Adaptation of porcine reproductive and respiratory syndrome virus to modifications in CD163

by

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease of pigs caused by porcine reproductive and respiratory syndrome virus (PRRSV), a virus endemic to most pork producing countries. CD163, a surface receptor expressed on porcine alveolar macrophages (PAMs) is required for infection. Genetically modified pigs with a complete CD163 knock out are non-permissive for PRRSV infection. CD163 is also involved in the regulation of the innate immune response and the removal of hemoglobin-haptoglobin complexes. Therefore, genetically modified pigs lacking complete CD163 may suffer health consequences. Scavenger receptor cysteine rich domain 5 (SRCR5), one of the nine extracellular domains of CD163, forms the point of interaction between CD163 and PRRSV. Mutations in domain 5 were created by inserting proline-arginine (PR) dipeptides in the SRCR5 genome along the domain 5 polypeptide. Constructs were expressed in HEK cells and infected with PRRSV. Mutations were placed in three groups: 1) infection levels similar to WT-CD163; 2) mutations that produced a severe effect on infection; and 3) mutations that resulted in a moderate reduction of infection. The hypothesis tested in this project is that serially passaging PRRSV on HEK cells expressing modifications on SRCR5 of CD163 will result in adaptation of viruses to the CD163 modifications. Furthermore, adaption will be the result of mutations in PRRSV surface envelope glycoproteins, GP2, GP3 or GP4. To test this hypothesis, PRRSV was repeatedly passaged in cells expressing modifications in CD163. Viral sequences were analyzed for the presence of mutations and viral growth patterns were assessed. After six passages, adaptations to PR-22 and PR-58 mutant CD163 constructs were located in non-surface region of the PRRSV matrix (M) protein. The mutation in PR-22 was a threonine to isoleucine substitution at position 141 (T141I) in the endodomain region. The mutation in PR-58 was a tyrosine to histidine substitution at position 86 (Y86H) in the third

transmembrane domain region of M. The results show that mutations in PRRSV occur in response to serial passage on cells expressing modified CD163 proteins; however, mutations were not located in the surface glycoproteins, but in the nonglycosylated M protein.

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Dedication

To my beloved grandmother, Naemi Tuli-mevava Amutenya and my mother, Helena Tuli-mekondjo Lutombi who have sacrificed a lot to give me an education.

1 Literature review

1.1 PRRSV

Porcine reproductive and respiratory syndrome virus (PRRSV) infection results in one of the most economically straining diseases of domestic pigs, called porcine reproductive and respiratory syndrome (PRRS). The economic impact is estimated to be over 600 million dollars annually in the US alone (Holtkamp, Kliebenstein, Neumann, Zimmerman, Rotto, Yoder, Yeske, 2013). The virus is endemic in most pork producing counties. A severe form of the disease, known as highly pathogenic PRRSV circulates in China (Shi, Holmes, Brar, Leung, 2013).

PRRSV is a positive-sense, single-stranded RNA virus, belonging to the Arteriviridae family, which also contains lactate dehydrogenase-elevating virus (LDV) of mice, simian hemorrhagic fever virus (SHFV), and equine arteritis virus (EAV) (Meulenberg & Snijder , 1998). Together with corona, toro and roniviruses, arteriviruses belong to the order Nidovirales (Meulenberg & Snijder, 1998). Two PRRSV genotypes exist: PRRSV-1 and PRRSV-2 also known as North American and European PRRSV genotypes respectively. Phylogenic analysis shows that the two genotypes only share around 70% genetic identity (Darwich, Gimeno, Sibila, Diaz, Torre, Dotti, Mateu, 2011). The greater variability between the two genotypes is illustrated by the glycoprotein 5 (GP5) which shares only 51-55% genetic identity (Murtaugh, Elam, Kakach, 1995).

The PRRSV genome contains seven open reading frames (ORFs): ORF1a, b and ORFs 2-7 (Kutish, Lewis, Doster, Rock, Ali, Allende, Lu, 1999; Meulenberg, Hulst, De Meijer, Moonen, Den Besten, De Kluyver, Moormann, 1993; Snijder, Van Tol, Pedersen, Raamsman, De Vries, 1999). ORFs 1a and 1ab encode for the two large polyproteins: polyprotein 1a and 1ab, which are

proteolytically cleaved into 14 non-structural proteins (nsps) named Nsp1α, Nsp1β, Nsp2 to 6, Nsp7α, Nsp7β, and Nsp8 to Nsp12. ORFs 2-7 on the 3' end are smaller and represent about 20% of the PRRSV genome. These code for the surface proteins, GP2 to GP5, the membrane protein (M) and the nucleocapsid (N) protein. The M and N proteins are non-glycosylated.

GP5, M and N proteins are the major structural proteins, while GP2 to GP4 are known as the minor structural proteins (Wu, Fang, Rowland, Lawson, Christopher-Hennings, Yoon, Nelson, 2005). All PRRSV structural proteins are required for infectivity (Wissink, Kroese, Van Wijk, Rijsewijk, Meulenberg, Rottier, 2005). Even though the PRRSV minor surface proteins, GP2, GP3 and GP4 are not required for virus assembly, they form a heterotrimeric complex that makes the viral particles infectious (Wissink, Kroese, Van Wijk, Rijsewijk, Meulenberg, Rottier, 2005). These results are similar to those observed for the assembly of equine arteritis virus (Wieringa, De Vries, Van Der Meulen, Godeke, Onderwater, Rottier, 2004). Both studies reported that the formation of the heterotrimeric complex is a pre-requisite for the transportation from the rough endoplasmic reticulum to the Golgi apparatus before the assembly of the virions.

The N protein of PRRSV-1 is about 123 amino acids long, while PRRSV-2 has 128 amino acids. A study on Arterivirus N protein topology estimated the transmembrane protein weights 14-15 kDa (De Vries, Chirnside, Horzinek, Rottier, 1992) N form 20-40% of the virion. A morphological study shows that the virus consists of spherical enveloped particles with an isometric core (Dea, Gagnon, Mardassi, Pirzadeh, Rogan, 2000; Mardassi, Athanassious, Mounir, Dea, 1994). Western

immunoblot of PRRSV's major proteins reveal a 29kDa N protein, suggesting that it exists as a homodimer (Mardassi, Helmi, Massie, Dea, 1996).

The M protein is approximately 18-19 kDa (Bautista, Meulenberg, Choi, Molitor, 1996; Mardassi, Helmi, Massie, Dea, 1996; Meulenberg, Petersen-Den Besten, De Kluyver, Moormann, Schaaper, Wensvoort, 1995) and is the most conserved structural protein (Kapur, Elam, Pawlovich, Murtaugh, 1996). The protein has membrane spanning regions crossing the membrane three times (Meulenberg & Snijder, 1998). A region of about 10 to 18 amino acids is predicted to be exposed on the surface of the virion (Dea, Gagnon, Mardassi, Pirzadeh, Rogan, 2000). Similar to EAV (De Vries, Post, Raamsman, Horzinek, Rottier, 1995) and lactate dehydrogenase-elevating viruses (Faaberg, Even, Palmer, Plagemann, 1995), the PRRSV M protein collects in the endoplasmic reticulum and forms a disulfide-linked heterodimer with GP5 (Mardassi, Helmi, Massie, Dea, 1996). Dimerization of defective GP5 or M protein results in retention in the endoplasmic reticulum, suggesting that the formation of the GP-M dimer is essential for transport (Snijder, Dobbe, Spaan, 2003). Formation of the dimer is due to intermolecular bonds at cysteine residues located in the ectodomain regions of GP5 and M (Snijder, Dobbe, Spaan, 2003). M protein also exist as a homodimer in EAV (De Vries, Chirnside, Horzinek, Rottier, 1992b; De Vries, Post, Raamsman, Horzinek, Rottier, 1995) as well as in PRRSV (Mardassi, Helmi, Massie, Dea, 1996). It has been reported that the M-M dimers are not incorporated into the PRRS virion (Mardassi, Helmi, Massie, Dea, 1996). At the initial steps of PRRSV infection, a weak interaction occurs between heparan sulfate, a host protein and the viral M protein (Delputte, Costers, Nauwynck, 2005; Dokland, 2010).

The most variable structural protein is GP5, with about 55% genetic identity between PRRSV -1 and PRRSV-2 genotypes (Kapur, Elam, Pawlovich, Murtaugh, 1996; Murtaugh, Elam, Kakach et al., 1995). The protein is estimated to be approximately 30-42 kDa (De Vries, Chirnside, Horzinek, Rottier, 1992b). GP5 has a 19 amino acid ectodomain followed by hydrophobic regions that cross the membrane three times, terminating in a 70 amino acid endodomain (Chirnside, De Vries, Mumford, Rottier, 1995; Meulenberg , 2000; Meulenberg & Snijder, 1998). The hydrophobicity profile is conserved among all arteriviruses (Meulenberg & Snijder, 1998). Neutralizing antibodies directed against a 24 to 42kDa protein were reported in a study where mice were inoculated with LDV (Cafruny, Chan, Harty, Yousefi, Kowalchyk, McDonald, Plagemann, 1986) and in EAV (Balasuriya, Rossitto, DeMaula, & MacLachlan, 1993; Chirnside, De Vries, Mumford, Rottier, 1995).

PRRSV GP2, GP3 and GP4 interact with each other forming a non-covalent heterotrimeric complex, a pre-requisite for their transportation to the Golgi (Wissink, Kroese, Van Wijk, Rijsewijk, Meulenberg, Rottier, 2005). Additionally, GP4 also interacts with GP5. This interaction is reported to be stronger than those of the other structural proteins (Das, Dinh, Ansari, De Lima, Osorio, Pattnaik, 2010). The same author reported that the structural protein interactions are mediated by GP4. The protein was shown to be crucial for infectivity when a lack of ORF3 expression in an EAV cDNA resulted in no virus production (Van Dinten, den Boon, Wassenaar, Spaan, Snijder, 1997).

