RESEARCHREPORT

SWINE HEALTH

Title: Adaptation of PRRSV to modifications in CD163 – **NPB #17-160**

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Industry Summary: In 2016 our lab showed that genetically edited pigs lacking expression of the CD163 protein on macrophages fail to support the replication of PRRSV. This was the first clear demonstration of how genetic modification can be used to prevent PRRS. This technology has since been used to demonstrate resistance to other swine diseases. Since CD163 is required for scavenging excess hemoglobin from the blood and participates in the regulation of inflammation, deletion of the entire CD163 protein has important negative consequences for the pig. Additional work, funded by the NPB and USDA NIFA, is directed at refining the genetic modification of CD163 to identify the smallest modifications in CD163 that confer resistance to PRRS without affecting other important functions. Since PRRSV undergoes rapid mutations in the field, the goal of this research is to determine if PRRSV can adapt successfully to different modifications in CD163; and if so, where the mutations for the adapted viruses are located within the surface genes of the viral genome. The experimental approach was to repeatedly passage PRRSV on HEK cell lines expressing mutations in SRCR domain 5 of CD613. Domain 5 is one of the critical regions required for PRRSV infection. The results showed that several mutations supported virus infection to some level. The repeated passage of PRRSV resulted in the appearance of mutations in the ORF6 protein. Overall, the results indicate that PRRSV remains relatively stable when passaged on cells possessing mutations in CD163.

Keywords: PRRSV, CD163, virus adaptation, genetic modification, PRRSV resistance

Scientific Abstract

Expressed on the surface of porcine macrophages, CD163 functions as the principal receptor for porcine reproductive and respiratory syndrome virus (PRRSV). Genetically modified (GM) pigs lacking CD163 expression on macrophages fail to support infection with Type 1 and Type 2 viruses. Furthermore, we demonstrated that domain 5 of CD163 is critical for virus recognition. The participation of CD163 in the removal of hemoglobin-haptoglobin (HbHp) complexes from

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the blood, along with the regulation of immunity, suggest that the complete removal of CD163 will create unintended consequences to other aspects of pig health. In our recent work using recombinant cell lines, several mutations in domain 5 of CD163 that reduce or prevent virus infection were identified. The hypothesis tested in this proposal is that repeated passage of PRRSV on HEK cells expressing mutated CD163 proteins will result in adaptation of the virus to the CD163 mutations. Furthermore, the adaptations will be reflected in mutations in the surface viral proteins, such as GP2, GP3, GP4, GP5, and M. The first goal was to prepare stable cell lines that expressed CD163. Mutations in CD163 were created by introducing single proline-arginine (PR) dipeptides along the length of the 102 amino acid SRCR5 polypeptide. A PRRSV-RFP (red fluorescent protein) virus was serially passed on cells expressing mutations at SRC5 amino acids 9 (PR-9), 22 (PR-22), 32 (PR-32), 38 (PR-38), 42 (PR-42), 55 (PR-55), 58 (PR-58), 67 (PR-67), and 100 (PR-100). After initial screening, three constructs, PR-22, PR-55, and PR-58 were able to maintain PRRSV infection for at least six passages. Sequencing ORFs 2-6 identified one mutation in each virus grown on constructs PR-22 and PR-58. Both mutations were located in the 174 amino acid non-glycosylated ORF6 protein or matrix (M) protein, which is highly conserved. The PR-22 change, a tyrosine to isoleucine, was located at amino acid 141, which is in the internal domain of the protein. The PR-58 mutation, a change from tryptophan to histidine, was located at position 86, within a transmembrane domain. Passing the virus on a wild-type (WT) CD163 showed no amino acid changes. The location of both mutations near the C-terminal half of the M protein suggest that M is important for the interaction of CD163 with PRRSV. However, it remains to be determined how mutations located internal to the virion envelope participate in infection. Together, these data provide information on how PRRSV interacts with CD163. Further research is needed to develop a mechanistic understanding of the interaction between PRRSV and CD163.

