RESEARCH REPORT
SWINE HEALTH

Title: Determination of the PRRSV minor glycoproteins contribution to antigenicity and protection - **NPB # 15-153**

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Industry Summary:
Porcine reproductive and respiratory syndrome virus (PRRSV) has been the most significant disease affecting swine in the U.S. for decades. Contemporary epidemiological methods rely on sequencing of the GP5 gene which encodes the major envelope glycoprotein GP5. While some studies have corroborated the importance of GP5 for PRRSV immunity, numerous studies have suggested that the minor glycoproteins encoded by GP2a, GP3, and GP4, play critical roles in PRRSV immunity and pathogenesis, although a paucity of studies have investigated the minor glycoproteins as compared to GP5. Here, using reverse genetics, we replaced the region of the genome encoding either the minor (GP2a-GP3-GP4) or major (GP5-M) proteins of the lab strain SD95-21 with various alleles determined from contemporary circulating PRRSV. Using indirect immunofluorescence and serum neutralization assays, we show that replacement of either the minor or major proteins of PRRSV variably affects the antigenicity of PRRSV. These results illustrate the importance of considering regions of the PRRSV genome besides GP5 when evaluating PRRSV epidemiology and immunity.

Keywords: PRRSV, sequencing, glycoprotein, antigenicity, immunity

Scientific Abstract:

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important diseases in swine caused by porcine reproductive and respiratory syndrome virus (PRRSV). Sixty-six near complete genome sequences were obtained using metagenomic sequencing of serum samples collected in the U.S. in 2014 to explore contemporary PRRSV genetic diversity. Phylogenetic analysis found three viral lineages with a majority of strains (46) being related to the reference NADC30. Only 16 strains were more closely related to the historical U.S. strain VR2332 and commercial vaccine strains and four strains had unresolved phylogeny. Phylogenetic analysis of the genes encoding the structural glycoproteins (GP) identified four to eight distinct lineages with >87% intraclade identity. To explore the effect of the observed genetic diversity on antigenicity, the genome region encoding either GP2a-GP3-GP4 or GP5-M in strain SD95-21 were replaced with alleles from each of eight distinct PRRSV strains using reverse genetics. The GP2a-GP3-GP4 region from only four of the eight strains yielded viable recombinant virus. When viable, both GP2a-GP3-GP4 and GP5-M variably affected antigenicity. A

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strain-dependent significant loss in cross reactivity was variably observed by indirect immunofluorescence assays using antisera from pigs vaccinated with commercial modified-live vaccines following replacement of GP2a-GP3-GP4 or GP5-M. Significantly reduced neutralization titers were similarly measured using antisera from naturally PRRSV-exposed pigs. These results illustrate the need to consider genomic regions besides GP5 for PRRSV epidemiology and vaccination.

Introduction:

Despite research and intervention for greater than 20 years, porcine reproductive and respiratory syndrome (PRRS) remains one of the most economically significant diseases affecting the U.S. swine industry. Both inactivated and modified live vaccines have long been used in an attempt to control PRRS however often are not effective. The vast amount of genetic variability between PRRS viruses (PRRSV) and the consequent poor match between vaccine strains and field viruses negatively impacts vaccine efficacy. The viral RNA dependent RNA polymerase of PRRSV has a low proof reading ability, leading to rapid viral evolution via accumulation of point mutations. Additionally, multiple PRRSV co-infecting a pig can recombine, leading to exchanges of large sections of the genome. Coupled with either naïve or animals vaccinated with specific PRRSV vaccines that fail to generate neutralizing antibodies to the challenge of diverse field viral strains, these two mechanisms contribute to genetic drift and evolution (Chang et al., 2002; Martin-Valls et al., 2014).

Early after its discovery, the GP5 protein of PRRS became the focus for PRRS intervention. GP5 is the major membrane glycoprotein of PRRSV. It possesses an epitope that was thought to be targeted by virus neutralizing antibodies (Kim et al., 2013). Genetic characterization of PRRSV has almost exclusively focused on GP5, with early restriction fragment length polymorphism (RFLP) methods being replaced with the now conventional GP5 gene sequencing. Despite more than two decades of PRRSV research with thousands of GP5 sequences determined, we are still facing the challenge of controlling PRRSV.

Recent work studying the evolution of PRRSV suggested that immunity to PRRSV is multigenic (Nguyen et al., 2013). Pigs infected with PRRSV elicited antibodies that specifically bound to recombinant polypeptides containing PRRSV ectodomain neutralizing epitopes (a combination of GP5 and the membrane protein M) but their titer did not correlate with neutralizing antibody response and did not neutralize PRRSV infectivity (Li and Murtaugh, 2012). Virus neutralizing antibodies often bind to viral proteins required for cell receptor binding, thus preventing virus from attaching to host cells and initiating infection. The GP5-M heterodimer, which is the dominant peptide on the PRRSV outer membrane, was long thought to be the PRRSV cell receptor binding protein owing to biochemical studies that showed its interactions with porcine sialoadhesin and porcine alveolar macrophages. However recent work suggested a key role for the minor PRRSV glycoproteins GP2a, GP3 and GP4 for receptor binding and consequently genetic variability leading to escape from immunity (Vu et al., 2011). Replacement of the PRRS minor glycoproteins in a PRRS infectious clone with those of the related equine arteritis virus (EAV) generated a chimeric virus displaying broad cell tropism characteristic of EAV (Tian et al., 2012). Similarly, a chimeric EAV infectious clone bearing PRRSV N-terminal GP5 and M ectodomains retained its ability to infect EAV permissive cell lines and did not acquire the ability to infect PRRS permissive cells (Lu et al., 2012). Screening of a cDNA expression library derived from porcine alveolar macrophages in a PRRSV non-permissive cell line found that the receptor protein CD163 enabled PRRSV growth (Calvert et al., 2007). It was later shown that GP2a and GP4 specifically interact with CD163 (Das et al., 2010).

Besides playing key roles in cell binding, numerous epitopes have also been identified in the PRRS minor glycoproteins (Costers et al., 2010). Peptides encompassing GP3 residues Y51 to S106 were shown to

react with highly pathogenic PRRSV antiserum (Wang et al., 2014). Likewise, monoclonal antibodies generated using recombinant GP3 recognized peptides in the same region (Zhou et al., 2006). N-glycosylation of GP3 residue 131 was also shown to mediate immune evasion via glycan shielding (Vu et al., 2011). The GP4 protein of type I PRRSV was shown to contain a highly variable neutralizing epitope that is subject to immune selection (Costers et al., 2010). Importantly, little is known on the antigenic regions of GP2a.

Objectives:

Objective #1: Construct recombinant PRRSV viruses containing alternative genes encoding GP2a, GP3 and GP4. Using an established PRRSV reverse genetics system, recombinant viruses bearing alternative genes for GP2a, GP3 and GP4 genes will be rescued.

Objective #2: Evaluate the contribution GP2a, GP3 and GP4 on PRRSV antigenicity using serum neutralization assays. Viruses rescued from Objective #1 will be assayed against reference PRRSV neutralizing antisera generated using three different commercial modified live vaccines.

Materials & Methods:

Note: PRRSV genome sequences were previously determined under NPB grant 14-204

Phylogenetic Analysis

Nucleotide sequences for the sixty-six near complete genomes and ORF2a –ORF6 genes determined here were aligned in MEGA6 with Muscle under default parameters. Each alignment was then evaluated for the best-fit model of evolution and phylogenetic trees were reconstructed in MEGA6 under the maximum likelihood approach using the best-fit model of nucleotide substitution. Nodal support was evaluated by 500 bootstrap replicates. Reference sequences, as proposed by Shi et al. [1] were also included, when available, as follows: lineage 1 MN184c (EF488739), lineage 3 FJ-1 (AY881994), lineage 5.1 VR2332 (EF536003) and Ingelvac MLV (EF484033), lineage 5.2 NADC-8 (U66394), lineage 6 NE35075 (U66380), lineage 7 PrimePac (AF066384), lineage 8 Ingelvac ATP2014 (EF532801), lineage 9 17198-6 (EF442776), a high-pathogenic Chinese strain JXA1 (EF112445), a U.S. reference strain NADC30 (JN654459), the parent of Ingelvac2014ATP, JA-142 (EF442773), and U.S. strain SDSU95-21 (KC469618). Not all reference strains had full genome sequences available.