PRRSV GP2 is about 29-30kDa (Meulenberg & Petersen-den Besten, 1996; Van Nieuwstadt, Meulenberg, Van Essen-Zanbergen, Petersen-den Besten, Bende, Moormann, Wensvoort, 1996) and a class I transmembrane protein (De Vries, Raamsman, Van Dijk, Horzinek, Rottier, 1995; Meulenberg & Snijder, 1998). For EAV, GP2 consists of four monomeric units (De Vries, Raamsman, Van Dijk, Horzinek, Rottier, 1995). GP2b is about 73 amino acids in length and is embedded within the GP2 ORF (Wootton, Yoo, Rogan, 2000). GP2b is required for PRRSV infectivity but is dispensable for replication and transcription. The small protein likely serves as an ion channel (Lee & Yoo, 2006). GP2 is about 256 amino acids long (Das, Dinh, Ansari, De Lima, Osorio, Pattnaik, 2010), and together with GP4 forms an interaction with the host receptor, CD163 (Das, Dinh, Ansari, De Lima, Osorio, Pattnaik, 2010). GP3 is around 40-45 kDa (Van Nieuwstadt, Meulenberg, Van Essen-Zanbergen, Petersen-den Besten, Bende, Moormann, Wensvoort, 1996). PRRSV GP3 was initially reported to be a PRRSV-2 non-structural protein (Gonin, Mardassi, Gagnon, Massie, & Dea, 1998). However, later studies demonstrated that GP3 is indeed a PRRSV-2 minor structural protein (De Lima, Ansari, Das, Ku, Martinez-Lobo, Pattnaik, Osorio, 2009). Monoclonal antibodies against LDV GP3 are not virus neutralizing (Cafruny, Chan, Harty, Yousefi, Kowalchyk, McDonald,, Plagemann, 1986). PRRSV GP4 is 31-35 kDa (Meulenberg & Petersen-den Besten, 1996; Van Nieuwstadt, Meulenberg, Van Essen-Zanbergen, Petersen-den Besten, Bende, Moormann, Wensvoort, 1996) and monoclonal antibodies directed against this protein possess neutralizing activity (Van Nieuwstadt , Van Essen-Zanbergen, Petersen-den Besten, Bende, Moormann, Wensvoort, 1996)

1.2 CD163

CD163 is a membrane protein that contains a 49 amino acid cytoplasmic region, a 24 amino acid transmembrane domain region, and an extracellular region composed of nine repeating domains (Law, Micklem, Shaw, Zhang, Dong, Willis, Mason, 1993). It belongs to the scavenger receptor cysteine rich (SRCR) family and is expressed on myeloid cells, such as macrophages and monocytes (Fabriek, Dijkstra, Van den Berg, 2005). CD163 is responsible for binding hemoglobin-haptoglobin complexes in blood Fabriek, Dijkstra, Van den Berg, 2005) although it does not bind free hemoglobin (Kristiansen, Graversen, Jacobsen, Sonne, Hoffman, Law, Moestrup, 2001). Due to the presence of greatly conserved domains, it has been suggested that members of the SRCR family are involved in the innate immune responses (Sarrias, Gronlund, Padilla, Madsen, Holmskov, Lozano, 2004). Surface CD163 can be released into the cytoplasm as soluble CD163 by the action of phorbol 12-myriaste 13-acetate. Soluble CD163 is involved in regulating inflammation (Droste, Sorg, Högger, 1999).

The function of CD163 as a PRRSV receptor was first reported when non-permissive BHK-21 cells became permissive to PRRSV infection after being transfected with porcine CD163 cDNA. However, CD163 constructs without the C terminal end could not render these cells permissive to infection. In addition, FK-A6 cells expressing human CD163 and treated with anti-goat anti-CD163 were able to block infection (Calvert, Slade, Shields, Jolie, Mannan, Ankerbauer, Welch, 2007). With the development of new techniques, such as CRISPR-Cas9, it possible to generate genetically modified pigs completely lacking CD163 (Whitworth, Rowland, Ewen, Trible, Kerrigan, Cino-Ozuna, Prather, 2016). It was also shown that PAMs and pigs without CD163 are not permissive to both PRRVS-1 and PRRSV-2 (Wells, Bardot, Whitworth, Trible, Fang,

Mileham, Rowland, 2017). Of the nine CD163 extracellular domains, domain five, also known as SRCR5 is indispensable for PRRSV infection. This was confirmed by the inability of PRRSV to infect HEK cells transfected with a recombinant CD163 lacking SRCR5 (Gorp, Breedam, Doorsselaere, Delputte, Nauwynck, 2010). Similar results were found in study where PAMs and peripheral blood derived monocytes lacking SRCR5 did not support PRRSV infection (Burkard, Lillico, Reid, Jackson, Mileham, Ait-Ali, Archibald, 2017). Moreover, macrophages and pigs with the entire SRSC5 knocked out do not support PRRSV-1 or PRRSV-2 infection (Burkard, Opriessnig, Mileham, Stadejek, Ait-Ali, Lillico, Archibal, 2018).

GP2a and GP4 but not GP3 and GP5 were found to be the points of PRRSV interaction with CD163 (Das, Dinh, Ansari, De Lima, Osorio, Pattnaik, 2010). Pull-down assays showed that the 223 amino acids located at the C-terminal region of CD163 do not interact with PPRSV. These findings are strengthened by another study that used a cDNA of EAV to demonstrate that arterivirus minor structural proteins are important for virus attachment during infection (Tian, Wei, Zevenhoven-Dobbe, Liu, Tong, Snidjer, Yuan, 2012).

1.3 Other factors associated with PRRSV infection

Several other factors have been associated with PRRSV infection. Adding heparinase, a heparin-degrading enzyme to Marc 145 cells drastically minimizes PRRSV infection (Jusa, Inaba, Kouno, Hirose, 1997). SDS-PAGE results showed that PRRSV-1 and PRRSV-2 membrane proteins interact with heparin during the earlier steps of infection (Delputte, Vanderheijden, Nauwynck, Pensaert, 2002).

Sialodhesin, a protein expressed on the surface of macrophages, facilitated the internalization of both PRRSV-1 and PRRSV-2 into naturally non-permissive PK-15 cells transfected with sialodhesin cDNA (Vanderheijden, Delputte, Favoreel, Vandekerckhove, Van Damme, Van Woensel, Nauwynck, 2003). In a study which used soluble sialodhesin receptor from pigs to analyze the interactions between sialodhesin and the siliac acids on the PRRSV structural proteins, it was shown soluble sialodhesin coated on beads and then incubated with PRRSV lysates resulted in only the GP5-M being bound. These results indicate that sialodhesin interacts with the siliac acids on GP5 of the heterodimeric complex but not with GP3 or GP4 (Van Breedam, Van Gorp, Zhang, Crocker, Delputte, Nauwynck, 2010). During the early stages of infection, PRRSV was reported to interact with heparin sulfate followed by a progressive interaction with sialodhesin (Delputte, Costers, Nauwynck, 2005). A study with pigs lacking for the sialodhesin gene showed that this protein is not necessary for PPRSV infection, as these pigs were as permissive as those expressing the gene (Prather, Rowland, Ewen, Trible, Kerrigan, Bawa, Green, 2013). Moreover, MARC 145 cells, which do not express sialodhesin, are permissive to PRRSV infection (Jusa, Inaba, Kuono, Hirose, 1997).

Non-muscle myosin heavy chain 9 (MYH9) is one of the three isoforms of non-muscle myosin II (Vicente-Manzanares, Ma, Adelstein, Horwitz, 2009). The protein was reported to act as bridge between PRRSV and CD163 during the initial steps of infection (Gao, Xias, Wang, Zhang, Zhao, Zhou, 2016). The same author reported that even though MYH9 and MYH10 were reported to be structurally similar, MYH10 plays only a very small role in PRRSV infectivity.

Vimentin is a type II intermediate filament in the cytoskeleton (Kim, Fahad, Shanmukhappa, Kapil, 2006). BHK-21 and CRFK cells transfected with vimentin became permissive to PRRSV infection, suggesting that this protein is involved in PRRSV infection. This was confirmed by the fact that the use of antibodies against vimentin blocked PRRSV infection on MARC 145 (Kim, Fahad, Shanmukhappa, Kapil, 2006).

CD151 a protein expressed on the surface of cells of the hematopoietic lineage, belongs to the transmembrane 4 superfamily and functions in a complex of signaling complexes (Fitter, Sincock, Jolliffe, Ashman, 1999). CD151 was shown to interact with PRRSV at the 3′ UTR viral RNA. Transfection of BHK-21 cells with CD151 cDNA resulted in the ability of PRRSV to infect these naturally non-permissive cell lines (Shanmukhappa, Kim, Kapil, 2007). It has been shown that transfection of SiRNA against CD151 into MARC145 cells as well as treating PRRSV infected cells with an antibody directed against CD151 resulted in a reduction or inhibition of PRRSV infection.

1.4 Clinical disease

The transmission of PRRSV is mainly through the respiratory route (Larochelle, D'Allaire, Magar, 2003). The virus was also reported to be acquired by ingesting contaminated food as well as sexually (Pileri & Mateu, 2016). In a study were boars were infected with PRRSV, viral RNA was detected in semen (Christopher-Hennings, Nelson, Hines, Nelson, Swenson, Zimmerman, Benfield, 1995; Swenson, Hill, Zimmerman, Evans, Landgraf, Wills, Ciszewski, 1994). It was

reported that PRRSV reduces the quality of semen when numerous spermatozoa defects were detected in PRRSV infected boars in comparison to non-infected boars (Schulze, Revilla-Fernández, Schmoll, Grossfeld, Griessler, 2013). The clinical signs of the disease includes short-lived fever, respiratory disease and abortions in pregnant sows (Hopper, White, Twiddy, 1992; Terpstra, Wensvoort, Pol, 1991). An "atypical" PRRSV was reported in China.

PRRSV interacts with other infectious pathogens and the environment to cause a combinational disease condition known as porcine respiratory disease complex (Opriessnig, Giménez-Lirola, Halbur, 2011). Some of the pathogens include viruses like porcine circovirus 2 (PCV2) and swine influenza, as well as bacterial pathogens such as *Mycoplasma hyopneumoniae* and *Pasteurella multocida*. PRRSV and PCV2 cause a disease complex known as porcine circovirus associated disease where PRRSV was reported to magnify the replication of circovirus (Niederwerder, Bawa, Serao, Trible, Kerrigan, Lunney, Rowland, 2015).

Clinical manifestations of PRRS are more evident in piglets that have just been weaned, as well as in pigs who had just entered an endemic herd (Balasch, Fort, Taylor, Calvert, 2018). PRRSV GP5 antibodies can be detected after one week of vaccination, while antibodies directed against the membrane and nucleocapsid proteins are only detected after 14 days (Loemba, Mounir, Mardassi, Archambault, Dea, 1996). These results correlate with those found by another study on the generation of PRRSV antibodies which demonstrated that pigs infected with various isolates of PRRSV produced neutralizing antibodies only after one week of infection, with a range of 7-28

days. They did not detect any neutralizing antibodies against the nucleocapsid protein (Plagemann, 2006).

A vaccine study based on live and killed vaccines used in the field, reported that protective immunity was only generated after inoculation with the live vaccine and not with the killed vaccine (Zuckermann, Garcia, Luque, Christopher-Hennings, Dorster, Brito, Osorio, 2007). However, there has been concerns on the possibility of reversion to virulence of some modified live vaccines (Storgaard, Nielsen, Stadejek, Botner, Oleksiewicz, Forsberg, 2001). Various other vaccine platforms such as DNA vaccines and recombinant DNA vector vaccines have been investigated; however, none of these have proven successful (Kimman, Cornelissen, Moormann, Rebel, Stockhofe-Zurwieden, 2009).

