrome Virus, PRRSV

Abstract

The Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the etiological agent of one of the most important swine diseases with a significant economic burden worldwide. Unfortunately, available vaccines are partially effective highlighting the need of novel approaches. Previously, antigenic viral proteins were described in serum-derived extracellular vesicles (EVs) from pigs previously infected with PRRSV. Here, a targeted-pig trial was designed to determine the safety and immunogenicity of such extracellular vesicles enriched fractions. Our results showed that immunizations with EV-enriched fractions from convalescence animals in combination with montanide is safe and free of virus as immunizations with up-to two milligrams of EV-enriched fractions did not induce clinical symptoms, adverse effects and detectable viral replication. In addition, this vaccine formulation was able to elicit specific humoral IgG immune response in vaccinated animals, albeit variably. Noticeably, sera from vaccinated animals was diagnosed negative when tested for PRRSV using a commercial ELISA test; thus, indicating that this new approach differentiates vaccinated from infected animals. Lastly, after priming animals with EV-enriched fractions from sera of convalescence animals and boosting them with synthetic viral peptides identified by mass spectrometry, a distinctive high and specific IFN- γ response was elicited. Altogether, our data strongly suggest the use of serum EV-enriched fractions as a novel vaccine strategy against PRRSV.

Introduction

Recent estimates calculate that the world human population will reach near 9,6 billion people by 2050 and the United Nations Food and Agriculture Organization (FAO) estimates that in order to feed this population the overall food production will need to increase by 70% (Roth, 2011). Undoubtedly, veterinary vaccines that preserve animal health and improve production will play an essential role in reaching this goal. Moreover, they will reduce the use of antibiotics as they are escalating into a global health crisis. To reach this goal, novel vaccination approaches are desperately required as classical and live-attenuated vaccines are far from being totally safe and efficacious in animal diseases (McFarland et al., 2011).

The Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) causes one of the most important diseases of veterinary medicine (Pileri and Mateu, 2016). Recent economical estimates in the United States, where the disease is highly prevalent, indicate more than 600 million \$ loses each year (Neumann et al., 2005). Current vaccines against PRRSV use modified live or attenuated virus, small peptides, vectored vaccines, inactivated virus and subunit vaccines (Nan et al., 2017). Nevertheless, available vaccines have serious limitations such as little protective immunity, possible reversion to virulence, inability to induce long lasting and heterologous protection among European and American genotypes, and high antigenic and genetic differences of strains. All together, these limitations indicate that new alternatives to conventional vaccines are needed trying to control and eventually eradicating PRRSV.

Extracellular vesicles (EVs) are gaining increase scientific attention as novel vaccines against infectious diseases, including animal diseases of veterinary importance (del Cacho et al., 2016; Marcilla et al., 2014; Montaner-Tarbes et al., 2016; Schorey and Bhatnagar, 2008). Our group previously determined that EVs obtained from serum of animals that had overcome a PRRSV infection were free of virus as detected by a commercial and sensitive qRT-PCR test and contained antigenic viral-specific cargo (Montaner-Tarbes et al., 2016). Here, we report a targeted-pig safety and immunogenicity trial by immunizing pigs with serum-derived EV-

enriched fractions from convalescence animals. Our results revealed that these EV-enriched fractions are safe, free of virus and contained immunogenic viral peptides capable of eliciting specific humoral and cellular immune responses. Moreover, this approach seems capable of differentiating vaccinated from infected animals (DIVA).

Materials and methods

Ethical statement for experimental procedures.

All studies were approved by the ethical committee of Universitat de Lleida and the Departament d'Agricultura, Ramaderia, Pesca, Alimentació I Medi rural (Section of Biodiversity and hunting) under licence DAAM 7700. All procedures and experiments in this research were performed in accordance with guidelines and regulations of University of Lleida and the Department of Animal Sciences of this University under the supervision of a veterinary.

Serum samples

Serum was obtained from five individual large white-Landrace pigs of 80-100 kg of body weight that had suffered a PRRSV natural infection. Animals were anesthetized and approximately 1L of peripheral blood from each animal was collected by venous puncture. Afterwards, animals were humanely euthanized according to procedures approved by the University of Lleida. Blood was collected into 50 mL Falcon tubes to facilitate separation of sera and minimize haemolysis. Viral as well as serological status of animals against PRRSV antigens were analysed, by an independent laboratory specialized in diagnosis of porcine diseases (Grup de Sanejament Porci (<http://www.gsplleida.net/cat>) using RT-PCR TaqMan® NA/EU PRRSV Reagents (Applied Biosystems) and IDEXX PRRS X3 Antibody Test (IDEXX) following their own standard operational procedures. All those animals were negative by PCR and positive for antibodies against PRRSV in serum.

EVs enrichment and isolation

Enrichment of serum-derived EVs was obtained through ultracentrifugation (Théry et al., 2006) followed by Size Exclusion Chromatography (SEC) using our own standard methodologies (de Menezes-Neto et al., 2015). Sera samples were centrifuged for 2 hours at 100.000 x g at 4°C and resuspended in ten mL of PBS (final volume) before loading into PuriFlash Dry Load Columns 80G (Interchim) filled with 100 mL of sepharose CL-2B (separation matrix) and five mL fractions collected for further analysis. Nanoparticle tracking analysis (NTA), flow cytometry (FACS / culture supernatant monoclonal antibodies against EVs tetraspanins CD9, CD63, CD81 and CD5L from Abcam ab45408) and proteomic analyses (liquid chromatography/mass spectrometry: LC-MS/MS) were carried out as previously described (Montaner-Tarbes et al., 2016). Protein concentration was determined by bicinchoninic acid assay protein assay (BCA Pierce protein quantification assay – Thermo Scientific) (Smith et al., 1985) and used as vaccine dose unit. All those tests are recommended as standard proxy for characterization of extracellular vesicles and exosomes (Lötvall et al., 2014).

Mass spectrometry

Liquid chromatography (nanoLCULTRA-EKSIGENT) followed by mass spectrometry (LTQ Orbitrap Velos - Thermo Fisher) was used to identify viral proteins associated to EVs enriched fractions as previously described (Montaner-Tarbes et al., 2016). Briefly, protein identification was done using RAW data from five different animals from which EVs enriched fractions were analysed by LC-MS/MS and Maxquant software v1.5 excluding those hits identified by reverse

database, marked as contaminant and identified by modifications. Only those proteins with 2 or more unique peptides and 1% FDR were used for further analyses.

Synthetic peptides

After LC-MS/MS identification of PRRSV proteins associated with serum-derived EVs enriched fractions (supplementary table T1), two different algorithms were used to examine matching regions between our identified peptides (LC-MS/MS) and possible B-cell epitopes or antigenic regions within the protein (Kolaskar and Tongaonkar, 1990; Larsen et al., 2006). Peptides corresponded to the Envelope glycoprotein (ENV), Nucleocapsid (N) and polyprotein 1a (pp1a) viral proteins. Matching regions were detected and then evaluated for suitable Swine leukocyte antigen binding (SLA) by NetMHCpan 4.0 (Jurtz et al., 2017; Welner et al., 2017). Those with higher score were used to synthesize 35aa peptides (Department of Experimental and Health Sciences – Peptide Synthesis Facility of Universitat Pompeu Fabra at Centre for Genomic Regulation (Barcelona – Spain) according to their own standard operation procedures. Characterization of each synthetic peptide was carried out using an A-HPLC with a Column Luna C18 (4.6x50mm, 3um; Phenomenex), a Gradient: Linear B (0.036% TFA in MeCN) into A (0.045% TFA in H₂O) over 15 minutes with a flow rate of 1mL/min and detection at 220nm. All peptides were 90% pure and were resuspended in ultrapure H₂O (MiliQ water), aliquoted and stored in -20°C until use.

Safety pig targeted trial.

Fifteen PRRSV negative pigs were divided into groups with food and water *ad libitum* in an experimental farm (Centre d'Estudis Porcins, Torrelameu, Spain) under veterinarian supervision. EV-enriched fractions from animals that had overcome the disease were combined with Montanide ISA 206 VG (SAFIC-ALCAN, batch. T83571 - gently provided by SEPPIC) in a 1:1 ratio using three different EV-enriched fraction protein concentrations (0.3, 0.5 and 1mg) and injected intra-muscularly (IM) in one side of the neck. Each animal received 2mL of the formulation in two different time points. Serum samples were drawn at 0, 21 and 51 days post initial vaccination (dpv) (Table I). Collection of samples, maintenance and culling of pigs were performed as approved by the animal use and care committee protocol of the Universitat de Lleida and the Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi rural (Section of Biodiversity and hunting) under licence DAAM 7700.

Table I. Experimental groups for exosome vaccination safety trial.

Group	Pigs	Antigen	Total ug (1 mL)	Adjuvant (1mL)	Route
Exosomes 1mg+M	3	Exosomes	1000	Montanide	IM
Exosomes 1mg	3	Exosomes	1000	N/A	IM
Exosomes 0.5mg+M	3	Exosomes	500	Montanide	IM
Exosomes 0.5mg	3	Exosomes	500	N/A	IM
Exosomes 0.3mg+M	3	Exosomes	300	Montanide	IM

Immunogenicity pig-targeted trial and challenge.

A heterologous prime-boost vaccination approach (Lu, 2009) was performed using serum derived EV-enriched fractions as a prime antigen (2 doses) and a boost with the viral synthetic peptides identified by LC-MS/MS. First, thirty-three PRRSV negative pigs were divided into groups with food and water *ad libitum* in an experimental farm (Centre d'Estudis Porcins, Torrelameu, Spain) under veterinarian supervision. To test immunogenicity, 14 animals

received one immunization and a boost containing 2mL with 1mg of EV-enriched fractions in different timepoints (Group A: completely naïve animals / Group B: previously received vaccines against circovirus and porcine ileitis). The second boost given to all animals consisted of a 900 ug peptide mix corresponding to 300 ug of each viral peptide. As a control of a classical vaccination approach, two groups (7 pigs each) were vaccinated only with synthetic peptides (0.5mg of each peptide) in combination with two different adjuvants (Group C: Montanide ISA201 and Group D: Montanide ISA206) in two different time-point doses and a boost with 300ug of the same peptide mix with the respective adjuvant and as a negative control, only PBS was injected to 5 animals (Figure 1; Table II).

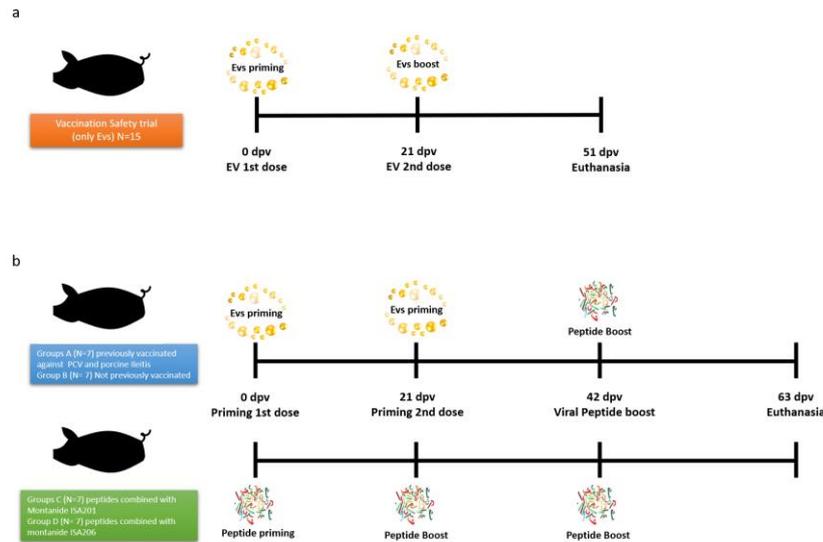


Figure 1. Scheme of safety and immunogenicity targeted-pig trial. Timeline of immunizations was the same for both strategies. (a) For safety trial, animals receive two doses of EVs injected intramuscularly (Day 0 and 21) and euthanised at day 51. (b) For immunogenicity trial, animals received either two doses of EV-enriched fractions or synthetic viral peptides (day 0 and 21) and all then boosted with synthetic viral peptides (day 42). All animals were euthanised at day 63. Serum samples were collected in all timepoints.

Serum samples were taken at 0, 21, 42 and 63 days post initial vaccination (dpv). Whole blood in EDTA tubes was collected at days 0 and 63. Collection of samples, maintenance and euthanasia of pigs were performed as approved by the animal use and care committee protocol of Universitat de Lleida and the Departament d'Agricultura, Ramaderia, Pesca, Alimentació I Medi rural (Section of Biodiversity and hunting) under licence DAAM 7700.

Table II. Experimental groups for immunogenicity trial.

<i>Group</i>	<i>Pigs</i>	<i>Priming antigen</i>	<i>Boost antigen</i>	<i>Adjuvant</i>	<i>Route</i>
<i>PBS</i>	5	PBS	PBS	N/A	IM
<i>Group A</i>	7	Exosomes (1mg) / two doses	Viral peptides (300ug each) / one dose	Montanide ISA 206vg	IM
<i>Group B</i>	7	Exosomes (1mg) / two doses	Viral peptides (300ug each) / one dose	Montanide ISA 206vg	IM
<i>Group C</i>	7	Viral peptides (500ug each / two doses)	Viral peptides (300ug each) / one dose	Montanide ISA 201 vg	IM
<i>Group D</i>	7	Viral peptides (500ug each / two doses)	Viral peptides (300ug each) / one dose	Montanide ISA 206 vg	IM

ELISA tests

All sera samples were also blindly evaluated by a commercial ELISA (IDEXX PRRS X3 Antibody Test, IDEXX) to detect PRRSV antibodies following their own standard operation procedures (Grup de Sanejament Porcí – Lleida, Spain. <http://www.gsp lleida.net/cat>).

Circulating IgG antibodies from vaccinated pigs in the immunogenicity trial were also evaluated by an indirect ELISA test against the synthetic peptides (ENV, N and pp1a). Plates were coated overnight at 4°C with each peptide (5ug/mL diluted in 50mM Carbonate-Bicarbonate buffer, pH 9.6). Sera samples (1/100) were incubated for 1h at room temperature, washed and incubated with secondary antibody Goat anti-Pig IgG (Fc): HRP (AbSerotec AAI41P) at 1/10000 dilution. Optical density was measured at 450nm using Varioskan equipment (Thermo Scientific).

Antibodies were also examined using attenuated virus as coating antigen. Briefly, viral particles were diluted to reach 10^4 particles per 50uL with Carbonate bicarbonate buffer (pH 9.6). Plates were incubated overnight at 4°C to ensure particle viral attachment to the plates. Plates were washed and then blocked with PBS 1X/ 5% non-fat dry milk. After 3 washes, 1/100 dilution of all sera were incubated at room temperature for 1h, then washed 3X and incubated with secondary antibody Goat anti-Pig IgG (Fc): HRP (AbSerotec AAI41P) at 1/10000 dilution. Optical density was measured at 450nm using Varioskan equipment (Thermo Scientific).

IFN- γ ELISPOT

For evaluation of IFN- γ production, ten mL aliquots of whole blood in EDTA tubes were collected. Peripheral mononuclear cells (PBMCs) were isolated by gradient centrifugation using Ficoll Hystopaque 1077 (Sigma-Aldrich) and washed twice with PBS 1X /2% FBS after which cell density and viability were measured by trypan blue staining. Cells were resuspended in complete media “CM” (RPMI 1640, 10% FBS, 1% penicillin/Streptomycin) to have a final concentration of approximately 5×10^6 cells/mL.

For ELISPOT, plates (Millipore, Cat n° MAHAS4510) were coated with 1/100 dilution of capture antibody in 50mM carbonate/bicarbonate coating buffer pH 9.6 (Anti pig IFN- γ antibody clone P2G10 – BD Biosciences, cat n° BD-559961) and incubated overnight at 4°C. Plates were washed twice with CM and blocked with 200uL of CM as blocking buffer for 2 hours at room temperature. Blocking buffer was discarded and all stimuli (synthetic viral peptides) were diluted in CM and added to the plate including a positive control (Lectin from *Phaseolus vulgaris* – Sigma Aldrich) and a negative control (CM alone). 100uL of cell suspension was loaded to the plate and incubated for 48 hours at 37°C and 5% CO₂. Later, cells were washed out with 200uL MilliQ water and plates washed 3 times with 1X PBS/ 0.05% Tween 20. One hundred uL of 1/250 dilution of a biotinylated Mouse anti-pig IFN- γ clone P2C11 was loaded in the plate (BD Biosciences, Cat. n° BD-559958) and incubated 2 hours at room temperature. Detection was carried out using a 1/100 dilution of Streptavidin-HRP conjugate (BD Biosciences cat n° BD-557630) after which plates were washed four times with 1X PBS/ 0.05% Tween 20 and incubated for 20 min with 100uL of non-soluble substrate AEC reagent (BD Biosciences cat n° BD-551951) to reveal spots. Developing of spots was

stopped by washing plates with distilled water 3 times and IFN- γ spots were counted in an AID ELISPOT Reader. Production of IFN- γ was expressed as spot forming colonies per million of PBMCs in each well.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7. The significance level (α) was set at $p < 0.05$. In the ELISPOT, significant differences between groups were determined using two-way analysis of variance followed by Sidak's multiple-comparison test. For the ELISA results, Kruskal-Wallis test for non-paired data was applied, comparing the ranks of each group in a particular timepoint and statistically significant differences were expressed as $p < 0.05$ (*) and $p < 0.01$ (**).

Results

Characterization of serum-derived EV-enriched fractions from non-viremic animals

Size-exclusion chromatography of EV-enriched fractions eluted in fractions 7-10, were analysed by bead-based flow-cytometry to determine the presence of CD63 and CD81, two tetraspanins widely used as exosomal markers. As shown in Figure 2A, high MFI values confirmed their presence associated with EV-enriched fractions. To further validate these results, two other tetraspanins, CD9 and CD5L, were evaluated showing similar results (supplementary figure 2). Of interest, CD5L gave the highest MFI of all tetraspanins tested. As previously reported (Montaner-Tarbes et al., 2016), NTA analysis revealed a mean size EVs distribution of 100-200 nm (Figure 2B) and cryo-TEM confirmed this size distribution (Figure 2C). These data thus indicate the large heterogeneity of plasma-derived EVs. Scaled-up production of serum derived EV-enriched fractions, allowed to accurately and precisely determine protein concentration from individual SEC fractions by BCA. We calculated circa 3mg of protein associated to EVs enriched fractions for every 180mL of serum.

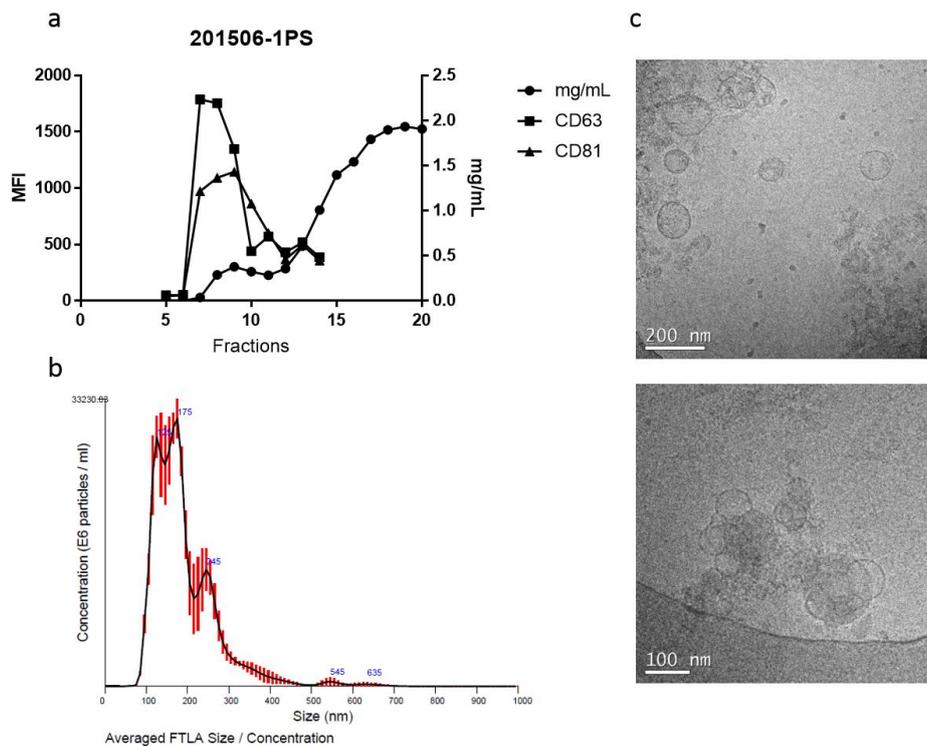


Figure 2. Characterisation of serum-derived enriched EV-fractions from scale-up process. (A) Flow cytometry analysis of CD63 and CD81. MFI, Median Fluorescence Intensity. Protein concentration by the Pierce bicinchoninic acid assay (BCA assay) is shown mg/mL. (B) NTA profiles of EV-enriched fractions from size exclusion chromatography (SEC). Concentration is shown in particle/ μ L. (C) Electron microscopy. (C) Representative TEM images. Scales in nanometers (nm).

Proteomic analysis

Individual proteomic analysis of serum-derived EVs enriched fractions from five animals used in these vaccination trials revealed peptides corresponding to viral proteins, pp1a, Envelope glycoprotein and Nucleocapsid proteins (Figure 3 and supplementary table T1). Of note, other viral proteins were detected in the preparations but were discarded due to our filtering criteria (FDR<1% and at least two peptides from individual proteins).

After viral peptide identification, we used two different algorithms to predict their immunogenicity (Jespersen et al., 2017; Jurtz et al., 2017; Kolaskar and Tongaonkar, 1990; Larsen et al., 2006). Interestingly, we found immunogenic regions, possible B-cell epitopes and decamers that fit as strong binders in several SLA pockets for those proteins matching with peptides detected in EVs enriched fractions. Figure 3 shows a schematic representation of the identification pipeline.