Pairwise Sequence Comparisons

Nucleotide alignments for the genomes and ORF2a-6 were imported into DNASTAR Navigator 13 for pairwise comparisons to determine percent identity. Sequences were trimmed to uniform length before pairwise comparison was calculated for the full genome analysis, as some sequences were missing the extreme 5'-end.

Nucleotide Accession Numbers

Sixty-six PRRSV genomes were deposited in Genbank under accessions KT257944-KT258009.

Cells, viruses and Serum

MARC145 (American Type Culture Collection) cells and BHK21 cells (American Type Culture Collection) were cultured in minimal essential medium (MEM) with 10% fetal bovine serum (Invitrogen) at 37 °C, 5% CO_2. Primary porcine alveolar macrophages (PAM) were obtained from PRRSV-negative pigs and cultivated in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) and a mixture of antibiotics at 37 °C in a humidified atmosphere with 5% CO_2 [25, 26]. Field virus isolates were

cultivated on PAM and then adapted to grow in Marc145 cells. The US type 2 PRRSV isolate SD95-21 (GenBank accession no. KC469618) was used as parental virus for the infectious clone. Pig serum samples 65B (49dpi) and 69B (49dpi) were a gift from Dr. Eric Nelson at South Dakota State University and are antisera collected from naturally PRRSV-exposed pigs which were previously found to have neutralizing antibody titers to PRRSV isolate 23983. For measurement of antibody titers by indirect immunofluorescence, antiserum samples SD95-21 (41days post infection (dpi); a gift from Dr. Ying Fang at Kansas State University), Ingelvac MLV and JA142 (21dpi; a gift from Dr. Eric Vaughn from Boehringer Ingelheim), Fostera (28dpi; a gift from Dr. Jay Calvert from Zoetis), and PrimePac (42dpi; a gift from Drs. Fernando Osorio and Hiep Vu from the University of Nebraska-Lincoln), were used.

Construction of PRRSV cDNA clones

The ORF2-ORF4 (G24) and ORF5–ORF6 (GM) gene fragments of the ISU28, ISU32 and 14-79 strains were amplified from the virus stock by extraction of viral RNA and RT-PCR using Pfu Ultra High-fidelity DNA Polymerase (Agilent, Santa Clara, CA). The G24 fragments of the ISU10, ISU23, ISU39, ISU94 and ISU49 were commercially synthesized, as was the GM of ISU10 (Genscript Inc.). Subsequently, fragments G24 and GM were assembled into the pSD95-21 infectious clone vector (a gift from Dr. Ying Fang, Kansas State University) by replacement of the corresponding parental gene regions using the DNA recombination method following the instructions of the NEBuilder® HiFi DNA Assembly Cloning Kit (New England BioLabs, Ipswich, MA).

Chimeric Virus Rescue and Identification

To rescue the chimeric viruses, eight chimeric clones (four clones swapping the G24 gene region of SD95-21 and four clones swapping the GM gene region of SD95-21) were transfected into the BHK-21 cells. At 48-60 h post-transfection, the supernatant of the transfected cells was harvested and subsequently passaged onto MARC145 cells. Immunofluorescence assay (IFA) was used to identify the rescued viruses by using anti-PRRSV N monoclonal antibody SDOW17 (Rural Technologies Incorporated, Brookings, South Dakota). The G24 and GM gene regions from the rescued chimeric viruses were amplified and sequenced to confirm that the chimeric viruses were successfully rescued.

In vitro Growth Characterization of the Chimeric Viruses

To analyze if the chimeric viruses with the replaced G24 and GM genes have altered growth kinetics *in vitro,* MARC145 cells were infected with the parental backbone strain SD95-21 and chimeric viruses containing the heterologous G24 and GM genes at a multiplicity of infection (MOI) of 0.1. The infectious titers of the viruses harvested at 12, 24, 36, 48, 60, and 72 hours post-infection (hpi) were determined by IFA in MARC145 cells and quantified as 50% tissue culture infective dose per ml ($TCID_{50}$/ml).

Virus plaque assay

MARC145 in six-well plates were inoculated with 10-fold serial dilutions of chimeric viruses. After 1 h, the inoculum was replaced with an overlay of 1% low-melting-point agarose in Eagle's minimal essential medium (EMEM) containing 2% FBS. When the agarose overlay had solidified, the plate was inverted and placed in an incubator at 37°C for 4 days. The resulting plaques were fixed in 4% (vol/vol) formaldehyde in distilled water for 1 h and were then stained with crystal violet (5% [wt/vol] in 20% ethanol).

Indirect immunofluorescence (IFA) assays

To analyze antigenic differences of eight chimeric viruses and SD95-21, indirect IFA were performed to detect anti-PRRSV antibodies in swine sera. MARC145 cells were infected with chimeric viruses and SD95-21 at 0.01 MOI and incubated for 48hrs. The PRRSV infected Marc145 cells were then fixed with 4% paraformaldehyde and treated with 0.1% triton-X100. PRRSV serum samples were initially diluted at

16 and then serially 2-fold diluted in phosphate buffered saline (PBS). The diluted serum samples were added onto fixed PRRSV infected Marc145 cells and incubated for 1 hr at 37°C. The cells were stained with an anti-PRRSV antibody SDOW17 and FITC-conjugated goat anti-mouse IgG. IFA antibody titer endpoints were determined as the highest dilution that showed specific intracytoplasmic fluorescence. A serum sample showing positive fluorescence at 1:16 dilution against negative antigen was excluded.

Serum neutralization (SN) assays

Neutralizing antibody (NA) titers were determined for eight chimeric viruses, parental backbone strain SD95-21 and field strains of PRRSV (ISU28, ISU32, and 14-79) for pig serum samples collected at 49 days post infection (dpi). Serum samples were serially 2-fold diluted in MEM media supplemented with 2% FBS. The diluted serum samples, starting at 1:2 or 1:4 dilutions, were mixed with an equal volume of 200 $TCID_{50}$ PRRSV and incubated for 1 h at 37°C. The mixtures were then added to a 96-well cell culture plate with confluent monolayers of MARC145 cells and incubated for another 1 h at 37°C. After removing the inoculum and washing the cells with MEM media, 100 µl of fresh Dulbecco's minimal essential medium (DMEM) supplemented with 2% FBS was added to each well. After incubation for 20 h at 37°C, the cells were fixed with 4% paraformaldehyde and stained with an anti-PRRSV antibody SDOW17 and FITC-conjugated goat anti-mouse IgG. The neutralizing antibody titers were expressed as the highest dilution that showed more than 90% reduction in the numbers of fluorescent foci compared to that of the serum samples from the negative control group in the same dilution.

Statistical Analyses

The differences in titer between the chimeric viruses and SD95-21 were evaluated by unpaired Student's t test, with 95% confidence level.

Results:

Phylogenetic and pairwise comparison analysis

Phylogenetic analysis of the complete genomes and ORF2a-ORF6 were performed separately. Phylogenetic analysis of the sixty-six full genome sequences and references found that a majority (46 strains) were included in a well-supported lineage which included the sole reference NADC30 (Fig 1). MN184c occupied an ancestral position in the tree and failed to have a strong evolutionary relationship to other viruses. Sixteen field strains were included in a monophyletic lineage which included VR2332/Ingelvac MLV references as well as the JXA1 and Ingelvac ATP2014 references. Six strains (14-76, 14-78, 14-79, 14-81, 14-95, and 14-96) were closely related to the Ingelvac ATP2014 vaccine strain and four strains (14-60, 14-64, 14-67, and 14-68) were closely related to SDSU95-21, the Ingelvac MLV vaccine and its parent strain VR2332. Other field strains in this lineage include 104194, ISU81, ISU82, ISU84, ISU86, and ISU87. Strains ISU91, ISU90, SDSU47, and 103837 formed a separate lineage with unresolved phylogeny to the VR2332 and NADC30 lineages. The genome-wide pairwise comparison yielded 78.2-99.9% identity of all field strains and references.