Controlling PRRSV in the field has been challenging because the introduction of the virus into a heard is crafty, the virus is easily transmittable between pigs both vertically and horizontally, it can be persistently maintained in the herd at low levels and the virus can interact with opportunistic microorganisms to cause complex multifactorial syndromes.

Even though vaccines against PRRSV do exist, they are not efficient in controlling PRRSV as they do not provide protection against all PRRSV strains. Moreover, the modified live vaccines have a potential to revert to virulence and can result in persistent shedding. Even though the PRRSV proteins interacting with CD163 are known, the exact PPRSV amino acid sequences interacting with it are not yet known. An in-depth knowledge and understanding of the amino acid sequences

interacting between PRRSV and CD163 will create opportunities for research on novel vaccine development.

The objective of this study was to determine if propagating PRRSV-2 on HEK cells expressing modifications in CD163 (*in vitro* system) will result in mutations in PRRSV which will allow the virus to adapt to growth on these cells. Our overall hypothesis was that repeated passaging of PRRSV on cells expressing mutations on SRCR5 of CD163 will result in the adaptation of the virus to growth on the modified CD163. Furthermore, adaptation will be evident by mutations in the viral glycoproteins. The approach was to serially passage PRRSV-2 expressing a red fluorescent protein (PRRS-RFP) on HEK cells expression modified CD163 fused to an enhanced green fluorescent protein (CD163-EGFP). The structural genes RT-PCR amplified and sequenced, and the genetic sequences compared to the parent PRRSV-RFP. Adaptation of PRRSV-RFP to growth on cell lines expressing modifications in CD163 would be evident by the appearance of amino acid substitutions in structural glycoproteins, GP2, GP3, GP4 and possibly GP5 and by an increase in the level of infection with increasing passaging.

2 Materials and methods

2.1 Cells and virus

The porcine CD163 HEK cell lines used in this project were prepared previously. CD163 cDNA variants were made by inserting proline-arginine (PR) residues at position 497, 507, 513, 517, 529, 530, 538 and 575 of CD163 to produce the SRCR5 variants PR-9, PR-22, PR-32, PR-38, PR-42, PR-58, PR-55, PR-68 and PR-100, respectively. All mutations were within SRCR5 of CD163 as shown in Figure 2-1. For a positive control, a wild type CD163 was included. The synthesized DNA segments were subcloned into a mammalian expression vector that contained a puromycin selection cassette. The plasmids were then transfected into HEK 293 cells. The cells were maintained and passaged in a selection medium containing puromycin for about three weeks. Expression of CD163 was confirmed by immunostaining using anti-CD163 monoclonal antibody and flow cytometry. After three weeks, the cells were confirmed to express CD163.

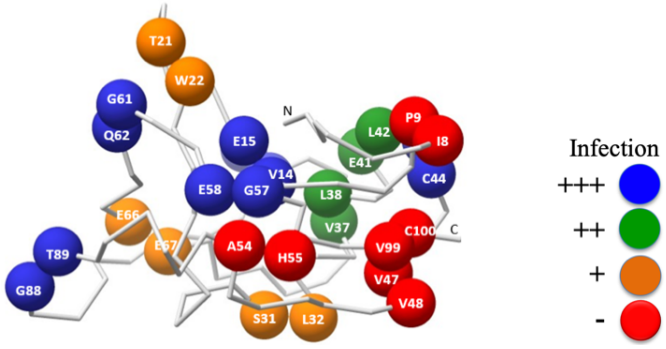


Figure 2-1: Heat map representation showing the Locations of peptide mutations along SRCR5 of CD163.

Cells were propagated in a T-25 tissue culture flask containing 5mL of maintenance media with Minimum Essential Medium Eagle 1X with Earle's salts & L-glutamine (Coning Cellgro, catalog

#: 10-010-CM), 7% Fetal bovine serum heat inactivated (Midsci catalog #: USDAFBSHI), 1.5% Penicillin Streptomycin (Thermo Fisher Scientific catalog #: 15070-063 100) and 1% Amphotericin B (Thermo Fisher Scientific, catalog # 15290018). Cells were incubated at 37ºC and 5% CO_2 and passaged when 90% confluent. The cell monolayer was washed once with 5mL sterile PBS and trypsinized using 0.5ml of 0.25% Trypsin-EDTA (Thermo Fisher Scientific, catalog #: 25200-072) and re-suspended with maintenance media. For cryopreservation, cells from a T-25 flask were re-suspended in 3.5 ml freezing media (50% MEM media, 50% FBS and 10% Dimethyl Sulfoxide, ACS MP Biomedicals, catalog no: 191418 of MEM and FBS volume). The cells were frozen in the Mr. Frosty™ Freezing Container (Thermo Fisher Scientific, catalog no: 5100-001) at -20ºC for 24 hours and finally conserved in liquid nitrogen.

PRRSV-RFP was made from an infectious clone as described in (Pei et al. 2009, 91-99), which possessed a green fluorescent protein (GFP) inserted between the stop and start codons of ORF1b and ORF2. A transcription regulatory sequence (TRS) was inserted between twenty-two nucleotides downstream and seventeen nucleotides upstream of the GFP and the start of ORF2. To make PRRSV-RFP, the GFP was substituted with RFP. PRRSV-RFP was propagated on MARC-145 cells in a T-25 tissue culture flask. The virus was harvested after 72 hours of infection, aliquoted into 1.5mL cryogenic tubes (Thermo Fisher Scientific, catalog # 5000-1010) and stored at -80ºC. Virus titration was done on confluent MARC 145 cells on a 96-well plate using a 1:10 serial dilution and incubated at 37ºC and 5% CO_2 for 72 hours. Virus concentration was estimated using the Reed-Munch Infectivity Calculator program and was expressed as tissue culture infectious dose 50 ($TCID_{50}$). The level of infection observed as red fluorescent cells under a Nikon

eclipse TE2000-S microscope. Three cell lines plus a positive control with the highest level of infection were selected for further studies.

2.2 Immunofluorescent staining for surface CD163

Cell monolayers were washed with sterile PBS and fixed with 4% paraformaldehyde solution, EM GRADE solution (Electron Microscopy Sciences, catalog no:15710-S) for 5 minutes. The solution was removed, and cells allowed to dry for 10 minutes. Cells were incubated with mouse anti-pig CD163 (Bio Rad, catalog # MCA2311GA) diluted 1:100 in PBS, for 1 hour at room temperature. The monolayers were washed with PBS and incubated with Alexa fluor™ 488 goat anti-Mouse IgG (Thermo Fisher Scientific, catalog #: A-11001) diluted 1:400 in PBS. After 1 hour at room temperature, cells were washed twice with PBS and incubated with 0.1% Triton-x 100 T-8532 to permeabilize the cells. After a wash with PBS, a single drop of NucBlue™ (Thermo Fisher Scientific, catalog #: R37606) was added to each well to stain the nuclei. Green fluorescence indicated CD163 expression on the cell surface membranes, while the nuclei were stained with blue.

2.3 Adapting PRRSV to cells expressing CD163 SRCR5 mutations

The PRRSV parent stock was adapted to growth on the selected cell lines by serial passage. Serial 1:2 dilutions were placed across a 24-well plate and incubated at 37ºC in 5% CO_2. At three hours post-infection, the monolayers were washed and replaced with fresh media. The levels of infection, as determined by the percent fluorescent cells, were recorded at 24- and 48-hours post-infection.

Supernatants were harvested and pooled and dilutions repeated on a fresh 24 well plate of cells. Viruses were passaged this way for at least six times

2.4 Sequencing

After harvesting each virus from infected cells, Lysis/Binding Solution Concentrate (Thermo Fisher Scientific, catalog # B8500G5) was added to each well. The cells were scrapped off the cell culture plate surface with a pipette tip, dispensed into eppendorf tubes and preserved at -80ºC. Total RNA was isolated from the infected cells using the MagMAX™ -96 Viral RNA Isolation Kit (Thermo Fisher Scientific catalog #: AMB18365) according to the manufacturer's instructions. Viral RNA concentrations were measured using the Nanodrop machine and temporarily preserved at -4ºC. Viral RNA was amplified using Superscript™ III One-Step-PCR System with Platinum™ Taq DNA Polymerase kit (Thermo Fisher Scientific, Catalog no: 12574-026). The RNA primers used for amplification and sequencing were designed based on the PRRSV-P129 GenBank sequence (AF494042.1) viewed with CLC Mainbenchwork 8 software. The properties of the primers were analyzed using the OligoAnalyzer tool (Integrated DNA Technologies). Three fragments were amplified from each virus. Fragment 1 consisted of 1502 nucleotides and was amplified using the primers ORF1b F and ORF3 R. Fragment 2 was amplified using the primers ORF4 F and ORF5 R and was 1150 nucleotides long. Fragment 3 had 1221 nucleotides and was amplified using the primers ORF5 F and ORF7 R. Primer sequences are listed in Table 2-1. RT-PCR was carried out in a final volume of 50µL per reaction. Each reaction tube contained 0.8µg viral RNA, 25µL 2X Reaction Mix, 1 µL forward primer, 1µL reverse primer and 2µL Taq polymerase. Fragment 1 reaction was set at 50ºC for 30 minutes and 94ºC for 2 minutes (1 cycle) for cDNA synthesis and pre-denaturation, followed by 40 cycles of 94ºC for 15 seconds for

denaturation, 60ºC for 30 seconds for annealing and 68ºC for 1.5 minutes for extension. Fragments 2 and 3 reaction settings were as above except that the extension time was set at 68ºC for 60 seconds. The PCR products were run on a 1% Agarose electrophoresis gel. The gel was run at 150 volts for 30 minutes and results were observed with a GelDoc-It™ Imaging System (UVP). The sizes of the PCR products were determined by correlating the positions of the bands in the gel to sizes on GeneRuler™ 1kb DNA Ladder (Thermo Fisher Scientific, catalog no: SM1333). PCR products were excised from the gel using a scalpel blade and transferred to appropriately labelled 1.5 mL eppendorf tubes.

The excised PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, catalog #: A9282) as per manufacturer's instructions. DNA concentration was measured the Nanodrop machine and stored at -4˚C. The sequencing primers are listed in Table 2-1 and the PRRSV genetic sequence they cover is shown in Figure 3-2. Sequences were assembled using the CLC Mainbenchwork 8 software and compared to the parent GenBank sequence (AF494042.1).

Table 2-1: PCR and sequencing primers.