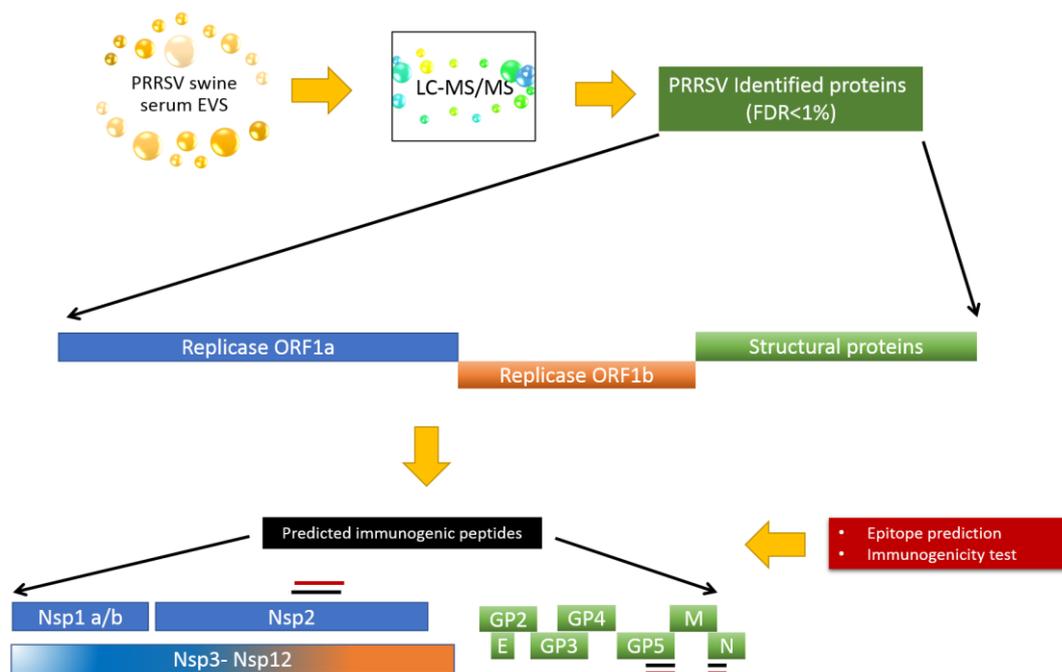


Figure 3. Protein identification pipeline from PRRSV convalescent sera EV-enriched fractions. Liquid chromatography (nanoLCULTRA-EKSIGENT) followed by mass spectrometry (LC-MS/MS) of EV-enriched fractions identified viral peptides with FDR <1% and at least two peptides from the same protein. Two different algorithms were used to examine matching regions between our identified peptides (LC-MS/MS) and possible B-cell epitopes or antigenic regions within the protein (Kolaskar and Tongaonkar, 1990; Larsen et al., 2006)

Safety

Three different doses of EV-enriched fractions derived from PRRSV convalescent swine sera were formulated in montanide and tested in nine animals and two different doses without adjuvant were injected in six animals to assess the risk of viral presence and adverse effects of

these preparations (Table I). As a first safety control, batches were tested prior to vaccinations by a sensitive RT-PCR. None of the batches obtained individually from 180mL of sera revealed the presence of viral particles. Moreover, none of the animals showed clinical signs associated to PRRSV, nor secondary effects due to vaccine/adjuvant preparation even after two doses of 1mg of EV-enriched fractions. Importantly, EV-enriched fractions containing viral proteins did not induce a positive result to the gold standard ELISA test (IDEXX X3 PRRSV) suggesting that this vaccination approach could allow to differentiate vaccinated from infected animals (DIVA).

Immunogenicity

To test the immunogenicity of serum derived EV-enriched fractions, fourteen animals were immunized twice with 1mg of EV-enriched fractions formulated in montanide and boosted a third time with viral peptides (identified by LC-MS/MS). To compare the immunological response, two groups using a classical vaccination approach with peptides and adjuvants were used (Figure 1). As expected from the safety data, neither clinical signs related to PRRSV infections, nor secondary effects were observed in any of these fourteen animals. Moreover, similar to the safety trial, all animals remained negative for the gold standard ELISA test for PRRSV virus, indicating that epitopes present in EVs enriched fractions and in our *in vitro* synthetic viral peptides are capable of differentiating animals infected with the virus (positive results in the ELISA test) and those vaccinated with our novel strategy.

To assess humoral immune response, blood was taken from all animals at days 0, 21, 42 and 63 post-vaccination. IgG levels against the different synthetic peptides used in our immunisation approach were determined by ELISA. As expected, all animals were negative for antibodies against these viral peptides at day 0 (Figure 4). At 21dpv, only groups C and D showed a response to the ENV peptide. At day 63dpv, higher antibody immune responses were detected in animals vaccinated and boosted with peptides as opposed to EVs-EVs-peptide ones (heterologous prime-boost) even though Groups A and B started to show a response against ENV and N but lower than Groups C and D.

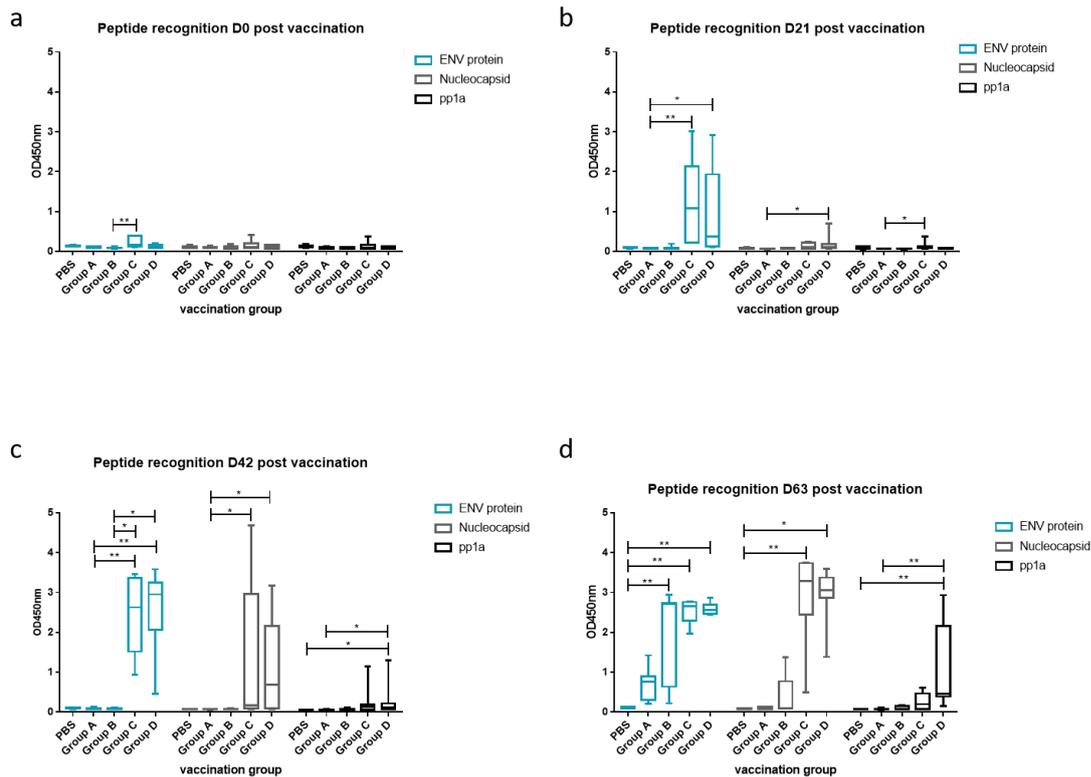


Figure 4. Evaluation of antibody immune response against peptides from D0 to D63 in swine sera. PRRS viral peptides were used in ELISA tests. Graphs refer to immune recognition of viral peptides after vaccination at different immunization days post vaccination. (A) Day zero, (B) Day 21, (C) Day 42, (D) Day 63. Vaccination with PBS was used as a control during whole experiment. (*) $p < 0.05$, (**) $p < 0.01$. OD, optical density

Moreover, based on these results, the ENV peptide seemed the most immunogenic from the ones tested, followed by the N peptide of peptide and the pp1a peptide (Figure 4). We took advantage of the immune sera from animals that had seroconverted against the ENV and Nucleocapsid proteins to demonstrate their association with EVs used in these trials. Thus, western blot analysis of EVs used in vaccinations were performed using pre-immune and immune sera from two animals primed with EVs and boosted with peptides. These animals seroconverted after the last boost to the nucleocapsid and ENV proteins, albeit variably. Noticeably, only immune sera specifically recognized these proteins (Supplementary Fig S3). These results thus demonstrate that viral sequences from these peptides were associated with EVs used in vaccinations. Experiments to identify residues within the 35 aa synthetic peptides representing these proteins in the context of SLA can be considered as part of a next series of targeted-pig trials including challenge.

To assess cellular immunity, numbers of cells producing IFN- γ were measured by ELISPOT. Excepting for one animal in group D, remaining animals in groups C and D did not produce IFN- γ . Noticeably, there was a significant increase in number of IFN- γ producing cells with values from 60 to 350 SFCx10⁶ PBMCs on groups A and B vaccinated with EV-enriched fractions obtained from convalescence animals (Figure 5).

IFN- γ ELISPOT secreting cells on immunogenicity trial

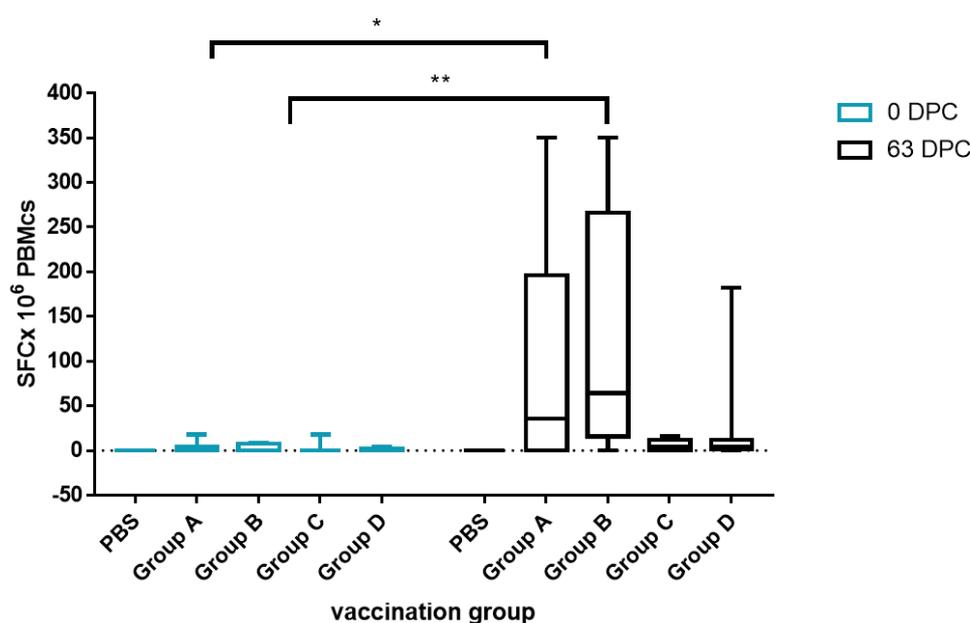


Figure 5. IFN- γ production after stimulation of swine PBMCs with viral peptides (mix) at days 0 and 63 post vaccination. IFN- γ producing cells were measured by ELISPOT at day zero and 63DPV. Results were compared (0dpv vs 63dpv response) using two-way ANOVA multiple comparison test. Statistically significant differences were observed only in exosome vaccinated groups between pre-vaccinated sera and 63dpv sera. (*) $p < 0.05$, (**) $p < 0.01$.

Discussion

Here, immunization of pigs with up-to two milligrams of EV-enriched fractions from sera of PRRSV convalescence animals demonstrated that they were free of virus and did not cause adverse effects. In addition, immunization of pigs with EV-enriched fractions followed by boosts with predicted immunogenic synthetic peptides from the ENV, N and pp1a proteins elicited specific humoral IgG immune responses not detected by a widely used PRRSV-diagnostics commercial test; thus, suggesting that this vaccination approach is able of differentiating infected from vaccinated animals. Lastly, such prime-boost approach elicited high and specific IFN- γ responses in comparison with immunizations using peptides alone.

Previous studies identified viral peptides corresponding to the Nucleocapsid, and pp1a proteins in serum-derived EV-enriched fractions from pigs that had overcome PRRSV infections (Montaner-Tarbes et al., 2016). Here, we confirm such results as we identified viral peptides from these same two proteins, in addition to peptides from envelope GP5 protein, in individual mass spectrometry studies of five different convalescence animals. Our data strongly indicate that there is selective viral antigen-cargo associated with EV-enriched fractions from convalescence animals. The Nucleocapsid protein is an important structural protein which after cytoplasmic synthesis migrates to the nucleus suppressing expression of type I interferons (Wongyanin et al., 2012; Wulan et al., 2015). Moreover, interactions between this structural protein and antigen presenting cells in the presence of viral induced IL-10 can lead to and alteration of APC functions (Wongyanin et al., 2012). pp1a encodes 10 non-structural proteins, NSPs, and expression of PRRSV nsp1 α/β and nsp2 were found to exert strong inhibitory effects on IFN- β promoter activation (Fang and Snijder, 2010). In addition, it has been recently discovered that the pp1a region of highly pathogenic PRRSV strains plays an important role in

inducing neutralizing antibodies in piglets (Leng et al., 2017). The GP5 protein contains epitopes involved in antibody dependent virus neutralization, protection, cell recognition and binding (Music and Gagnon, 2010). However, it remains to be studied which EV component(s) are responsible for the immunogenicity presented in this work. EVs include sets of immune related proteins such as MHC that could contribute to elicit a better immune response (Anand, 2010). Recently, we have analyzed the peptides present in EV by NetMHCspan and our preliminary results identified peptide sequences with the theoretical ability to bind to certain MHC (SLA in the case of swine). Moreover, these peptides were predicted as strong binders for different SLA alleles (data not shown). If these predictions are confirmed, such antigenic peptides in the EV cargo could participate in developing specific immune responses. Further experiment will determine whether antigenic peptides play a role in immune responses elicited by EVs in pigs.

Our data thus strongly suggest that novel viral antigenic peptides can be discovered in serum EV-enriched fractions from animals that were free of virus in peripheral circulation. As it is known that PRRSV infection can last for months in internal tissues (Allende et al., 2000; Pileri and Mateu, 2016; Wills et al., 2003), it is tempting to speculate that such cryptic infections in lymphoid tissues might release into circulation EV-enriched fractions with selected cargo eliciting immune responses capable of maintaining animals with undetected viremias in peripheral circulation and with no clinical symptoms.

To scale-up the production of EV-enriched fractions for the safety and immunogenicity trials, serum samples were concentrated 100-times through ultracentrifugation and size exclusion chromatography (see Methods). To demonstrate that these preparations were free of virus, a sensitive qRT-PCR capable of detecting 100 particles/mL (Applied Biosystems) was firstly performed showing that they did not contain viral RNA. Next, we injected increasing doses of EV-enriched fractions into nine different animals in safety trial and fourteen animals in the immunogenicity trial (Figure 1). Results demonstrated that injections of up to two milligrams of serum-derived EV-enriched fractions from these animals did not cause any clinical symptoms and were free of virus (Table I and Table II). Of interest, a recent publication suggested that exosomes from PRRSV-infected cells and free of virus act as vehicles for intercellular transmission (Wang et al., 2017). Whether these seemingly discrepant results are due to *in vitro* vs *in vivo* studies, remains to be determined.

Current vaccine approaches against PRRSV have met little success in developing broad neutralizing antibodies and no correlate of protection is presently available with antibody levels (Lunney et al., 2016). Moreover, available tests for measuring neutralizing antibodies are difficult to interpret as the PRRSV isolate used for testing are not field strains but cell-cultured adapted (Christopher-Hennings et al., 2002). Our data demonstrated that our prime-boost approach elicited specific IgG antibodies. However, even though all animals seroconverted at the end of the study, titers and recognition against individual peptides varied significantly among groups (Figure 3). Whether these antibodies contain high quality neutralizing antibodies remains to be determined. Serum-derived EV-enriched fractions represent a complex mixture of vesicles release from widely different type of cells (Raposo and Stoorvogel, 2013). Therefore, it is reasonable to speculate that serum EVs containing viral peptides represent a very small percentage of circulating EVs, partly explaining the low seroconversion rate in EV-vaccinated groups when compared with peptide vaccination added to individual variability observed in this trial.

Highly pathogenic PRRSV strains are emerging all over the world with cases mainly in Asia, America and some in Europe through recombination of wild type viruses with vaccine strains

^{30,31} making a top priority to find a test that allow identification of vaccinated animals from those that overcome the disease (DIVA). As several current vaccination approaches are using modified live viruses, deletion mutants and chimeric viruses are being tested with the idea of solving the above mentioned problem (Vu et al., 2017) Noticeably, based on a gold standard commercial method (IDEXX PRRSV x3 Ab test), sera from all animals that seroconverted were diagnosed free of virus suggesting that this vaccination approach can differentiate infected from vaccinated animals.

Priming with EV-enriched fractions and boosting with viral peptides induced a high and specific cellular IFN- γ immune response above 300 SFCx10⁶ PBMCs in EVs vaccinated groups, that was not present in the peptide-vaccinated group. The fact that viral peptides alone were not able to stimulate immune cells *in vivo* and EV-peptide vaccination approach could, made this a new and exciting opportunity to evaluate the role of EV during and post natural infection, as well as their role in viral clearance and immunity. Our result was remarkable when considering that in natural PRRSV infections, T cell immune responses are usually weak and delayed (Mateu and Diaz, 2008), as well as variable appearing 4-6 weeks after infection (Mulupuri et al., 2008). Moreover, infected pigs show an important decrease in CD8+ T-cells and IFN- γ production and a reduction of 50 to 80% of NK cell cytotoxicity from day 7 to 24 post infection maybe due to increased IL-4 levels (Dwivedi et al., 2012b).

Interestingly, exosomes are vehicles of proteins and nucleic acids acting in intercellular communication and antigen presentation (Chaput and Théry, 2011; Mathivanan et al., 2010; Robbins and Morelli, 2014; Simons and Raposo, 2009). Thus, serum-derived EVs from convalescence sera, bearing viral proteins and other immune-related proteins could be delivered to sites where memory cells are present in higher frequencies and activate effector memory T cells. In the absence of supporting data, this hypothesis remains to be proved. Such mechanism, however, has been recently shown to be elicited by reticulocyte-derived exosomes from infections in an experimental rodent malaria model (Martín-Jaular et al., 2016).

Because of their ability to modulate the immune response, exosomes are presently being explored as novel therapeutic agents against infectious diseases. Pioneering studies demonstrated that macrophages infected with *Mycobacterium bovis* secreted exosomes inducing bacterial-specific pro-inflammatory activity (Bhatnagar et al., 2007). Similar results were obtained with exosomes from macrophages infected with *Mycobacterium tuberculosis* and this response was also evident in other infectious diseases caused by intracellular pathogens, *Salmonella typhimurium*, *Toxoplasma gondii*, *Plasmodium yoelii* as well as in other parasitic diseases from worms and protozoa (Bhatnagar et al., 2007; Martín-Jaular et al., 2011). Remarkably, using serum-derived CD80+ and CD80- enriched exosomes from *Eimeria* infected chicken induced increased numbers of intestinal and spleen cells secreting Th1 (IL-2, IL-6, IFN- γ) and Th2 (IL-4) cytokines, compared with unimmunized controls. In addition, in the case of *Eimeria*, CD80+ EVs induced a stronger immune response with increased numbers of IFN- γ and IL-2 producing cells (in gut and spleen as well) and greater protective immunity following *E. tenella* challenge, as measured by weight gain, feed efficiency, parasite shedding, and intestinal lesions (del Cacho et al., 2016). Altogether, the data from several studies strongly support the use of EVs and exosomes as a novel vaccine approach against veterinary diseases of economic importance.

Competing interests

All the authors declare that they have no competing interests.

Authors' contributions

SMT performed the experiments. EN and VT performed all the analyses to discard virus in samples (Commercial ELISA and qRT-PCR). SMT, MM, FEB, LF and HAP designed the research study and analysed data. SMT, LF and HAP drafted the manuscript. All authors read and approved the final manuscript. LF and HAP coordinated the study.

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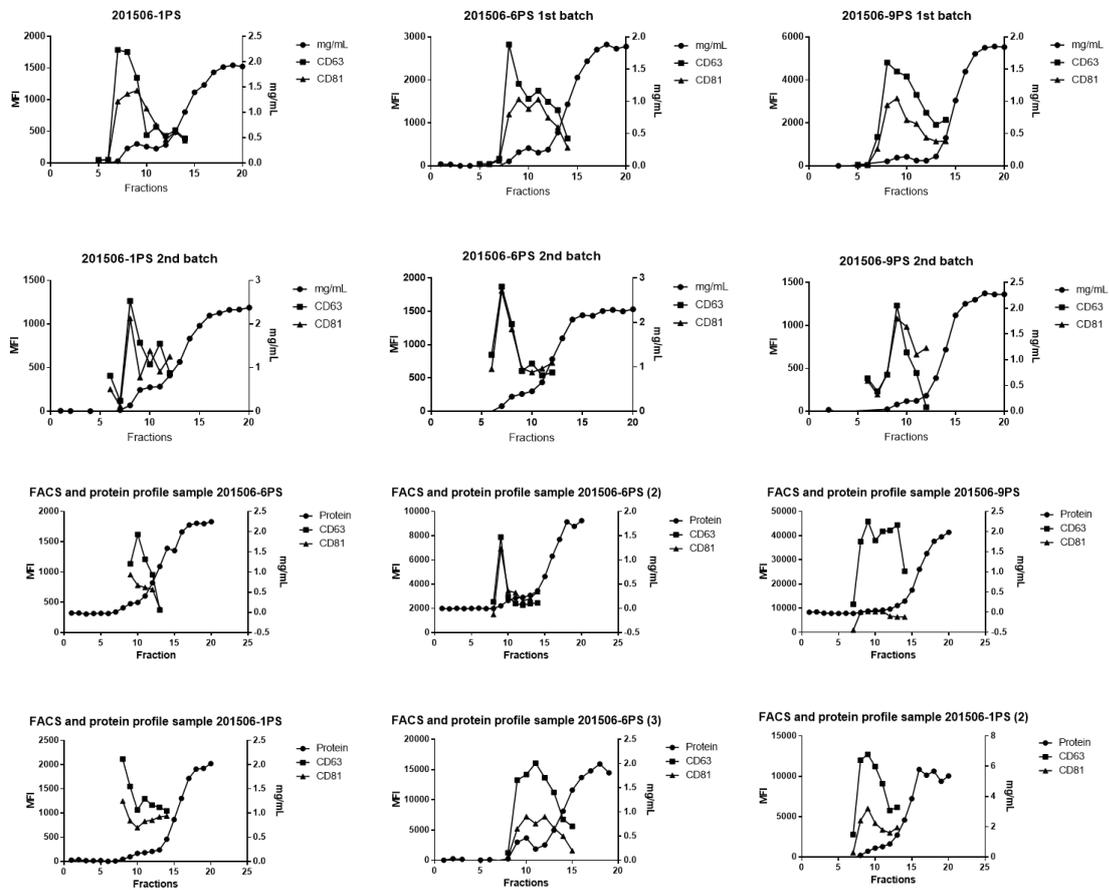
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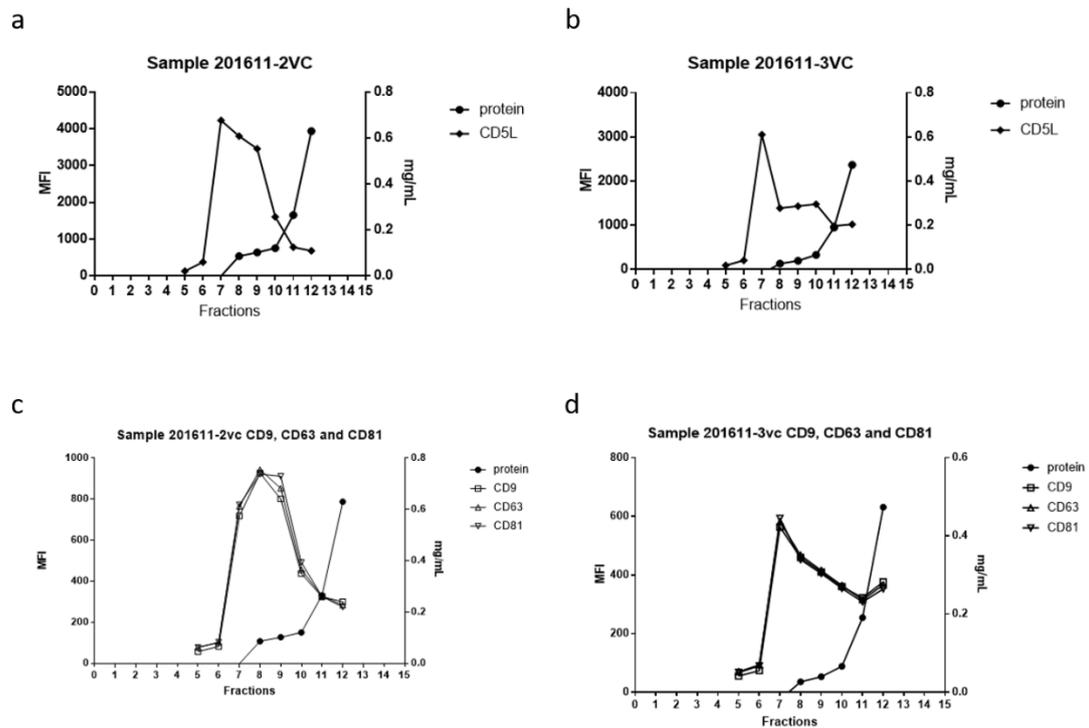
Supplementary material.