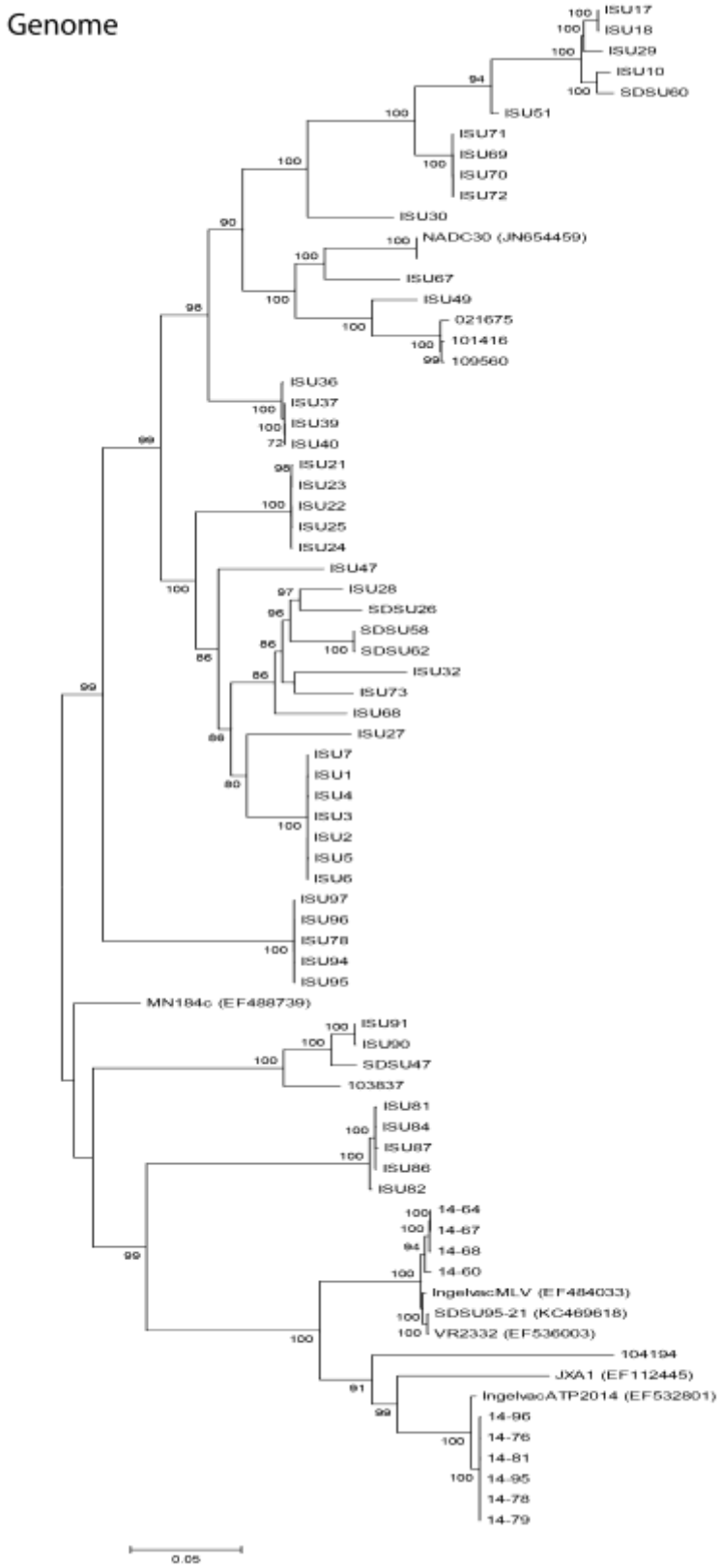

Figure 1. Phylogenetic analysis of porcine reproductive and respiratory syndrome virus genome nucleotide sequences. Maximum-likelihood analysis in combination with 500 bootstrap replicates was used to derive trees based on the nucleotide sequences. A scale representing the number of nucleotide changes is shown in each panel and clades with bootstrap values >70 are labeled.

Phylogenetic analysis of ORF2a revealed four well supported clades (bootstrap values≥94) with overall percent identity ranging from 83.4-100% (Figure 2). Intraclade identity ranged from 87.0-100%. One clade contained numerous PRRSV lineage references, including vaccine strains Ingelvac ATP2014, Ingelvac MLV PRRS and PrimePac PRRS [1], while two of the other clades each contained a single lineage reference (NADC30 and MN184c). The remaining clade containing 20 strains was genetically novel.

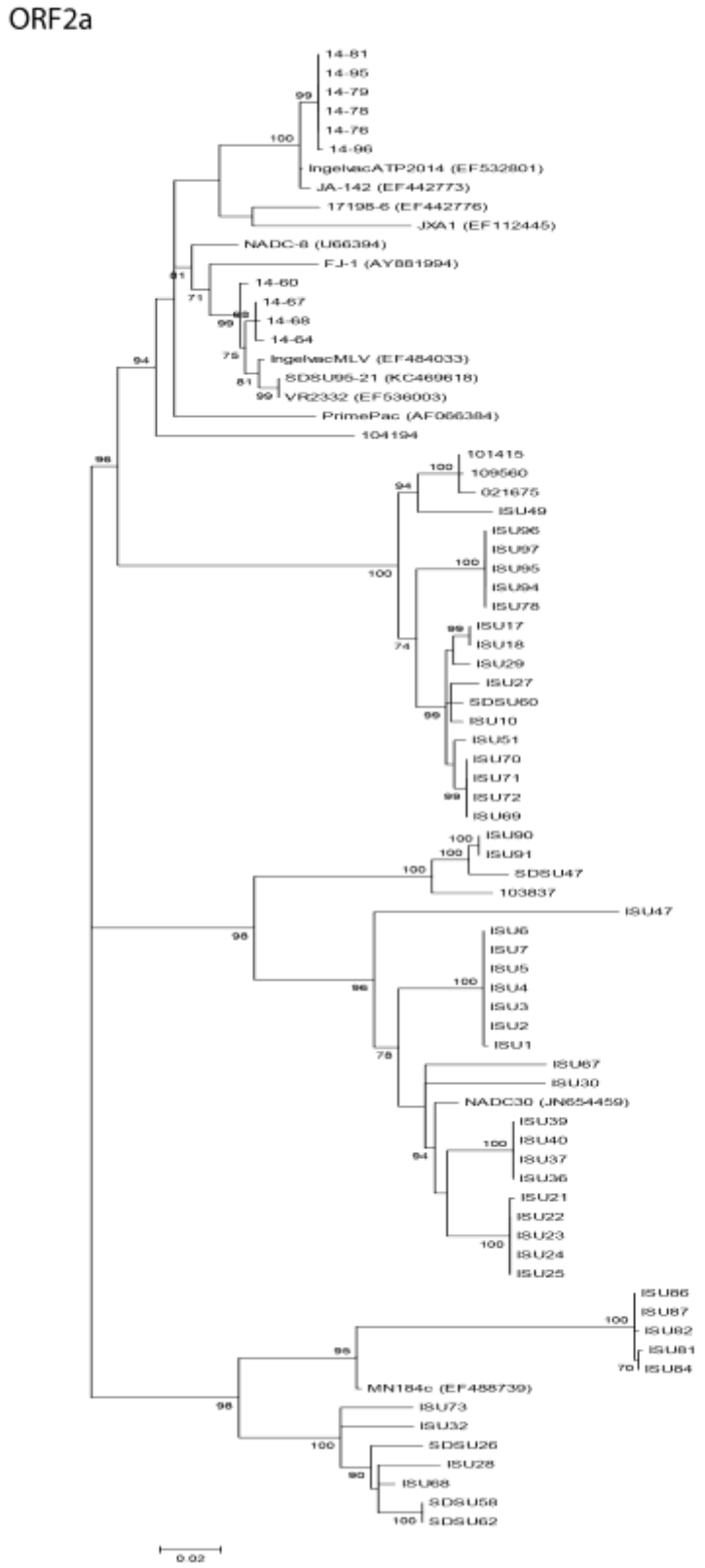

Figure 2. Phylogenetic analysis of porcine reproductive and respiratory syndrome virus ORF2a nucleotide sequences. Maximum-likelihood analysis in combination with 500 bootstrap replicates was used to derive trees based on the nucleotide sequences. A scale representing the number of nucleotide changes is shown in each panel and clades with bootstrap values >70 are labeled.