Primer	5' Sequence 3'	Nucleotide location
ORF1b F	ATGATGCGT TTCGTGCGCGCC	11 943 - 11 963
ORF2only R	TGCTGAAAATCATGAAGCTTTGGC	12 656 - 12 679
ORF2 F	ATGAAATGGGGTCTATACAAAGCC	12 057 - 12 080
ORF2 R	TCACCATGAGTTCGAAAGAAAAAT	12 804 - 12 827
ORF3 F	ATGGCTAATAGCTGTACATTCCTC	12 680 - 12 703
ORF3 R	CTATCGCCGTGCGGCATT	13 427 - 13 444
ORF4 F	ATGGCTGCGTCCCTTCTTT	13 225 - 13 243
ORF4 R	TCAAATTGCCAGTAGGATGGCAAA	13 738 - 13 761
ORF5 F	ATGTTGGGGAAATGCTTGACC	13 772 - 13 792
ORF5 R	CTAGAGACGACCCCATTGTTC	14 354 - 14 374
ORF6 F	ATGGGGTCGTCTCTAGACGACTTT	14 359 - 14 382
ORF6 R	TTATTTGGCATATTTGACAAGGTT	14 860 - 14 883
ORF7 R	TCCCTTGCCTCTGGACTGGTT	14 972 - 14 992

The table contains primer names, their sequences and the regions they cover on the PRRSV genome sequence.

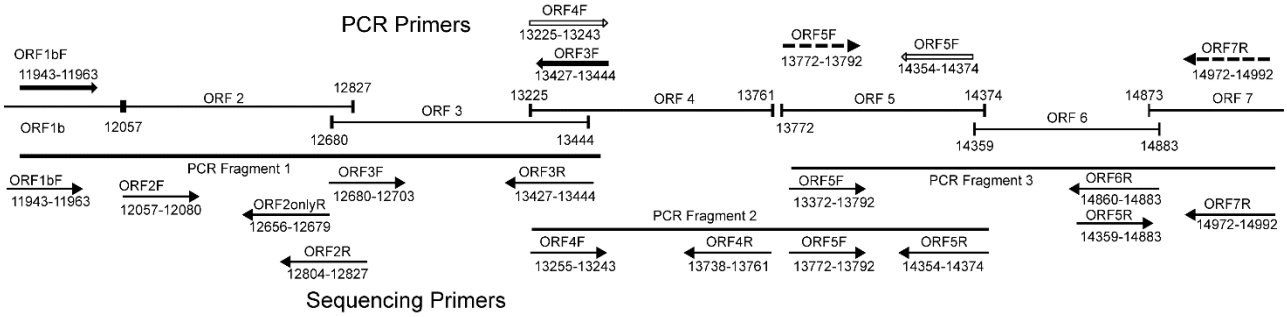


Figure 2-2: Primers used for PCR amplification and sequencing of ORFs 2 through 6. The top of the figure shows the primers used for PCR amplification. The result was the amplification of three PCR fragments. The bottom of the figure shows the primers used for sequencing

3 Results

3.1 Screening the cell lines for infection:

The PRRSV-RFP had a titer of 5 620 $TCID/_{50}$ (log_{10} concentration of 3.7). The results for the nine cell lines are presented in Table 3-1. Overall, the cell lines PR-9, PR-32, PR-38, and PR-100 showed very low levels of infection and were excluded from further study. PR-42 and PR-67 showed the highest level of infection; whereas, PR-9, PR-22 and PR-58 cells showed intermediate levels of infection.

Table 3-1:Infection of CD163 constructs with PRRSV-RFP*

CD163	1/virus dilution			
Construct	10	40	160	640
PR-9	++	+	+	-
PR-22	++	++	+	-
PR-32	-	-	-	-
PR-38	-	-	-	-
PR-42	++	+	+	+
PR-55	+	+	-	-
PR-58	++	+	+	-
PR-67	++	+	-	++
PR-100	+	+	-	-

* Relative levels of infection were determined by the presence of PRRSV RFP fluorescence at passage one: (-) no red fluorescence; (+) low to medium positive for RFP fluorescence; (++) high positive for RFP expression. Highlighted rows identify CD163 constructs selected for virus adaptation. Infected cells were observed at 48 hours after infection. The gray highlighted cells continued to passage two.

3.2 Serially passaging of PRRSV on cells expressing mutated CD163

PRRSV-P129 RFP was serially passaged on the cells expressing modified CD163 at least 6 times. All viruses showed comparable levels of infection to the wild type CD163 with at least 70% infected cells up until the third passage. From passage 5 the number of infected PR-22 cells had a noticeable decline where the highest percent of infected cells being about 50%. At passage three in PR-58 cells, less than 50% of infected cells were observed and infection level continued to decline until no infection was observed at passage seven. PR-67 cells had an infection level of less than 50% at passage five and between 15%-20% at passage six. See Table 3 for presence and absence of infection in specific cell lines at different passages and figure 4 for infection images.

Table 3-2:Serial passage of PRRSV-RFP on CD163 constructs expressed in HEK cells

Cell line	**Virus Passage number**[*1]								
	P1	P2	P3	P4	P5	P6	P7	P8	P9
PR-22	+	+	+	+	+	+	+	+	ND[*2]
PR-42	+	-							
PR-55	+	-							
PR-58	+	+	+	+	+	+	+	-	
PR-67	+	+	+	+	+	+	ND[*2]		
WT*3	+	+	+	+	+	+	+	+	ND[*2]

*1. The results shown as positive (+) or negative (-) for the presence of PRRSV-RFP fluorescence.

*2. ND, Not Done

*3. WT, cells that express the wild-type CD163

Examples of CD163 staining are shown in Figure 3-1 for WT, PR-22, PR-58, and PR-67. The results showed that all cells were positive for CD163. The exception of PR-67 which showed only a few positive cells.

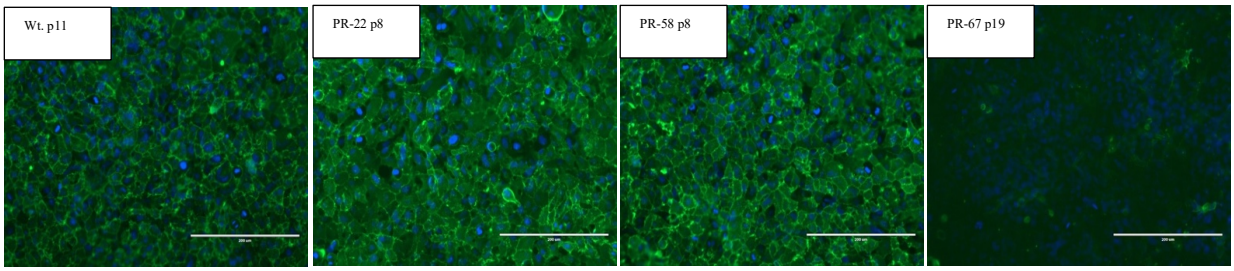


Figure 3-1: IFA for CD163 expression.

The image shows IFA results for the cell lines used to adapt P129-RFP on. HEK cells expressing wild type CD163 (wt.) passage 11, constructs PR-22 and PR-58 passage eight had similar levels of CD163 expression on their surface.

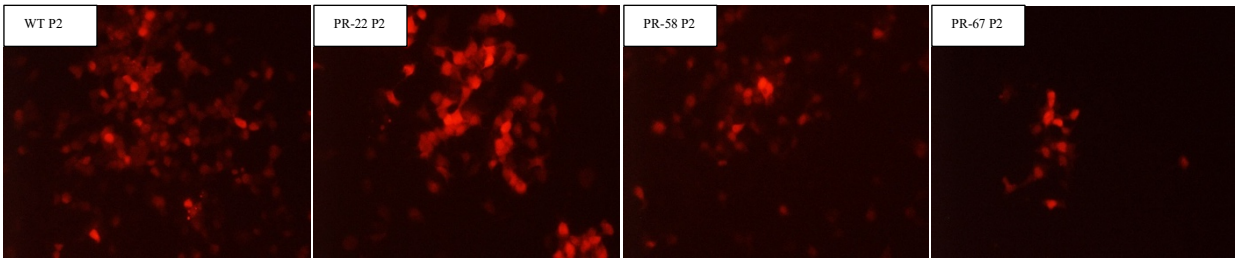


Figure 3-2: Examples of infection of modified CD163 cells with PRRSV-RFP. The cells were viewed under a fluorescence microscope at 2 days post infection using the same dilution of virus.

3.3 Peptide sequence differences between the parent stock and GenBank sequence.

PRRSV P129 surface proteins are 256, 254, 178, 200 and 174 amino acids long for glycoprotein 2 (GP2), GP3, GP4, GP5 and the M protein, respectively. Only a partial sequence could be obtained for GP2 from the parent stock. Compared to the Genbank sequence, the parent stock GP2 has mutations at amino acid positions 118 and 189 where isoleucine was substituted for threonine (I118T) and glutamine was substituted for arginine (Q189R) respectively resulting into a 99.32% sequence identity. The parent stock's GP3 amino acid sequence in comparison to the Genbank

sequence had mutation at amino acid position 96, 102 and 143 where proline was substituted for serine (P96S), serine was substituted for glycine (S102G) and phenylalanine for leucine (F143L) respectively, resulting into a 99.48% identity. The GenBank sequence and parent stock GP2 and GP3 sequence alignments are shown in Figure 3-3. GP4, GP5 and the M protein of the Genbank and parent stock sequences were 100% identical.