• Supplementary figure S1.



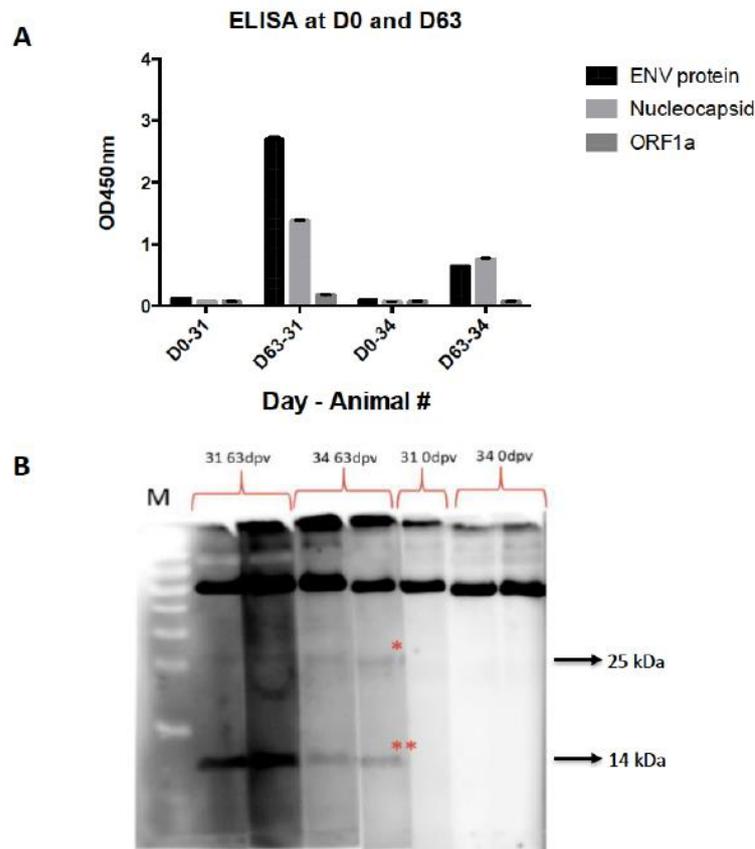
Supplementary figure S1. Individual FACS and protein elution profile of all prepared batches of isolated exosomes from convalescent swine sera for PRRSV.

- **Supplementary figure S2.**



Supplementary figure S2. FACS and protein elution profile for evaluation of tetraspanins from serum EVs enriched fractions (CD5L, CD9, CD63 and CD81). **(a, c)** Serum sample 201506-1PS **(b, d)** Serum sample 201506-6PS.

- Supplementary figure S3



Supplementary figure S3. Western blot analyses of EVs used for vaccination. (A) **ELISA.** PRRS viral peptides (GP5, Nucleocapsid and ORF1a) were used in ELISA tests. Graphs refer to immune recognition from individual pigs primed with EVs and boosted with peptides at day 63 post-vaccination. OD, optical density. (B) **WESTERN BLOT.** Twenty micrograms of EVs from the same batches used in vaccinations were resolved on 12% SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose membrane was cut and individual strips were incubated for 1h with preimmune and immune sera at 1:100 dilution. Secondary antibodies were used at 1:5000 and 1:10000 dilutions. All strips were evaluated together during developing process at the same time and exposure. Signals were detected on a chemiluminescence BOX Syngene device using standard measurement of exposure and 1 photo per minute are shown below since minute one. GP5 (*) and Nucleocapsid protein (**). Molecular weight in kiloDaltons (kDa).

CHAPTER 3.3. Challenge study of serum derived extracellular vesicles from PRRSV convalescent animals. (confidential data / not published)

Challenge trial in a targeted-pig trial using serum derived EVs enriched fractions from PRRSV convalescent animals. CONFIDENTIAL by “sponsor’s request”.

Different targeted pig trials have been described in the previous chapter. In those experiments, it was clearly demonstrated that serum derived extracellular vesicles (from PRRSV convalescent swine) were safe (virus free) and immunogenic. In this chapter, the degree of protection was determined using a homologous challenge trial, where PRRSV challenge strain was the same with which pigs were infected and their extracellular vesicles obtained after recovery from infection.

Materials and methods

Immunogenicity pig-targeted trial and challenge.

First, thirty-five PRRSV negative pigs were divided into six groups (control, four vaccinated groups and sentinels) in an experimental farm (Centre d’Estudis Porcins, Torrelameu, Lleida, Spain) under veterinarian supervision. Food and water were provided *ad libitum*. Details of the experimental groups for immunogenicity trial are provide in Table II. Briefly, negative control pigs (5 animals) receive PBS as priming and boost antigen for immunization every 21 days. On the other hand, a prime-boost vaccination approach (Lu, 2009) was performed using serum derived EV-enriched fractions as a prime antigen (2 doses) and a boost with the viral synthetic peptides identified by LC-MS/MS as detailed in previous chapters. Thus, 14 animals received two immunizations with EVs (2 mL with 1 mg of EV-enriched fractions) every 21 days and the final boost consisted of a 900 ug peptide mix corresponding to 300 ug of each viral peptide 21 days later. It was necessary to split these 14 pigs in two groups because the sow farm applied routinely vaccines against circovirus porcine type 2 (PCV2) and porcine ileitis at weaning in piglets. Thus, 7 pigs were completely naïve animals that did not receive any vaccine before the experimental ones (group A) whereas other 7 pigs had received vaccines against PCV2 and porcine ileitis at weaning (group B). As a control of a classical vaccination approach, two groups (7 pigs each) were vaccinated only with synthetic peptides (0.5 mg of each peptide) in combination with two different adjuvants (Group C: Montanide ISA201 and Group D: Montanide ISA206) in two different time-point doses and a boost with 300 ug of the same peptide mix with the respective adjuvant (Table I). Moreover, 2 pigs were maintained as sentinels and they do receive neither vaccine nor virus challenge.

Sixty-three days after beginning the immunization process, animals were challenged with a wild type PRRSV virus (Sat la Vall strain, Lleida, Spain) using 2×10^6 viral particles / animal by intramuscular route. Serum samples were drawn at 7, 21, and 28 days post challenge (dpc). Sample collection, maintenance and euthanasia of pigs were performed at 28 dpc as approved by the animal use and care committee protocol of the Universitat de Lleida and the Departament d’Agricultura, Ramaderia, Pesca, Alimentació I Medi rural (Section of Biodiversity and hunting) under licence DAAM 7700.

Table I. Experimental groups for immunogenicity and challenge trial.

<i>Group</i>	<i>Pigs</i>	<i>Priming antigen</i>	<i>Boost antigen</i>	<i>Adjuvant</i>	<i>Route</i>	
<i>PBS</i>	5	PBS	PBS	N/A	IM	
<i>Group A</i>	7	Exosomes (1mg) / two doses	Viral peptides (300ug each) / one dose	Montanide 206vg	ISA	IM
<i>Group B</i>	7	Exosomes (1mg) / two doses	Viral peptides (300ug each) / one dose	Montanide 206vg	ISA	IM
<i>Group C</i>	7	Viral peptides (500ug each / two doses)	Viral peptides (300ug each) / one dose	Montanide vg	ISA 201	IM
<i>Group D</i>	7	Viral peptides (500ug each / two doses)	Viral peptides (300ug each) / one dose	Montanide vg	ISA 206	IM

Diagnostic tests (PRRSV ELISA and RT-PCR)

All sera samples were blindly evaluated by a commercial RT-PCR (TaqMan PRRSV Reagents and Controls) and commercial ELISA test (IDEXX PRRS X3) to detect PRRSV viral load and antibodies, respectively following the standard operation procedures of the Grup de Sanjament Porcí, Lleida, Spain. (<http://www.gsplleida.net/cat>). For a better understanding of the PRRSV viraemia dynamics in the present study, Area under the curve (AUC) was calculated using the time of sampling and its correspondent PRRSV viral load quantified using the trapezoidal method from 0 to 28 dpc, as previously described (Lopez Soria et al, 2014). Thus, AUC can be used as an indicator of the viral exposure over time. Also, tonsils were evaluated by RT-PCR at 28 dpc to test the presence of the virus in this secondary lymphoid tissue.es.

Circulating IgG antibodies from vaccinated pigs were also evaluated by an indirect ELISA test against the synthetic peptides (ENV, N and pp1a) as previously described in the former chapter. Briefly, plates were coated overnight at 4°C with each peptide (5 ug/mL diluted in 50mM Carbonate-Bicarbonate buffer, pH 9.6). Sera samples (1/100) were incubated for 1h at room temperature, washed and incubated with secondary antibody Goat anti-Pig IgG (Fc): HRP (AbSerotec AAI41P) at 1/10000 dilution. Optical density was measured at 450nm using Varioskan equipment (Thermo Scientific).

Temperature and body condition.

Fever has been analyzed as the main clinical end-point. This parameter was defined as an ordinal variable: 0 (temperature <39.5°C), 1 (39.5-40.5°C) and 2 (>40.5°C). Body condition was also defined as an ordinal variable (from 1 to 4) where the highest value (4) corresponded to the best body condition whereas the lowest values (1) were associated to the worst condition from the clinical point of view.

Histopathology examination after challenge.

Macroscopic and microscopic examination was blindly performed by Dr Gustavo A. Ramírez, (DVM; PhD, DiplECVP) from the Service of Research and Veterinary anatomopathology diagnosis in ETSEA - Campus Agroalimentari, Forestal i Veterinari lof the Universitat of Lleida using his own standard operational procedure. Samples from lungs, spleen and tonsils were collected at 28dpc and an anatomopatological analysis was carried out focused on the detection of lesions compatible with viral or bacterial infections. Samples were fixed in 10% buffered paraformaldehyde, cut in a Vogel VO-5-8100 station and processed by dehydration, and infiltration in a tissue processor Myr STP120. Later, tissue blocks were done by paraffin inclusion in a Myr EC 500 and preparations were cut (5um thick) in a rotation microtome (Thermo-Fisher) model HM325. Hematoxyline-eosine staining was used to characterize cell types and structures.

Statistical analyses.

All statistical analyses were carried out using SAS V.9.1.3 (SAS institute Inc, Cary, NC, USA). For all analyses, the individual pig was used as the study unit. The significance level (p) was set at 0.05. The variables included in the statistical analyses were classified as nominal (experimental group), ordinal (fever and body condition) or continuous (antibody titer, viral load and AUC). Shapiro Wilk's and Levene tests were used to evaluate the normality of the distribution of the continuous variables and the homogeneity of variances, respectively. Contingency tables (Chi-square or Fisher exact tests) were used when the association between nominal and ordinal variables was assessed. To study the association between nominal variables with the continuous non-normally distributed variables, the kruskal-wallis test (with the Dunn's multiple comparison test to compare all pair of values) was used. To analyse the association between continuous normally distributed variables and nominal or ordinal variables, an ANOVA test (with Student's t-test to compare each pair of values) was used. Spearman correlation test was used to evaluate the effect of antibody values pre-challenge and AUC post-challenge. Finally, a logistic regression analysis was also carried out to decipher the effect of the experimental groups in body temperature considering the pig as a random factor.

Results.

Clinical parameters and clinical score.

It was not observed any general clinical outbreak after challenge pigs with the wild PRRSV strain. Thus, the outcome of the challenge could be described as subclinical in general terms.

Temperature and body condition.

As mentioned before, it was analyzed temperature and body condition using an ordinal variable. Figure 1 depicted the timeline mean temperature of each group until 23dpc. All groups displayed a moderate increase in body temperature after inoculation with the PRRSV wild type strain. The increase of the temperature in some animals, belonging to groups A and B, was moderate without reaching values higher than 40.5°C throughout the study. Interestingly, some pigs vaccinated with peptides or PBS (control group) showed a marked increase in temperature from 3 to 10 dpc reaching in some animals, values higher than 40.5°C (green bars in Figure 1). It was not observed statistically significant differences between groups comparing these values each day but, if the temperature is analyzed using a logistic regression model, it was observed that body temperature was affected by the experimental group in a statistically significant manner ($p < 0,0001$).

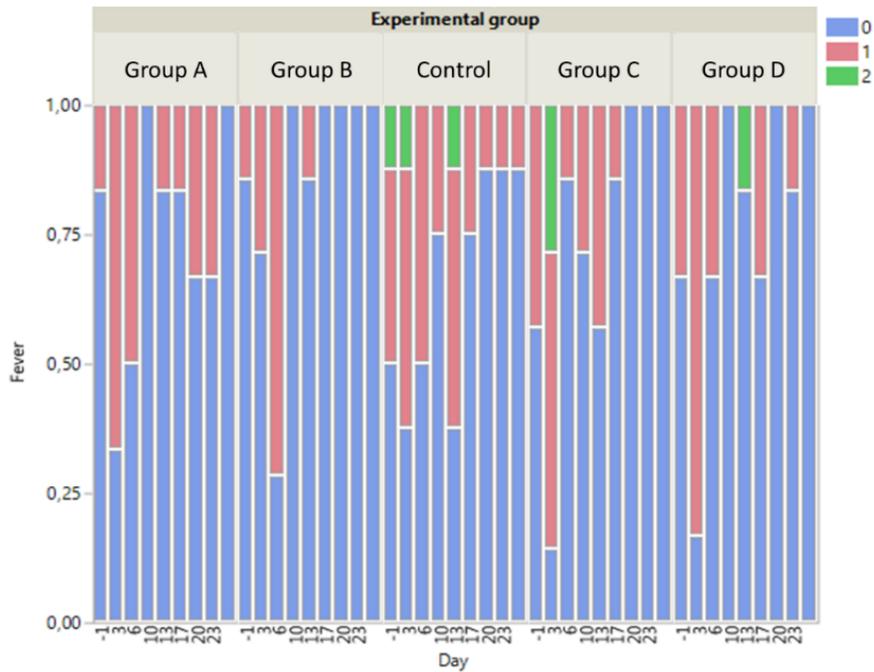


Figure 1. Temperature measure timeline from one day before the challenge to 23 dpc. Each bar represents the mean temperature for each group per day and was defined as an ordinal variable divided as 0 (temperature $<39.5^{\circ}\text{C}$), 1 ($39.5-40.5^{\circ}\text{C}$) and 2 ($>40.5^{\circ}\text{C}$).

The body condition however did not show any statistically significant differences between vaccinated groups. However, animals in the control group did show a worse body condition than vaccinated animals as showed in Figure 2 but without detecting statistically significant differences.

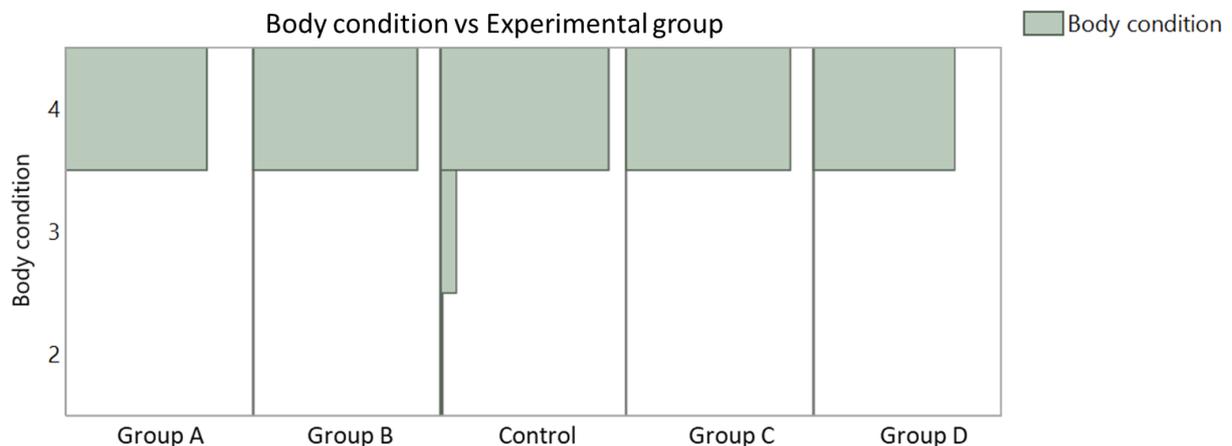


Figure 2. Body condition of vaccinated groups and controls 28dpc. All vaccinated groups showed values of 4 indicating a good body condition. Control (PBS) group showed some values of 3 indicating worse body condition than all other groups.

Antibody immune response after challenge.

First, animals were evaluated using the commercial gold standard technique (IDEXX X3 PRRSV ELISA) to detect antibody levels against European strains of PRRSV from 63 days post vaccination (day of the challenge) until 28 days post challenge, each line representing an animal timeline during the whole trial (Figure 3). As expected, all animals were negative for

antibodies at the day of the challenge (63dpv) and started to show a sample/positive ratio with positive outcome after 14dpc (S/P positive > 0.4) with values increasing gradually until 28dpc in all the experimental groups. Nevertheless, some animals showed a decrease in antibody titer after 21dpc independently of the experimental groups. There were no statistically significant differences in any timepoint in all examined groups using this diagnostic procedure.

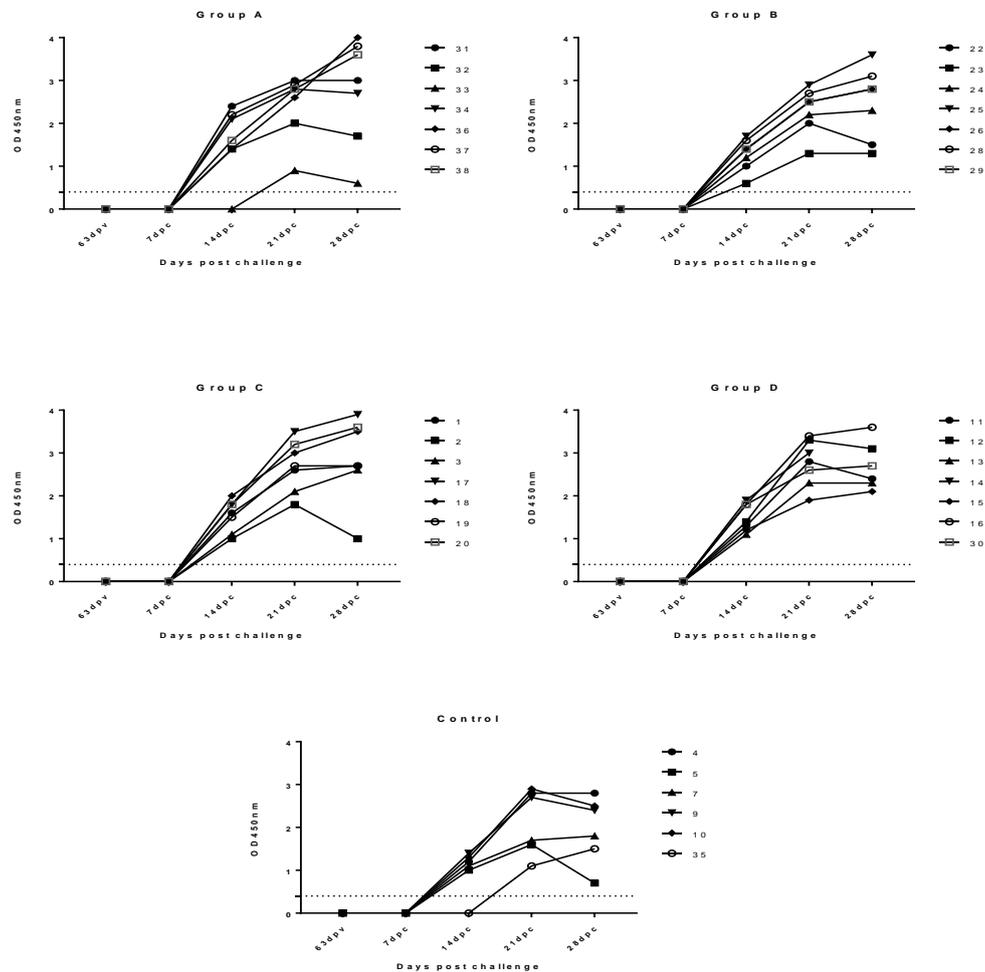


Figure 3.- ELISA test IDEXX X3 PRRSV for antibody detection of herd exposure to the virus. (S/P > 0.4 is considered as positive). Each line represents an animal during the challenge trial in different sampling points.

To assess specific antibody immune response against the viral peptides used for vaccination, IgG levels were determined by ELISA for each one of the three peptides. When response against envelope protein was evaluated (Figure 4), only the groups C and D showed statistically significant differences when compared with the control group during the whole challenge trial ($p < 0.05$). In the groups vaccinated with EVs, there was no statistically significant differences maintained during the challenge with other groups. Nevertheless, Group A showed higher antibody titers ($p < 0.01$) when compared with the control group at challenge day (63dpv).

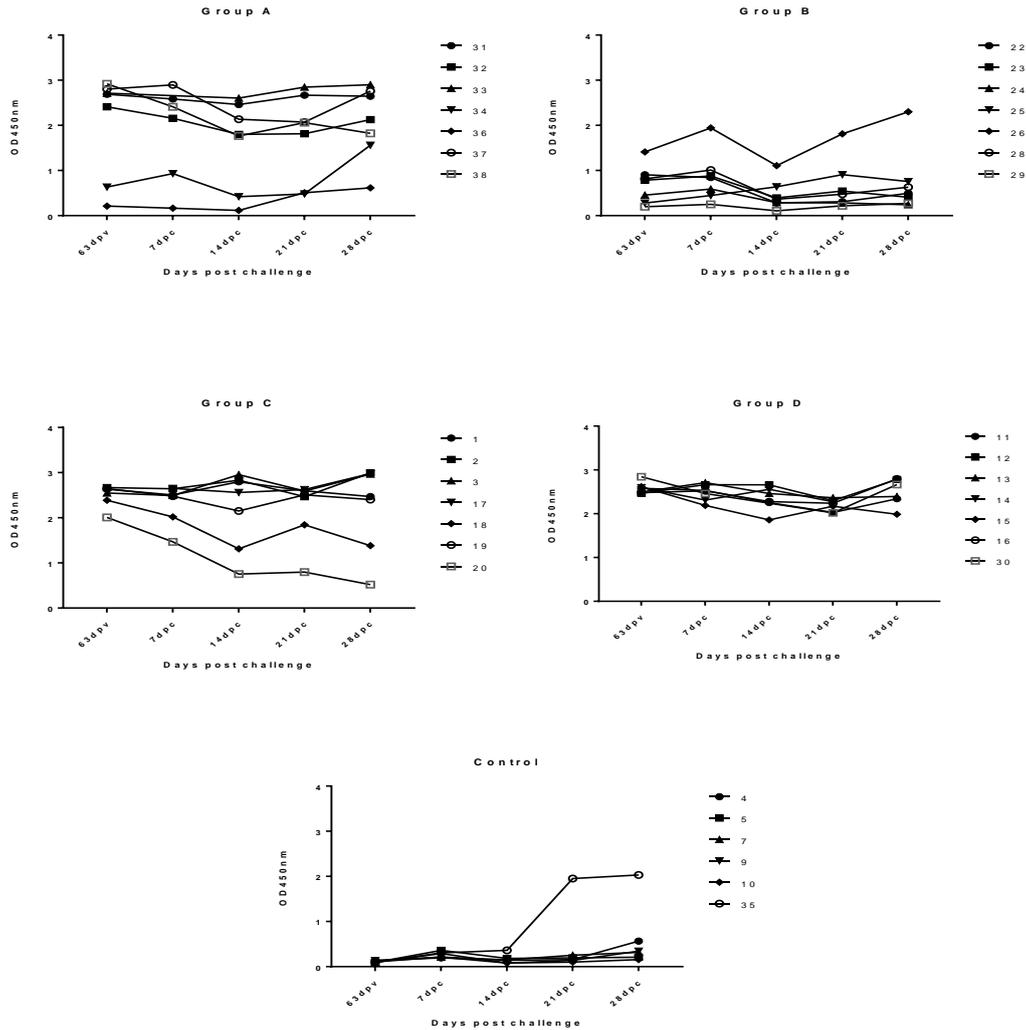


Figure 4. Evaluation of antibody immune response against envelope viral peptide from 0dpc (63 days post initial vaccination) to 28dpc in swine sera. Graphs refer to immune recognition of viral peptides and each line represents an individual animal during challenge at different sampling days.