Introduction: As we first reported in Nature Biotechnology in 2016, "CD163 facilitates both entry and replication of porcine reproductive and respiratory syndrome virus", we demonstrated that genetically edited pigs lacking surface expression of the CD163 protein on macrophages fail to support the replication of PRRSV. This observation is the first clear demonstration of how genetic modification can be used to prevent PRRS and other swine diseases. CD163 is a 130 kDa type 1 membrane protein composed of nine scavenger receptor cysteine-rich (SRCR) domains and two proline serine threonine (PST) domains. Surface expression of CD163 is restricted to cells of the monocyte-macrophage lineage. The normal function of CD163 is to bind and remove excess hemoglobin-haptoglobin (HbHp) complexes from the blood (Kristiansen et al., 2001; Fabriek et al., 2005). Free Hb is released by disrupted RBCs following tissue damage caused by physical trauma or infection. The metabolic degradation of Hb by macrophages results in the release degradation products which possess potent anti-inflammatory activities. This feedback loop is important for wound healing. Therefore, deletion of the entire CD163 protein potentially has important negative consequences to the pigs.

A currently funded NPB project "Genetic modifications in CD163 that confer complete resistance of pigs to infection with PRRSV" is directed at refining the genetic modification of CD163 to identify the smallest peptide sequence modification that confers resistance to PRRS without affecting CD163's other important functions. Since PRRSV is one of the most genetically diverse viruses on the planet, constructing pigs possessing small modifications in CD163 raises important concerns. The first concern is that PRRSV will naturally adapt to the

modified CD163 proteins. A second related concern is that the newly adapted viruses will possess different virulence properties.

The overall objective of the proposed research is the application of an *in vitro* system to study how PRRSV adapts to mutations in CD163. The experimental approach is the use of a red fluorescent protein (RFP)-labeled virus (PRRSV-RFP) to infect cells that express modified CD163 proteins. The mutations in CD163 are located in the domain 5 region, which is known to be critical for PRRSV infection. We already possess a panel of constructs with CD163 modifications which confer varying degrees of resistance to infection. Viruses that adapt to growth on cells expressing the modified receptors are sequenced. The second objective is to identify the mutations in the virus that are involved in adaptation.

Pigs with genetically modified CD163 provide a practical and realistic method for eliminating PRRS. Additional benefits are derived from the elimination of the costs associated with PRRS vaccination, diagnostic testing, and PRRSV-specific biosecurity measures, such as barn filtration. Besides understanding the capacity for PRRSV to mutate and adapt to modified CD163, important benefits come from understanding how the PRRSV genome functions; i.e. the “functional genomics” of the virus. Another benefit from this research is the characterization of viral genes and amino acids involved in the interaction between PRRSV and CD163, which may be useful as protective antigens in vaccine formulations. And finally, identifying mechanisms for the interruption of the CD163-PRRSV interaction by small molecules would create opportunities to explore alternative therapeutics, such as antivirals.

Objectives as originally stated in the proposal: Genetic modification of virus receptors creates a means to produce pigs that are resistant to PRRSV and other swine diseases. Other benefits of genetic modification include eliminating vaccination, diagnostic testing, and agent-specific biosecurity. Current refinements in preventing PRRSV infection through genetic modification include introducing small changes in CD163 SRCR5 domain 5 that confer resistance PRRSV infection while retaining normal CD163 function. However, the concern is that PRRSV might mutate and adapt to the subtle CD163 modifications. Therefore, the goal of this project is to determine if the propagation of PRRSV on HEK cells expressing modifications in CD613 results in mutations in PRRSV that allow the virus to adapt to growth on cells possessing modified CD163 receptors.

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1          11         21         31         41         51
PRLVGGDIPC SGRVEVQHGD TWGTVCDSDF SLEAASVLCR ELQCGTVVSL LGGAHFGEGS
        *              *          *     *    *             *  *
        9              22         32    38   42            55 58

61         71         81         91
GQIWAEEFQC EGHESHLSLC PVAPRPDGTC SHSRDVGVVC S
     *                                   *
     67                                 100
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Figure 1. Location of mutations in CD163 SRCR domain 5. The asterisks show the location of proline-arginine (PR) insertions within the domain 5 polypeptide sequence. The CD163 mutant proteins were expressed in HEK cells and then infected with PRRSV-RFP

Objective 1. Adapt PRRSV to growth on transfected HEK cells expressing genetic modifications in CD163. The experimental approach is the repeated passage of PRRSV-RFP on HEK cell lines expressing modified CD163 proteins. The modifications are located in SRCR domain 5 region critical for PRRSV infection. The CD163 domain 5 modifications to be tested

for adaptation include the constructs PR-9, PR-22, PR-32, PR-38, PR-42, PR-48, PR-55, PR-58, PR-67, PR-100 (see Figure 1).