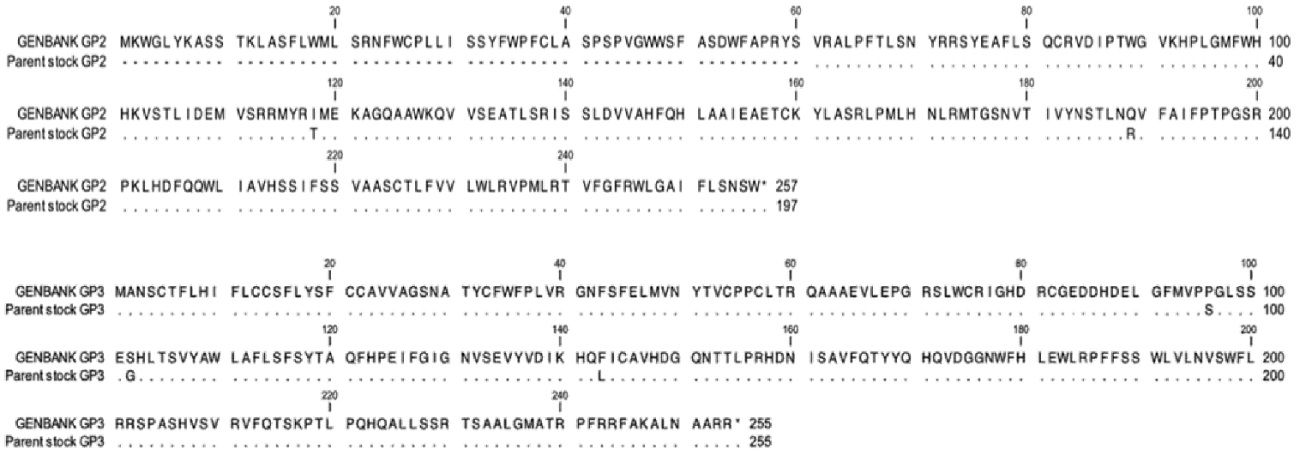
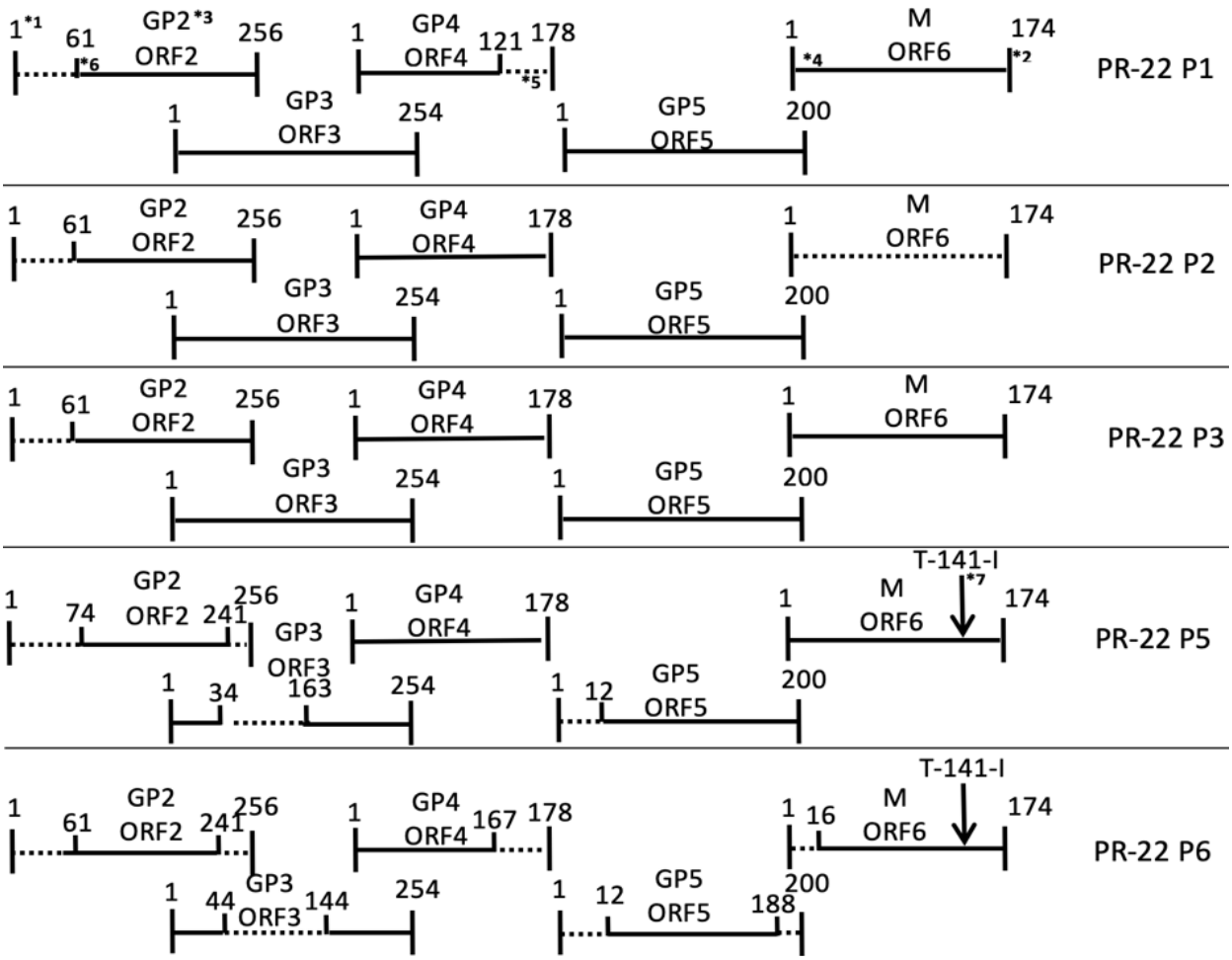


Figure 3-3: GP2 and GP3 amino acid sequence alignments between the GenBank sequence and the parent stock virus. The parent stock sequence is partial with the first 60 amino acids missing and has mutations. Compared to the Genbank sequence, the parent stock partial sequence has mutations at the 118th and the 189th amino acids where isoleucine was substituted for threonine (I118T) and glutamine was substituted for arginine (Q189R) respectively.

3.4 Peptide sequences during serial passage of adapted viruses

Passages five and six of PR-22 and PR-58 sequences were "noisy" and therefore only partial sequences could be retrieved. The last fifteen amino acids of GP2 for virus PR-22 passages five and six, and virus PR-58 passage five were missing. At least eighty-three amino acids in the middle

of the GP3 sequence were missing in the passage five and six sequences of viruses PR-22 and PR-58. In GP4 at least ten last amino acids were missing, affecting passages one and six of virus PR-22 and passages five and six of virus PR-58. In GP5, the first twelve amino acids are missing in both passages five and six of viruses PR-22 and PR-58, and at least twelve last amino acids missing in passage six of virus PR-22 and passage five of virus PR-58. In the M protein at there were at least nine missing amino acids in passage six of virus PR-22 and passage five of PR-58, while no sequence was retrieved for passage two of virus PR-22. PR-22 and PR-58 sequence results are shown in Figure 3-4.



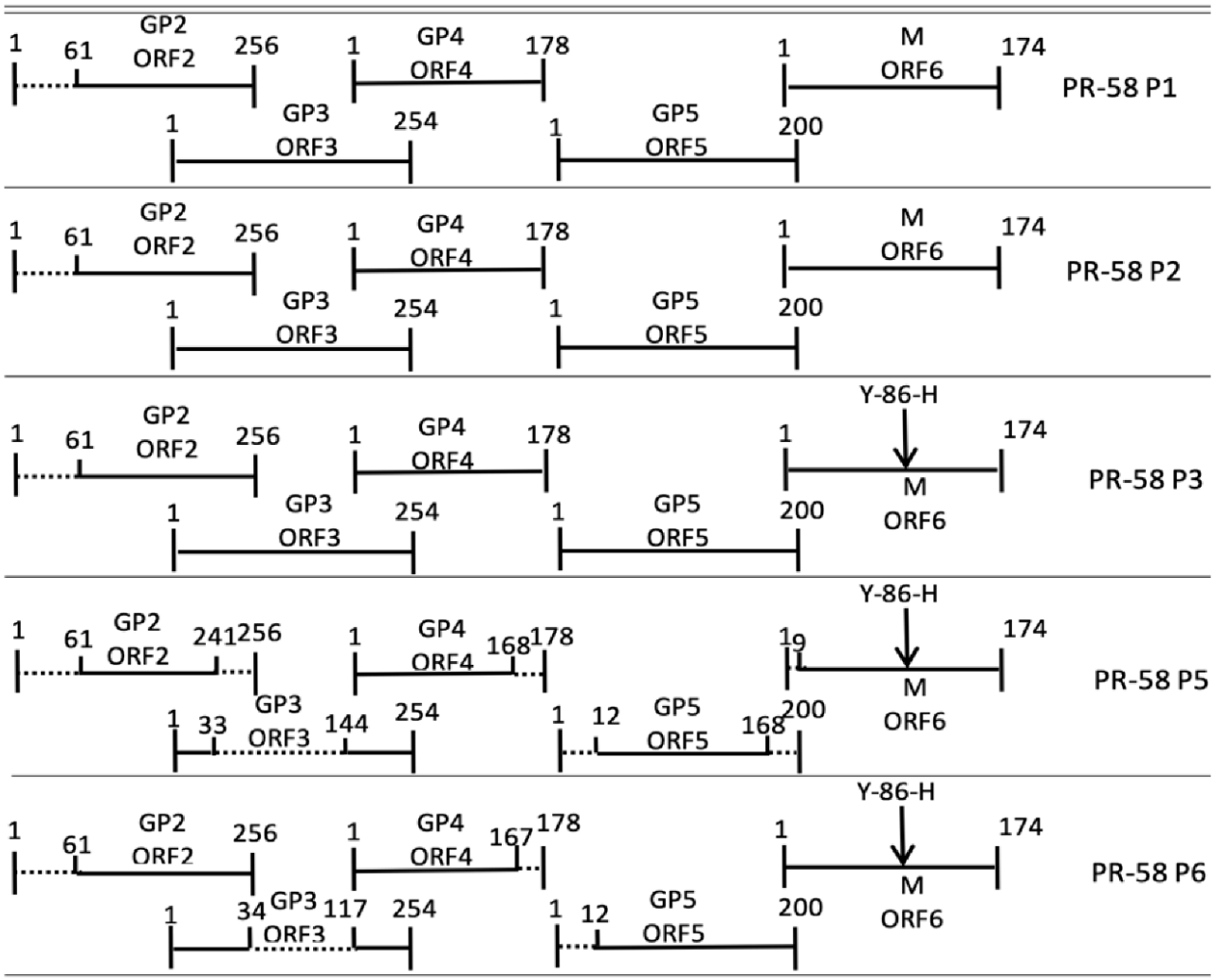


Figure 3-4: ORF2-6 sequence results for PRRSV-P129 during serial passage on PR-22 and PR-58 CD163 cell lines. *1. Amino acid position. *2 beginning or end of glycoprotein amino acid sequence. *3 Open reading frame and the surface protein it encodes for. *4 Length of amino acid sequence. *5 no sequence data. *6 beginning or end of a partial amino acid sequence. *7 Location of mutation. Amino acid mutations were observed in M protein of viral sequence PR-22 at passages 4 and 5 where threonine was substituted for isoleucine, and M protein viral sequence PR-58 at passages 3, 4 and 6 where tyrosine was substituted for histidine.

In comparison to the parent stock, GP2, GP3, GP4 and GP5 sequences of viruses adapted to growth on PR-22, PR-58 and the only sequenced passage of PR-67 cells had no mutations. The M protein of virus PR-58 had a synonymous mutation at amino acid position 33 located in the transdomain region: thymine in the codon GCT (located at position 97 to 99) was substituted for cytosine to make the codon GCC. GCT and GCC both code for alanine. Non-synonymous mutations were seen in the M protein of both virus PR-22 and PR-58. Passages five and six of virus PR-22 had the codon ACT coding for threonine (amino acid position 141) changed to ATT coding for isoleucine (T141I). Passages three, five and six of virus PR-58 had the codon TAC, which codes for tyrosine (amino acid position 86), changed to the codon CAC which codes for histidine (Y86H). The non-synonymous mutation observed in virus PR-22 is in the endodomain region, while the that observed in virus PR-58 is located transdomain region. The locations of the mutations in the M region are shown in the model in Figure 3-5.