For the nucleocapsid peptide (Figure 5), the antibody profiles behaved similarly between groups where the same vaccination strategy was used (either EVs or peptides). Thus, groups vaccinated with EVs (Group A and B) showed responses around zero, except for two animals in group A (one with increasing values of OD from 1.5 to 2 and other with starting values around 0.8 and decreasing in time) and one animal in the group B with increasing OD values from 14dpc until the end of the study. No animal in the control group showed positive OD values during the challenge study. In the groups vaccinated with peptides, all animals showed OD values that were decreasing from the day of the challenge until 28dpc. Although differences were observed graphically, only Groups C and D maintain statistically significant differences with the control group during the whole challenge trial. In addition, Group B showed differences ($p < 0.05$) in particular timepoints when compared with Groups C and D (at 0, 7 and 21 dpc).

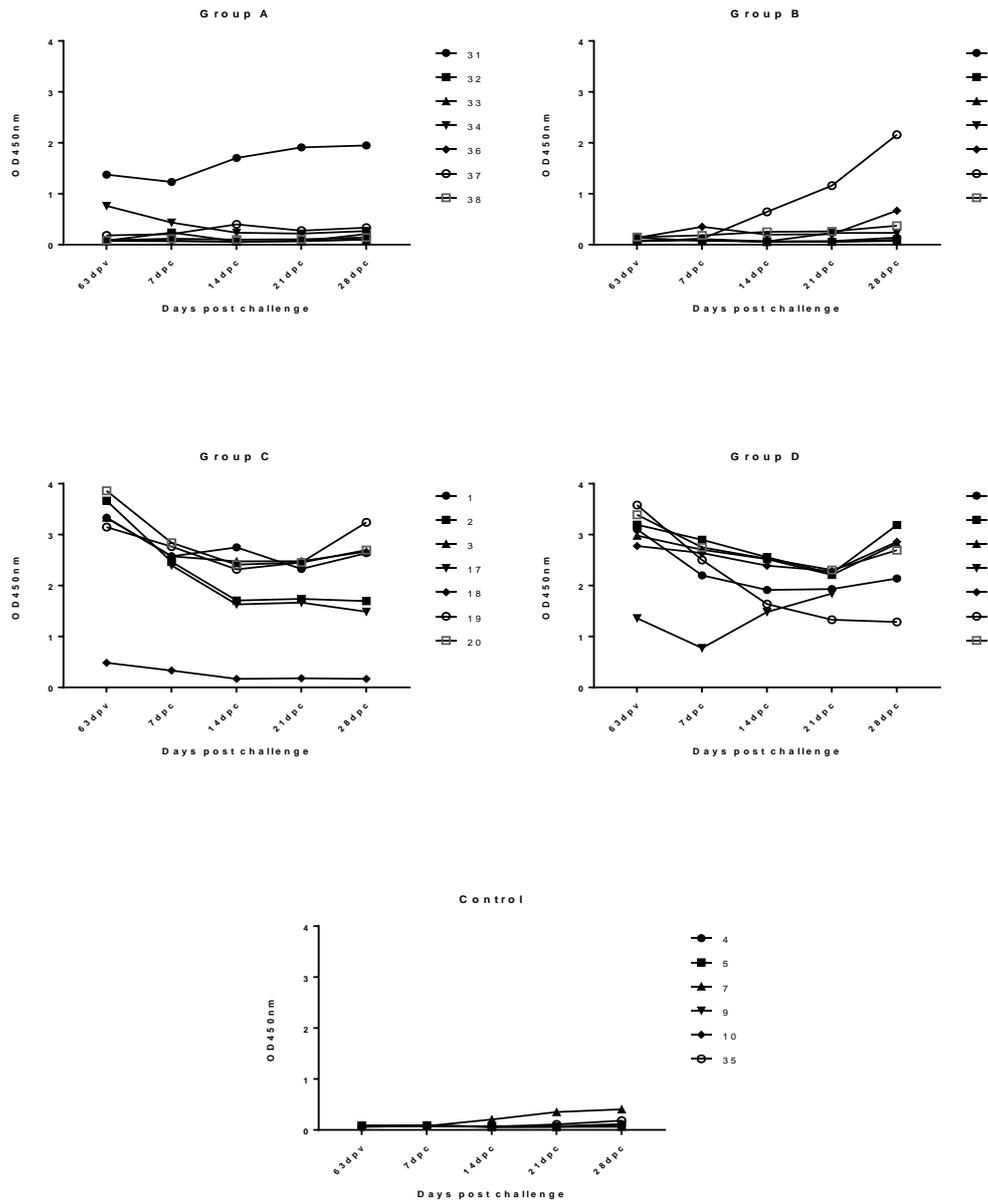


Figure 5. Evaluation of antibody immune response against nucleocapsid viral peptide from 0dpc (63 days post initial vaccination) to 28dpc in swine sera. Graphs refer to immune recognition of viral peptides and each line represents an animal during challenge at different sampling days.

When sera were evaluated against the ORF1a peptide (Figure 6), there were different results for each experimental group, although some common responses were observed. Thus, group A showed a small increase in OD in all animals except one, which after 14dpc, its OD value increased until 2 rapidly and stabilized until 28dpc. Group B showed a slight increase in OD in four animals reaching a maximum OD between 1 and 1.5. In Group C, most animals were around OD values of 1-1.5 similarly to group B, however, statistically significant differences were observed only when compared those values at 21-28dpc with the control group ($p < 0.05$) in which values were negative during the whole trial. Group D represents the most different in terms of antibody response to the ORF1a peptide and the only one with statistically significant

differences when compared with controls (in all timepoints) and other vaccinated groups like group B at 0dpc and 7dpc.

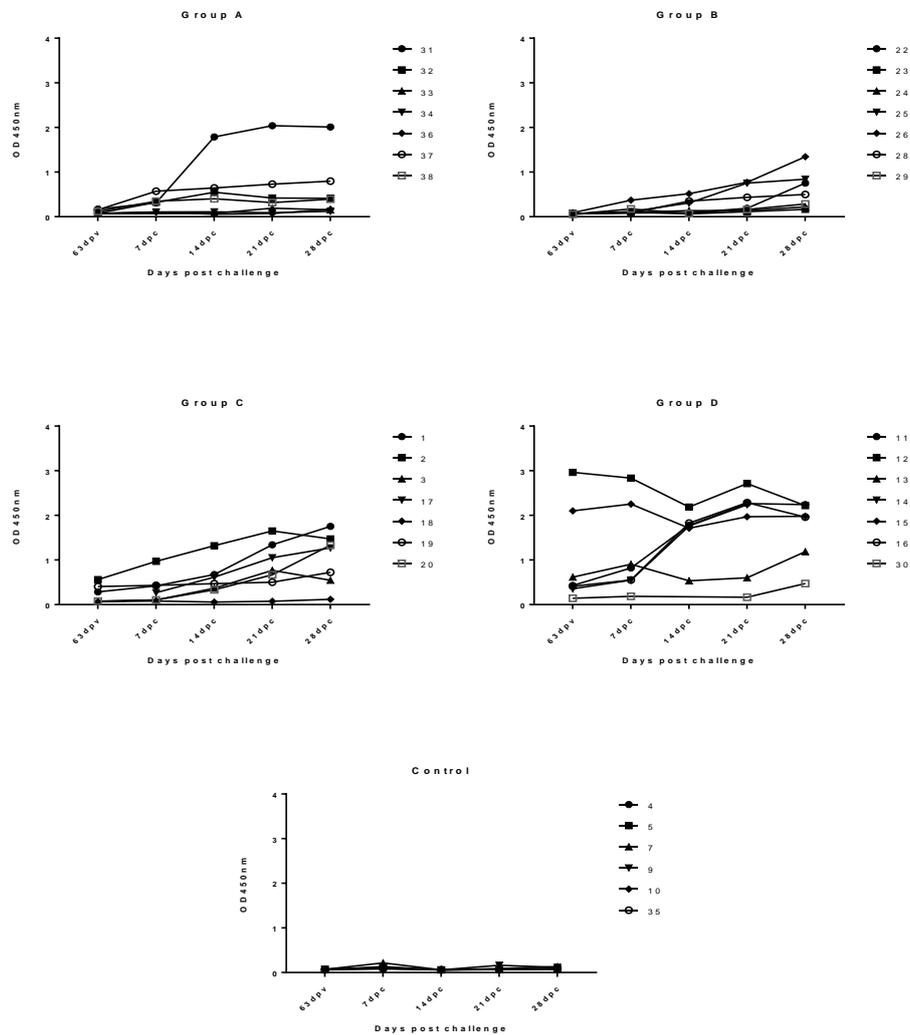


Figure 6. Evaluation of antibody immune response against ORF1a viral peptide from 0dpc (63 days post initial vaccination) to 28dpc in swine sera. Graphs refer to immune recognition of viral peptides for each animal challenge at different sampling days.

Viral load in serum during challenge trial.

To determine the degree of protection in all vaccinated groups, the main proxy was to calculate the viral load present in serum during the challenge (gold standard to determine vaccine efficacy) and in tonsils at 28dpc to evaluate the presence of the virus in lymphoid tissues and its potential for possible transmission to other pigs. As seen in figure 7, animals in groups A and B showed less dispersion for viral load when compared to other groups and controls at 7dpc (Figure 7a). At 14dpc and 21dpc, all groups had similar viral loads except for Group D (14dpc) which showed higher values. Finally, at 28dpc almost all animals in group A and B were negative for PRRVS in serum but controls, Group C and D had at least three different animals still detectable for PRRSV in serum. Importantly, sentinel animals remained negative during the whole study.

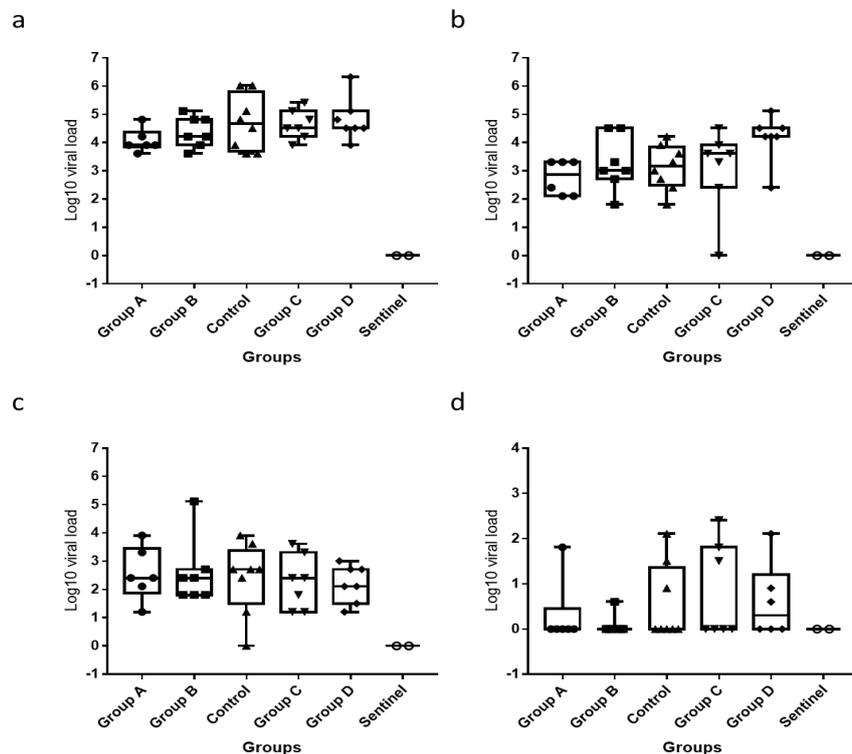


Figure 7.- Viral load in swine serum samples after challenge. Each box represents a sampling day to determine viral load in all animals' sera by qRT-PCR. (a) 7 days post challenge (b) 14 days post challenge (c) 21 days post challenge (d) 28 days post challenge (culling day).

When the area under the curve was calculated to determine the reduction in percentage for viral load during the whole study, there were no statistically significant differences at 21dpc. However, it was clear that at 28dpc Group A showed a reduction of 9.4% in the viral load when compared to the control group (Figure 8 but not statistically significant). Nevertheless, there were statistically significant differences ($p < 0.05$) between Group A (EVs vaccinated) and Groups C and D (viral peptides vaccinated), where the lasts had the highest values. The reduction of viral load in Group A was 8.2% and 12.9% when compared to Groups C and D, respectively. These latter groups were vaccinated with peptides and different adjuvants.

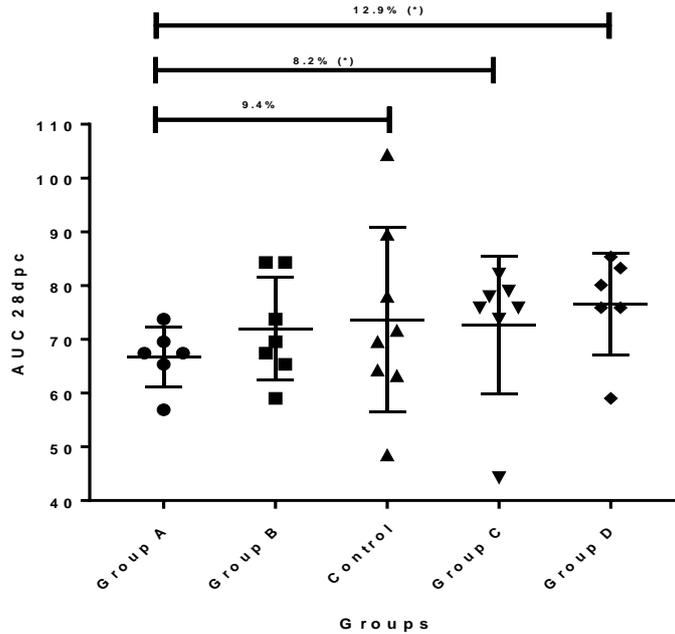


Figure 8.- Area under the curve calculated by 28dpc for viral load concentration per time. Statistically significant differences (* p<0.05).

After evaluating the reduction in viral load and different immunological responses post challenge, it was studied the relationship between antibody values or number of IFN- γ secreting cells previous challenge and the viral load kinetics after it.

As seen in figure 9a, before challenge, only cells from animals of groups A and B were stimulated by viral peptides to induce IFN- γ secretion *in vitro* with statistically significant values when compared with pre-immune cells (day 0) in the ELISPOT assay. In contrast, only one animal of group D showed values of IFN- γ producing cells when it was stimulated. To evaluate differences according to IFN- γ production and viral load, 60 IFN- γ spots secreting cells per million PBMCs (SFC) was established a relevant level for cellular immunity against PRRSV. When animals with more than 60 SFC were compared to those with less than 60 SFC, statistically significant differences were observed in terms of AUC for each group. Thus, animals with more than 60 SFC had a smaller AUC that means less viral load in serum during the trial (Figure 9b; $p = 0.0237$). Moreover, antibodies generated pre-challenge were compared with viral load to evaluate possible correlations as seen with IFN- γ producing cells. There was no correlation between antibody values (OD) and viral load in all the groups; Remarkably, only in Group A, it was possible to establish a negative correlation between antibodies against GP5 viral peptide before challenge and AUC values at 28dpc close to be significant (Figure 9c; $p = 0.0581$ $\rho = -0.7383$).

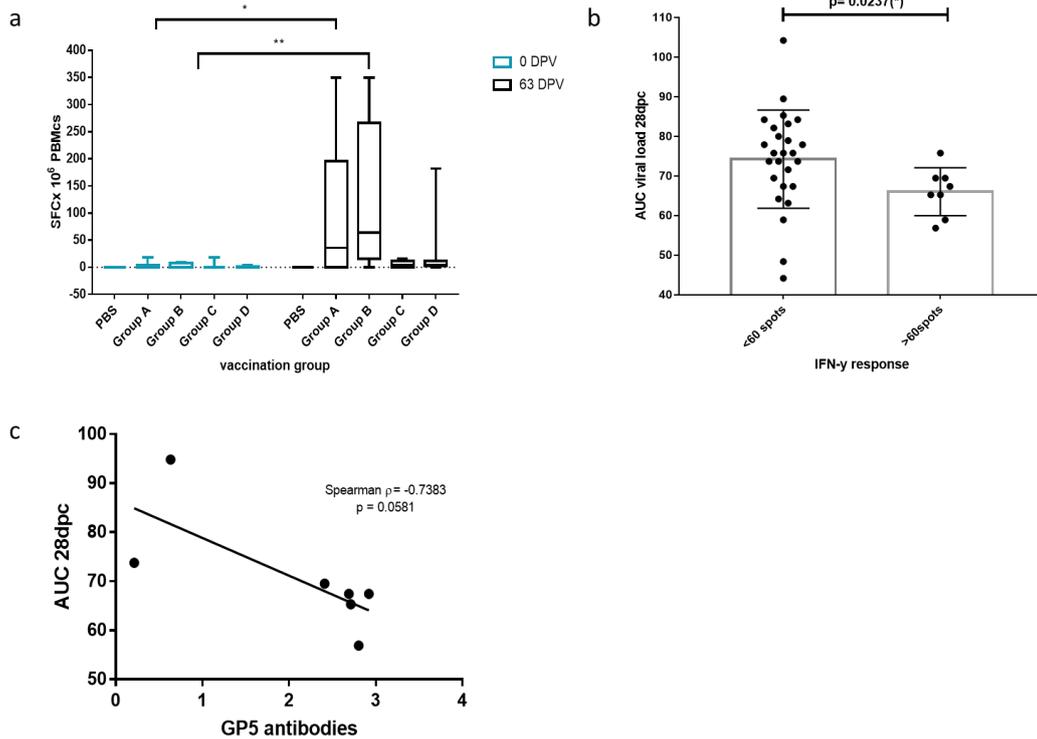
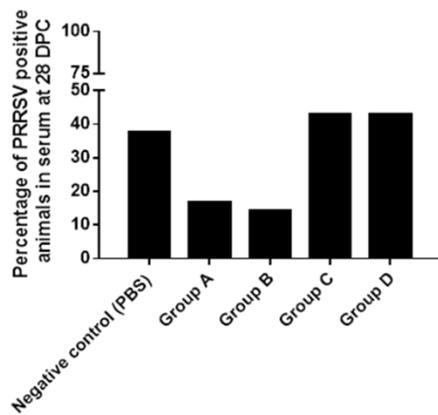


Figure 9. Comparison IFN- γ secreting cells with challenge outcome as AUC at 28 days post challenge. (a) Cellular immune response pre-challenge (63 days post vaccination – previous chapter) for all vaccination groups. (b) AUC at 28dpc in relation with IFN- γ producing cells. (c) Correlation of antibody titers and viral load by Spearman correlation test. $p < 0.05$ (*) $p < 0.01$ (**).

Finally, percentage of PRRSV positive animals in serum and tonsils at 28 dpc was determined for each group (figure 10). Controls, Group C and Group D had around 40% of the animals with detectable viral load in serum (Figure 10A). In contrast, groups vaccinated with EVs enriched fractions showed only a 15% of all animals still positive for viral particles in serum (Groups A and B). When viral load was determined in tonsil samples (Figure 10B), control group showed, as expected, the highest percentage of positive animals for this tissue, as viral particles were detected in all animals from this group. Groups B and D had around 40% of animals with detectable viral particles on their tonsils. Interestingly, Groups A and C showed no detectable viral particles in any animal of each group, although both were still positive for viral particles in serum. Although clear differences are observed in viral presence in serum and tonsils, those are not statistically significant due probably to our small number of animals per group. Importantly, sentinel animals showed negative results for viral load in serum and tonsils during the whole trial.

A



B

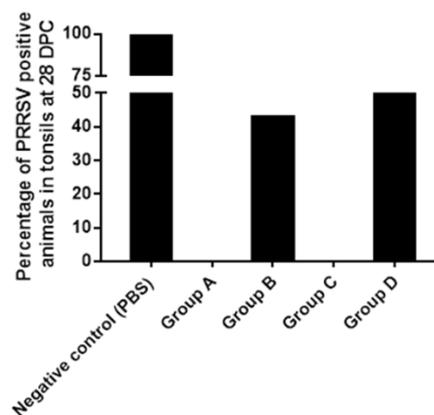


Figure 10. Presence of PRRSV viral particles in serum and tonsils at 28dpc. (A) Percentage of positive animals for viral particles in serum where Groups A and B showed the lowest values, around 15% of positive animals in each group. (B) Calculated percentage of positive animals for viral particles in tonsils. Control group shows a 100% of animals with tonsillar infection, followed by Groups B and D with around 50% of all animals infected in this tissue and Groups A and C with no detectable viral particle in tonsils.

Histopathological analyses at 28 days post challenge.

Macroscopic lesions.

Macroscopic lesions compatible with interstitial pneumonia were observed only in an individual belonging to the negative control group suggesting that the PRRSV strain used in the challenge study was not virulent. This observation agrees with the scarce of clinical symptoms showed by the animals in any group after the challenge. The group of sentinels has been excluded of statistical comparisons (and graphic representations) due the low number of animals included in it (low statistical potency).

A similar percentage of animals showed an increase in the size of the mediastinal lymph nodes (29.7%) and the mesenteric lymph nodes (27%) at the end of the experiment. Curiously, this percentage was not evenly distributed neither across experimental groups nor lymph nodes. Thus, the percentage of increase of mediastinal lymph nodes for group A (83.3%) was significantly higher ($p < 0.05$) than for the rest of experimental groups with values observed between 14.3 to 25%. However, the percentage of increase of mesenteric lymph node for group A and B was close to 50% and higher than for the rest of experimental groups (12.5 to 14.3%) but without showing statistically significant differences between them ($p > 0.05$).