Phylogenetic analysis of ORF3 likewise identified four clades with ≥97% bootstrap support along with strain 104194 which failed to show an clear evolutionary relationship to any other strains (Figure 3.). Overall percent identity ranged from 77.6-100% and intraclade identity ranges from 87.7-100%. As seen with ORF2a, one clade contained several PRRSV lineage references described by Shi et al.[1], including vaccine strains Ingelvac ATP2014, Ingelvac MLV PRRS and PrimePac PRRS, while two of the other clades each contained a single lineage reference (NADC30 and MN184c). The remaining clade containing 20 strains was genetically novel and contained the same 20 strains as the novel clade identified for ORF2a. Field strain 104194 failed to show a strong evolutionary relationship to any other field strains or references.

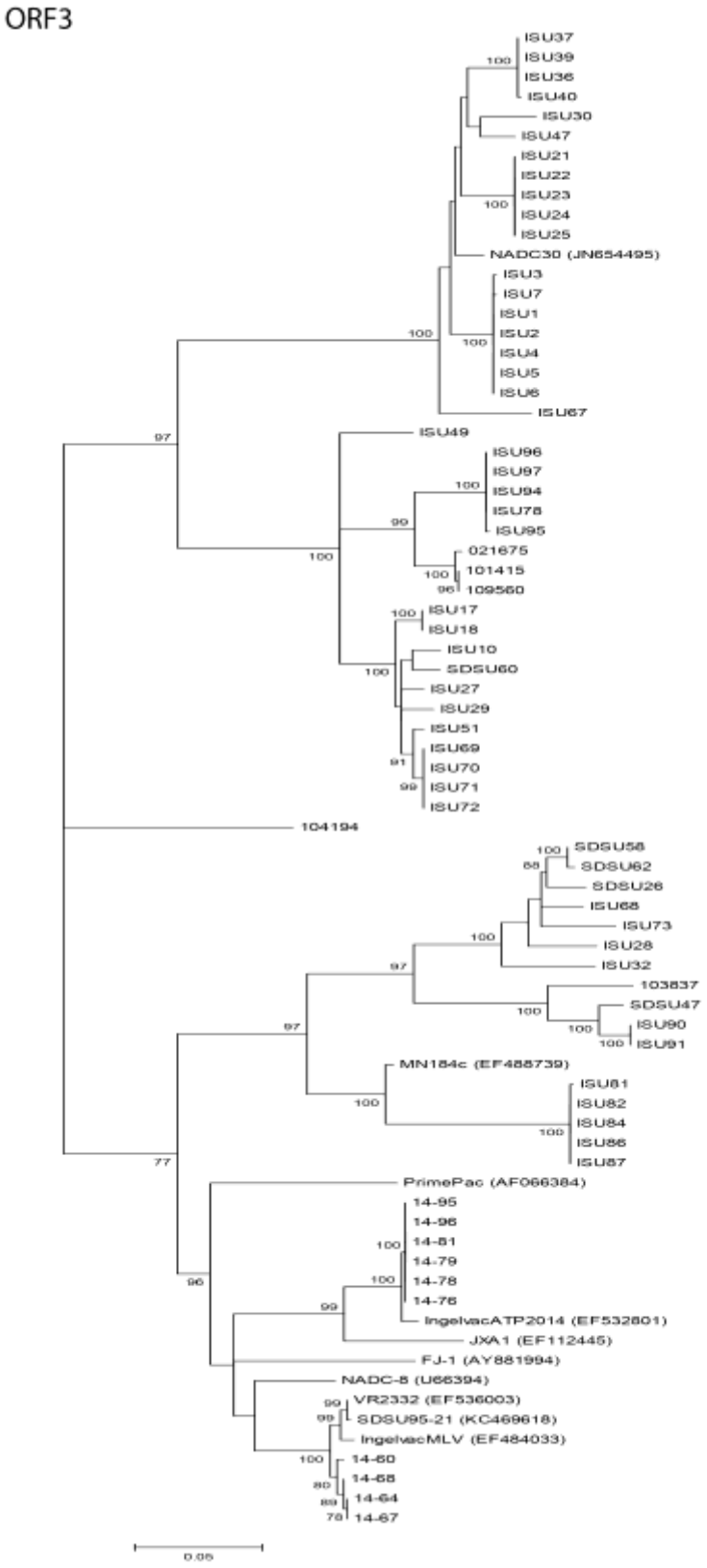

Figure 3. Phylogenetic analysis of porcine reproductive and respiratory syndrome virus ORF3 nucleotide sequences. Maximum-likelihood analysis in combination with 500 bootstrap replicates was used to derive trees based on the nucleotide sequences. A scale representing the number of nucleotide changes is shown in each panel and clades with bootstrap values >70 are labeled.

The phylogeny of ORF4 was less resolved, with seven clades showing nodal support values of 70 or higher (Figure 4.). Overall identity was 83.6-100% between strains for ORF4 while intraclade percent identity ranged from 90.3-100%. The field strain 104194 and references FJ-1 and PrimePac did not have well defined evolutionary relationships to other strains in the tree. Nearly half (n=31) the strains formed a clade with NADC30 while three of the clades failed to contain a reference sequence. One clade contained reference strains VR2332, SDSU95-21, Ingelvac MLV, and NADC-8 along with four strains determined here while its sister clade contained references JXA1, JA-142, 17198-6 and Ingelvac ATP along with 6 field strains. The remaining clade consisted of five strains along with reference MN184c.

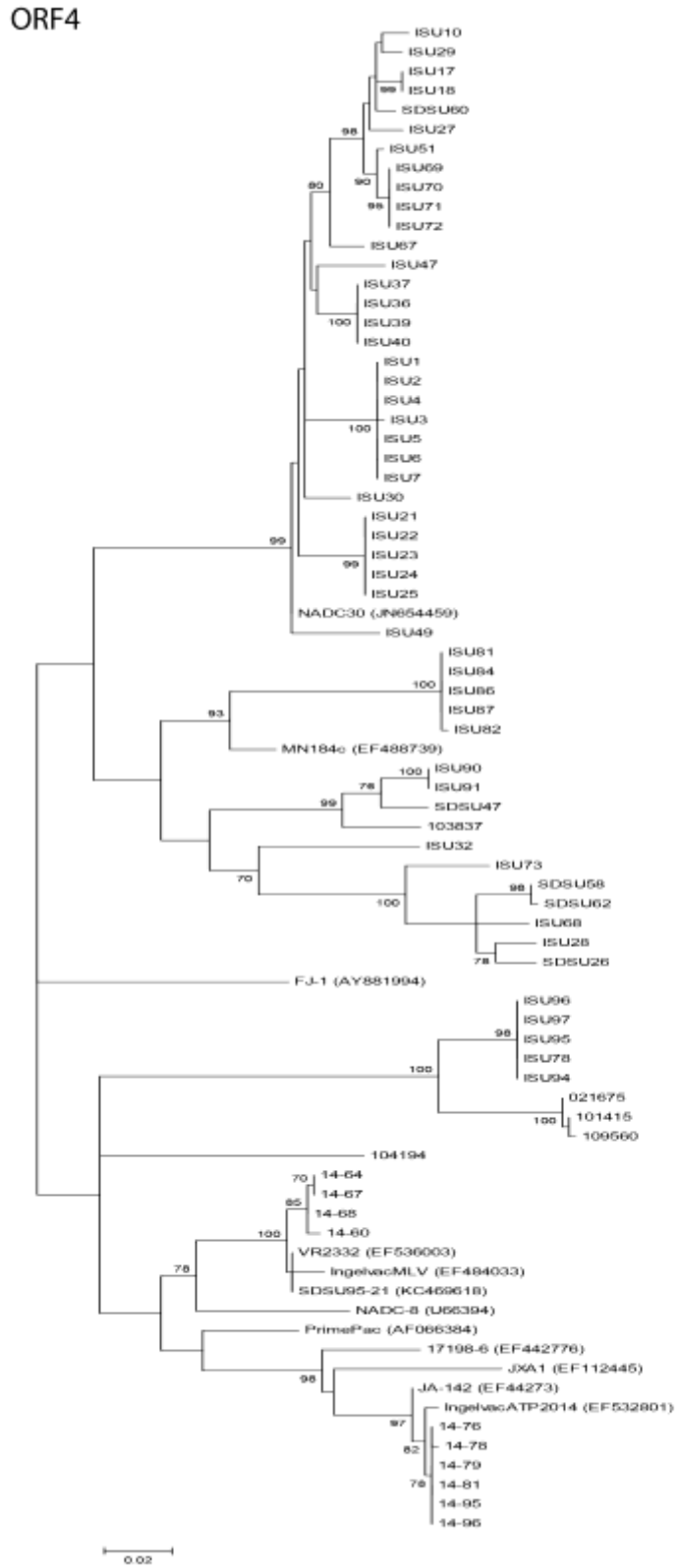

Figure 4. Phylogenetic analysis of porcine reproductive and respiratory syndrome virus ORF4 nucleotide sequences. Maximum-likelihood analysis in combination with 500 bootstrap replicates was used to derive trees based on the nucleotide sequences. A scale representing the number of nucleotide changes is shown in each panel and clades with bootstrap values >70 are labeled.