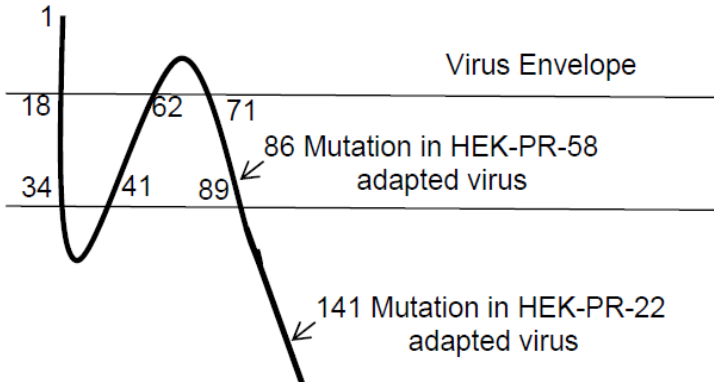


Figure 3-5: Model of PRRSV M protein in relation to the location of PRRSV-RFP mutations. The numbers identify the amino acids in the M protein peptide sequence. The portion above the Virus envelope is the ectodomain region which is followed by the transdomain region between the two solid lines and finally the endodomain region. Virus PR-22 mutation at amino acid position 141is located in the endodomain region and Virus PR-58 mutation at amino acid position 86 is located in the transdomain region.

Viruses PR-22, PR-58 and PR-67 were passaged at least six times, their growth properties are summarized in Table 3-3. Virus PR-22 maintained a relatively high level of infection throughout passaging as compared to the other two viruses. However, all viruses had a general decrease in the level of infection

Table 3-3: Table 4 Growth of adapted viruses on mutant CD163 cell lines

	Passage Number											
Virus dil.	**PR-22**				**PR-58**				**PR-67**			
	P2	P4	P6	P8	P2	P4	P6	P7	P2	P4	P5	P6
10	#[*1]	#	#	#	#	#	#	#	#	#	#	#
10^2	#	#	#	#	#	#	#	#	700	180	1110	400
10^3	368	1371	682	1281	1600	1201	263	305	58	0	87	88
10^4	0	3	32	0	0	0	0	0	0	0	0	0
10^5	0	0	0	0	0	0	0	0	0	0	0	0
$TCID_{50}$/ML	1580	15800	15800	1580	1580	1580	1580	1580	1580	158	1580	1580
Conc (log_{10})	3.2	4.2	4.2	3.2	3.2	3.2	3.2	3.2	3.2	2.2	3.2	3.2

The results are presented as the total RFP fluorescent cells in a single well of a 24 well plate for wild type, PR-22, PR-58 and PR-67 cells**.** Data were made at 72 hours post infection. Therefore, the presence of fluorescence approximates the third round of virus replication. The numbers of infected cells per well were counted and the Reed-Munch Infectivity calculator was used to calculate the $TCID_{50}$/mL and the concentration in log.

*1. Cells were too many too count

4 Discussion

Even though it is generally considered that PRRSV GP2 and GP4 directly interact with the primary receptor CD163, the exact PRRSV amino acids involved remain unknown. The goal of this study was to determine if the propagation of PRRSV-RFP on HEK cells expressing modifications on CD163 will result in mutations in PRRSV which will allow the virus to adapt to growth on these cells. We serially passaged PRRSV-2 RFP on HEK cells expressing mutations in SRCR5 of CD163 at least six times and sequenced the viruses at various passages. Our overall hypothesis was that repeated passaging of PRRSV on cells expressing mutations on SRCR5 of CD163 will result in the adaptation of the virus to the modified CD163. Adaptation of PRRSV would be evident by mutations within GP2, GP3, GP4 and possibly GP5, and by an increase in the level infection.

Unexpectedly, all mutations observed in our study were within ORF6, which codes for the virus matrix (M) protein. A synonymous mutation was observed in virus PR-58 at the 98tth nucleotide position where thymine in the codon GCT was substituted for cytosine to make the codon GCC both of which code for alanine. This amino acid is located at the 33rd amino acid position in the transmembrane domain region. Non-synonymous mutations appeared in PR-22 and PR-58. The mutation in virus PR-22 occurred in the endodomain region at the 141st position where threonine was substituted for isoleucine (T141I). The PR-58 virus occurred in the transdomain region at the 86th position where tyrosine was substituted for histidine (Y86H) (Figure 4-5). This was unexpected because the M protein is not known to directly interact with CD163 and is a relatively conserved protein. In a study where PRRSV strain HuN_4-F_{112} was serially passaged on MARC 145 cells, GP2 to GP5 proteins had higher mutation rates than the highly conserved M protein

(An et al., 2011). This is in contrast to our study where after at least six passages on HEK cells expressing CD163 mutations, only the M protein had mutated.

Out of the nine PR- cell lines only three, PR-22, PR-58 and PR-67, were able to sustain infection with PRRSV-2 P129 for at least six passages (Table 4-1). In addition, we observed varying PRRSV growth patterns on these three cell lines: virus PR-22 had maintained a higher level of infection compared to the other two viruses until passage eight where the number of infected cells sharply declined. Viruses PR-58 and PR-67 had a progressive decline in the level of infection where virus PR-58 disappeared at passage seven (Table 4-2). Throughout the study, PRRSV grown on cells expressing wild type CD163 maintained a higher level of infection than virus grown on other cells (data not shown).

This data suggests that the mutations in viruses PR-22 and PR-58 were not sufficient to completely adapt these viruses to growth on cells expressing mutated CD163. In a study where PRRSV was serially passaged on feline kidney cells transfected with MARC CD163 and porcine kidney cells transfected with susCD163, at least ten serial passages were required to adapt PRRSV to growth on those cells (Calvert, Slade, Shields, Jolie, Mannan, Ankenbauer, Welch et al., 2007). In our study, the highest number that PRRSV was passaged and sequenced was six. It is possible that the virus would have adapted in passages higher such as passages ten and above. A mutation in the same protein was found in a previous study on PRRSV mutations involved in virus neutralization: a deletion of tryptophan at position 10 (Y10 deletion). Interestingly, that mutation was located within ectodomain region (Popescu, Trible, Chen, Rowland, 2017) while in this study we found mutations in the endodomain and transdomain regions. We saw no evidence of increasing PRRSV

replication throughout serial passaging PRRSV on HEK cells expressing CD163 modifications which suggests that the mutations observed in the M protein were not sufficient for virus adaptation.

HEK cells are naturally not permissive to PRRSV infection. In our study PRRSV had to overcome two factors: both the cell line and the mutations on CD163 expressed by that cell line. Future studies can be done using a naturally permissive cell line such as MARC 145 cells or the primary cell line, porcine alveolar macrophages (PAMS) where only the mutations in CD163 will be a factor the virus will need to overcome. Future studies should also be done using a parent virus with a higher titer to give the virus a chance to reach ten and higher passages to give the virus ample passaging to result in adaption. In addition, future studies can be done to adapt both PRRSV-1 and PRRSV-2 to growth on porcine alveolar macrophages expressing the same CD163 modifications.

5 Bibliography

An, T.-Q., Tian, Z.-J., Zhou, Y.-J., Xiao, Y., Peng, J.-M., Chen, J., … Tong, G.-Z. (2011). Comparative genomic analysis of five pairs of virulent parental/attenuated vaccine strains of PRRSV. *Veterinary Microbiology*, *149*(1–2), 104–112. https://doi.org/10.1016/j.vetmic.2010.11.001

Balasch, M., Fort, M., Taylor, L. P., & Calvert, J. G. (2018). Vaccination of 1-day-old pigs with a porcine reproductive and respiratory syndrome virus (PRRSV) modified live attenuated virus vaccine is able to overcome maternal immunity. *Porcine Health Management*, *4*, 25. https://doi.org/10.1186/s40813-018-0101-x

Balasuriya, U. B. R., Rossitto, P. V., DeMaula, C. D., & MacLachlan, N. J. (1993). A 29K envelope glycoprotein of equine arteritis virus expresses neutralization determinants recognized by murine monoclonal antibodies. *Journal of General Virology*, *74*(11), 2525–2529. https://doi.org/10.1099/0022-1317-74-11-2525

Bautista, E. M., Meulenberg, J. J. M., Choi, C. S., & Molitor, T. W. (1996). Structural polypeptides of the American (VR-2332) strain of porcine reproductive and respiratory syndrome virus. *Archives of Virology*, *141*(7), 1357–1365. https://doi.org/10.1007/BF01718837

Burkard, C., Lillico, S. G., Reid, E., Jackson, B., Mileham, A. J., Ait-Ali, T., … Archibald, A. L. (2017). Precision engineering for PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. *PLOS Pathogens*, *13*(2), e1006206. https://doi.org/10.1371/journal.ppat.1006206

Burkard, C., Opriessnig, T., Mileham, A. J., Stadejek, T., Ait-Ali, T., Lillico, S. G., …

Archibald, A. L. (2018). *Pigs Lacking the Scavenger Receptor Cysteine-Rich Domain 5 of CD163 Are Resistant to Porcine Reproductive and Respiratory Syndrome Virus 1 Infection*. https://doi.org/10.1128/JVI

Cafruny, W. A., Chan, S. P. K., Harty, J. T., Yousefi, S., Kowalchyk, K., McDonald, D., … Plagemann, P. G. W. (1986). Antibody response of mice to lactate dehydrogenase-elevating virus during infection and immunization with inactivated virus. *Virus Research*, *5*(4), 357–375. https://doi.org/10.1016/0168-1702(86)90029-8

Calvert, J. G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G., & Welch, S.-K. W. (2007). CD163 Expression Confers Susceptibility to Porcine Reproductive and Respiratory Syndrome Viruses. *Journal of Virology*, *81*(14), 7371–7379. https://doi.org/10.1128/JVI.00513-07

Calvert, Jay G., Slade, D. E., Shields, S. L., Jolie, R., Mannan, R. M., Ankenbauer, R. G., & Welch, S.-K. W. S.-K. W. S.-K. W. (2007). CD163 Expression Confers Susceptibility to Porcine Reproductive and Respiratory Syndrome Viruses. *Journal of Virology*, *81*(14), 7371–7379. https://doi.org/10.1128/JVI.00513-07

Chirnside, E. D., de Vries, A. A. F., Mumford, J. A., & Rottier, P. J. M. (1995). Equine arteritis virus-neutralizing antibody in the horse is induced by a determinant on the large envelope glycoprotein GL. *Journal of General Virology*, *76*(8), 1989–1998. https://doi.org/10.1099/0022-1317-76-8-1989