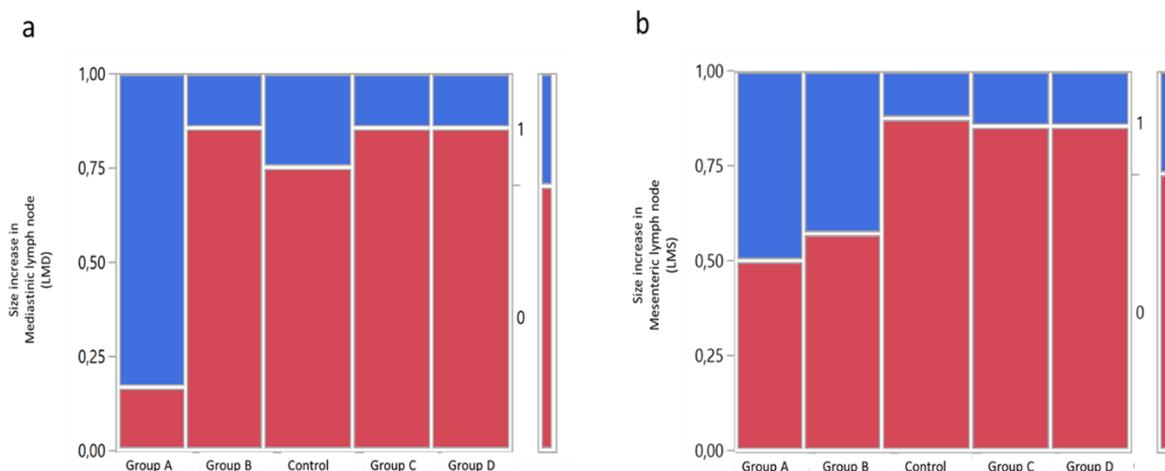


Figure 10. Percentage of animals in which MLD and MSLD size is increased at 28dpc. (a) Evaluation of mediastinal lymph node (MLD) size increase. (b) Evaluation mesenteric lymph node (MSLD) increase in vaccination groups.

Microscopic lesions.

In spite of the low presence of macroscopic lesions in the animals, a microscopic analysis was carried out by a pathologist in a blinded way for the lung, lymph nodes, spleen and tonsils.

Microscopic lung examination

There was a lymphohistocytic thickening of the alveolar septa in the lungs of 70.3% of the animals submitted to the study, from mild to moderate, and with focal or multifocal distribution. Only one animal showed lesions of greater intensity, corresponding to the same one animal (control group) with macroscopic interstitial pneumonia. In addition, 32.4% of the animals presented peribronchial and perivascular cuffs, 27% presented hypertrophy and hyperplasia of type II pneumocytes and 24.3% presented macrophages and necrotic debris (karyorrhexis) in the alveolar spaces. These observations are compatible with a viral infection and, in particular, with a PRRSV infection. This result highlights the success of the challenge. In the following table (Table II), it is detailed the percentage of microscopic lesions in the lung by experimental group. It was not observed any significant difference between experimental groups in any of the microscopic lesions observed.

Table II. Microscopic lung lesions after challenge.

Experimental group	Thickening of the alveolar septa	Peribronchial and perivascular cuffs	Hypertrofia and hyperplasia of type II pneumocytes	Macrophages and necrotic debris in alveolar spaces
Group A	83.3 a	33.3 a	33.3 a	33.3 a
Group B	71.4 a	14.3 a	14.3 a	14.3 a
Control	50 a	50 a	25 a	37.5 a
Group C	71.4 a	57.1 a	14.3 a	0 a
Group D	85.7 a	14.3 a	42.9 a	28.6 a

Different letters in a column means significant differences ($p < 0.05$)

Microscopic lymph node examination

Mediastinal lymph node

Regarding the lesions present in mediastinal lymph nodes, generalized hyperplasia was observed in 88.9% of pigs, with follicular hyperplasia in 66.7% of them. In 27.8% of the animals, pyknotic lymphocytes or macrophages with individualized phagocytosed cell debris were observed, however, necrotic foci were absent in all the animals. These observations are compatible with an immune response to cope with an infection at lung level and it reinforces the success of the challenge (Table III). In the following table, it is detailed the percentage of microscopic lesions in the mediastinal lymph node by experimental group. It was not observed any significant differences between experimental groups in any of the microscopic lesions evaluated with the exception of follicular hypertrophy and hyperplasia. For this parameter, the group A, B and C showed significant less lesions ($p < 0.05$) than the control and D group.

Table III. Microscopic lesions in the mediastinal lymph node after challenge.

Experimental group	Follicular hypertrophy and hyperplasia	Proteinaceous fluid-filled spaces	Pyknotic macrophages/lymphocytes	Lymph node hyperplasia
Group A	40a	20 a	60 a*	80 a
Group B	42.3 a	0 a	14.3 a*	85.7 a
Control	100 b	0 a	25 a	87.5 a
Group C	57.1 a	0 a	14.3 a*	100 a
Group D	71.4 b	0 a	42.9 a	85.7 a

Different letters in a column means significant differences ($p < 0.05$)

Statistical tendency (*) between Group A and B and C for pyknotic macrophages/lymphocytes

Mesenteric lymph node

Regarding the lesions present in mesenteric lymph nodes, generalized hyperplasia was observed in 97% of the animals, with follicular hyperplasia in 82.9% of them. In 25% of the animals, pyknotic lymphocytes or macrophages with individualized phagocytosed cell debris were observed, however, necrotic foci were absent in all the animals. These observations are compatible with an immune response to cope with a generalized infection and it reinforces again the success of the challenge. In table IV, it is detailed the percentage of microscopic lesions in the mesenteric lymph node by experimental group. It was not observed any significant difference between experimental groups in any of the microscopic lesions with the exception of pyknotic macrophages/lymphocytes. For this parameter, the group A showed significant more cells with these characteristics ($p < 0.05$) than the control and C group.

Table IV. Microscopic lesions in the mesenteric lymph node after challenge.

Experimental group	Follicular hypertrophy and hyperplasia	Proteinaceous fluid-filled spaces	Pyknotic macrophages/lymphocytes	Lymph node hyperplasia
Group A	80 a	16.7 a	66.7 a	100 a
Group B	71.4 a	0 a	28.6 a,b	100 a
Control	100 a	0 a	14.3 b	85.7 a
Group C	100 a	0 a	0 b	100 a
Group D	71.4 a	14.3 a	28.9 a,b	100 a

Different letters in a column means significant differences ($p < 0.05$)

Spleen

22.2% of the animals presented follicular and periarterial lymphatic sheath hyperplasia, but necrotic foci were absent in all the animals. The negative control group showed the highest values of hyperplasia (37.5%) but without showing statistically significant differences with the other groups (between 14.3 to 28.9%).

Tonsil

In tonsils, 83.3% of the individuals presented hyperplasia and hypertrophy of the follicles. Necrotic debris mixed with inflammatory cells were observed in 27.8% of cases and expanded lymphoreticular tissue in 58.3% of the animals without observing necrotic foci in any animal. The negative control group showed the highest percentage of animals with hypertrophy and hyperplasia of the follicles but without observing significant differences with other experimental groups (Table V). This result again reinforces the success of the experimental challenge.

Table V. Microscopic lesions in the mediastinal lymph node after challenge.

Experimental group	Necrotic crypt debris intermixed with mixed inflammatory cells	Hypertrophy and hyperplasia of follicles	Expanded lymphoreticular tissue
Group A	60 a*	80 a	60 a
Group B	14.3 a*	71.4 a	71.4 a
Control	37.5 a	100 a	75 a
Group C	14.3 a*	85.7 a	42.8 a
Group D	28.9 a	85.7 a	42.9 a

Different letters in a column means significant differences ($p < 0.05$)

Statistical tendency (*) between Group A and B and C for necrotic crypt debris intermixed with mixed inflammatory cells

Discussion.

As previously reported in the immunogenicity trial, the groups vaccinated with EVs enriched fractions exhibited a different immune response than those vaccinated with viral peptides regardless the adjuvant used; in terms of antibodies previous to challenge, peptide vaccination elicit higher responses, however, IFN- γ producing cells and specific antibodies against viral proteins contained in EVs (GP5 and Nucleocapsid) were detected only in EVs vaccinated animals. In that sense, it was necessary to evaluate the degree of protection by means of immune response, histopathological changes and viral load after challenge with a wild type PRRSV-1 strain that circulates in swine farms in the province of Lleida (Catalonia) and corresponding to the same strain that infected the pigs used for the isolation of EVs (vaccination antigen).

Several vaccines has been tested during PRRSV history, nevertheless, few of them have met the standards for safety and immunogenicity desired to control this pathogen, and none of them conferred complete protection (Hu and Zhang, 2014). Our criteria for evaluation of effectiveness of this new vaccine proposal was based on antibodies response, viral load, body condition, clinical signs and histopathological analyses at macroscopic and microscopic level. In any case, the main criteria for efficacy is the PRRSV viral load. Thus, the lower the PRRSV viral load after challenge, the better the protection conferred by the vaccine.

Results obtained with the most used ELISA test for PRRSV diagnosis (IDEXX X3 PRRSV), no differences between groups were observed after challenge, as all animals generated antibodies against the nucleocapsid protein (antigen used in this ELISA assay) at 14dpc that increased until 28dpc (Sattler et al., 2015). Compared with other challenge trials, our vaccination approach does not improve the onset of antibodies against this recombinant nucleocapsid protein. Infection with different viral strains or vaccination with MLV vaccines did not influence the appearance of antibodies and regardless the virus, animals seroconverted 6 to 14dpc (Martínez-Lobo et al., 2013; Molina et al., 2008; Opriessnig et al., 2002).

During PRRSV infection, antibody response seems to be controversial as most antibodies generated appear in early stages of infection and are not neutralizing (Lopez and Osorio, 2004), however, their role during viral clearance is not elucidated and mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent complement-mediated cytotoxicity (CDC) could be some of the strategies by which those non-neutralizing antibodies intervene and collaborate in the whole swine immune response against PRRSV infection (Rahe, Michael C. and Murtaugh, 2017). We were not able to determine the titer of neutralizing antibodies in the vaccination groups and this is one limitation of the study; nevertheless, we identified specific antibodies that recognize nucleocapsid and gp5 proteins presented in EVs by western blot using immune sera post vaccination only in the animals that received EVs in the prime-boost (Groups A and B). In addition, it was possible to establish a negative correlation, close to significant values, between antibodies against Gp5 and the area under the curve after 28dpc only in those animals vaccinated with EVs without previous vaccinations (Group A) indicating that high values of antibodies in this group before challenge may be related to low values of viral load (AUC) at the end of the study. In that sense, we hypothesized that antigen presentation (mediated by EVs) and priming moment (no previous vaccination) are key features to understand the cell and antibody immune responses obtained that translated into reduction of viral load. Neutralizing antibodies techniques are laborious and based on a non-porcine cell line and usually cell-adapted viral strains that differ from wild type isolations from farms (Osorio et al., 2002) causing some contradictory results depending of the virus isolate used. However, it is clear enough that antibody (Ig) fraction from serum of hyperimmune animals is capable of conferring sterilizing immunity and inhibition of reproductive failure in sows in passive-transfer experiments (Osorio et al., 2002). We hypothesize that Ig fraction precipitated and used in passive transfer studies could contain more than only Ig and, possibly, extracellular vesicles that could be playing a role on protective immune response activation during infection. This hypothesis paved the way for further experiments to decipher the real role of extracellular vesicles in the generation of an immune response. In this sense, a new research line can be open to find out the role that could have previous vaccinations with other vaccines with the immune response generated with exosomes vaccines. This a new field research in veterinary science that could be probably developed in the future.

Our vaccination approach with EVs enriched fractions (group A) reduced viremia significantly ($p < 0.05$) when compared with a classical vaccination approach with viral peptides (Group C: 8.21% and D 12.85% - almost 1log₁₀ reduction in some timepoints). Importantly, these results indicate that some protective antigens could be related to EVs and are presented to the immune system in a different manner than peptides alone. Most inactivated vaccines available in the market, which contain no replicating virus (as our EVs), are able to reduce viral load in 1-2 log₁₀ or induce early clearance (Renukaradhya et al., 2015), but most used vaccines available are MLV and those contain replicating viral particles that could revert to a virulent state (Chareerntantanakul, 2012) or even recombine with circulating wild type isolates (Wenhui et

al., 2012). Our vaccination approach seems to induce some degree of protection with the addendum of being a virus-free approach. Important to notice, because of the variability inside the control group it was difficult to obtain statistically significant differences when compared with experimental groups regardless the vaccination strategy applied although a lower viral load in EVs vaccinated groups and higher viral load in peptide vaccinated ones is consistently observed.

When viral load was evaluated at the end of the study, Groups A and B had almost all individuals negative for PRRSV in serum while Groups C and D still had detectable virus. In addition, Group A (EVs vaccinated) and Group C (peptides) showed negative results in tonsils. With some modified live vaccines (MLV) were tested during challenge trials, less than 40% of the pigs remained positive after three weeks post vaccination, and there was no detectable virus genome in almost all lymphoid organs (Martínez-Lobo et al., 2013). Surprisingly, another study using the same MLV reported at least two positive pigs and other showing positive results for MLV and the wild type strain at 47 and 51 days post vaccination (Pileri et al., 2015). As mentioned previously, Group A showed the best result in terms of viral load in serum and tonsils in a time period shorter than those studies previously explained. If these results with shorter viremias are associated to the wild type strain used in our study (low pathogenicity), it remains to be proved with other strains, however it seems that EVs without previous vaccination induces better anti-viral response than the other groups.

Interestingly, sentinel pigs were also evaluated for viral load during the challenge phase, remaining negative which indicates no viral dissemination through pens. Some studies reported that sentinel pigs usually gets infected during challenge trial due to virus secretion by oral-nasal route contact, with an average of 10 days of detectable viremia (Martínez-Lobo et al., 2013; Pileri et al., 2015). It seems that our vaccination approach could confer herd immunity by delaying or inhibiting PRRSV transmission by oral secretions, however due to small number of animals per group and the short time of the trial (28 days post challenge) it is difficult to establish statistically significant differences to support our hypothesis. In addition, PRRSV persistence on infected and vaccinated animals had been demonstrated until 250 days approximately (Wills et al., 2003) with intermittent shedding periods in the absence of viremia in serum. These data support that further experiments are required to evaluate viral clearance during a longer time period using EVs as vaccination antigens. Moreover, these experiments should be designed to allow quantifying precisely the PRRSV transmission between vaccinated animals with this new vaccine approach and naïve pigs.

Although groups C, D and controls displayed the highest fever during the challenge trial, there was a great proportion of animals with moderate temperature between 3 and 6dpc, which corresponded to the initial peak of viremia observed in serum. No statistically significant differences were observed in terms of fever and body condition between vaccinated groups probably due that the viral strain used for this study seems to induce mild to moderate clinical symptoms.

PRRSV infection begins by replication in alveolar macrophages, located in the lungs, accompanied by damage in lung epithelia, affecting later the muco-cilliary transport that impedes the control and elimination of respiratory pathogens (Gomez-Laguna et al., 2013). In our work, gross and microscopic analyses from lung tissues revealed damages associated with interstitial pneumonia in only one individual from control group confirming that strain used for challenge had low pathogenicity. Due to variability and the low statistical potency (relative low number of animals), only some parameters allowed to detect statistically significant

differences. Thus, group A showed less hypertrophy and hyperplasia (mediastinal lymph node) and higher number of pyknotic lymphocytes associated to mesenteric lymph nodes.

The effect of PRRSV-1 pathogenicity has been studied in different viral strains with variable degree of virulence (Low virulence Lelystad virus, UK 215-06 strain and highly virulent SU1-Bel) finding that no statistically significant differences were observed at 35dpi in terms of macroscopic damage or histological lesions in the mediastinal lymph nodes (García-Nicolás et al., 2015), although all viral strains in this study induced measurable changes at 3 and 7dpi. Another study reported mild to moderate size increase from 10 days post-infection until the end of the study (Rodríguez-Gómez et al., 2013, 2014). Nevertheless, our analyses were performed only at 28dpi using a low virulent field strain, making difficult to find any differences between vaccinated animals and controls at macroscopic level. In another case, lesions like moderate, multifocal, tan-colored consolidation of the lungs and severe enlargement of the lymph nodes were detected at necropsy as common during PRRSV infections with marked lymphoid follicular hyperplasia and follicular necrosis in the tonsil, spleen, and lymph nodes (Halbur et al., 1995).

Although was possible to find statistically significant differences in some between control group and vaccinated ones (enlargement of lymph nodes, follicular hyperplasia of mediastinal and mesenteric lymph nodes), due to small animal number these results do not allow to infer the effectiveness of the new vaccination approach in comparison with the control taking into account histopathological findings. In addition, as mentioned previously, will be necessary to use mid to high virulence strains of PRRSV to have more statistical potency when compared different vaccination schemes in terms of improving body condition, fever and histological lesions after challenge.

In conclusion, extracellular vesicle enriched fractions remain as a safe and potent new strategy to be further explored for antigen discovery and vaccination for PRRSV. This proposal of vaccination is safe (virus free), capable of inducing strong cell mediated IFN- γ secretion, capable of activation of lymph nodes (determined by size increase) and reducing viral load in serum almost 10% compared to control and classical vaccination with peptides. Finally, absence of viral load in tonsils could potentially block the dissemination of the pathogen in the herd (sentinel animals remained negative for viral load during the whole challenge) although this latter finding must be confirmed in new experiments specially designed to measure the transmission of PRRSV.

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**CHAPTER 3.4. Application of EVs in other animal diseases, African Swine
Fever case: PROOF OF CONCEPT**

Serum-derived extracellular vesicles from African Swine Fever virus infected pigs selectively recruit viral and porcine proteins related to each viral strain.

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Introduction

African swine fever is a virus (ASFV) belonging to the *Asfarviridae* family. ASFV affects domestic pigs and wild boars but also some other African suids in which clinical signs during infection vary considerably from acute forms, with mortality rates up to 90-100%, to inapparent courses like infections in bushpigs (*Potamochoerus* sp.) and warthogs (*Phacochoerus* sp.) which intervene in the sylvatic cycle with a tick vector *Ornithodoros* sp. (Jori et al., 2013). The mature viral particle has icosahedral morphology with a size range of 180-200nm in diameter composed of multiple layers (core shell, inner envelope, viral capsid and external envelope when the virus egress from the cell). The genome is a double-stranded DNA molecule located in the inner core with approximately 170 to 193kbp codifying for 151 to 167 ORFs most differences in genome size are associated to multigene family (MGF) (Dixon et al., 2013; Galindo and Alonso, 2017). Mature viral particle infects primarily monocytic/macrophage by clathrin-mediated endocytosis and egress is completed by transport of mature viral particle by microtubule mechanisms and finally budding from the plasma membrane (Revilla et al., 2018).

Several attempts have been made on vaccination for ASFV comprising inactivated vaccines, subunit approaches and live attenuated viruses, includes recombinant mutants. However, in most of the cases vaccine candidates have demonstrated only partial protection against experimental infections with homologous, and occasionally heterologous virulent isolates of ASFV (Arias et al., 2017; King et al., 2011; Lacasta et al., 2015). Developing a safe and effective DIVA vaccine against ASFV is nowadays one of their animal health.

Since 2007, ASFV have been reintroduced to Europe after eradication in the beginning of 1990 (Rowlands et al., 2008). Due to those recent outbreaks it has been even more important to develop control and vaccination strategies to avoid possible pandemic as wild boars suffer the disease and spread the virus by moving across borders without control, entering in contact with domestic pigs (Ge et al., 2018; Olesen et al., 2018).

Most of the current research is focused on attenuated viral models trying to elucidate pathogen and immunological mechanisms of protection in the host. Part of research strategies to find a effective vaccine have been linked to the use of natural attenuated strains and deletion mutants, allowing also to study the immune response of domestic pigs after challenge. One example is the non-virulent non-haemadsorbing Portuguese isolate OURT 88-3 which belongs to the genotype I (Chapman et al., 2008), and when combine with a boost of a close related haemadsorbing Portuguese isolate (OURT 88-1) can induce homologous and heterologous protection from 60 to 100% with almost none clinical signs neither detectable viremia pre-challenge. (King et al., 2011). Another case is the deletion mutant Benin DMGF, originated from wild type isolate Benin97/1 from which several genes related to IFN response were inactivated by deletion of interruption of transcription (MGF360-10L, 11L, 12L, 13L, 14L and MGF530/505-1R, 2R and 3R were deleted and MGF360-9L and MGF530/505-4R). This deletion mutant is capable of inducing homologous protection against parental isolate, however, in this case clinical signs are observed and detectable viremia detected in infected pigs (Reis et al., 2016). Nevertheless, several biosafety problems could arise when immunization with attenuated viral strains is translated to the field because of possible recombination events with wild type viruses and in some cases, animals developed reactions to vaccination with those attenuated or deletion mutants such as pneumonia, necrotic foci,

abortion and even death after vaccination, making the search of new virus-free strategies for vaccination a priority in the field (Arias et al., 2017).

On the other hand, extracellular vesicles have been proven an excellent new vaccination platform for veterinary diseases in situations where conventional approaches have failed, such as PRRSV (Montaner-Tarbes et al., 2016, 2018). Extracellular vesicles are small round 50-400nm in diameter vesicles secreted by different cell types and classified as microvesicles if formed by direct budding of the plasma membrane and exosomes if derived from late endosome trafficking and multivesicular bodies formation and finally release to the extracellular space (Théry et al., 2009). Importantly, exosome-like vesicles share the same pathway of formation as some viruses with multivesicular bodies and viral factories involved, by which viruses send proteins and nucleic acids to the extracellular space that later on can trigger immune responses against or in favour of viral replication (Dias et al., 2018; Nolte-‘t Hoen et al., 2016; Sadeghipour and Mathias, 2016).

Given the abilities of extracellular vesicles for inducing immunological responses in pigs together with the fact that conventional vaccine approaches have been only partially successful for ASFV, the main objective of this work was to characterize extracellular vesicles on serum from infected animals using two different ASFV viruses (OURT 88-3 and Benin DMGF), corresponding to a naturally attenuated virus and a deletion mutant respectively, in order to determine possible differences in swine and viral protein content on EVs enriched fractions that could contribute to better understanding of ASFV pathogenesis and immune/protective responses in the host.

Materials and methods.

Cells and viruses

Both low virulence non-haemabsorbing genotype I ASFV isolate OURT88/3 and attenuated deletion mutant Benin Δ MFG, obtained from virulent genotype I isolate Benin97/1, were grown in primary macrophage cultures derived from bone marrow. The preparation of viruses and virus titration was carried out as previously reported (Chapman et al., 2008; Guinat et al., 2014; Reis et al., 2016). Results are presented as the amount of virus infecting 50% of the macrophage cultures (TCID₅₀/ml).

Experimental design, sampling, clinical evaluation and viremia.

Experiments were conducted in SAPO4 high containment facilities at The Pirbright Institute and regulated by the Animals (Scientific Procedures) Act UK 1986 (Project License 70/8852). Large White and Landrace crossbred female pigs, 9 to 10-week-old (21-25 kg), from a high health status herd were used. Pigs were separated in two groups of six pigs each. In group A (pigs A1 to A6), animals were immunised by intramuscular route (IM) in the neck muscles with 1 ml containing 10⁴ TCID₅₀/ml of OURT88/3 isolate, whereas in group B (pigs B1 to B6), animals were immunised using the same route and dose with deletion mutant Benin Δ MFG. Immunization day was defined as day 0 (0 dpi). EDTA blood and serum samples from jugular vein were collected from all pigs at day 7 and 24 pi. Rectal temperatures and clinical signs were monitored daily as described (King et al., 2011). DNA was extracted from blood samples taken at days 7 and 24 pi and analysed for ASFV genome detection by quantitative PCR (qPCR) (King et al., 2003).

Extracellular vesicles isolation: size-exclusion chromatography.

Isolation of serum-derived extracellular vesicles (EVs) by size exclusion chromatography (SEC) was performed as previously described with swine sera samples from 7dpi and 24dpi (Montaner-Tarbes et al., 2016). Briefly, Sepharose CL-2B (Sigma-Aldrich, St. Louis, MO, USA) was packed in 10 mL syringes to a final volume of 10 mL and equilibrated with PBS-Citrate 0,32% (w/v). Frozen serum samples were thawed, centrifuged at 500 x g for 10 minutes at room temperature to remove cellular debris, and 2 mL aliquots were applied to each column. Collection of 20 fractions of 0.5 mL each started immediately using PBS-citrate as the elution buffer. Protein content of each fraction was analyzed using Bradford protein quantification assay according to manufacturer's instructions (Pierce BCA protein quantification assay kit, Thermo-Fisher).

