Genetic and biologic characterization of a porcine reproductive and respiratory syndrome virus isolate attenuated by cell culture passage

Susan Kay Schommer
Iowa State University
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Genetic and biologic characterization of a porcine reproductive and respiratory syndrome virus isolate attenuated by cell culture passage

by

Susan Kay Schommer

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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2000
This is to certify that the Doctoral dissertation of

Susan Kay Schommer

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Committee Member

Signature was redacted for privacy.

Co-Major Professor

Signature was redacted for privacy.

Co-Major Professor

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For the Major Program

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For the Graduate College
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<thead>
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<th>Full Form</th>
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<tr>
<td>CDCD</td>
<td>colostrum-deprived-cesarean-derived</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>DPI</td>
<td>days post-infection</td>
</tr>
<tr>
<td>EAV</td>
<td>equine arteritis virus</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>GP</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>gRNA</td>
<td>genomic ribonucleic acid</td>
</tr>
<tr>
<td>IFA</td>
<td>indirect fluorescent antibody test</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IPMA</td>
<td>immunoperoxidase monolayer assay</td>
</tr>
<tr>
<td>ISH</td>
<td><em>in situ</em> hybridization</td>
</tr>
<tr>
<td>LDV</td>
<td>lactate dehydrogenase-elevating virus</td>
</tr>
<tr>
<td>LV</td>
<td>Lelystad virus, European PRRSV isolate</td>
</tr>
<tr>
<td>M</td>
<td>membrane protein</td>
</tr>
<tr>
<td>MLV</td>
<td>modified live virus vaccine</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>N</td>
<td>nucleocapsid protein</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>nsp</td>
<td>non-structural protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>p</td>
<td>passage (in cell-culture)</td>
</tr>
<tr>
<td>PAMs</td>
<td>porcine alveolar macrophages</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PRRS</td>
<td>porcine reproductive and respiratory syndrome</td>
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<tr>
<td>PRRSV</td>
<td>porcine reproductive and respiratory syndrome virus</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SHFV</td>
<td>swine hemorrhagic fever virus</td>
</tr>
<tr>
<td>sgmRNA</td>
<td>subgenomic messenger ribonucleic acid</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen free</td>
</tr>
<tr>
<td>ST</td>
<td>swine testes cell line</td>
</tr>
<tr>
<td>SVN</td>
<td>serum virus neutralization test</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% tissue culture infectivity dose</td>
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GENERAL INTRODUCTION

Objectives of Dissertation Research

In 1987, a new disease syndrome in pigs was recognized as causing both respiratory disease and reproductive failure in United States swine herds (Hill, 1990). The causative agent, porcine reproductive and respiratory syndrome virus (PRRSV), was first isolated in the Netherlands in 1991 on primary porcine alveolar macrophages (PAMs) and was designated Lelystad virus (LV) (Wensvoort et al., 1991). The first PRRSV in the United States was isolated shortly after that on a continuous cell line (Collins et al., 1992; Kim et al., 1993; Meng et al., 1996a). The virus spread rapidly and is now found throughout Europe, North America, and Asia (Chueh et al., 1998; Lindhaus and Lindhaus, 1991; Murakami et al., 1994; Paton et al., 1991; Plana et al., 1992). In 1991, seroprevalence of PRRSV was estimated to be as high as 60-80% in U.S. herds (Zimmerman et al., 1997).

Comparisons of the European and U.S. PRRSV strains show that they are structurally and antigenically related, but represent genetically distinct groups (Meng et al., 1995a; Nelson et al., 1999; Wensvoort et al., 1992). Differences have been demonstrated in both reproductive and respiratory pathogenicity among U.S. PRRSV isolates and in comparison to isolates from Europe (Halbur et al., 1996; Halbur et al., 1995; Mengeling et al., 1996; Park et al., 1996). These differences have been attributed to a complex of factors including those associated with environment, host susceptibility, and virus strain differences. Genetic differences among isolates of varying pathogenicities have been well
established, but the regions of the genome responsible for these differences in virulence are unknown (Goyal, 1993; Halbur et al., 1995; Meng et al., 1995b; Meng et al., 1996b).