The phylogeny of ORF5 consisted of eight clades with ≥83% bootstrap support (Figure 5). ORF5 pairwise alignments found 82.3-100% identity between strains with intraclade percent identities from 89.6-100%. References MN184c and FJ-1failed to show clear evolutionary relationships to any clade, as did field strain 104194. Five of the ORF5 clades did not contain any reference strains. Eleven strains were most closely related to most of the references and commercial vaccine strains, with four field strains closely related to VR2332 and six field strains closely related to IngelvacATP2014. Included in this alignment were references described by Shi et al. (17198-6, FJ-1, VR2332, NADC-8, PrimePac) and a highly pathogenic Chinese strain JXA1, that represent different ORF5 lineages based on a comprehensive phylogenetic analysis of 8,624 ORF5 sequences [1]. The remaining clade consisted of 13 strains and reference NADC30.

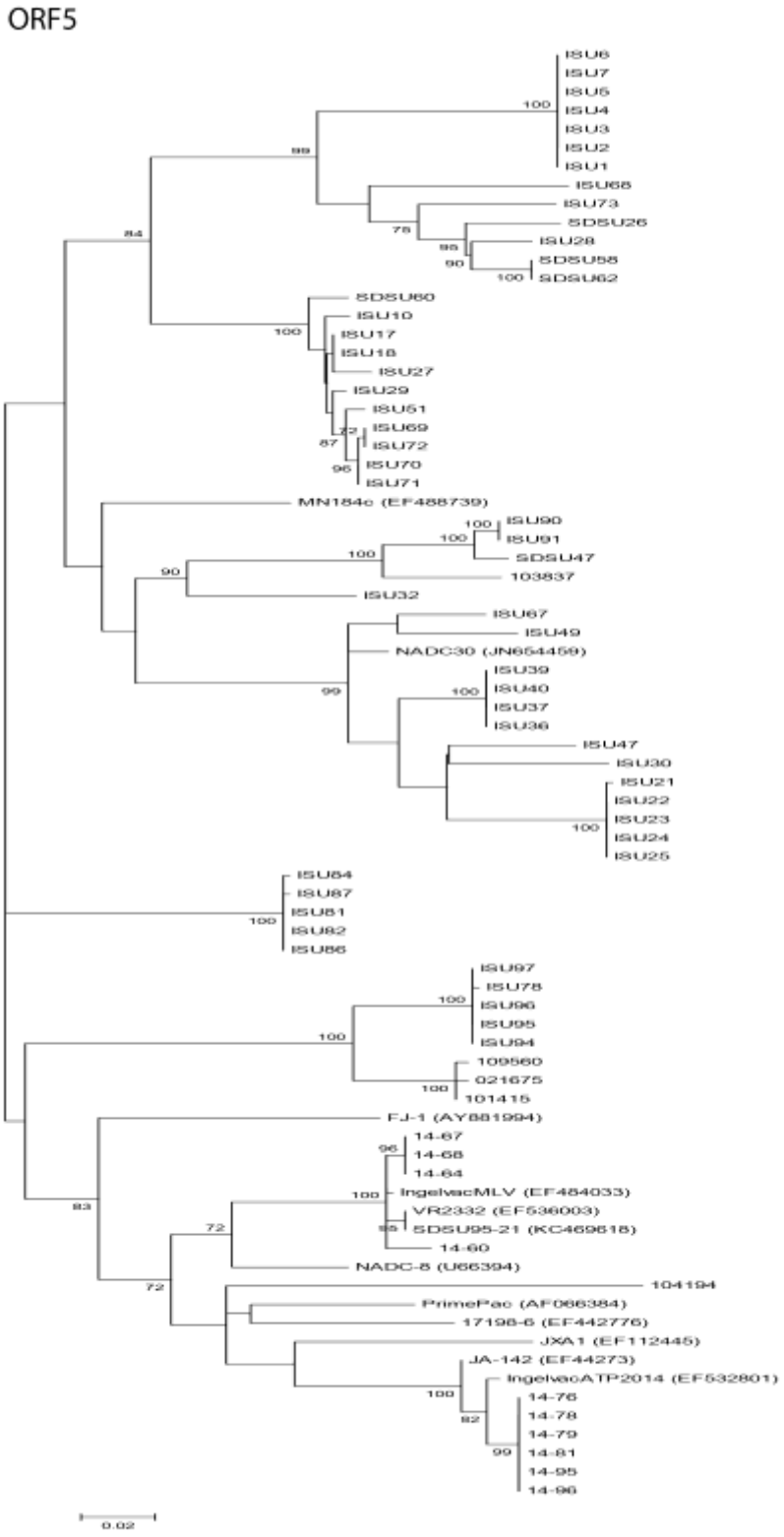

Figure 5. Phylogenetic analysis of porcine reproductive and respiratory syndrome virus ORF5 nucleotide sequences. Maximum-likelihood analysis in combination with 500 bootstrap replicates was used to derive trees based on the nucleotide sequences. A scale representing the number of nucleotide changes is shown in each panel and clades with bootstrap values >70 are labeled.

The ORF6, which encodes the matrix protein, consisted of six clades and a number of strains with unresolved evolutionary history (Figure 6; ISU32, 104194 and references JXA1, 17198-6, FJ-1, and PrimePac). Overall percent identity ranged from 87.0-100% and intraclade identity ranged from 90.5-100%. Nearly half the strains (n=32) formed a clade including NADC30. Ten strains formed a clade with MN184c while two clades were genetically unique in lacking inclusion of a reference strain. The remaining two clades contained strains closely related to either NADC-8, VR2332, SDSU95-21 and Ingelvac MLV or JA-142 and IngelvacATP2014.