Flow cytometry analysis of molecular markers associated with extracellular vesicles.

A bead-based assay for detection of classical exosome markers, CD63 and CD81 and two new ones (CD5 and CD163) was used with some modifications to allow the use of this protocol in a BSL-3 environment. Briefly, EVs enriched fractions were coupled with Aldehyde/Sulfate Latex Beads, 4% w/v, 4 μ m (Invitrogen) and then blocked with PBS 1X / BSA 0.1% (Sigma-Aldrich) / NaN₃ 0.01% (Sigma-Aldrich). Fractions were incubated in microtest conical bottom 96-well plates for 30 minutes at 4°C with anti-CD63 (Clone H5C6), anti-CD81 (Clone JS-81) antibodies (BD Biosciences) at 1:100 dilution, anti-CD5 (clone PG114A, Kingfisher Biotech) at 1:200 or anti-CD163 (clone 2A10 gently given by Dr. Javier Dominguez). After washing, a 1/100 dilution of secondary antibody FITC (Southern Biotech) was incubated for 30 minutes

at 4°C. After removal of unbound secondary antibodies by centrifugation and wash with PBS/BSA, beads were suspended 4% paraformaldehyde for 30 minutes to inactivate any possible contamination with ASFV particles. Then, plate was centrifuged, and beads resuspended in PBS and analyzed by flow cytometry using MACSQuant Analyzer 10 equipment (Miltenyi Biotec). Median Fluorescence Intensity (MFI) and beads count data were obtained by FlowJo analysis Software of every sample-reading file.

Detection of viral proteins in surface of extracellular vesicles using Bead based assay.

A bead-based assay for detection of viral proteins was used to phenotypically identify SEC fractions containing EVs and viral proteins (Montaner-Tarbes et al., 2016). Briefly, EVs enriched fractions were coupled with Aldehyde/Sulfate Latex Beads, 4% w/v, 4 µm (Invitrogen) and then blocked with PBS 1X / BSA 0.1% (Sigma-Aldrich) /NaN3 0.01% (Sigma-Aldrich). Fractions were incubated in microtest round bottom 96-well plates for 30 minutes at 4°C with anti-p30, anti-p54 and anti-p72 antibodies (The Pirbright Institute) at 1:100 dilution. After washing, a 1/100 dilution of secondary antibody FITC (Southern Biotech) was incubated for 30 minutes at 4°C. After removal of unbound secondary antibodies by centrifugation, beads were suspended 4% paraformaldehyde for 30 minutes to inactivate any possible contamination with ASFV particles. Then, samples were centrifuged, and beads resuspended in PBS and analyzed by flow cytometry using MACSQuant Analyzer 10 equipment (Miltenyi Biotec). Median Fluorescence Intensity (MFI) and beads count data were obtained by FlowJo analysis Software of every sample-reading file.

Transmission Electron Microscopy and negative staining.

Ten microliters of each sample were placed on Formvar coated, glow discharged copper grids for one minute. After removing excess sample with filter paper, the grids were briefly placed on droplets of distilled water, and the excess removed. The grids were placed on droplets of 3% aqueous uranyl acetate for one minute before excess stain was removed and the grids allowed to dry. Samples were imaged using a FEI T12 TEM at 100kV with a Tietz F214 camera. For size distribution, all images were evaluated using ImageJ software and plotted in size-range percentages.

Mass spectrometry

Extracellular vesicle fractions from size exclusion chromatography had to be heat inactivated at 65°C for 2 hours for biosafety issues and send to Biological Mass Spectrometry Facility of University of Sheffield. Liquid Chromatography (nanoLCULTRA-EKSIGENT) followed by mass spectrometry (nanoLC-MS/MS) was performed on a LTQ Orbitrap Velos (Thermo Fisher). Briefly, samples were reduced with 10 mM DTT (Dithiothreitol), alkylated with 55 mM iodoacetamide and precipitated by 10% TCA. After washing with acetone, 2 µL of 8 M urea were added and samples brought to a final concentration of 1.6 M urea. One microgram of trypsin (*Sus scrofa*) was added and digestions were carried overnight at 37 °C. The reaction was stopped with 1% formic acid. The amount of sample submitted to mass spectrometry analyses was based on nanoparticle tracking analysis (see below) and ranged from 9.8×10^7 to 3.9×10^8 particles/mL among all samples analysed. MS/MS was performed in the LTQ using data dependent dynamic exclusion of the top 20 most intense peptides using repeat count = 1, repeat duration = 30 s, exclusion list size of 500 and exclusion list duration = 30s as parameters. The top 20 most intense peptides were isolated and fragmented by low energy CID, 35% collision energy.

Database search and protein identification.

Raw spectral data from Xcalibur™ (Thermo Scientific, v2.1) was searched against a custom database compiled from in FASTA format for uploading it into Maxquant 1.6. The databases were obtained from UniProtKB and contained complete and partial sequences of ASFV (4125 sequences – reviewed 806) and *Sus scrofa* (40713 complete proteome sequence). The sequence for default contaminant database was also included in the search carried out with Maxquant 1.6 software. Contaminants and proteins identified only by site modification were filtered out from the list. Proteins found in all groups were scored positive if they had at least two unique peptides and 1% False Discovery Rate (FDR) for protein and peptide identification. In ASFV proteins analyses, the identification also included the criteria of being present on infected samples and absent in control samples to avoid false identification hits due to the small number of proteins in ASFV UniprotKB proteomic database. ASFV proteins were evaluated only in terms of presence/absence while swine proteins were compared using a relative quantification approach.

Statistical Analysis

Pig protein hits were evaluated using Perseus v1.6. Contaminants, reversed identified hits and identified by site modification proteins were filtered out of the analysis. Matrix was reduced by eliminating proteins identified with less than 1 unique peptide and 1% FDR. Then, sample groups of Controls and infected swine proteins identified in EVs enriched preparations were compared using two sample student t-test for the mean normalized intensity (LFQ) by permutation-based FDR method. Differentially expressed proteins were marked as significant if $p < 0.05$ (red) and $p < 0.01$ (volcano plot above threshold). After filtering, proteins of each group were compared in a Venn diagram using Venny 2.1 (Oliveros) software to determine which proteins were unique and shared among groups.

Gene Ontology analysis.

Proteins statistically significant by student t-test were evaluated for gene ontology. For that, identified hits were examined using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (Huang et al., 2009) to identify significant enrichment in terms of Biological processes, molecular functions and cellular components using $-\log(p \text{ value})$ obtained previously. Results were expressed in categories belonging to each enrichment term.

Results

Viremia and clinical signs of ASFV.

As reported previously (Sánchez-Cordón et al., 2017), OURT88/3 immunised pigs did not show changes in rectal temperatures (Figure 1). However, except for pig B6, a transient increase in rectal temperature above 40.5°C for 1 or 3 days was observed in all pigs immunised with Benin Δ MGF between days 3 and 5 pi. Nevertheless, no other clinical signs were observed along with increased temperatures.

Blood samples were taken at day 7 and 24 for EVs purification. Those samples were tested for ASFV DNA content by quantitative PCR (qPCR) (King et al., 2003). All pigs at day 7dpi showed viral load in blood and could not be used for EVs isolation and enrichment. However, at 24dpi none of the pigs immunised with OURT88/3 (group A) showed detectable levels of virus genome in blood (data not shown), while in all pigs immunised with Benin Δ MGF (group B) moderate levels of ASFV DNA were detected as previously reported (Reis et al., 2016). As some EVs and viral particles have similar size, it was relevant to establish whether ASFV could be present in extracellular vesicles enriched fractions to avoid possible false identifications of viral proteins in further analyses.

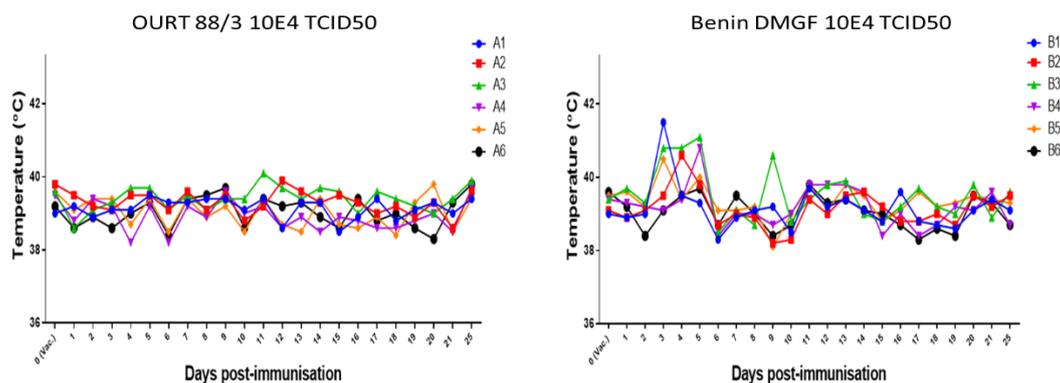


Figure 1. Rectal temperatures in pigs. Rectal temperatures were assessed at different days after immunization with two different ASFV strains: (a) OURT-88/3 and (b) Deletion mutant BeninDMFG by intramuscular route. This graph was gently given by ASFV Research group at Pirbright Institute as a part of the collaboration research project.

Extracellular vesicles in ASFV infected sera.

Both naïve (Figure 2A to 2C) and ASFV infected (Figure 2D to 2F) sera at 7dpi were used to obtain EVs enriched fractions to standardize all the procedures. As described previously (Montaner-Tarbes et al., 2016), swine EVs eluted in fractions 7-10 from the exclusion chromatography column, displayed CD63 and CD81 high MFI values and exhibited a mean size distribution of 100-200 nm measured by Cryo-TEM (Figure 2C and 2F).

In addition, there was no difference in the elution profile in terms of protein and FACS for the molecular markers, independently of the experimental group (naïve or infected swine serum from both groups) and the virus used; however Cryo-TEM pictures showed differential

structures observed in the background field of EVs enriched fractions from ASFV infected animals independently of the virus strain used at 7dpi but not in all preparations (Figure 2F).

In addition, presence of two new molecules were studied in our EV fractions. CD5 was examined because it was previously present in the proteomic analyses for swine enriched EVs fractions (Montaner-Tarbes et al., 2016). Also, the presence of CD163 was analyzed as it is a surface molecule (scavenger receptor and soluble extracellular form) related to macrophages and it is thought to be involved in ASFV viral entry pathway (Sánchez-Torres et al., 2003). Both molecular markers were present in EVs with higher levels than those showed for swine EVs previously, therefore, we selected them for our forwarding experiments.

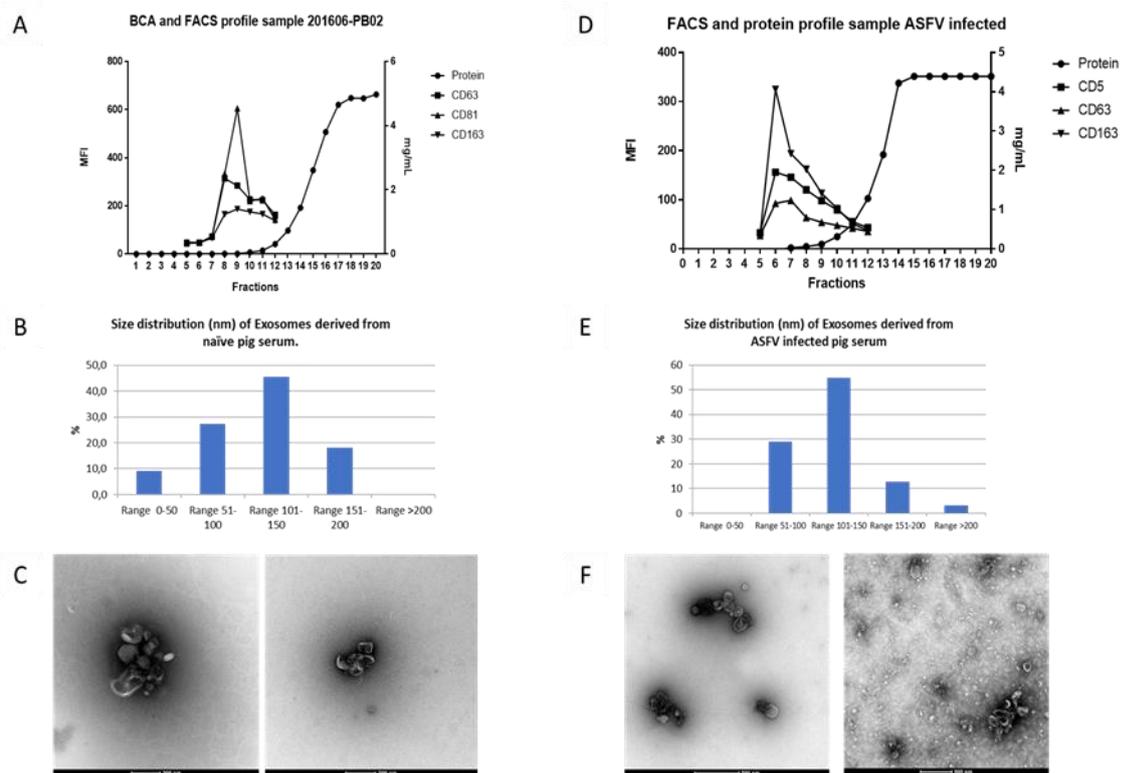


Figure 2. Characterization of extracellular vesicles derived from uninfected and ASFV infected swine sera. (a) (d) FACS analyses for molecular markers for swine serum Evs (b) (e) Size distribution as measured by Electron microscopy (c) (f) Electron microscopy of uninfected and infected simples respectively.

ASFV proteins in EVs enriched fractions.

Next, using bead-based assay and flow cytometry, we wonder whether ASFV surface proteins were present or not in our EVs enriched fractions. Thus, CD163 and CD5 expression were combined with three ASFV proteins p30, p54 and p72.

To differentiate between viral particles and EVs, viral load was evaluated in serum samples as already mentioned, grouping them in those negative for ASFV in serum (OUR 88-3 Group A) and positive in serum (Benin DMGF Group B) at 24 days post-infection. As observed in Figure 3a, group A at 24DPI and without detectable viral genomes in serum, gave measurable MFI values to viral proteins evaluated in EVs enriched fractions, decreasing as later fractions are eluted from the size exclusion column and not present in the control sera from abattoir (Figure 3b). P72 is the viral protein with high MFI value in almost all EVs enriched preparations.

On the other hand, samples from group B at 24DPI showed detectable viremia and positive MFI values as observed in figure 3c, as expected due to viral particles present in serum samples that could be capture in the assay. As seen with OURT88/3, p72 is the viral protein with highest MFI values of all three evaluated. Importantly, control samples (negative sera) showed MFI values for exosome markers but not for viral proteins confirming specificity of antibodies used in this assay.

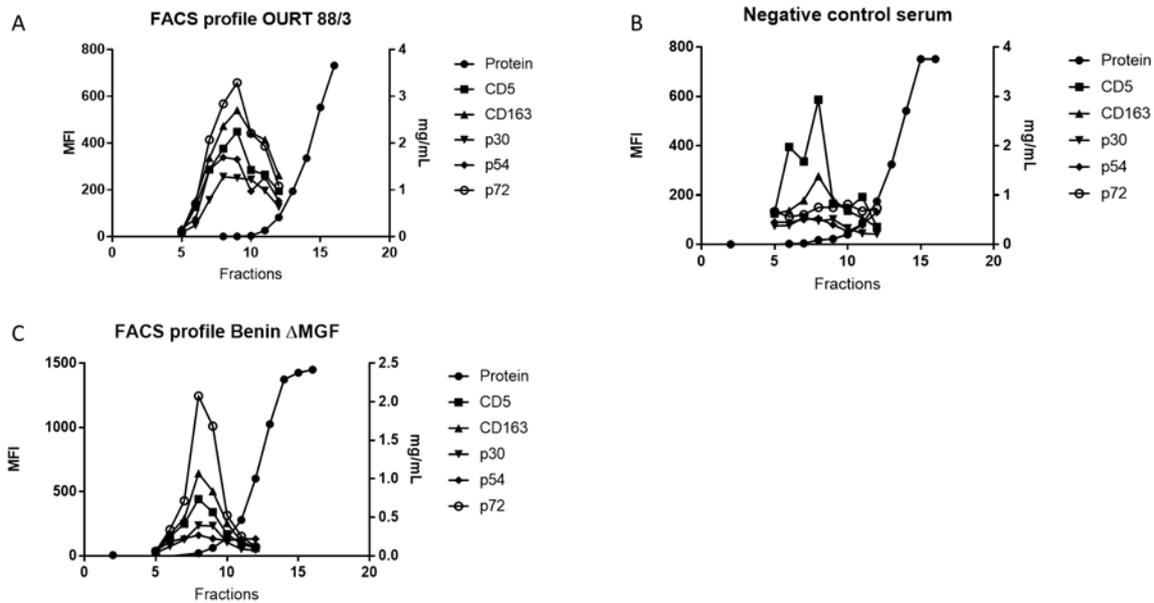


Figure 3. FACS analyses on infected EVs enriched fractions and control. (a) Evs from sera of infected animals with OURT 88-3 at 24dpi (b) Evs profile from uninfected swine sera (c) Evs FACS profile from animals infected with Benin DMGF virus. In all samples, molecular markers CD5 and CD163 were used as control markers for EVS and three different viral proteins evaluated (p30, p54 and p72).

Proteomic analyses of ASFV infected serum derived EVs.

Protein composition in serum derived EVs fractions was analysed in terms of ASFV viral proteins and possible changes in pig proteins due to infection. First, independent groups (Group A and Group B) were compared with controls and only viral proteins identified with 1% FDR and 2 or more unique peptides and absent in controls were marked as positive identifications. As seen in table I and II, some viral proteins were identified in EVs enriched fractions, only for Benin DMGF viral strain.

In group A, there were no protein identified by mass spectrometry following the exclusion criteria applied to the analyses. Later, proteins at least present in samples but not in controls were evaluated and only 3 proteins were identified, two of them in animals at 7dpi and one in one animal at 24dpi. Although, viral proteins were identified by FACS by coupling EVs enriched fractions to beads, we were not able to detect them by LC-MS/MS.

In group B (infected with Benin DMGF) EVs enriched fractions contained at least one protein identified by LC-MS/MS following out exclusion criteria, but two more proteins were detected

in this preparation with a less stringent analyses with 1 unique peptide and shared among samples. The best hit of this analysis was protein structural protein p72 (NP_042775.1) Table II. As viral particles were present in this group (detected by PCR), protein hits could be associated to viral particles that range in the size of EVs. P72 represented the protein with the highest MFI in our FACS analyses for EVs enriched fractions from sera of group B, confirming the presence of this proteins by two different methods.

When swine proteins fold change was evaluated, a total of 943 filtered swine proteins were identified by analysis of LFQ intensities and some of them showed statistically significant differences in relative expression in a virus strain dependent manner when compared with uninfected control. Animals of Group A (OURT-88/3), had only seven proteins in EVs enriched fractions with a significant fold change ($p < 0.05$), and from those, four significant at $p < 0.01$ including Platelet factor 4, Ficolin, Coagulation factor VIII and one uncharacterized pig protein (F1S3H9_PIG) that has extracellular region location (Figure 4a and table III).

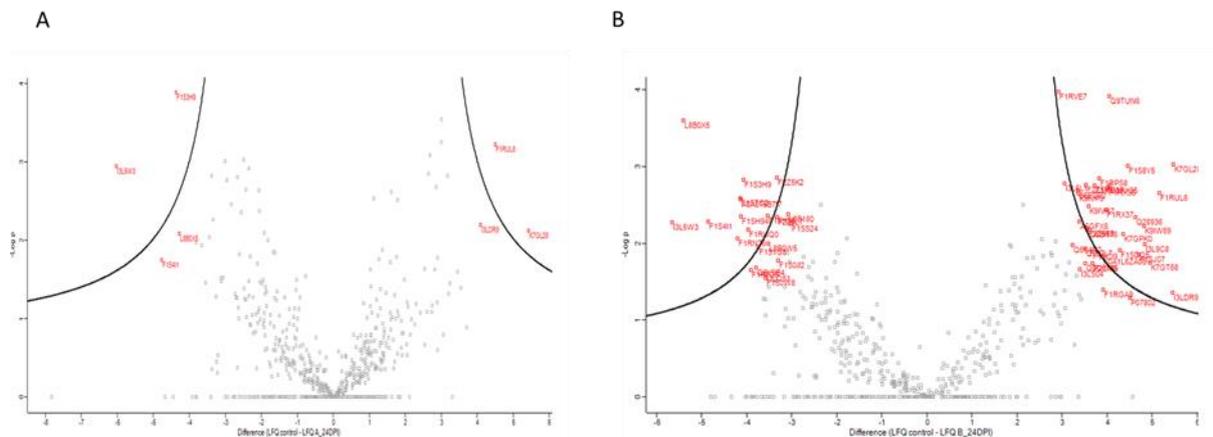


Figure 4. Swine proteins on Evs enriched fractions evaluated at 24 days post infection. (red proteins significant at $p < 0.05$) – above volcano plot threshold ($p < 0.01$). (a) Proteins differentially expressed in Evs enriched fractions from group A (infected with OURT 88-3). (b) Proteins differentially expressed in Evs enriched fractions from group B (Infected with Benin DMGF).

When pigs were infected with Benin Δ MGF, 56 proteins were differentially expressed in EVs enriched fractions (Figure 4b, $p < 0.01$) including Platelet factor 4, HSP70, Integrin beta, cAMP-dependent protein kinase, Lymphocyte antigen 6 complex, Fibrinogen beta chain, different types of IgG, Calcium-transporting ATPase, Coagulation factor VIII and high quantity of uncharacterized pig proteins (Table IV). In addition, when compared using a venne diagram, all proteins identified as significant in EVs from group a (OURT 88-3) were identified also on EVs preparations from Benin Δ MGF, however, there were 49 proteins identified as unique in group b representing an 87.5% of all significant proteins from this group (Figure 5).

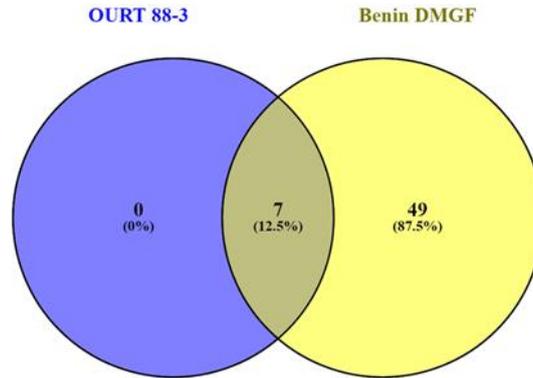


Figure 5. Venn diagram for comparison of significantly expressed swine proteins in a virus dependent manner detected in serum derived EVs.

On EVs extracted from pigs infected with OURT88/3, there were not enough proteins to do further analyses as only 4 swine proteins were differentially expressed. However, the situation for EVs from Benin Δ MGF infected pigs was a different case, as 56 proteins from a total of 942 proteins (selected with 1% FDR and 2 or more unique peptides) were differentially expressed between controls and infected animals at least in the extracellular vesicles enriched fractions. All significant proteins were further analyzed using gene ontology (Figure 6). The most enriched categories were threonine endopeptidase activities (molecular functions), extracellular exosome and blood microparticles (cellular components) and integrin mediated signalling pathway and platelet activation/aggregation (biological processes).