The overall objective of this dissertation was to establish a model of high and low virulence strains from the same lineage. Since there is substantial nucleotide diversity among North American PRRSV strains, it has been difficult to correlate changes in specific regions of the genome with changes in pathogenicity (Andreyev et al., 1997; Kapur et al., 1996; Meng et al., 1995b; Murtaugh et al., 1998). In this study, the strain to strain genetic variations are removed by using two passages from the same original virus isolate. The overall hypothesis of this research was that serial passage of PRRSV isolate VR-2385 in a continuous cell line, CRL11171, would result in genetic and phenotypic changes that lead to a decrease in virulence. The specific aims for validating this hypothesis were:

1. Determine if serial cell culture passage resulted in a decrease in virulence by observing the clinical and pathological signs resulting from experimental infection with VR-2385 passage 8 (p8) and passage 85 (p85) in nursery pigs;
2. Determine the genome sequence of VR-2385 p85 and compare it to the sequence of the parent isolate;
3. Determine the effect of serial passage of VR-2385 on virus growth in a continuous cell line and primary cells.

The initial objective was to compare the pathogenicity of VR-2385 p8 and p85. After VR-2385 p8 and p85 were found to significantly differ in their ability to cause respiratory disease in young pigs, these strains were examined for a genetic and/or biologic correlate of virulence. The sequence of VR-2385 p85 was determined to differ from the parent strain by less than 1% at the nucleotide level and regions potentially important for
virulence were identified. Once these objectives were met, the next goal was to identify a biological characteristic or marker that could be used to differentiate isolates of different pathogenicity. Prior to doing this, PRRSV replication in cell culture was examined and optimized. The replication ability of the two passages of VR-238S was then examined in a continuous cell line and primary cells to determine if in vitro growth characteristics correlated with the in vivo differences in virulence.

Dissertation Organization

This dissertation begins with a general introduction. A literature review relevant to this dissertation precedes the first manuscript. There are three separate manuscripts contained in this dissertation, each prepared according to the guidelines of the journal to which it has or will be submitted. The Ph.D. candidate, Susan Schommer, is the primary author for all three manuscripts. The first manuscript, "Comparison of porcine reproductive and respiratory syndrome virus (PRRSV) growth in fetal bovine serum and a serum replacement supplemented media" has been submitted to the Journal of Veterinary Diagnostic Investigation and describes the findings from the optimization of PRRSV growth in cell culture. All of the work included in this paper was completed by the Ph.D. candidate. The second manuscript, "Porcine reproductive and respiratory syndrome virus genome changes during cell culture attenuation" will be submitted to the journal Archives of Virology. Sequencing of the VR-2385 p85 genome was completed by the primary author, Susan Schommer, while sequencing of VR-2385 p7 was previously performed by Drs. Igor Morozov and Xiang-Jin Meng. The third manuscript, "Biological characterization
of a cell culture-attenuated porcine reproductive and respiratory syndrome virus (PRRSV) isolate will be submitted to the journal Virus Research and discusses the in vivo and in vitro growth characteristics of the two passages of VR-2385. For this study, gross and pathologic examination of tissues was performed by Dr. Patrick Halbur and clinical disease was monitored by Ryan Royer. The Ph.D. candidate assisted with animal care and monitoring, as well as completed all of the cell culture work including inoculum preparation. The three manuscripts are followed by general conclusions and the references for the general introduction, literature review and general conclusions.
LITERATURE REVIEW