Christopher-Hennings, J., Nelson, E. A., Hines, R. J., Nelson, J. K., Swenson, S. L., Zimmerman, J. J., … Benfield, D. A. (1995). Persistence of Porcine Reproductive and Respiratory Syndrome Virus in Serum and Semen of Adult Boars. *Journal of Veterinary Diagnostic Investigation*, *7*(4), 456–464. https://doi.org/10.1177/104063879500700406

Darwich, L., Gimeno, M., Sibila, M., Diaz, I., de la Torre, E., Dotti, S., … Mateu, E. (2011). Genetic and immunobiological diversities of porcine reproductive and respiratory syndrome genotype I strains. *Veterinary Microbiology*, *150*(1–2), 49–62. https://doi.org/10.1016/J.VETMIC.2011.01.008

Das, P. B. (2010). THE GLYCOPROTEINS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND THEIR ROLE IN INFECTION AND IMMUNITY. In *INFECTION AND IMMUNITY*. Retrieved from http://digitalcommons.unl.edu/vetscidisshttp://digitalcommons.unl.edu/vetscidiss/3

Das, P. B., Dinh, P. X., Ansari, I. H., De Lima, M., Osorio, F. A., & Pattnaik, A. K. (2010). The Minor Envelope Glycoproteins GP2a and GP4 of Porcine Reproductive and Respiratory Syndrome Virus Interact with the Receptor CD163. *JOURNAL OF VIROLOGY*, *84*(4), 1731–1740. https://doi.org/10.1128/JVI.01774-09

de Lima, M., Ansari, I. H., Das, P. B., Ku, B. J., Martinez-Lobo, F. J., Pattnaik, A. K., & Osorio, F. A. (2009). GP3 is a structural component of the PRRSV type II (US) virion. *Virology*, *390*(1), 31–36. https://doi.org/10.1016/J.VIROL.2009.04.017

De Vries, A. A. F., Chirnside, E. D., Horzinek, M. C., & Rottier, P. J. M. (1992a). Structural Proteins of Equine Arteritis Virus. In *JOURNAL OF VIROLOGY* (Vol. 66). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC240121/pdf/jvirol00042-0064.pdf

De Vries, A. A. F., Chirnside, E. D., Horzinek, M. C., & Rottier, P. J. M. (1992b). Structural Proteins of Equine Arteritis Virus. In *JOURNAL OF VIROLOGY* (Vol. 66). Retrieved from http://jvi.asm.org/

de Vries, A. A., Post, S. M., Raamsman, M. J., Horzinek, M. C., & Rottier, P. J. (1995). The two major envelope proteins of equine arteritis virus associate into disulfide-linked

heterodimers. *Journal of Virology*, *69*(8), 4668–4674. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7609031

de Vries, A. A., Raamsman, M. J., van Dijk, H. A., Horzinek, M. C., & Rottier, P. J. (1995). The small envelope glycoprotein (GS) of equine arteritis virus folds into three distinct monomers and a disulfide-linked dimer. *Journal of Virology*, *69*(6), 3441–3448. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7745690

Dea, S., Gagnon, C. A., Mardassi, H., Pirzadeh, B., & Rogan, D. (2000). Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates Brief Review. In *Arch Virol*. Retrieved from https://link.springer.com/content/pdf/10.1007/s007050050662.pdf

Delputte, P. L., Costers, S., & Nauwynck, H. J. (2005). Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. *Journal of General Virology*, *86*(5), 1441–1445. https://doi.org/10.1099/vir.0.80675-0

Delputte, P. L., Vanderheijden, N., Nauwynck, H. J., & Pensaert, M. B. (2002). Involvement of the matrix protein in attachment of porcine reproductive and respiratory syndrome virus to a heparinlike receptor on porcine alveolar macrophages. *Journal of Virology*, *76*(9), 4312–4320. https://doi.org/10.1128/jvi.76.9.4312-4320.2002

Dokland, T. The structural biology of PRRSV. , 154 Virus Research § (2010).

Droste, A., Sorg, C., & Högger, P. (1999). Shedding of CD163, a Novel Regulatory Mechanism for a Member of the Scavenger Receptor Cysteine-Rich Family. *Biochemical and Biophysical Research Communications*, *256*(1), 110–113. https://doi.org/10.1006/bbrc.1999.0294

Faaberg, K. S., Even, C., Palmer, G. A., & Plagemann, P. G. (1995). Disulfide bonds between two envelope proteins of lactate dehydrogenase-elevating virus are essential for viral infectivity. *Journal of Virology*, *69*(1), 613–617. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7983766

Fabriek, B. O., Dijkstra, C. D., & van den Berg, T. K. (2005). The macrophage scavenger receptor CD163. *Immunobiology*, *210*(2–4), 153–160. https://doi.org/10.1016/j.imbio.2005.05.010

Fitter, S., Sincock, P. M., Jolliffe, C. N., & Ashman, L. K. (1999). Transmembrane 4 superfamily protein CD151 (PETA-3) associates with beta 1 and alpha IIb beta 3 integrins in haemopoietic cell lines and modulates cell-cell adhesion. *The Biochemical Journal*, *338 (Pt 1)*, 61–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9931299

Gao, J., Xiao, S., Xiao, Y., Wang, X., Zhang, C., Zhao, Q., … Zhou, E. M. (2016). MYH9 is an Essential Factor for Porcine Reproductive and Respiratory Syndrome Virus Infection. *Scientific Reports*, *6*. https://doi.org/10.1038/srep25120

Gonin, P., Mardassi, H., Gagnon, C. A., Massie, B., & Dea, S. (1998). A nonstructural and antigenic glycoprotein is encoded by ORF3 of the IAF-Klop strain of porcine reproductive and respiratory syndrome virus. *Archives of Virology*, *143*(10), 1927–1940. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9856081

Gorp, H. Van, Breedam, W. Van, Doorsselaere, J. Van, Delputte, P. L., & Nauwynck, H. J. (2010). Identification of the CD163 Protein Domains Involved in Infection of the Porcine Reproductive and Respiratory Syndrome Virus †. *JOURNAL OF VIROLOGY*, *84*(6), 3101–3105. https://doi.org/10.1128/JVI.02093-09

Holtkamp, D. J., Kliebenstein, J. B., Neumann, E., Zimmerman, J. J., Rotto, H., Yoder, T. K., …

Yeske, P. E. (2013). Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers): 72. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. In *Journal of Swine Health and Production* (Vol. 21). Retrieved from http://lib.dr.iastate.edu/econ_las_pubs/50

Hopper, S., White, M., & Twiddy, N. (1992). An outbreak of blue-eared pig disease (porcine reproductive and respiratory syndrome) in four pig herds in Great Britain. *Veterinary Record*, *131*(7), 140–144. https://doi.org/10.1136/vr.131.7.140

Jusa, E. R., Inaba, Y., Kouno, M., & Hirose, O. (1997). Effect of heparin on infection of cells by porcine reproductive and respiratory syndrome virus. *American Journal of Veterinary Research*, *58*(5), 488–491. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9140556

Kapur, V., Elam, M. R., Pawlovich, T. M., & Murtaugh, M. P. (1996). Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. In *Journal of General Virology* (Vol. 77). Retrieved from www.microbiologyresearch.org

Kim, J.-K., Fahad, A.-M., Shanmukhappa, K., & Kapil, S. (2006). Defining the Cellular Target(s) of Porcine Reproductive and Respiratory Syndrome Virus Blocking Monoclonal Antibody 7G10. *JOURNAL OF VIROLOGY*, *80*(2), 689–696. https://doi.org/10.1128/JVI.80.2.689-696.2006

Kimman, T. G., Cornelissen, L. A., Moormann, R. J., Rebel, J. M. J., & Stockhofe-Zurwieden, N. (2009). Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. *Vaccine*, *27*(28), 3704–3718. https://doi.org/10.1016/j.vaccine.2009.04.022

Kristiansen, M., Graversen, J. H., Jacobsen, C., Sonne, O., Hoffman, H.-J., Law, S. K. A., & Moestrup, S. K. (2001). Identification of the haemoglobin scavenger receptor. *Nature*, *409*(6817), 198–201. https://doi.org/10.1038/35051594

Kutish, G. F., Lewis, T. L., Doster, A. R., Rock, D. L., Ali, A., Allende, R., … Lu, Z. (1999). North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. *Journal of General Virology*, *80*(2), 307–315. https://doi.org/10.1099/0022-1317-80-2-307

Larochelle, R., D'Allaire, S., & Magar, R. (2003). Molecular epidemiology of porcine reproductive and respiratory syndrome virus (PRRSV) in Québec. *Virus Research*, *96*(1–2), 3–14. https://doi.org/10.1016/S0168-1702(03)00168-0

Law, S. K. A., Micklem, K. J., Shaw, J. M., Zhang, X.-P., Dong, Y., Willis, A. C., & Mason, D. Y. (1993). A new macrophage differentiation antigen which is a member of the scavenger receptor superfamily. *European Journal of Immunology*, *23*(9), 2320–2325. https://doi.org/10.1002/eji.1830230940

Lee, C., & Yoo, D. (2006). The small envelope protein of porcine reproductive and respiratory syndrome virus possesses ion channel protein-like properties. *Virology*, *355*(1), 30–43. https://doi.org/10.1016/J.VIROL.2006.07.013

Loemba, H. D., Mounir, S., Mardassi, H., Archambault, D., & Dea, S. (1996). Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. *Archives of Virology*, *141*(3–4), 751–761. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8645111

Mardassi, H, Athanassious, R., Mounir, S., & Dea, S. (1994). Porcine reproductive and respiratory syndrome virus: morphological, biochemical and serological characteristics of

Quebec isolates associated with acute and chronic outbreaks of porcine reproductive and respiratory syndrome. *Canadian Journal of Veterinary Research = Revue Canadienne de Recherche Veterinaire*, *58*(1), 55–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8143254

Mardassi, Helmi, Massie, B., & Dea, S. (1996). Intracellular Synthesis, Processing, and Transport of Proteins Encoded by ORFs 5 to 7 of Porcine Reproductive and Respiratory Syndrome Virus. *Virology*, *221*(1), 98–112. https://doi.org/10.1006/VIRO.1996.0356

Meulenberg, J. J. M. (2000). PRRSV, the virus. *Veterinary Research*, *31*(1), 11–21. https://doi.org/10.1051/vetres:2000103

Meulenberg, J. J. M., Hulst, M. M., de Meijer, E. J., Moonen, P. L. J. M., den Besten, A., de Kluyver, E. P., … Moormann, R. J. M. (1993). Lelystad Virus, the Causative Agent of Porcine Epidemic Abortion and Respiratory Syndrome (PEARS), Is Related to LDV and EAV. *Virology*, *192*(1), 62–72. https://doi.org/10.1006/VIRO.1993.1008