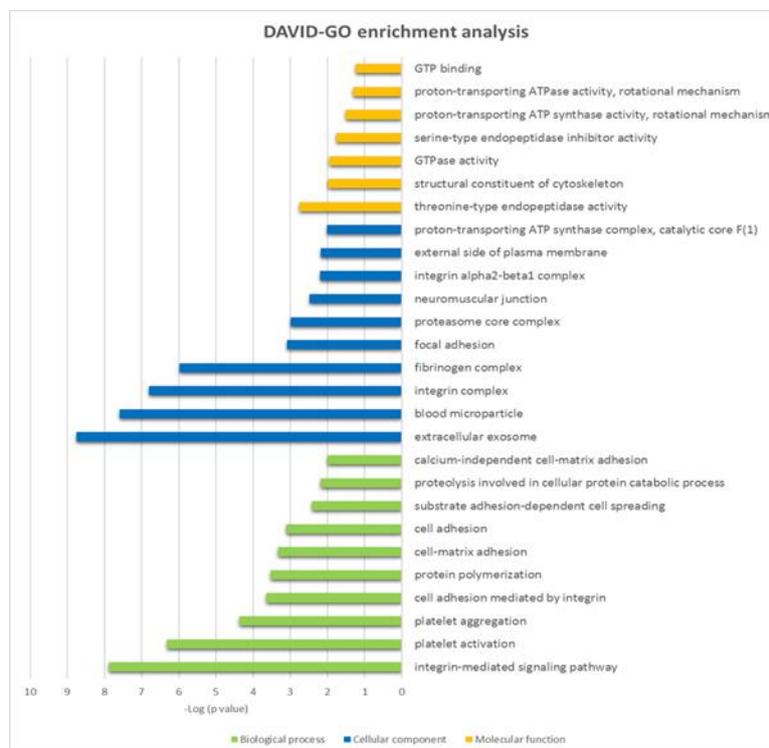


Figure 6. Gene Ontology Analyses of Benin Δ MGF Evs enriched fraction where differentially expressed swine proteins were classified according to the biological process, cellular component or molecular function.

Discussion.

Here we presented the first proteomic comparison of EVs enriched fractions obtained from two different swine infections with attenuated strains of ASFV. Following previous experimental work from swine sera (Montaner-Tarbes et al., 2016), naïve and ASFV infected pigs EVs enriched fractions were evaluated with tetraspanins reported to be present as a surface marker of swine EVs (Montaner-Tarbes et al., 2016). Interestingly, infection with ASFV did not alter the elution profile neither the size distribution of extracellular vesicles within enriched fractions 7 to 10 (Figure 2). Most of the vesicles measured were around 100-150nm in diameter but in the case of ASFV infected group, round small structures were observed in some preparations (figure 2F), believing that could correspond to protein aggregates related to infection that are not seen in all infected samples (viral capsid fragments or other structures related to viral particles). Importantly, in naïve samples, those protein aggregates were not observed at all.

As ASFV infects macrophages we decided to include CD163 as a macrophage-related marker, complemented by CD5, related to T-cells and B-cells. As expected, those markers allowed to differentiate extracellular vesicles enriched fractions independently of the virus used for infection. Although, these two molecules are associated to membranes in cells such as lymphocytes and macrophages, the expression of them after infections in extracellular vesicles derived from serum samples is variable and possibly associated to interactions with viral mechanisms, but warrants, that at least part of the whole group of EVs was produced by cells of those lineages. Interestingly, CD163 was first thought to be one of the main receptors related to viral entry in ASFV, but recent work using knockout pigs lacking the receptor and challenge with virus strain Georgia2007/1 were not protected from infection, demonstrating that CD163 is not necessary for viral entry at least with this strain (Popescu et al., 2017).

In our analyses of EVs enriched fractions, ASFV proteins were detected by FACS in both groups using antibodies against viral proteins p30, p54 and p72, although, MFI values obtained in group B preparations were higher than those in group A. Those three proteins had been reported as structural proteins of early (p30/p54) and late (p72) synthesis and related to viral attachment, viral internalization and capsid formation (Dixon et al., 2013; Muñoz-Moreno et al., 2015; Netherton and Wileman, 2013). Extracellular vesicles has been reported to contain viral proteins in some studies related to ASFV (Afonso et al., 1992; Carrascosa et al., 1985; Esteves et al., 1986). In the particular case of p30, direct translocation through the plasma membrane and release of vesicles containing virally induced proteins (structural and non-structural) had been proposed as secretion mechanisms involved in extracellular transport of this protein, although unknown mechanisms are not discarded (Afonso et al., 1992). Suggested pathways for protein release mentioned in this work supports the idea of Golgi-independent pathway as brefeldin treatment does not inhibit p30 release to the culture media. Although vesicles are mentioned as an alternative route for protein release, (Mittelbrunn et al., 2011) it has been proved that exosomes-like EVs secretion is partially blocked by brefeldin (quantified indirectly by expression of CD81 in western blot, CD63-GFP expression by FACS and microRNA). A deep understanding of secretion mechanisms during viral infections is needed to evaluate further if vesicles release although partially inhibited, are the main responsible for viral protein transport to the extracellular environment.

Several viral proteins had been identified as potential target for vaccination and capable of inducing neutralizing antibodies including these three (p30, p54 and p72) (Arias et al., 2017; Gómez-Puertas et al., 1998). Nevertheless, antibodies for those proteins are not enough to protect pigs against viral replication in a challenge trial, as measured by delay in appearance of clinical symptoms and reduced levels of viremia when compared with the control group (Neilan et al., 2004).

Extracellular vesicles had been studied during viral infections and seems their role is still controversial depending on interactions between the virus and the host. In some cases, viral proteins associated to these vesicles facilitate viral infectivity and impair the immune response in the host, also, are able to transfer cell receptors necessary infections to cells that were previously incapable of interacting with the virus (Chahar et al., 2018; Dias et al., 2018; Pleet et al., 2018; Rozmyslowicz et al., 2003; Vora et al., 2018). It has also been reported that EVs from virus-infected cells, such as HSV-1, could induce the opposite effect and inhibit viral infection by activation of different immune mechanisms on EVs receptor cells (Deschamps and Kalamvoki, 2018; Zhu et al., 2014b). Whether the vesicles derived from ASFV have a role in pathogenesis or immune response is yet to be examined.

Few viral proteins were identified by mass spectrometry in both EVs preparations (group A and B) and only p72 was selected by our exclusion criteria of two unique peptides. Nevertheless, other viral proteins were detected in more than one animal but with 1 unique peptide, making those identifications potential candidates for further evaluation. We suspect that small number protein identification could be related to lack of information of protein sequences for ASFV virus. It is important to mention that ASFV protein database available at UniprotKB has presently 4419 protein sequences, a small number compared with other viruses such as PRRSV (around 25000 sequences) or Influenza virus (788906 sequences in UniprotKB). Another limitation in protein identification is associated directly to biosafety protocols for analysing BSL-3/SAPO4 samples of ASFV. In our case, EVs enriched fractions needed to be heat inactivated at 60°C for 2 hours. This process could contribute to protein degradation and loose of information within EVs enriched samples. Taken together, those results indicate that EVs could be a source of new viral antigens that could explain pathogenicity and immune response during infection with different viral strains.

In addition, it was necessary to evaluate swine proteins on EVs cargo and how viral infection with different strains could modify protein content of those EVs. Interestingly, we identified - 943 swine proteins in all EVs preparations (controls, OURT 88-3 and Benin DMGF infected), however, OURT 88-3 virus infection minimally modified EVs swine proteins, as only seven proteins were differentially expressed. OURT 88-3 has been reported to be a low virulent strain (naturally attenuated) with few to none clinical signs and no detectable viremia post immunizations (Reis et al., 2016) and this low pathogenicity with almost no effect in the host could be reflecting the few modifications of porcine protein expression detected in the EVs enriched fractions when compared with control (non-infected). Importantly, the infections with this attenuated strain protects against a lethal challenge from the same genotype I and even other genotypes (Boinas, 2004; King et al., 2011). By the contrary, Benin DMGF is a deletion mutant of some multigene family genes related to IFN expression similar to those genes absent in OURT 88-3 but there is detectable viremia and clinical signs post-infection possibly associated to another features of this particular deletion mutant (Reis et al., 2016), although it has been characterized as an inductor of protection. By this reason, we hypothesized that there is a relation between viral proteins detected in the EVs enriched fractions we examined and the modifications in swine proteins associated those fractions of EVs (56 significant proteins) including those proteins identified as differentially expressed (7 proteins) on OURT 88-3 infections. Most of these proteins are related to threonine endopeptidase activities, integrin and fibrinogen complexes and platelet activation and integrin mediated signalling pathways and could be associated to virus-host cell interactions involved in the pathogenesis of this deletion mutant of ASFV.

Concluding remarks.

Here, we presented new evidence of different strains of ASFV modifies one aspect of host biology related to extracellular vesicles. Several viruses are able to exploit the EVs formation and secretion pathway in different ways to either interfere on immune responses (Dias et al., 2018; Pleet et al., 2018) or induce blocking of viral entry and replication (Deschamps and Kalamvoki, 2018; Zhu et al., 2014b). We think that exploring new aspects of ASFV infections such as EVs enriched fractions content (viral and host proteins) will contribute to understand how different viral strains interacted with host cells modifying expression of immune response genes and proteins. Recent research works are contributing in this aspect by exploring proteomics of viral particles and intracellular proteome of this virus (Alejo et al., 2018; Oleaga et al., 2017). Our work is more focus on extracellular vesicles enriched fractions as those have shown importance for several viral infections and recently as vaccination strategy in other important swine disease, PRRS virus (Montaner-Tarbes et al., 2018).

As mentioned, ASFV is a complex virus that is causing major outbreaks in Europe and Asia (Cisek et al., 2016; Ge et al., 2018; Olesen et al., 2018), with no vaccine or treatment available for this pathogen but only slaughtering as effective control measure (Rock, 2017). Several genotypes (Bastos et al., 2003) and immune-suppression genes (Correia et al., 2013), long term viral infections (Carrillo et al., 1994; Petrov et al., 2018), gaps in knowledge in biology and immunology and several spreading players such as wild boars and *Ornithodoros spp.* ticks (Gallardo et al., 2018; Kleiboeker and Scoles, 2001; Nurmoja et al., 2017; Pietschmann et al., 2016) generates an urgent need to cover those aspects less explored of this virus to control or prevent pandemics that could affect global economy in animal production.

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Supplementary Information

Table I. Identified viral proteins in OURT 88-3 group of porcine serum derived EVs enriched fractions at 24 days post infection.

Identified protein (by unique peptides)	A1D7	A2D7	A1D24	A2D24	A4D24	A5D24	A6D24	CONTROL 1	CONTROL 2
pL57L	0	1	0	0	0	0	0	0	0
ASFV_G_ACD_00330	0	1	0	0	0	0	0	0	0
VF602_ASFM2 Protein B602L	0	0	1	0	0	0	0	0	0

Table II. Identified viral proteins in Benin DMGF group of porcine serum derived EVs enriched fractions at 24 days post infection.

Identified protein (by unique peptides)	B1D7	B2D7	B1D24	B2D24	B3D24	B4D24	B5D24	B6D24	CONTROL 1	CONTROL 2
gi 858945434 gb AKO62698.1 pJ328L	0	0	0	0	0	1	0	1	0	0
Structural protein p72	1	1	1	2	0	0	0	1	0	0
Hypothetical protein AFSV47Ss_0158	1	1	0	1	0	1	0	1	0	0
VF354_ASFWA Uncharacterized protein B354L	0	1	1	0	1	0	0	0	0	0

Table III. Differentially expressed pig proteins in serum derived EVs fractions from OURT-88/3 infected pigs at 24 days post infection.

Gene name	Protein ID	Protein Name	-Log p-value control vs A_24DPI	Difference control Vs A_24DPI
F8	K7GL28	Coagulation factor VIII	2.123919902	5.42533493
PPBP	F1RUL6	C-X-C motif chemokine	3.219079808	4.493174871
SDPR	I3LDR9	Caveolae associated protein 2	2.191007299	4.085711161
IGHG	L8B0X5	IgG heavy chain	2.084611488	-4.282530149
LOC100517145	F1S3H9	Complement C3 (LOC100517145)	3.885740476	-4.364484406
GOLM1	F1S4I1	Golgi membrane protein 1	1.746130664	-4.767168681
FCN2	I3L5W3	Ficolin-2	2.937884686	-6.029483795

Table IV. Differentially expressed pig proteins in serum derived EVs fractions from Benin DMGF infected pigs at 24 days post infection.

Gene name	Protein ID	Protein Name	-Log p-value control vs A_24DPI	Difference control Vs A_24DPI
F8	K7GL28	Coagulation factor VII	3.02428948	5.472725296
CAVIN2	I3LDR9	Caveolae associated protein 2	1.354488605	5.447482745
PPBP	F1RUL6	C-X-C motif chemokine	2.657114869	5.152716955

ITGA6	K7GT68	Integrin subunit alpha 6	1.749338143	4.950547854
ILK	I3L9C8	Integrin linked kinase	1.988120564	4.824102084
ATP2A3_tv1	K9IW69	Calcium-transporting ATPase	2.223654113	4.816053391
PLEK	F1SJ07	Pleckstrin	1.857273097	4.696280479
N/A	Q28936	Fibrinogen A-alpha-chain	2.342393148	4.619222005
PRKAR1A	P07802	cAMP-dependent protein kinase type I-alpha regulatory subunit	1.288915259	4.504089355
PGAM1	F1S8Y5	Phosphoglycerate mutase	3.006333036	4.45399793
ITGA2	K7GPK0	Uncharacterized protein	2.124166667	4.362858772
EMILIN1	F1SDQ5	Elastin microfibril interfacier 1	1.907341643	4.285336018
FGG	F1RX35	Uncharacterized protein	2.747714093	4.054901441
GPIIb; ITGA2B	Q9TUN6	Glycoprotein IIb	3.917758116	4.045817693
LOC100514666	F1RX36	Uncharacterized protein	2.717573188	4.01283741
FGB	F1RX37	Fibrinogen beta chain	2.431243123	3.964152972
CORO1C	F1RGA9	Coronin	1.391760825	3.911675453
LY6G6F	A0A1L6ZA05	Lymphocyte antigen 6 complex locus G6F	1.826127125	3.890546799
ATP5A1	F1RPS8	ATP synthase subunit alpha	2.847389826	3.819492817
FERMT3	F1RQ01	Fermitin family member 3	2.748417874	3.717049599
GGT1	P20735	Glutathione hydrolase 1 proenzyme	1.736149213	3.669027328
STXBP2	F1SCI9	Syntaxin-binding protein 2	1.886460976	3.653300285
CD61;GPIIIa	D6BR76	Integrin beta	2.180958059	3.606406848
WDR1	K9IVR7	WD repeat domain 1	2.478896272	3.582942327
TUBA4A	F2Z5S8	Tubulin alpha chain	2.185756575	3.549171448
MYL12B	F2Z5V6	Uncharacterized protein	2.763041986	3.532177544
ATP5B	Q0QEM6	ATP synthase subunit beta	1.739728871	3.515419006
TUBB	Q767L7	Tubulin beta chain	1.933344877	3.510186513
DELETED	I3LS04	Uncharacterized protein	1.660923531	3.393268585
TUBB1	A5GFX6	Tubulin beta chain	2.279609551	3.379505157
MYH9	K9IVP5	N-myosin-9	2.648360079	3.367460569
GPIbA	B6ECP2	Glycoprotein Ib platelet alpha subunit	2.681522725	3.341304588
HSPA1B	Q6S4N2	Heat shock 70 kDa protein 1B	1.974503015	3.234610558
RAP1A	I3L5L1	RAP1A, member of RAS oncogene family	2.781650335	3.057108879
ITGB1	F1RVE7	Integrin beta	3.972272372	2.923595746
FN1	F1SS24	Fibronectin 1	2.250441913	- 3.020938555
IGHG	L8B180	IgG heavy chain	2.373289221	- 3.076951027
C4BPA	F1S0J2	Uncharacterized protein	1.775641255	-3.30101649
PSMA1	F2Z5L7	Proteasome subunit alpha type	2.338316455	- 3.325544357
PSMA5	F2Z5K2	Proteasome subunit alpha type	2.851858881	- 3.328734716
IGHG	L8B0W5	IgG heavy chain	2.000232471	- 3.521834691
ITIH1	F1SH96	Inter-alpha-trypsin inhibitor heavy chain H1	2.356516145	- 3.534379196
PSMB1	I3LQ51	Uncharacterized protein	1.601525029	- 3.579036713
SERPING1	F1SJW8	Uncharacterized protein	1.551996553	- 3.582340876
AOC3	F1S1G8	Amine oxidase	1.945752564	- 3.751467133

ACDC;ADIPOQ;APM1	Q6V9B4	Adiponectin	1.68103827	- 3.785552979
THBS4	F1RF28	Thrombospondin 4	1.652059272	- 3.914692879
JCHAIN	F1RUQ0	Joining chain of multimeric IgA and IgM	2.174132721	- 3.966109276
LOC100517145	F1S3H9	Uncharacterized protein	2.822347213	- 4.074431229
DELETED	A0A075B7I7	Uncharacterized protein	2.567990759	- 4.125251452
ITIH3	F1SH94	Uncharacterized protein	2.35014255	-4.13372612
N/A	F1STC2	Uncharacterized protein	2.585282992	- 4.145962079
CD5L	F1RN76	Uncharacterized protein	2.057836202	- 4.201869329
GOLM1	F1S4I1	Golgi membrane protein 1	2.28597367	- 4.857357025
IGHG	L8B0X5	IgG heavy chain	3.60062972	- 5.399693807
FCN2	I3L5W3	Ficolin-2	2.274392861	-5.66368707

CHAPTER IV. GENERAL DISCUSSION

Veterinary vaccines represent approximately 25% of global animal health market and it is continuedly growing due to new approaches in vaccine development as a consequence to cope with new and re-emergent diseases and the sudden increase of the incidence of antimicrobial resistance (Meeusen et al., 2007; Roth, 2011). In that sense, veterinary vaccines are a cost-effective method to prevent animal diseases and nowadays have an important role to improve animal welfare and increase production of livestock animals. Moreover, vaccines are able to dramatically reduce the need for pharmaceutical compounds such as antibiotics (Roth, 2011) and increase the efficiency of food production to cover the necessities for the 7 billion people on earth.

In viral diseases, vaccination have been the cornerstone to overcome infections and the economic burden associated to livestock epidemics; as example, some important viral pathogens controlled using vaccines, with different strategies, are Porcine Circovirus 2 (Blanchard et al., 2003), Pseudorabies virus (Ferrari et al., 2000), Classical swine fever (van Aarle, 2003), West Nile virus (Afzal and Jubelt, 2011), Avian influenza virus and Newcastle disease (Park et al., 2006) among others. Nevertheless, vaccines against 47 major animal pathogens (according to information of the European Union and the European Medicines Agency) have some important gaps in availability. These diseases are caused not only for viruses but also for bacteria and parasites as well. Some examples are African swine fever, tuberculosis, contagious bovine pleuropneumonia (CBPP), Campylobacteriosis, coccidiosis, cryptosporidiosis, cysticercosis, echinococcosis/hydatidosis, hepatitis E, leptospirosis in sheep and horses, liver fluke, Nipah virus, peste des petits ruminants, transmissible spongiform encephalopathies, swine vesicular disease and trypanosomosis (Videnova and Mackay, 2012) There are two main reasons for this lack of vaccines for some diseases: there is no vaccine available due failure of all candidates to provide protection against the pathogen and secondly, there are effective vaccines outside de EU/EEA but insufficient financial support to authorize their use in the European Union. Moreover, we believe there is a third reason to seek new vaccination approaches that is also applicable to re-emerging diseases such as the failure or lack of protection by vaccines available in the market due to highly variable pathogens in which PRRSV virus is included (Vu et al., 2017). We decided to explore new antigen discovery and vaccination approaches for veterinary important diseases mainly focusing on PRRSV to pave the way to increase the availability of vaccines for new or emergent animal diseases and extracellular vesicles were selected for their potential and novel features in terms of cell communication and modulation of immune responses (Simons and Raposo, 2009).

In the beginning, exosomes (a subclass of extracellular vesicles) had been described as vehicles for garbage disposal and protein recycling (Johnstone et al., 1984; Pan and Johnstone, 1983) but 20 years later, new features have been proposed, giving them an important role to play in cellular communication, modulation of immune responses and antigen presentation (Simons and Raposo, 2009; Théry et al., 2002; Yáñez-Mó et al., 2015) and, from that point, several pathogens were in the scope for evaluation of its extracellular vesicle content, adding new information to classical known pathogen-host interactions (Schorey and Bhatnagar, 2008) Extracellular vesicles are now considered as a source of antigens that could be used to produce vaccines and achieve protection by stimulation of immune responses against bacteria, parasites and finally viruses (Anticoli et al., 2018; Kulp and Kuehn, 2010; Montaner et al., 2014).

Extracellular vesicles and virus share similarities such as size, density, cell pathway for biogenesis and a selected mixture of proteins and nucleic acids related to both virus and host (Chahar et al., 2015). Some important examples by which viruses exploits EVs biogenesis pathways are Epstein Barr virus LMP1 protein with immunosuppressive capacities secreted in

exosomes (Dukers et al., 2000), HIV-1 particles incorporating host molecules like tetraspanin CD81, CD63 and molecules such as MHC-II and ICAM-1 in combination with extracellular vesicles secreted by infected cells that contain viral antigens (Gag, Env, Nef and VPU) (Dias et al., 2018) and non-enveloped viral particles incorporated inside extracellular vesicles to be protected from degradation and to increase the infection rate in new susceptible host cells (Santiana et al., 2018).

Therefore, separation of both viral particles and extracellular vesicles seems nowadays a challenge but necessary task to achieve as this is probably the main limitation for using EVs in the context of viral diseases either for antigen discovery or vaccination approaches. Some attempts have been carried out, but no standard and secure method is available today. For example with *in vitro* infections of HIV-1, culture supernatant EVs were separated by immunodepletion using CD45 and acetylcholinesterase antibodies (Cantin et al., 2008); another approach based on combination of flow-through and heparin affinity chromatography, allows separation of HIV-gag virus-like particles and exosomes (Reiter et al., 2018) concluding that this method solves the crucial problem for separation of VLP and extracellular vesicles with concomitant quantification, on a scalable robust platform with chromatography; however, these VLPs are not infectious and do not contain complete viral proteome that could interfere with separation do to shared host lipids and proteins as mentioned previously.

To overcome this problem, it was noticed that there are some pathogens with the ability to cause cryptic infections, in which there is no detectable infectious agent in blood but cells in different tissues could still contain replicating virus at suboptimal rate, integrated viral genomes or dormant stages. In that sense, previous reports indicate that is possible to detect presence of hidden infectious agents by means of EVs content; one example is described for cryptic liver infections caused by *Plasmodium vivax*, in which hypnozoites or dormant stages can induce secretion of EVs from hepatocytes (where the parasite is located) with host and parasite proteins that can be detected by analyzing plasma-derived EVs when the parasite is not present in blood circulation (Gualdrón-López et al., 2018).

Based on these results, we hypothesized that this approach could facilitate isolation and purification of EVs from a viral infection with similar cryptic infections, and we started comparing acute phase and convalescent swine sera for PRRSV infected pigs, as it was previously described that viral particles disappeared rapidly (weeks) from serum, spleen, lungs and mediastinal lymph nodes but persisted in tonsils for longer periods of time (Lamontagne et al., 2003). Animals would be PRRSV PCR negative and PRRSV antibody positive if they had overcome the disease whereas pigs would be PRRSV PCR positive and antibodies against PRRSV positive or negative in an acute phase of the disease. Thus, the animal selection criteria during sera collection and presence of antibodies against PRRSV was a hallmark in this doctoral thesis. Only samples of pigs that had overcome the disease (PRRSV PCR negative and PRRSV antibody positive in serum) were used for enrichment of EVs and later characterization. Our working hypothesis was that pigs that cleared the virus from serum but present in other organs had specific infected cells that will be secreting extracellular vesicles containing viral antigens and other immune-related proteins that constantly stimulates host immune system to maintain a neutralizing and protective response against the virus until complete viral clearance.