History and Etiology of PRRS

A new disease syndrome in pigs was first recognized in the United States in 1987, characterized as causing both respiratory disease and reproductive failure in swine herds (Hill, 1990). Initially this syndrome was known as mystery swine disease, since the causative agent was unknown (Hill, 1990; Keffaber, 1989). In late 1990, a similar disease was reported in Germany and the Netherlands (Wensvoort et al., 1991). Since then, this syndrome has been reported throughout North America, Europe, and Asia including Canada, Germany, Spain, the United Kingdom, Czechoslovakia, Denmark, Taiwan, Japan, and Korea (Lindhaus and Lindhaus, 1991; Murakami et al., 1994; Paton et al., 1991; Plana et al., 1992; Valicek et al., 1997). This syndrome was referred to by various names throughout the world including swine infertility syndrome (SIRS) (Collins, 1991), mystery swine disease (MSD), blue ear disease (White, 1991), Heko-Heko and porcine epidemic abortion and respiratory syndrome (PEARS) (Meredith, 1992; Pol et al., 1991; Wensvoort, 1993). It was agreed to adopt the name porcine reproductive and respiratory disease syndrome (PRRS) in 1992 at the first international meeting on this disease (Collins, 1993).

In 1991, the agent causing this disease was found to be a virus and was isolated on primary swine alveolar macrophages at the Central Veterinary Institute at Lelystad, the Netherlands (Pol et al., 1991; Wensvoort et al., 1991). This first European PRRS virus (PRRSV) isolate, designated Lelystad virus (LV) was shown to cause PRRS in experimentally infected pigs and fulfilled Koch's postulates (Pol et al., 1991; Terpstra et
PRRSV was isolated in the United States in 1992 using a continuous cell line, ATCC CL2621, and was demonstrated to be the causative agent of PRRSV in the U.S. (Benfield et al., 1992; Collins et al., 1992). Since this time many other PRRSV isolates have been reported, and several other cell lines have been shown to support PRRSV replication including a subpopulation of MA-104 cells known as MARC 145 and the ATCC CRL11171 cell line (Kim et al., 1993; Meng et al., 1996a). PRRSV also replicates in primary swine alveolar macrophages and at least one European strain is able to grow in the swine testes (ST) cell line (Plana et al., 1992; Wensvoort et al., 1991). The Lelystad virus (LV) and U.S. isolates, collectively known as PRRSV, have been shown to be antigenically and genetically related, but represent two distinct members of a common family that can be readily differentiated by polyclonal and monoclonal antibodies, in situ hybridization (ISH), and the polymerase chain reaction (PCR) (Gagnon and Dea, 1998; Gilbert et al., 1997; Larochelle and Magar, 1997; Mardassi et al., 1994; Nelson et al., 1993; Oleksiewicz et al., 1998a; Wensvoort et al., 1992). Due to the substantial variation throughout the genome, it has been proposed that the European and U.S. PRRSV evolved independently on separate continents, though they may share a common ancestor (Murtaugh et al., 1995; Nelsen et al., 1999).

**Biologic and Physical Characteristics**

PRRSV is a small virus with a single positive-strand RNA genome. Infectivity of PRRSV is completely lost when pretreated with chloroform, suggesting the presence of a lipid envelope (Benfield et al., 1992; Meng et al., 1996a; Wensvoort et al., 1991). An
electron-translucent ring surrounding virus particles was also observed during electron microscopy, further indicating the presence of a lipid envelope (Benfield et al., 1992; Meng et al., 1996a). Virus particles were observed to be predominantly spherical and to have a diameter of 48-84 nm, with a nucleocapsid core of 20-35 nm (Benfield et al., 1992; Meng et al., 1996a; Wensvoort et al., 1991). Buoyant density of the PRRS virus was found to be 1.18-1.19 g/ml using a cesium chloride gradient (Benfield et al., 1992; Meng et al., 1996a; Wensvoort et al., 1991). The PRRSV was inactivated after 48 hours of incubation at 37 °C and 45 minutes at 56 °C, but infectivity was unchanged after 1 month at 4 °C and 4 months at -70 °C (Benfield et al., 1992). Viral infectivity was also affected by pH, and it dropped by 90% at a pH less than 5 or greater than 7 (Benfield et al., 1992). The virus was found to be nonhemagglutinating after testing with erythrocytes of several animal species (Benfield et al., 1992; Meng et al., 1996a; Wensvoort et al., 1991; Yoon et al., 1992b).