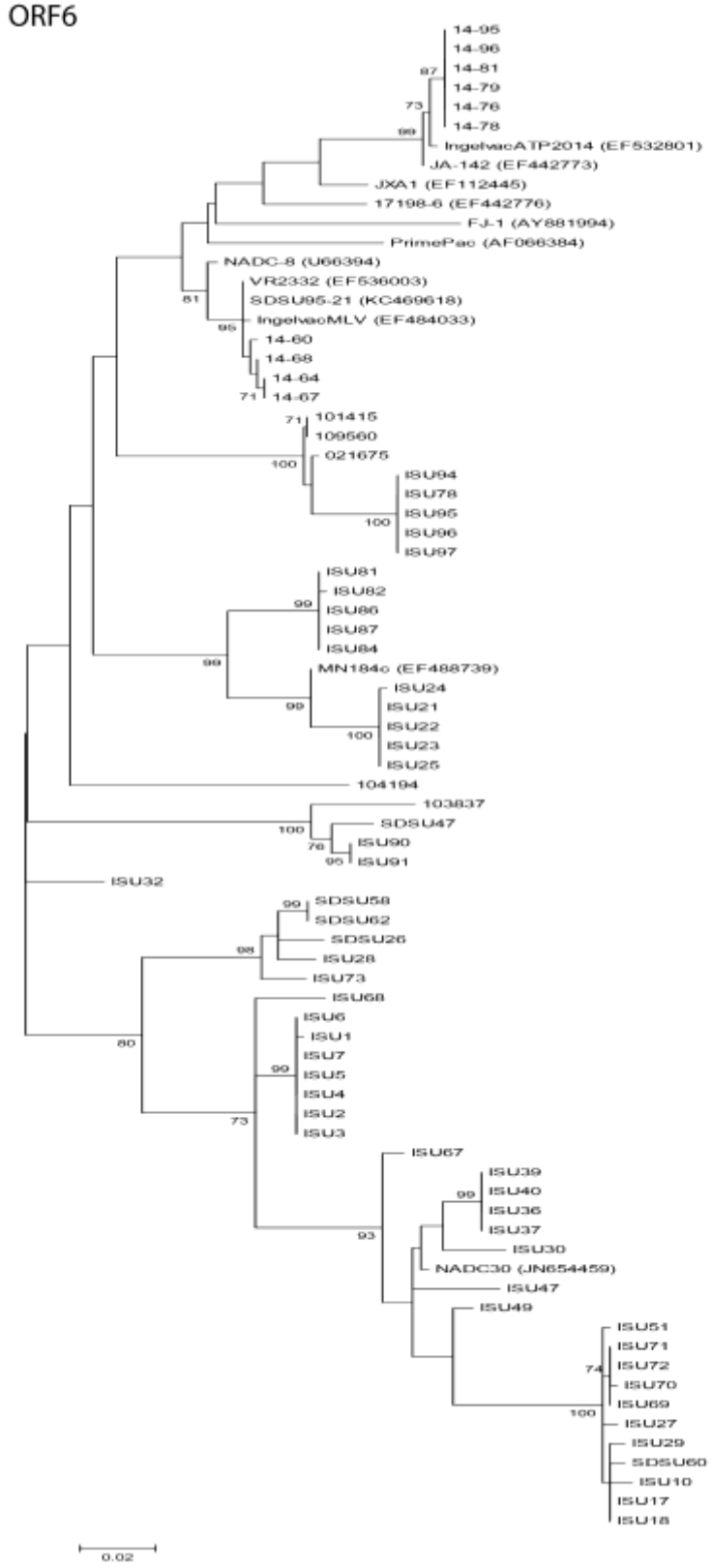

Figure 6. Phylogenetic analysis of porcine reproductive and respiratory syndrome virus ORF6 nucleotide sequences. Maximum-likelihood analysis in combination with 500 bootstrap replicates was used to derive trees based on the nucleotide sequences. A scale representing the number of nucleotide changes is shown in each panel and clades with bootstrap values >70 are labeled.

Construction of chimeric clones and recovery of viable chimeric viruses

Serum samples for PRRSV strains ISU28, ISU32, ISU10, ISU23, ISU39, ISU94, ISU49 and 14-79 were inoculated on PAM cells and then passaged to Marc145 cells. ISU28, ISU32 and 14-79 were recovered from cell culture however ISU10, ISU23, ISU39, ISU94 and ISU49 could not be propagated *in vitro*. Therefore, the G24 gene fragments were reconstructed synthetically for ISU10, ISU23, ISU39, ISU94 and ISU49 (Genscript, Piscataway, NJ). The GM region of the genome was additionally synthesized for ISU10. Then, the G24 and GM gene fragments were assembled onto SD95-21 infectious clone by replacing the corresponding regions of virus backbone and confirmed by sequencing (Figure 7a). Eight chimeric viruses containing heterologous G24 and GM genes from four different PRRSV strains were successfully rescued from BHK21 cells (SD95-ISU10-GM, SD95-ISU28-GM, SD95-ISU32-GM, SD95-14-79-GM) and (SD95-ISU10-G24, SD95-ISU28-G24, SD95-ISU32-G24, SD95-14-79-G24). Chimeric viruses with a SD95-21 backbone and G24 from ISU23, ISU39, ISU94 and ISU49 were not viable. The supernatants of BHK21 cells were used to infect MARC145 cells and the virus rescue was further confirmed by IFA with anti-PRRSV N antibody SDOW17 (Figure 7b). The chimeric virus clone stocks were analyzed by RT-PCR and sequencing, and a number of nucleotide changes and resulting amino acid alterations were observed (Table 1).

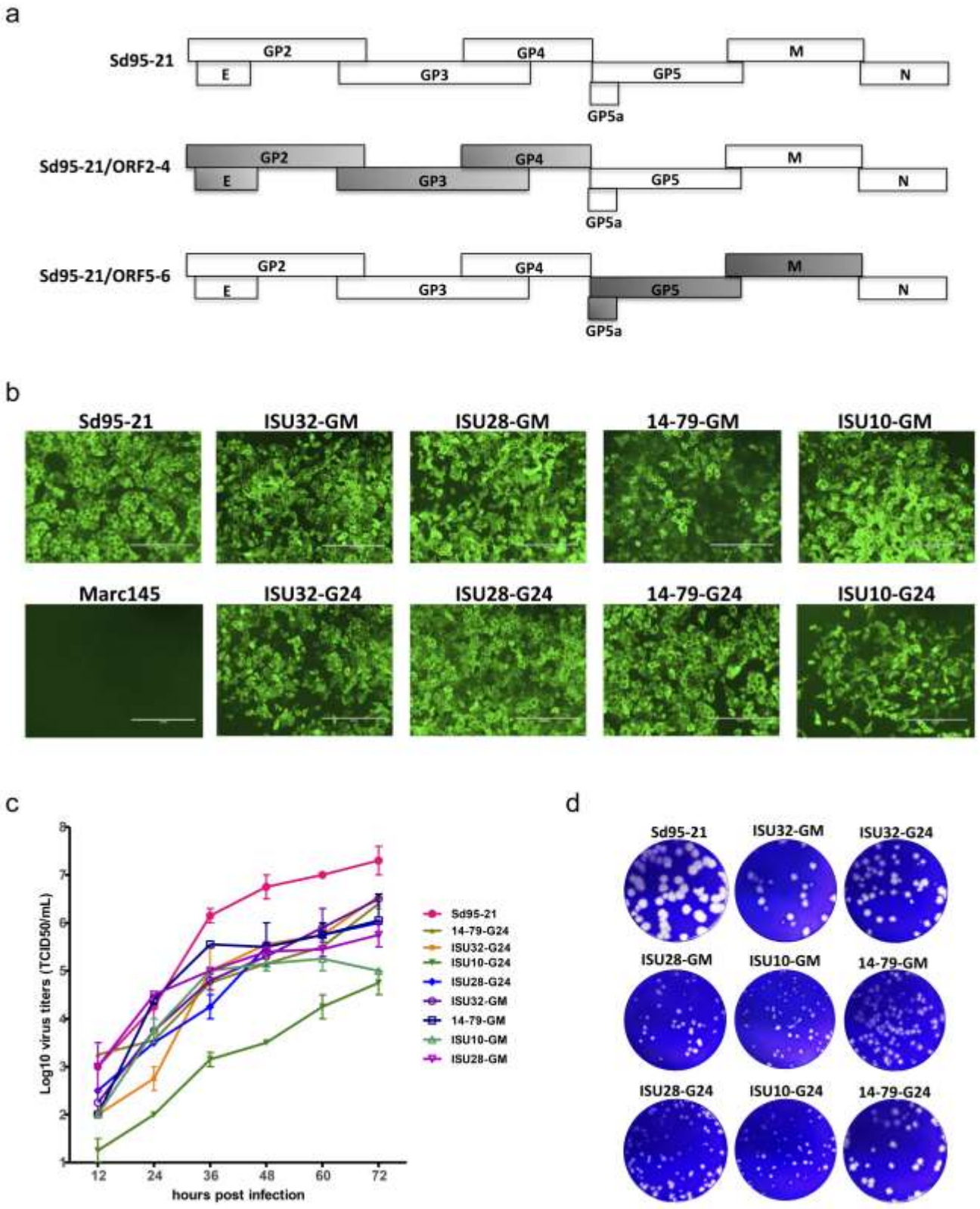

Figure 7. Recombinant PRRSV viruses were constructed via reverse genetics using a SD95-21 backbone (a) by replacing either genes ORF2a-ORF3-ORF4 encoding glycoproteins GP2-GP3-GP4 and the envelope (E) (G24), or genes ORF5-ORF6 encoding glycoprotein GP5-matrix (GM) (b) growth of recombinant viruses in Marc145 cells was verified by direct immunofluorescence using the monoclonal antibody SDOW-17 (c) *In vitro* growth kinetics were determined by

inoculating Marc145 cells at a multiplicity of infection (MOI) of 0.01 followed by titration on Marc145 cells (d) plaque morphology of recombinant PRRSV on Marc145 cells

Table 1. Mutations identified in constructed recombinant PRRS viruses.