Objective 2. Identify mutations in PRRSV responsible for adaptation of PRRSV-RFP to growth on HEK cells expressing modified CD163. Nothing is known regarding the amino acids on the surface of PRRSV proteins that form direct interactions with CD163. The purpose of this objective is to identify mutations in PRRSV proteins in viruses that appear after serial passage on HEK cells expressing modifications in CD163. The approach is to sequence the structural genes, ORFs 2-6, of the adapted viruses and compare the sequence to the parent virus.

Table 1. Infection of CD163 constructs with PRRSV-RFP*

CD163 Construct	1/virus dilution 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: (-) no red fluorescence; (+) positive for RFP fluorescence; (++) high positive for RFP expression. Highlighted rows identify CD163 constructs selected for virus adaptation. Infected cells were observed at 48 hrs after infection.

Table 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
PR-22	+	+	+	+	+	+	+	+
PR-42	+	-						
PR-55	+	-						
PR-58	+	+	+	+	+	+	-	
PR-67	+	+	+	+	+	ND*2		
WT*3	+	+	+	+	+	+	+	+

*1. The results are 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

Materials and Methods

Preparation of permanent HEK cell lines that express CD163. The stable cell lines used in this project were made by inserting the Sac II restriction enzyme sequence, CCG CGG, in the same reading frame into the CD163 domain 5 sequence (see Figure 1). The mouse kappa light chain was fused to sequence residues 47-1115 of porcine CD163 (NCBI Accession AAZ50616). CD163 cDNA variants were made by inserting proline-arginine residues to form the variants, PR-9, PR-22, PR-32, PR-38, PR-42, PR-48, PR-55, PR-58, PR-67, and PR-100. A WT CD163 was included as a control. The synthesized DNA segments were subcloned into a mammalian expression vector that contained a puromycin cassette. The vector was modified to contain HS4 chromatin insulators and Piggybac transposon ITR elements flanking the CD163 expression cassette, in order to increase expression of CD163. The plasmids were then transfected into HEK 293 cells along with a plasmid expressing Piggybac transposase. The cells were maintained in a selection medium containing puromycin, and the cells passaged in the selection medium for about three weeks. Expression of CD163 was confirmed by immunostaining using anti-CD163 monoclonal antibody and flow cytometry.

Adaptation protocol. HEK cells were plated on 24 well plates. The confluent cells were infected with different dilutions of the PRRSV-RFP virus. After three days, the media from the virus-positive wells - those which showed red fluorescence - were harvested and virus infection repeated on fresh cells. Passaging was performed for a maximum of eight times.

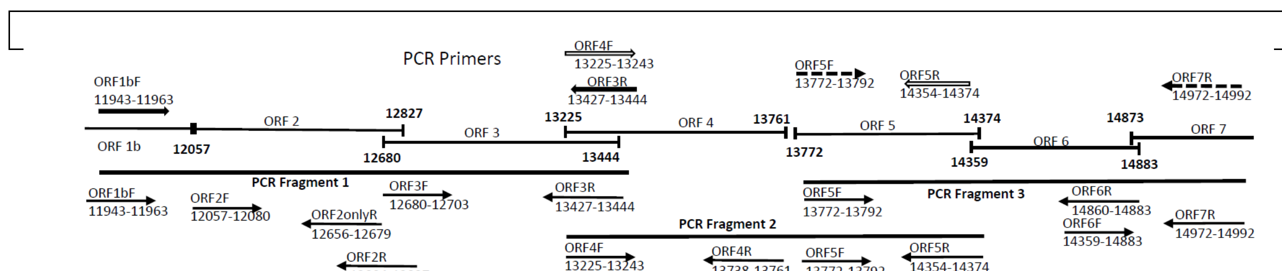

Figure 2. Primers used for PCR amplification and sequencing of ORFs 2 through 6. The top of the figure shows the primers used for PCR amplification. The result was the amplification of three PCR fragments. The bottom of the figure shows the primers used for sequencing