Meulenberg, J. J. M., Petersen-Den Besten, A., De Kluyver, E. P., Moormann, R. J. M., Schaaper, W. M. M., & Wensvoort, G. (1995). Characterization of proteins encoded by ORFs 2 to 7 of Lelystad virus. *Virology*, *206*(1), 155–163. https://doi.org/10.1016/S0042-6822(95)80030-1

Meulenberg, J. J., & Petersen-den Besten, A. (1996). Identification and characterization of a sixth structural protein of Lelystad virus: the glycoprotein GP2 encoded by ORF2 is incorporated in virus particles. *Virology*, *225*(1), 44–51. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8918532

Meulenberg, J. J., & Snijder, E. J. (1998). The molecular biology of arteriviruses. *Journal of General Virology*, *79*(5), 961–979. https://doi.org/10.1099/0022-1317-79-5-961

Murtaugh, M. P., Elam, M. R., & Kakach, L. T. (1995). Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Archives of Virology*, *140*(8), 1451–1460. https://doi.org/10.1007/BF01322671

Niederwerder, M. C., Bawa, B., Serao, N. V. L., Trible, B. R., Kerrigan, M. A., Lunney, J. K., … Rowland, R. R. R. (2015). Vaccination with a porcine reproductive and respiratory syndrome (PRRS) modified live virus vaccine followed by challenge with PRRS virus and porcine circovirus type 2 (PCV2) protects against PRRS but enhances PCV2 replication and pathogenesis compared to results for nonvaccinated cochallenged controls. *Clinical and Vaccine Immunology*, *22*(12), 1244–1254. https://doi.org/10.1128/CVI.00434-15

Opriessnig, T., Giménez-Lirola, L. G., & Halbur, P. G. (2011). Polymicrobial respiratory disease in pigs. *Animal Health Research Reviews*, *12*(2), 133–148. https://doi.org/10.1017/S1466252311000120

Pileri, E., & Mateu, E. (2016). Review on the transmission porcine reproductive and respiratory syndrome virus between pigs and farms and impact on vaccination. *Veterinary Research*, *47*(1), 108. https://doi.org/10.1186/s13567-016-0391-4

Plagemann, P. G. W. (2006). Neutralizing Antibody Formation in Swine Infected with Seven Strains of Porcine Reproductive and Respiratory Syndrome Virus as Measured by Indirect ELISA with Peptides Containing the GP5 Neutralization Epitope. *Viral Immunology*, *19*(2), 285–293. https://doi.org/10.1089/vim.2006.19.285

Popescu, L. N., Trible, B. R., Chen, N., & Rowland, R. R. R. (2017). GP5 of porcine reproductive and respiratory syndrome virus (PRRSV) as a target for homologous and broadly neutralizing antibodies. *Veterinary Microbiology*, *209*, 90–96. https://doi.org/10.1016/J.VETMIC.2017.04.016

Prather, R. S., Rowland, R. R. R., Ewen, C., Trible, B., Kerrigan, M., Bawa, B., … Green, J. A. (2013). An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. *Journal of Virology*, *87*(17), 9538–9546. https://doi.org/10.1128/JVI.00177-13

Sarrias, M. R., Grønlund, J., Padilla, O., Madsen, J., Holmskov, U., & Lozano, F. (2004). The Scavenger Receptor Cysteine-Rich (SRCR) domain: an ancient and highly conserved protein module of the innate immune system. *Critical Reviews in Immunology*, *24*(1), 1–37. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14995912

Schulze, M., Revilla-Fernández, S., Schmoll, F., Grossfeld, R., & Griessler, A. (2013). Effects on boar semen quality after infection with porcine reproductive and respiratory syndrome virus: a case report. *Acta Veterinaria Scandinavica*, *55*(1), 16. https://doi.org/10.1186/1751-0147-55-16

Shanmukhappa, K., Kim, J.-K., & Kapil, S. (2007). Role of CD151, A tetraspanin, in porcine reproductive and respiratory syndrome virus infection. *Virology Journal*, *4*, 62. https://doi.org/10.1186/1743-422X-4-62

Shi, M., Holmes, E. C., Brar, M. S., & Leung, F. C.-C. (2013). Recombination is associated with an outbreak of novel highly pathogenic porcine reproductive and respiratory syndrome viruses in China. *Journal of Virology*, *87*(19), 10904–10907. https://doi.org/10.1128/JVI.01270-13

Snijder, E. J., Dobbe, J. C., & Spaan, W. J. M. (2003). Heterodimerization of the Two Major Envelope Proteins Is Essential for Arterivirus Infectivity. *JOURNAL OF VIROLOGY*, *77*(1), 97–104. https://doi.org/10.1128/JVI.77.1.97-104.2003

Snijder, E. J., Van Tol, H., Pedersen, K. W., Raamsman, M. J. B., & De Vries, A. A. F. (1999).

Identification of a Novel Structural Protein of Arteriviruses. In *JOURNAL OF VIROLOGY* (Vol. 73). Retrieved from http://jvi.asm.org/

Storgaard, T., Nielsen, H. S., Stadejek, T., Bøtner, A., Oleksiewicz, M. B., & Forsberg, R. (2001). Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *Journal of General Virology*, *82*(6), 1263–1272. https://doi.org/10.1099/0022-1317-82-6-1263

Swenson, S. L., Hill, H. T., Zimmerman, J. J., Evans, L. E., Landgraf, J. G., Wills, R. W., … Ciszewski, D. K. (1994). Excretion of porcine reproductive and respiratory syndrome virus in semen after experimentally induced infection in boars. *Journal of the American Veterinary Medical Association*, *204*(12), 1943–1948. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8077142

Terpstra, C., Wensvoort, G., & Pol, J. M. A. (1991). Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad vims: Koch's postulates fulfilled. *Veterinary Quarterly*, *13*(3), 131–136. https://doi.org/10.1080/01652176.1991.9694297

Tian, D., Wei, Z., Zevenhoven-Dobbe, J. C., Liu, R., Tong, G., Snijder, E. J., & Yuan, S. (2012). *Arterivirus Minor Envelope Proteins Are a Major Determinant of Viral Tropism in Cell Culture*. https://doi.org/10.1128/JVI.06836-11

Van Breedam, W., Van Gorp, H., Zhang, J. Q., Crocker, P. R., Delputte, P. L., & Nauwynck, H. J. (2010). The M/GP(5) glycoprotein complex of porcine reproductive and respiratory syndrome virus binds the sialoadhesin receptor in a sialic acid-dependent manner. *PLoS Pathogens*, *6*(1), e1000730. https://doi.org/10.1371/journal.ppat.1000730

van Dinten, L. C., den Boon, J. A., Wassenaar, A. L., Spaan, W. J., & Snijder, E. J. (1997). An

infectious arterivirus cDNA clone: identification of a replicase point mutation that abolishes discontinuous mRNA transcription. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(3), 991–996. https://doi.org/10.1073/pnas.94.3.991

van Nieuwstadt, A. P., Meulenberg, J. J., van Essen-Zanbergen, A., Petersen-den Besten, A., Bende, R. J., Moormann, R. J., & Wensvoort, G. (1996). Proteins encoded by open reading frames 3 and 4 of the genome of Lelystad virus (Arteriviridae) are structural proteins of the virion. *Journal of Virology*, *70*(7), 4767–4772. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8676504

Vanderheijden, N., Delputte, P. L., Favoreel, H. W., Vandekerckhove, J., Van Damme, J., van Woensel, P. A., & Nauwynck, H. J. (2003). Involvement of sialoadhesin in entry of porcine reproductive and respiratory syndrome virus into porcine alveolar macrophages. *Journal of Virology*, *77*(15), 8207–8215. https://doi.org/10.1128/jvi.77.15.8207-8215.2003

Vicente-Manzanares, M., Ma, X., Adelstein, R. S., & Horwitz, A. R. (2009). Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nat Rev Mol Cell Biol*, *10*(11), 778–790. https://doi.org/10.1038/nrm2786

Wells, K. D., Bardot, R., Whitworth, K. M., Trible, B. R., Fang, Y., Mileham, A., … Rowland, R. R. R. (2017). Replacement of Porcine CD163 Scavenger Receptor Cysteine-Rich Domain 5 with a CD163-Like Homolog Confers Resistance of Pigs to Genotype 1 but Not Genotype 2 Porcine Reproductive and Respiratory Syndrome Virus. *Journal of Virology*, *91*(2). https://doi.org/10.1128/JVI.01521-16

Whitworth, K. M., Rowland, R. R. R., Ewen, C. L., Trible, B. R., Kerrigan, M. A., Cino-Ozuna, A. G., … Prather, R. S. (2016). Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. *Nature Biotechnology*, *34*(1), 20–22.

https://doi.org/10.1038/nbt.3434

Wieringa, R., De Vries, A. A. F., Van Der Meulen, J., Godeke, G.-J., Jos, ‡, Onderwater, J. M., … Rottier, P. J. M. (2004). Structural Protein Requirements in Equine Arteritis Virus Assembly The absence of E entirely prevented the incorporation of the GP 2b , GP 3 , and GP 4 proteins into viral particles. EAV particles lacking GP 2b , GP 3 , GP 4 , and E did not markedly differ from wild-type virions in buoyant density, major structural protein composition, electron microscopic appearance, and genomic RNA content. On the basis of these results, we propose a model for the EAV particle in which the GP 2b. *JOURNAL OF VIROLOGY*, *78*(23), 13019–13027. https://doi.org/10.1128/JVI.78.23.13019-13027.2004

Wissink, E. H. J., Kroese, M. V, van Wijk, H. A. R., Rijsewijk, F. A. M., Meulenberg, J. J. M., & Rottier, P. J. M. (2005). Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. *Journal of Virology*, *79*(19), 12495–12506. https://doi.org/10.1128/JVI.79.19.12495-12506.2005

Wootton, S., Yoo, D., & Rogan, D. (2000). Full-length sequence of a Canadian porcine reproductive and respiratory syndrome virus (PRRSV) isolate *. *Arch Virol*. Retrieved from https://link.springer.com/content/pdf/10.1007/s007050070022.pdf

Wu, W.-H., Fang, Y., Rowland, R. R. R., Lawson, S. R., Christopher-Hennings, J., Yoon, K.-J., & Nelson, E. A. (2005). The 2b protein as a minor structural component of PRRSV. *Virus Research*, *114*(1–2), 177–181. https://doi.org/10.1016/J.VIRUSRES.2005.06.014

Zuckermann, F. A., Garcia, E. A., Luque, I. D., Christopher-Hennings, J., Doster, A., Brito, M., & Osorio, F. (2007). Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon

challenge. *Veterinary Microbiology*, *123*(1–3), 69–85.

https://doi.org/10.1016/j.vetmic.2007.02.009