Virus	Position at pCMV-SD95-21*	Nucleotide Mutation	Amino acid Mutation
ISU28-GM	13888	G→A	Arg→Gln
14-79-GM	14885	C→T	silent
	14866-14867	AG→GA	Gln→Arg
ISU10-GM	none	none	none
ISU32-GM	None	none	none
ISU28-G24	12812	T→C	silent
14-79-G24	12473	G→A	Ala→Thr
ISU10-G24	none	none	None
ISU32-G24	12323	A→G	silent
	12747	G→A	Cys→Thr
	13626	G→A	Val-Ile

* GP5-M is located at nucleotides 13779-14900 and GP2-GP4 is located at nucleotides 12074-13778 in pCMV-SD95-21

In vitro growth properties of chimeric viruses

The growth kinetics of the chimeric viruses were compared with the parental viruses SD95-21 in MARC145 cells. Chimeric viruses displayed similar growth kinetics, but had relatively impaired growth ability compared to the parental virus (Figure 7c). Among the eight chimeric viruses, SD95-ISU10-G24 had the largest growth attenuation, with a final titer 2-3 $\log_{10}$ lower than SD95-21. Chimeric PRRS viruses produced plaques that were clearly smaller than SD95-21 with SD95-ISU10-G24 producing smallest plaques, suggesting that the heterologous ORF2-ORF4 genes or ORF5-ORF6 genes may significantly impair the replication ability of chimeric SD95-21 viruses in MARC145 cells (Figure 7d).

IFA antibody analysis

IFA antibody titers were examined in the sera from PRRSV inoculated or vaccinated pigs for SD95-21 and eight chimeric PRRS viruses (Figure 8). The IFA antibody titers were less than 16 in serum samples collected from PRRSV naïve pigs, but the titers of serum samples of PRRSV-infected pigs ranged between 2^9 (512) and 2^{11} (2048). Replacement of the SD95-21 G24 or GM with those from ISU28 or 14-79 significantly reduced cross reactivity with SD95-21 antiserum. Only the GM of ISU32 significantly reduced cross reactivity. Glycoproteins derived from ISU10 had no significant effect on antigenicity relative to parental alleles. None of the recombinant viruses reacted differently than parental virus using Ingelvac MLV antiserum. Using Fostera antiserum, recombinant viruses bearing alternate G24 alleles all demonstrated significantly decreased cross reactivity while replacement of GM had no effect on cross reactivity. Recombinant viruses containing ISU28 G24 and GM reacted less strongly with JA142 antisera as did virus containing GM of 14-79. Both the G24 and GM genes from ISU28 and ISU10 decreased cross reactivity with PrimePac antiserum as did GM alleles from ISU32 and 14-79.

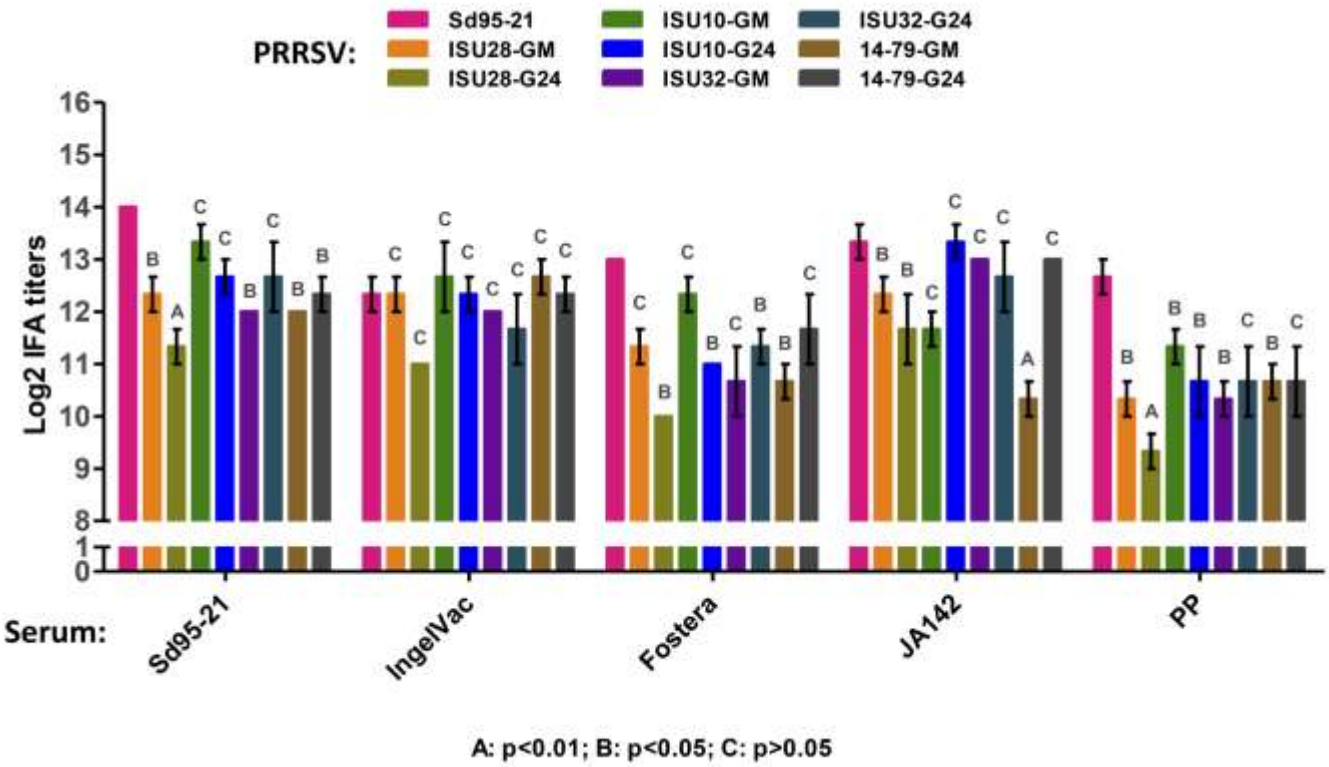

Figure 8. Changes in antigenicity were assessed for the recombinant viruses by indirect immunofluorescence assay using porcine antiserum generated against the parent of the reverse genetics system SD95-21 as well as antiserum from pigs inoculated with commercial modified-live viruses Ingelvac PRRS-MLV, Fostera PRRS, Prime Pac PRRS, and the reference virus JA142. Differences in IFA titer for recombinant viruses as compared to the parent SD95-21 were assessed by the student's t-test at the $P<0.01$ (a), $P<0.05$ (b), and $P>0.05$ level.

Neutralizing antibody analysis

To investigate if replacement of SD95-21 G24 or GM genes with heterologous genes derived from genetically diverse strains of PRRSV could alter the neutralizing antibody activity of pig serum samples against SD95-21, we determined and compared the anti-PRRSV specific neutralizing antibody titers in sera collected at 49 days post exposure with an unknown field virus by neutralizing antibody assay with each of the eight chimeric PRRS viruses, three available heterologous PRRSV field stains, and SD95-21 (Figure 9). We initially planned to use the same antisera tested by IFA however all IFA antisera lacked neutralizing titers >4. Antiserum 69B had a neutralizing titer ~14 to SD95-21 however showed a significant decrease in neutralizing titer to the four PRRSV isolated from clinical samples as well as recombinant viruses bearing GP24 or GM derived from the same viruses. In contrast, antiserum 65B failed to neutralize (titer>4) SD95-21 and field viruses or recombinant strains derived from ISU32 and ISU10. Antiserum 65B neutralized 14-79 and recombinant viruses containing 14-79 genes as well as ISU28 and a recombinant virus with ISU28-G24.