PCR amplification and sequencing. Superscript™ III One-Step-PCR System with Platinum™ Taq DNA Polymerase kit was used for RT-PCR amplification. As summarized in Table 1, three PCR products were amplified, which covered ORF2-3, ORF4-5 and ORF4-6. PCR was performed as a 50 ul reaction volume containing 0.8 ug of total RNA in a 0.2 ml Eppendorf tube. The calculated RNA volume was dispensed into the Eppendorf tube containing 25 ul reaction mix, 1 ul forward primer (10 nM/ml), 1 ul reverse primer (10 nM/ml), and 2 ul Taq polymerase. Nuclease free water was added to a final volume of 50 ul. Reverse transcription and pre-denaturation steps were performed at 50^{0}C for thirty minutes and 94^{0}C for two minutes. A total of 40 cycles were performed. For the ORF2-3 reaction, the extension time was 30 seconds at 68^{0}C, while for ORF4-5 and ORF5-7, the extension time was 60 seconds for at 68^{0}C. Denaturation was performed at 94^{0}C for 15 seconds and the annealing step done at 60^{0}C for thirty seconds.
PCR products were separated by electrophoresis on a 1% agarose gel. The bands were excised and placed in 1.5 mL Eppendorf tubes. DNA purification was performed using the Wizard® SV Gel and PCR Clean-Up System according the manufacturer's instructions. The cDNA concentrations (ug/ul) were read using the Nanodrop machine. Purified PCR products were temporarily stored – at 4˚C. Sequence analysis was performed using the CLC Main Workbench 8 software. The location of the primers for generating the PCR products and for sequencing are diagrammed in Figure 2.

Table 3. Growth of adapted viruses on mutant CD163 cell lines*

Virus	Passage Number					
	PR-22			PR-58		
Dilution	P1	P5	P7	P1	P5	P7
10	2343	3622	780	1507	130	0
10^2	324	858	59	666	0	0
10^3	28	98	0	19	0	0
10^4	0	0	0	45	0	0
Concentration (log)	4.3	4.9	3.8	4.5	3.1	-

*The results are presented as the total number of RFP fluorescent cells in a single well of a 24 well plate. Data were made at 24 hrs after infection. Therefore, the presence of fluorescence approximates the first round of virus replication.

Results

Objective 1. Infection of CD163 HEK cell lines. In the initial proposal, we proposed to perform the infection experiments in HEK cells that could only transiently express CD163. Under these conditions, we had difficulty passaging the virus. As an alternative, we convinced a colleague to prepare cell lines that permanently expressed the mutant CD163 proteins. The first experiment was to determine if the different CD163 HEK cells supported PRRSV infection. The results, presented in Table 1, showed that the different CD163 constructs supported virus

infection at different levels. Two constructs, PR-32 and PR-38 were negative for the presence of infection and were not subjected to further study. For the remainder of the constructs, we selected constructs that were highly permissive for infection, such as PR-22 and PR-42, as well as constructs that were less permissive, such as PR-55. The infection results for the modified CD163 cell lines are illustrated in Figure 3.

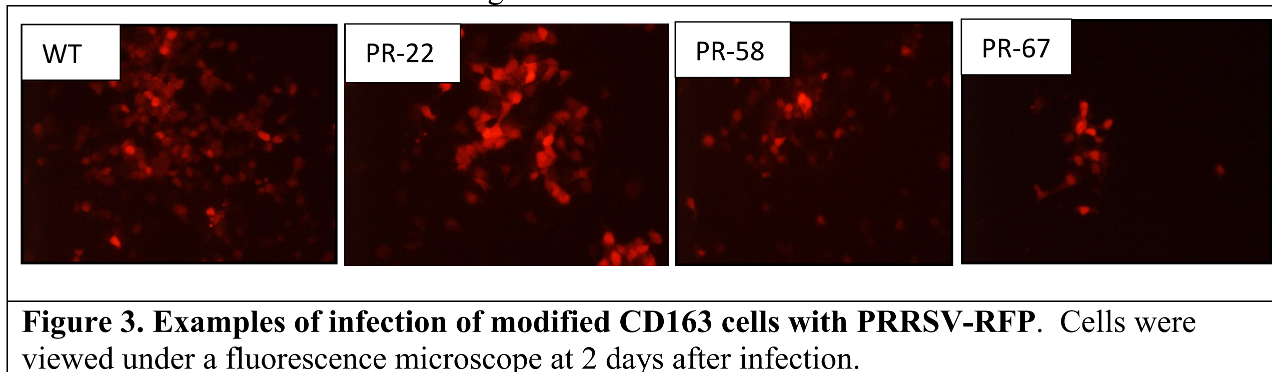

Figure 3. Examples of infection of modified CD163 cells with PRRSV-RFP. Cells were viewed under a fluorescence microscope at 2 days after infection.

PRRSV was serially passaged up to eight times on the remaining six cell lines. A summary of the passage results is shown in Table 2. For these experiments, we also included HEK cells that expressed a WT CD163. Two of the constructs, PR-43 and PR-55 were negative for the presence of infection at passage 2, and were not subjected to further study. Together the results showed that PRRSV can be adapted to growth on HEK cells that express recombinant CD163 proteins.