Importantly, few viral antigens were found associated to EVs of convalescent sera when no circulating virus was detected in serum, which suggests that virus could be still replicating in lymphoid tissues (Lamontagne et al., 2003; Morgan et al., 2016) but at suboptimal rate, and we

hypothesized that extracellular vesicles with specific viral cargo are released from infected cells to blood circulation and later interact with other immune cells by direct antigen presentation or internalization to induce cytokines or antibody production, causing viral clearance (Montaner-Tarbes et al., 2016). Previous research with other virus demonstrated the capacity of EVs to activate antiviral immune responses; naturally derived extracellular vesicles can block viral replication by activation of IFN innate immune responses; for example for *Herpesvirus* by transfer of components such as Stimulator of interferon genes (STING) or Interferon alpha and anti-HBV (mucin receptor 1 “TIM-1”) activities on hepatocytes (Yao et al., 2018) and for other viruses such as Dengue virus, EVs contain Interferon-inducible Transmembrane proteins capable of transmitting Interferon type I responses from infected cells to new non-infected recipient cells (Zhu et al., 2014b).

In addition to previous results, two different scale-up methods were evaluated to obtain enough protein associated to EVs enriched fractions for later vaccination purposes and to evaluate viral presence after concentration as main concern for safety and animal welfare. First, a methodology based on polyethylene glycol precipitation for preparation of large-scale retrovirus stocks (Cepko, 2001) was carried out in combination with size exclusion chromatography using serum as starting point. This protocol indicated yields of 100X concentrated infectious viral particles, meaning that this procedure does not alter functions of external membrane proteins in envelope virus (Cepko, 2001).

Moreover, a similar protocol for enrichment of extracellular vesicles and applied in a particular case of Graft versus host disease (Kordelas et al., 2014) by means of polyethylene glycol and repeated washes. Results of this research pointed to successful enrichment of EVs (exosomes and microvesicles as well) and improvement of clinical symptoms of the patient by reducing inflammatory responses by means of these mesenchymal stem cell EVs preparations (Kordelas et al., 2014) in which immunological properties of vesicles were maintained post-enrichment. Interestingly, with swine serum preparations concentrated up to 100 times and then separated by this method no virus was detected, increased values for EVs molecular markers were obtained and 10 to 1000 times more particles were quantified by NTA analyses, confirming the successful enrichment of extracellular vesicles on those preparations (Montaner-Tarbes et al., 2016). In relation to EVs membrane proteins, we concluded that enrichment did not alter epitope conformation as PRRSV immune swine sera similarly and specifically recognized them in a capture ELISA assay.

Nevertheless, polyethylene glycol treatment have proved to be immunosuppressive in some cases when used as irrigation solution in kidney and heart transplantation due to interactions between this polymer and the membrane lipids and antigens present in donor cells (Collins et al., 1991; Yandza et al., 2012). Moreover, it has been demonstrated that PEG is a powerful tool when cell membrane fusion is desired (Lentz, 2007; Lentz and Lee, 1999), and in the case of EVs, this fusion may alter molecular functions not related to surface proteins but modifying size, density and content (proteins and nucleic acids) associated to EVs, yet this remain to be proved. We hypothesized that PEG removing by size exclusion chromatography diminished possible side effects in terms of immune suppression mediated by this precipitation agent, although, for later vaccination purposes it was decided to combine other separation methods with similar results in terms of isolation and molecular markers that do not include chemical modifications such as differential ultracentrifugation and size exclusion chromatography.

Later, we decided to further explore the use of EVs in other diseases of veterinary importance at least using the same virus-swine model. In that sense, by examining the list of animal

pathogens with no-available vaccines in the European Union but posing as a possible risk to animal health, African swine fever virus (ASFV) was selected (Videnova and Mackay, 2012). Discovered in 1921, ASFV is the unique member of *Asfarviridae* family, it has icosahedral morphology with a size range of 180-200nm in diameter composed of multiple layers (core shell, inner envelope, viral capsid and external envelope when the virus egress from the cell). The genome is a double-stranded DNA molecule located in the inner core with approximately 170 to 193kbp codifying for 151 to 167 ORFs most differences in genome size are associated to multigene family (MGF) (Dixon et al., 2013; Galindo and Alonso, 2017). This virus is completely different from PRRSV but it shares that no efficacious vaccine is available and problems related with the use of modified live vaccines under field conditions (Arias et al., 2017).

Interestingly, using two different viral strains, one naturally attenuated and a deletion mutant, viral proteins were detected on EVs enriched fractions by two different methodologies (FACS and proteomics) in particular p30 and p72. There were some previous research regarding these results, but no further characterization of these vesicles was carried out in terms of host protein content, immune proteins and nucleic acids due to lack of molecular biology techniques different from western blot and electron microscopy (Afonso et al., 1992; Carrascosa et al., 1985; Esteves et al., 1986). In our case, host cell proteins were identified by mass spectrometry and compared between individuals infected with different viruses and specific modifications were detected in swine protein cargo on EVs in a virus strain-dependent manner showing that naturally attenuated virus (the one with less clinical signs and no viral load in circulation) cause less modification than the deletion mutant. Based on these results, we believe that there is a substantial need for basic research to evaluate the aspects of pathogen-host interactions in ASFV in the context of extracellular vesicles and immune responses. In addition, some new molecular markers were associated to EVs (CD5 and CD163), the last, a known entry receptor for several virus including strains of ASFV different from Georgia 2007/1 (Popescu et al., 2017) and more important, for PRRSV-1 virus (Yang et al., 2018).

As a proof of concept, it was possible to conclude that EVs constitute an important source of pathogen and host antigens in two different virus models (ASFV and PRRSV). In addition, it is possible to scale-up EVs isolation by different methods (chemically and physically) with no alterations of immune properties at least in terms of surface antigens, supporting the idea of further evaluation of EVs as vaccination platforms in a targeted pig-trial.

Lipid, protein and nucleic acid cargo of extracellular vesicles is not a random event and depends on the cell of origin and the state of this in terms of activation or infection by a pathogen (Théry et al., 2009), which will affect the immune response achieved by its interaction with other immune cells in the host (Chaput and Théry, 2011); while most cell types could produce EVs with molecules such as MHC-I that induce T CD8 activation, those derived from antigen presenting cells present functional peptide-MHC complexes in which activation of cytotoxic T CD8 cells is achieved by direct presentation (Andre et al., 2004). Some examples in viral diseases demonstrated that proteins of Ebola, Influenza, Crimean-Congo, West Nile and Hepatitis C virus when combined with an exosome anchoring protein, elicited antigen specific cytotoxic T CD8+ response enough to kill antigen loaded or antigen expressing syngeneic cells (Anticoli et al., 2018). By other side, EVs derived from tumour cell lines and viruses such as HIV-1 and EBV can induce the opposite effect by releasing EVs with signals that triggers suppression of immune responses including increase number of myeloid-derived suppressor cells, T-regulatory cells, and others (Dias et al., 2018; Dukers et al., 2000; Zhang and Grizzle,

2011). These results highlight that different populations of EVs or antigens related to those EVs can trigger the desired immune responses but, also can act synergistically with the pathogen to help to establish a long-lasting infection by suppression of protective immune responses and more important, in the case of PRRSV and other viruses like HCV, evidence showed possible transmission of viral genomes in these extracellular vesicles (Karamichali et al., 2018; Nour and Modis, 2014; Wang et al., 2017).

Two important examples using extracellular vesicles as vaccines to elicit protective immune responses are based on challenge trials with rodent malaria model *Plasmodium yoelii* and avian coccidia *Eimeria*, both Apicomplexa parasites (del Cacho et al., 2016; Martin-Jaular et al., 2011). The first, using exosomes derived from plasmodium infected reticulocytes in combination with a potent adjuvant, induced spleen dependent long-lasting immune protection when animals were challenged with a lethal strain of *P. yoelii*. The second, serum derived exosomes from *Eimeria* infected chicken increase body weight and food conversion efficiency, increased the numbers of IL-2-, IL-4-, IL-6-, and IFN- γ -secreting cells in the intestine and spleen and reduce parasite faecal shedding and gut lesion scores after challenge; Moreover, a subpopulation of EVs characterized as CD80+ elicited stronger immune responses than the CD80- population. In both cases, there are important results that highlights the potential of extracellular vesicles and exosomes to be used as source of antigens for vaccination purposes in animal health.

In bacterial infections, a kind of extracellular vesicles called outer membrane vesicles (OMV), being involved in enzyme transport, bacterial survival, bacterial stress response, biofilm development and in some cases related to pathogenesis (Kulp and Kuehn, 2010). As extracellular vesicles in mammalian cells, OMV protein cargo is specific of the bacterial specie and contain antigens that could be important for development of protective immune responses; more important, the main advantage of proteins being presented on this vesicles is their native conformational state, their natural adjuvant capacity as well as their native form from local environment making them suitable as stable and strong immunogen molecules that could be exploited as vaccination antigens (Kroniger et al., 2018) and some examples include Salmonella, Streptococcus, Staphylococcus and Neisseria (Howlader et al., 2018). Nevertheless, as mentioned before, not in all cases OMV induce protective immune responses, like *Clostridium* OMV that induces release of inflammatory cytokines like TNF- α , but no protection is conferred after vaccination (Jiang et al., 2014). As explained previously, similar situation with viruses and parasites derived EVs, the role of extracellular vesicles is controversial and need to be evaluated in terms of safety and immunogenicity before *in vivo* efficacy trials that finally will show their potential use as vaccines.

For new vaccine strategies in animal health, some features need to be fulfilled before arriving to the market (Knight-Jones et al., 2014). The legislation about immunological products is established in the European legislation (European Medicine Agency - Committee for Medical Products for Veterinary Use, 2016). In general terms, safety evaluation is necessary to ensure animal welfare due to possible vaccine contamination with external pathogens. Other important point is to minimize the presence of adverse effects. On the other hand, vaccine efficacy must be demonstrated carrying out studies under laboratory and field conditions. Thus, according to these guidelines, safety and efficacy must be also studied in field trials performed on the target species distributed in more than one premises. It is evident that this trial to demonstrate the efficacy of EVs as a platform against PRRSV has been carried out under laboratory conditions.

In the safety trial, animals were vaccinated in a dose-escalating scheme. EVs enriched fractions were used as antigen in this trial contained previously identified viral proteins (Montaner-Tarbes et al., 2016, 2018). As mentioned earlier, some *in vitro* studies indicated possible association between infectious viral genomes and extracellular vesicles (Karamichali et al., 2018; Nour and Modis, 2014; Wang et al., 2017). As quality control, all EVs enriched batches were evaluated by PCR for negative confirmation and then used for vaccination. We demonstrated by what we believe is the first *in vivo* targeted-pig trial that EVs enriched fractions obtained from convalescent swine sera were virus free because it was unable to reproduce the disease. Moreover, it causes neither adverse effect nor symptoms associated to respiratory disease on vaccinated animals and all pigs remained negative when serum was tested for presence of viral genome at different timepoints of the study. Remarkably, this vaccination approach seems to be DIVA, as all animals remained negative for the routine test to detect antibodies against PRRSV nucleocapsid protein adding great value to this proposal. To our knowledge, this the first time that a PRRSV vaccine, with a reasonable level of efficacy, is able to differentiate vaccinated from infected animals. From the epidemiological point of view, it is critical to have a DIVA vaccine for any disease because it allows monitoring the status of pig populations and using a vaccine at the same time.

After confirmation of safety using EVs for vaccination, experiments related to immunogenicity and protection were conducted using the same breed of pigs and maximum dose of EVs. In addition, to compare the outcome of this approach, we included a classical vaccination scheme (Montaner-Tarbes et al., 2018). As expected, synthetic peptides induced early and the highest antibody immune response in comparison with EVs. Particularly, the strongest response was associated to the envelope glycoprotein peptide (Gp5), one of the major envelope glycoproteins of PRRSV (Li and Murtaugh, 2012).

However, it was surprising when cellular immune response was evaluated through IFN- γ secretion after stimulation of PBMCs, that only those cells from EVs-vaccinated groups responded to the viral peptide stimuli; neither controls or peptide vaccinated responded after stimulation; furthermore, on EVs vaccinated animals, specific antibodies were detected by western blot against nucleocapsid protein (12-14kDa) and GP5 (25kDa) that were not present in other groups, confirming that our preparation contained at least two viral antigens, capable of priming porcine immune system to develop specific cellular and antibody immune response against PRRSV. It is tempting to speculate that although viral peptides detected on EVs preparation and synthesized *in vitro* were used as antigen, cellular and specific antibody immune responses obtained only from EVs vaccinated animals is associated to how antigens are presented on EVs context and not to the antigen itself, yet this remain to be proved.

To the best of our knowledge, there is no *in vivo* trial comparing the immunological properties of EVs and the properties of detected pathogen proteins without the EVs context. The most similar approach involving antigens and antigen-loaded exosomes has been carried out in a mouse model of *Mycobacterium tuberculosis* (TB) in which macrophages were either infected or pulsed with bacterial culture filtrated proteins and immune responses compared with the classical vaccine BGC. In that sense, EVs derived from macrophages were found to induce antigen-specific IFN- γ and IL-2-expressing CD4⁺ and CD8⁺ T cells in a TB mouse model; in addition, when compared with BGC classical vaccination approach, there was a similar TH₁ response but a more limited TH₂ response as necessary to induce protection against TB infection (Cheng and Schorey, 2013). Some examples in viral diseases using EVs loaded with specific cargo has been investigated (Anticoli et al., 2018), but there is an important gap in comparing the responses against the peptides alone and the immune activation measured when

EVs are used, although cytotoxic CD8⁺ immune responses were detected when primed cells were stimulated.

To test efficacy of this new vaccination approach, animals were challenge with a field isolate of PRRSV-1 already circulating in pig population. By that moment, most animals had developed antibody responses against viral peptides, however only EVs-vaccinated accompanied a good humoral with cellular immune response. Major findings after challenge were related to those groups vaccinated with EVs in which we detected a reduction in almost 10% of viral load in serum, no detectable virus in tonsils (at least in one group), accompanied by activation of mediastinal and mesenteric lymph nodes, no clinical signs and low macroscopic and microscopic lesions in different organs. More important, correlation was found between our EV vaccination approach and the number of IFN- γ producing cells and antibodies against GP5 peptide before challenge and the results of viral load after challenge. In this sense, these two different parameters could be predictive values related to efficacy observed at least in the group vaccinated with EVs.

In contrast, peptide vaccinated animals exhibited an increased viral load, hyperthermia, no activation of mediastinal and mesenteric lymph nodes and some clinical signs including cough and dyspnoea in one of the animals that died after challenge. These results confirmed that immune responses detectable in this trial specifically in the group of EVs vaccinated animals can be translated into better outcome after infection as previously demonstrated by viral load reduction and clinical parameters. Several attempts have been made using inactivated vaccines (EVs could classify in this group as no viral replication occurs after vaccination) with different combination of virus and adjuvants, including some commercially available with recorded immune responses similar to what we presented in this first challenge trial using EVs, with most of them pointing to poor immune responses obtained in vaccinated animals which is translated in small degree of protection after challenged (Hu and Zhang, 2014; Renukaradhya et al., 2015).

Nevertheless, there are some interesting approaches that reflects important degree of efficacy in peptide-based vaccines. For example, a study comparing prototype and commercial subunit vaccines against HP-PRRSV reported a maximum of 80 IFN- γ secreting cells PBMCs per million PBMCs, a significant reduce in viral load between vaccination approaches (prototype and commercially available) and fewer macroscopic and microscopic lesions compared to unvaccinated controls and survival of animals after challenge (DUY et al., 2018); moreover, virus neutralizing antibodies appeared later after challenge with titter values of 1:2, indicating that viral load reduction and protective response were not associated to neutralizing antibodies, and other mechanisms such as IFN- γ secreting cells but not solely, are responsible of viral clearance at least on HP-PRRSV infections (DUY et al., 2018). Interestingly, IFN- γ secreting cells in this study as in our experimental work represent a good indicator of partial reduction in viral load after challenge.

Vaccination trials using nanoparticle-based approaches have demonstrated interesting results. First, exosomes containing artificial micro RNA targeting sialoadhesin or CD163 have demonstrated to have an effect on viral replication by suppressing mRNA expression and later available cell receptors in targeted cells, supporting the hypothesis that exosomes can also serve as an efficient small RNA transfer vehicle for pig cells (Zhu et al., 2014a) as CD163 has the main role during PRRSV infections, and knockout of this receptor causes complete protection against infection (Whitworth et al., 2016). Another example, proved that chitosan particles are able to induce specific antibody immune responses but failed to induce strong antigen-specific

T cell responses, which most researchers pointed as one of the most important aspects for the effective control of PRRSV infection in the absence of neutralizing antibodies (Mokhtar et al., 2017). In this chitosan-based vaccination, there was no improvement in viremia resolution with either encapsulated viral particles or encapsulated peptides, but nanoparticles seem to be a good approach to facilitate antigen delivery to immune cells. Other nanoparticle compounds have been approved by the FDA for antigen and drug delivery, and one example is poly (lactide-co-glycolides) or PGLA (Aguado and Lambert, 1992), facilitating a gradual release of desired antigen over long periods of time. Using this particulate system, viral antigens obtained from sonicated virus were entrapped and used in a intra-nasal vaccination trial suggesting that optimal mucosal immunization induces protective immune response at both mucosal and systemic sites compared to systemic immunization (Dwivedi et al., 2012a). The most promising result of this nanoparticle-entrapped vaccine was to completely clear virus on immunized individuals in a heterologous challenge using a virulent strain of PRRSV-2 in just two weeks post-challenge, indicating the importance of the immunization route and the delivery system.

With all this evidence on vaccinology research about PRRSV in combination with our results in which we have demonstrated that extracellular vesicles represent a promising strategy to be used in vaccination and the role of EVs and nanoparticles in terms of antigen presentation and activation of specific immune responses, we think that efficacy in heterologous challenge, different immunization routes, and scalable production of serum-derived EVs remain as main goals for future pig-trials to try to demonstrate species-transcending protection and industrial development of this novel vaccination platform.

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CHAPTER V. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Concluding remarks (personal opinion and future perspectives).

The field of extracellular vesicles is growing fast, and it is exciting to think how our comprehension of cellular processes linked with EVs could improve human and animal health. From garbage disposal to immunological products and vaccine development, extracellular vesicles nowadays represent a strategy by which the immune system of the host, without almost any help will be able to overcome diseases such as cancer, parasitosis, bacterial and virus infections reducing the use of therapies like antivirals, antibiotics and chemotherapies and more important, as EVs reflects the biological condition of the cells that generated it, will be possible to evaluate the outcome of the treatment and prognosis of the disease.

However, there are some clear limitations in the field that are of our concern to improve research in this and future work. First, there is a substantial need on research for technology associated to characterization of EVs; nowadays we are able to separate an heterogeneous population of extracellular vesicles with a particular size range and characterize its protein and nucleic acid content, but we cannot do precise quantification as available instruments are not powerful enough to differentiate particles, lipoproteins, and extracellular vesicles, although some allow direct visualization of EVs (Cryo-TEM, Confocal microscopy and AFM). Secondly, there is still no a selective pan-marker for populations of extracellular vesicles (meaning microvesicles, exosomes, virus-like particles) that allows recruiting specific populations within biological samples in enough quantities for functional assays. To explain it better, virus and EVs share similarities such as size and density, even host and viral proteins are present in both structures due to shared biogenesis mechanisms inside the cell. In addition, it is difficult to associate immune responses to a particular EVs phenotype when those are used as stimuli to immune cells at least *in vitro*. In our research, we demonstrated that EVs obtained from convalescent sera can induce specific and protective immune responses in vaccinated pigs in a challenge trial against PRRSV-1, but how this response is triggered, how can we improve the outcome and which kind of EVs are involved in the process remains as the most important open question on the table. Future work will imply two steps directly associated to this project. First, to test this approach with other viral strains of PRRSV-1 to evaluate safety, immunogenicity and efficacy in homologous (EVs of convalescent sera from the same virus for challenge) and heterologous (EVs from other PRRSV-1 viral strain convalescent animals) challenges to demonstrate if this approach is strain transcending; and second, a better understanding of PRRSV-1 viral proteins and host proteins with selection of EVs populations from different cell types and its characterization to discover in that complex mixture that serum represents, which of them contributes to activate the desired protective immune responses, and how the virus avoid these mechanisms to survive in the host.

All results derived from this project had been achieved in collaboration between academic and industry research. Most people are used to think that applied research can only be fulfilled in an industrial setting, while basic research is more associated to academics. Nowadays, there is no successful research project, neither financial support if both sides of science do not work together with a common purpose. My experience inside a PhD scholarship program where academic and industrial support were linked, was interesting in what I think could be divided into two different aspects: First, most academic scientific knowledge seems to be related to do research without profit, with a constant struggle to get financial support in order to explain our ideas to the world by means of scientific publications and shared knowledge, and second,

industrial R+D+I (research, development and innovation) most likely is associated to win the race to be the first protecting important findings and receive compensation for what was invested.

In that sense, for me it was possible to discover through this project that both sides have a common goal, seek for knowledge (profitable or not), but it is more delicate how the industry side needs to prove and certify some sort of discovery to be delivered to the market in the final step and how stringent confidentiality, data management and discovery played the most important role in which knowledge dissemination is placed momentarily in the background, publishing is the second most important thing after patent and intellectual property protection. In the end by my experience in this combined research strategy, I think the best research project will be that in which flexibility for creativity and development of ideas is permitted but with the strictness thought that knowledge and research in any area is an investment of money and time, and it is necessary to keep in mind that the most valuable asset for academic and industrial research is the flow of new ideas (scientist) and the legal/financial support (Industry) facilitating in the end a complete translational research as I think this project was in all the aspects.

Conclusions

1. Extracellular vesicles can be isolated in scalable manner to be used as vaccination antigen after characterization.
2. Extracellular vesicles in swine sera from convalescent viral infections contained specific viral and host proteins related to pathogenesis and immune responses.
3. In African Swine fever virus, viral and host proteins are selected in a strain dependent manner. Naturally attenuated strain OURT 88-3 altered less the protein content in comparison with deletion mutant Benin DMGF.
4. Extracellular vesicles enriched fractions from PRRSV-1 convalescent swine sera demonstrated to be safe and immunogenic to be used as a vaccine strategy in a targeted-pig trial.
5. Extracellular vesicles enriched fractions elicit PRRSV-1 specific antibody and cellular immune responses in pigs, particularly against proteins N and GP5, different from those obtained only with synthetic viral peptides.
6. EVs vaccination approach demonstrated to allow differentiation between vaccinated and infected animals (DIVA) at least for PRRSV-1 virus.
7. Prime-boost strategy using EVs and synthetic viral peptides activate immune responses and reduced 10% the viral load in serum versus control group after challenge with a homologous PRRSV vaccine in naïve and previously vaccinated pigs with PCV2 and ileitis vaccine.
8. Prime-boost strategy using EVs and synthetic viral peptides showed no detectable virus and 40% of positive animals in tonsils of naïve pigs and previously vaccinated pigs with PCV2 and ileitis vaccine, respectively at 28 days post-challenge with a PRRSV homologous strain.
9. Synthetic viral peptides vaccinated animals did not showed improvement versus control group after challenge with a PRRSV homologous strain