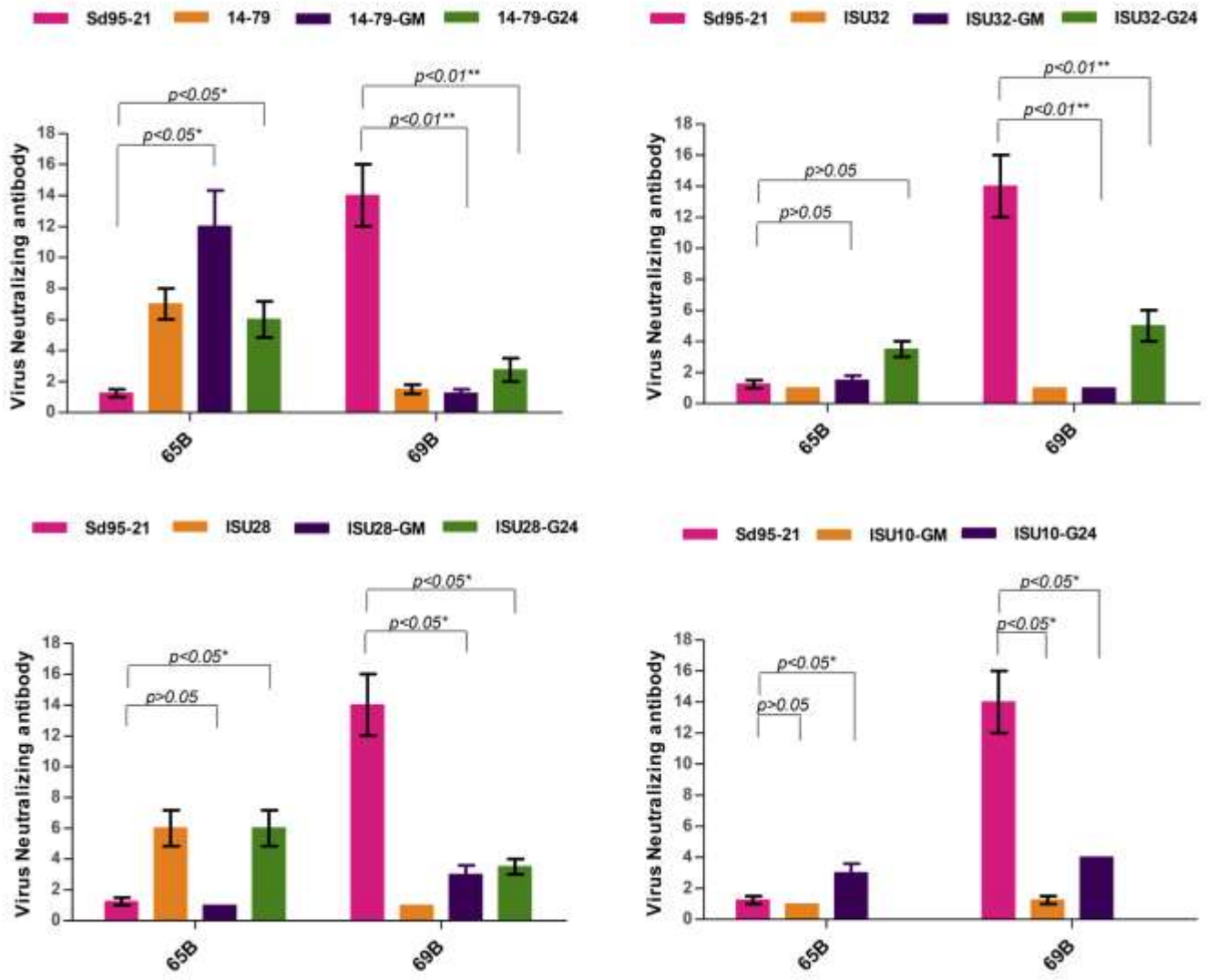

Figure 9. Changes in antigenicity were assessed for the recombinant viruses by serum neutralization (SN) assays using sera from pigs naturally exposed to an unknown, wild type PRRSV (65B and 69B). SN assays were performed with the reverse genetics backbone donor SD95-21, ORF2a-ORF3-ORF4 (G24) and ORF5-ORF6 (GM) donor strains 14-79, ISU32, ISU28 and ISU10, and recombinant viruses. Differences in SN titer for recombinant viruses as compared to the parent SD95-21 were assessed by the student's t-test at the $P<0.01$, $P<0.05$, and $P>0.05$ level.

Discussion:

PRRSV epidemiology and control efforts have largely focused on GP5 due to its high genetic variability and prominence on the virion. More recently, the minor structural glycoproteins of PRRSV were shown to control PRRSV cellular tropism [19]. While ~10-fold fewer PRRSV minor structural glycoprotein sequences have been determined as compared to GP5, genetic diversity for each of the structural glycoproteins is similar [27]. In order to gain further insight into the genetic and antigenic diversity of PRRSV, here we determined the genomes of 66 PRRSV strains directly from clinical specimens. As PRRSV has a very restricted cell tropism limited to porcine alveolar macrophages and Marc145 cells, we hypothesized that direct sequencing of PRRSV from clinical specimens would identify greater genetic diversity than previously described from genome sequences determined from isolates propagated *in vitro*, especially for the minor

glycoproteins GP2a-4 owing to their role in receptor binding. We observed 83.4-100%, 77.6-100%, 83.6-100% and 82.3-100% percent identity between strains for ORF2a, ORF3, ORF4 and ORF5, respectively, which demonstrates more diversity than previously reported (91-99%, 86-98%, 92-99% and 88-97% identity for ORF2a, ORF3, ORF4 and ORF5, respectively) [27]. It is unclear if this increased diversity is due to our methodology or if PRRSV has become increasingly diverse in the subsequent two decades between studies.

A large scale phylogenetic analysis including more than 8,000 GP5 sequences categorized type 2 PRRSV into nine lineages with less than 10% intraclade and more than 10% interclade genetic diversity [1]. Despite inclusion of references from Shi et al. into our analysis, we identified several novel, well supported monophyletic lineages using similar criteria for defining clades [1]. It is unclear whether these discrepancies are due to differences in phylogenetic analysis, PRRSV evolution between the two studies, epidemiology or sequencing methodology.

Recombination is a common evolutionary mechanism utilized by PRRSV to generate genetic diversity [8]. Recombination can lead to phylogenetic incongruencies with respect to tree topology depending on the gene used for analysis. For example, two strains of PRRSV that we used for generation of recombinant viruses, ISU28 and ISU32, had ORF2a, ORF3 and ORF4 genes belonging to the same lineage however ORF5 and ORF6 were in distinctly different lineages. Phylogenetic methods are designed to elucidate virus evolution vertically and horizontal sequence introduction, via recombination, can lead to incongruences [28]. Analysis of single or small numbers of concatenated genes led to a significant probability for topological incongruences while analysis of larger regions of the genome better resolved phylogeny and increased topological support [29]. Given the commonplace nature of recombination for PRRSV, phylogenetic analysis of full genome sequences is more likely to recapitulate viral evolution as compared to single gene phylogeny. As seen here, phylogenetic analysis using complete genome sequences gave nearly fully resolved evolutionary histories for the strains characterized.

Ten of the strains in this study (15%) had high identity to the commercial attenuated live vaccines IngelvacATP2014 and Ingelvac MLV. While full clinical histories were not available for all the samples included in this study, all samples were submitted to veterinary diagnostic laboratories for PRRSV qRT-PCR. Previous studies have isolated viruses with high identity to PRRSV vaccine strains, suggesting potential reversion to virulence [30, 31].

We generated recombinant viruses with a SD95-21 backbone with either ORF2a-ORF3-ORF4 or ORF5-ORF6 regions from four strains with different evolutionary histories. Antigenic analyses using IFA assays and antisera generated against the parent SD95-21 or from pigs vaccinated with commercial modified live viruses or reference viruses variably showed loss of cross reaction due to replacement of SD95-21 genes with those from strains ISU28, ISU32, ISU10 or 14-79, both due to donor strain differences and genes replaced. Similar results were obtained by serum neutralization assays. A characteristic of PRRSV infection is a delayed neutralizing antibody response [32]. The lack of neutralizing antibodies in our commercial vaccine reference antisera unfortunately prevented us from assessing the antigenic impact of GP2a-GP3-GP4 or GP5-M replacement in the chimera viruses against antibodies generated in a controlled manner. Despite this, the results suggest that both ORF2a-ORF3-ORF4 and ORF5-ORF6 regions of the PRRSV genome are important for antigenicity and immunity. Kim and Yoon previously reported that GPs 3, 5 and 6 played the largest role in determined cross reactivity between PRRSV strains and their

effects were additive [33]. Others have also reported that GP3 and GP4 are targeted by neutralizing antibodies [34, 35].

We were unable to rescue four of the eight chimera G24 viruses. It is unclear if this is due to genetically incompatible genotypes or if the G24 sequences determined directly from clinical specimens encode proteins not compatible with cultivation *in vitro*. A significant advantage of our sequencing methodology is elimination of the potential genetic bottleneck associated with cell culture. Increased user-friendliness and decreased costs associated with next generation sequencing make PRRSV genome sequencing directly from clinical specimens possible. Our results demonstrate this approach can uncover novel genetic diversity which is relevant to PRRSV antigenicity and suggest a need for more comprehensive PRRSV genetic analysis as part of epidemiological analyses.

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