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Genetic diversity in envelope genes of contemporary U.S. porcine reproductive and respiratory syndrome virus strains influences viral antigenicity

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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). Genome sequences of sixty-six PRRSV strains were obtained using metagenomic sequencing of serum samples collected in the U.S. in 2014 to explore contemporary genetic diversity. Phylogenetic analysis of the genes encoding the envelope proteins identified four to eight distinct lineages with > 87% intraclade identity. To explore the effect of the observed genetic diversity on antigenicity, the genome regions 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 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.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS), caused by porcine reproductive and respiratory syndrome virus (PRRSV), is one of the most economically significant diseases affecting the swine industry with near worldwide distribution (Shi et al., 2010). Clinical symptoms are variable, with respiratory disease, fever, lethargy and stunting due to systemic disease in growing pigs (Rossow, 1998). Reproductive failure is common in the breeding herd. Highly virulent PRRSV can lead to high morbidity and mortality. An extreme example was the highly pathogenic PRRSV identified in China in 2006 which resulted in the loss of millions of pigs (Tian et al., 2007). There are two genotypes of PRRSV, type 1 (European) and type 2 (North American) which share only ~60% nucleotide identity (Kappes and Faaberg, 2015). The viral RNA dependent RNA polymerase of PRRSV lacks proof reading ability, leading to rapid viral evolution *via* accumulation of point mutations (Lauber et al., 2013). Additionally, multiple PRRSV co-infecting a cell can recombine, leading to exchanges of large sections of the genome (Yuan et al., 1999). Together, these two mechanisms contribute to PRRSV evolution (Chang et al., 2002; Martin-Valls et al., 2014).

Early after its discovery, the GP5 protein of PRRSV became the focus for PRRSV epidemiology and intervention. GP5 is the major membrane glycoprotein of PRRSV and is genetically highly variable (Pirzadeh et al., 1998). GP5 possesses a conserved linear neutralizing epitope and amino acid mutations in the N-terminal ectodomain significantly influenced susceptibility of mutant viruses to 010 neutralizing antibodies

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(Plagemann et al., 2002; Ostrowski et al., 2002; Wissink et al., 2003; Kim et al., 2013). Additionally, changes in GP5 N-linked glycosylation influences virus replication and immunogenicity (Vu et al., 2011; Ansari et al., 2006; Wei et al., 2012). 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 (Wesley et al., 1998).

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 (Van Breedam et al., 2010). However, subsequent studies 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; Calvert et al., 2007; Das et al., 2010; 2011). 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). Variability in minor glycoprotein glycosylation was shown to affect viral replication and immunogenicity (Das et al., 2011).

PRRSV has an extremely limited cell tropism *in vitro*, with porcine alveolar macrophages and Marc145 being the principal non-genetically engineered cells for propagating PRRSV *in vitro*. To prevent genetic bias potentially introduced by cultivating PRRSV *in vitro*, here we characterize genome sequences of sixty-six PRRSV strains determined directly from porcine serum using metagenomic sequencing and explored the effect of the genetic diversity of GP2a, GP3, GP4, GP5 and M on antigenicity using reverse genetics.

2. Materials and methods

2.1. Ethics statement

Swine samples used in this study were collected by veterinarians as part of routine care of swine herds. The serum samples were collected by veterinarians not affiliated with this study. Serum samples were collected from naturally infected animals and were submitted to either Kansas State Veterinary Diagnostic Laboratory, Iowa State Veterinary Diagnostic Laboratory or the South Dakota Animal Research and Diagnostic Laboratory for diagnostic testing and consequently, Institutional Animal Care and Use Committee approval was not necessary. PAM cells were a kind gift from Dr. Ying Fang and were harvested from pigs as approved by the Kansas State University Institutional Animal Care and Use Committee protocol 3356. Other cell lines used were purchased from commercial sources.

2.2. Samples

Metagenomic sequencing was performed on 182 swine serum samples that were quantitative reverse transcript PCR (qRT-PCR) positive for PRRSV. The serum samples were submitted to Kansas State Veterinary Diagnostic Laboratory (KSVLD), Iowa State University Veterinary Diagnostic Laboratory or the South Dakota State University Animal Disease Research and Diagnostic Laboratory for PRRSV RT-PCR. The samples originated from thirteen states: Iowa (n = 35), Minnesota (n = 39), South Dakota (n = 2), Texas (n = 9), North Carolina (n = 18), Nebraska (n = 14), Kansas (n = 22), Oklahoma (n = 1), Illinois (n = 2), Indiana (n = 1), Missouri (n = 1), Arizona (n = 2), and Colorado (n = 4), Mexico (n = 4) and unknown (n = 28).

2.3. Sample preparation and sequencing

Serum samples were centrifuged for five minutes (min) at 5000 × *g* to remove particulate matter. Samples were prepared for sequencing as previously described (Hause et al., 2015). In brief, samples were treated with a cocktail of DNases and RNases to remove unprotected nucleic acids followed by viral RNA extraction. Samples were then reverse transcribed using random hexamers containing a known 20 bp at their 5′ ends followed by second strand synthesis. PCR was next performed using primers corresponding to the known 20 bp sequence incorporated into the random hexamer. Barcoded sequencing libraries were prepared individually for each sample using the Nextera XT library preparation kit according to the manufacturer's instructions and the libraries were assembled into four pools. Libraries were sequenced using the Illumina MiSeq and v2 reagents on four independent sequencer runs. Paired end reads were demultiplexed and fastq files were created with MiSeq Reporter software (Illumina). Onboard read analysis software removed poor quality reads.

2.4. Sequencing read assembly and annotation

Paired end reads for each sample were imported into CLC Genomics Workbench 7.0 software (Qiagen) and reads mapping to *Sus scrofa* were subtracted. The remaining reads were *de novo* assembled into contigs using default parameters. Consensus sequences for assembled contigs were compared against the non-redundant nucleotide database at NCBI using the BLASTn algorithm. When *de novo* assembly failed to yield a near complete genome, the GenBank accession number with the lowest expectation (*E*) value was used for reference-based assembly. Consensus sequences were extracted from the reference-based assemblies, open reading frames (ORFs) were determined in CLC Genomics Workbench, and PRRSV ORF2a–ORF6 sequences were extracted and used in phylogenetic analyses and sequence comparisons.

2.5. Phylogenetic analysis

Nucleotide sequences for the sixty-six strains' 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 1000 bootstrap replicates. Reference sequences, as proposed by Shi et al. (2010) were also included, when available, as follows: lineage 1 MN184c (EF488739) lineage 3 FJ-1 (AY881994), lineage 5.1 VR2332 (U87392) and RespPRRS MLV (AF066183), lineage 5.2 NADC-8 (U66394), lineage 7 PrimePac (AF066384), lineage 8 Ingelvac ATP2014 (EF532801), and lineage 9 17198-6 (EF442776). Other reference strains include a high-pathogenic Chinese strain JXA1 (EF112445), a U.S. reference strain NADC30 (JN654459), the parent of Ingelvac2014ATP, JA-142 (EF442773), U.S. strain SDSU73 (JN654458), Chinese strains CH1a (AY032626) and TP (EU864233), and recently identified virulent strains from the Midwest MN414 (KT581982) and NCV26 (KX192119), as well as U.S. strain SDSU95-21 (KC469618).

2.6. Pairwise sequence comparisons

Nucleotide alignments for the ORF2a-6 were imported into DNASTAR Navigator 13 for pairwise comparisons to determine percent identity.

2.7. Nucleotide accession numbers

Sixty-six PRRSV ORF2a-6 genomes were deposited in GenBank under accessions KT257944–KT258009.