Objective 2. **Growth and sequencing of adapted viruses**

PRRSV-RFP was passaged at least six times on three constructs, PR-22, PR-58 and WT. The growth properties of PR-22 and PR-58 are summarized in Table 3. On the PR-22 construct, the virus replicated to a relatively high level over the course of the passages. Sequence was obtained for all ORFs, except for the first 183 bp corresponding to the first 61 amino acids in ORF2. At the nucleotide level, this region is G-C rich, which is blocking the primers used to sequence the PCR products. We are currently cloning this region into a sequencing vector and will continue to sequence this region. No mutations were detected in PRRSV passaged on HEK cells expressing the WT CD163 receptor (data not shown). The sequence results for PRRSV passaged on the PR-22 and PR-58 constructs are summarized in Figure 4. For PRRSV grown on PR-22, no nucleotide changes were observed over the first three passages. However, at passage 5 a single mutation was found in ORF6 resulting in a tyrosine to isoleucine change at amino acid 141 of the M protein. The mutation was still present in the passage 6 virus. Overall, the quality of the sequence data for passages 5 and 6 was rather "noisy". The reason for the poor data quality for these samples is not known. The sequencing results for PRRSV grown on PR-58 showed no changes in sequence during the first two passages. At passage 3, a nucleotide change appeared in ORF6, which resulted in tryptophan to histidine change at amino acid 86 in the M protein. Similar to the results for virus grown on PR-22, there was an overall decrease in the quality of the sequence data as the passages progressed. The reasons for this remain unclear.

Discussion

CD163 is the principal receptor for PRRSV. It would be predicted that PRRSV should possess the ability to readily adapt to changes in CD163 that affect virus replication. Furthermore, conventional wisdom would predict that the mutations involved in adaptation of PRRSV to mutations in CD163 should locate to the surface of GP2 and/or GP4. Conversely, the results from this study show that the serial passage of PRRSV on HEK cells expressing mutant CD163 proteins resulted only in mutations in the ORF6 protein, M, a gene that is relatively stable and conserved. Furthermore, we observed different patterns related to the growth of PRRSV on the different HEK-CD163 cell lines. For example, the serial passage of PRRSV-RFP on HEK-PR-58 cell showed that the virus eventually disappeared over the course of passaging (see Table 2 and Table 3). A second pattern is illustrated by the results obtained for the virus passaged on HEK-PR-22. Over seven passages, the amount of virus was decreased by only about one log (see Table 3). In these experiments we found no evidence of increased replication of PRRSV maintained by passage on cells expressing different mutations in CD163 suggesting that viruses do not readily adapt. Future work will be directed determining if genetically modified pigs possessing similar mutations in CD163 remain resistant to PRRSV.

The presence of mutations in ORF6 M protein was unexpected. Also unexpected was the location of the mutations within the M protein. Figure 5 shows a model of the M protein as it exists in the envelope of the virion. M is predicted to be a three transmembrane protein with a short, non-glycosylated ectodomain protruding from the envelope and a long endodomain tail. The ectodomain possesses a conserved cysteine, which forms a disulfide bond with a cysteine in the ectodomain of GP5. The two mutations identified in this study are located at positions 86 and 141, which correspond to the transmembrane and endodomain regions of the protein. It is difficult to understand how these mutations in M influence the adaptation of PRRSV to mutations in CD163. In previous work, we studied mutations in PRRSV that were involved in the escape from virus neutralization. Interestingly, one important mutation located to M, but within the ectodomain region (Popescu et al., 2017b).

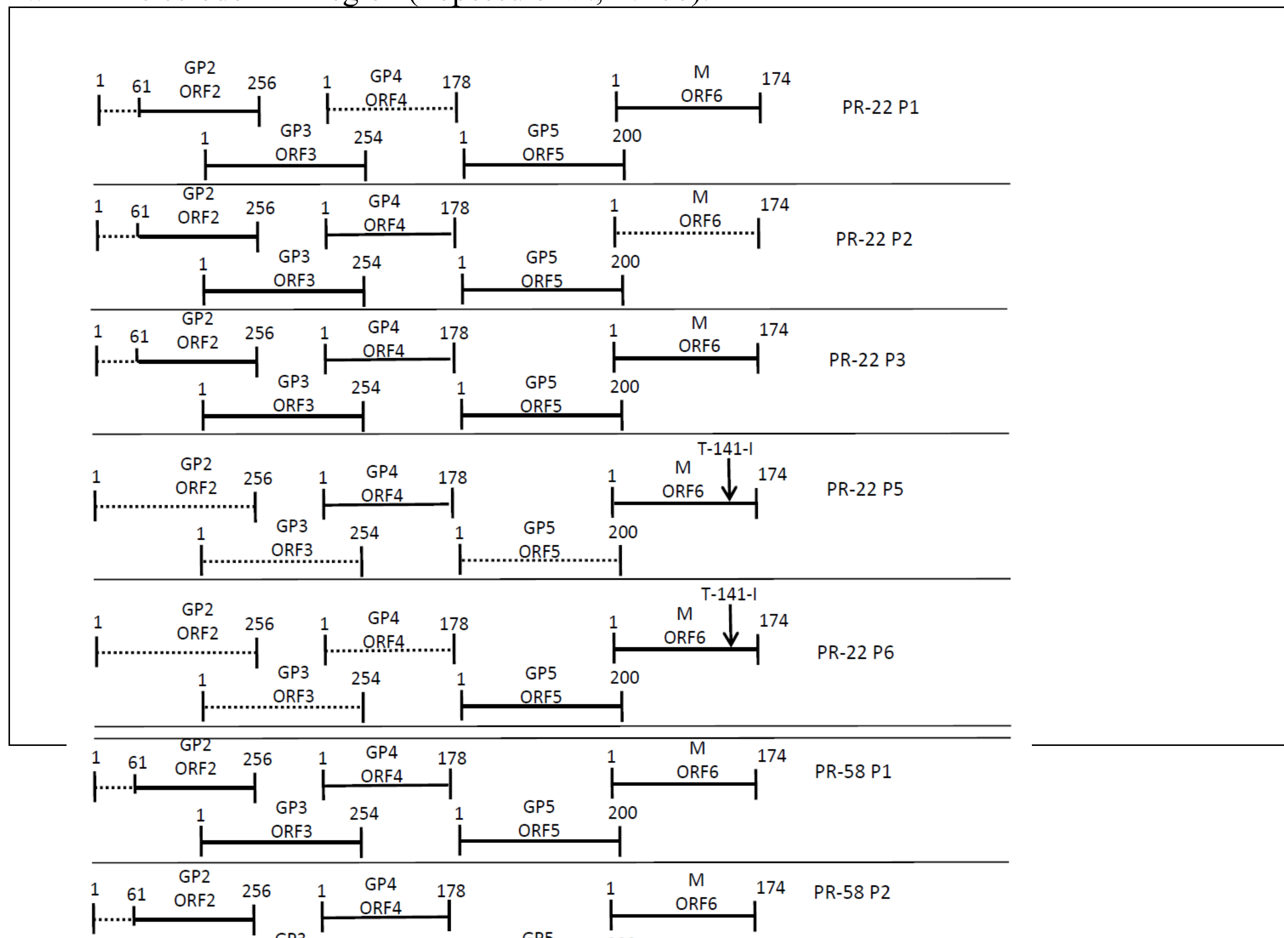

Figure 4. ORF2-6 sequence results for PRRSV-P129 during serial passage on PR-22 and PR-58 CD163 cell lines. The dotted lines are areas where there was no sequencing data. The arrows show the locations of mutations. The numbers refer to the peptide sequence.

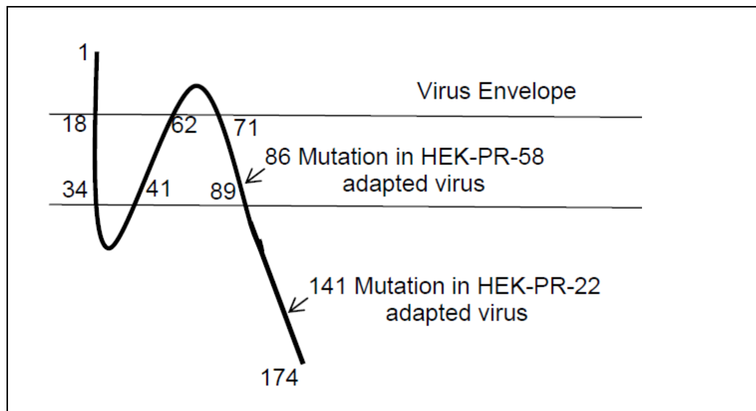

Figure 5. Model of PRRSV M protein in relation to the location of PRRSV-RFP mutations. The numbers identify the amino acids in the M protein peptide sequence.

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