Cytopathic effect (CPE) caused by the virus in continuous cell lines has been described by degeneration of cells, cell rounding and clumping of cells, eventually leading to complete destruction of the monolayer (Benfield et al., 1992; Meng et al., 1996a). The small round clumps of cells appear to be raised above the monolayer and are found regardless of the cell line used (Benfield et al., 1992; Kim et al., 1993; Meng et al., 1996a).

The tropism of PRRSV has been shown to be extremely limited, after testing more than 30 cell lines only continuous cell lines derived from MA-104 cells were able to support productive infections (Meng et al., 1996a; Wensvoort et al., 1991; Yoon et al., 1992b). Cell lines tested included others derived from monkey tissues as well as primary cells and continuous cell lines from pig tissue (Meng et al., 1996a). A study done on
PRRSV tropism found that Vero cells, another monkey kidney cell line, were able to bind and internalize U.S. prototype strain VR-2332 but could not replicate the virus (Kreutz, 1998). In the same study, PRRSV was unable to bind ST cells and could not replicate in this cell type even when the cells were transfected with viral RNA (Kreutz, 1998). This contrasted the previous finding that one European PRRSV isolate was able to infect and propagate in ST cells (Plana et al., 1992). This difference in ability to replicate in ST cells may be associated with the substantial difference in sequence between the European and U.S. PRRSV genotypes. Many U.S. PRRSV isolates have been found to replicate in either PAMs or a continuous cell line but not both, further suggesting there may be differences in receptor binding or replication mechanisms among isolates (Bautista et al., 1993b). Even in the primary cell type that PRRSV infects in the host, PAMs, there is only a subset of susceptible cells (Duan et al., 1997). Recent observations suggest that PRRSV enters cells via receptor-mediated endocytosis and the specificity of the receptor probably plays a role in the limited tropism (Duan et al., 1998; Duan et al., 1997; Kreutz, 1998). There must be other cell factors involved since not all cell types transfected with PRRSV RNA were able to replicate the virus (Kreutz, 1998).

Classification

Originally, PRRSV was grouped as a member of the Togaviridae based on virion size, genome size and its icosahedral nucleocapsid, but its lack of cross-reactivity with other members of the group led to the proposal that it was a member of a new genera (Benfield et al., 1992; Plagemann and Moennig, 1992). At the Xth International Congress
of Virology in Jerusalem, a new order of viruses was created, known as *Nidovirales*, so named because *nidus* means nest in Latin and all members form a 3' co-terminal nested set of subgenomic RNAs (Cavanagh, 1997). This order was comprised of two families, the *Coronaviridae* and the *Arteriviridae*, with PRRSV as a member of the genus Arterivirus, within the latter family. All members of the order *Nidovirales* have linear, non-segmented, positive single-strand polyadenylated RNA genomes, with the Arteriviruses having a much smaller genome than the Coronaviruses. The viruses in this order also share similar genome organization, with the genomic RNA functioning as the messenger RNA (mRNA) and the 5' gene product, the replicase, is directly translated.

Members of the *Arteriviridae* are different morphologically from the *Coronaviridae* with a lack of the prominent surface proteins characteristic of coronaviruses and an observable isometric nucleocapsid as opposed to the helical nucleocapsid of coronaviruses. Arteriviruses also share a tropism for cells of the macrophage lineage and are capable of producing subclinical and/or persistent infections (Plagemann and Moennig, 1992). There is no antibody cross-reaction among the members of the Arteriviridae, which includes lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Benfield et al., 1992; Chen and Plagemann, 1995; Keffaber, 1989). However, degenerate primers have been designed to amplify all of the different family members (Benfield et al., 1992; Chen and Plagemann, 1995; Keffaber, 1989). European and North American PRRSV isolates are genetically distant with only about 60% nucleotide identity, which is similar to PRRSV relatedness to LDV, while EAV and SHFV are more distantly related (Meng et al., 1995a; Murtaugh et al., 1995). There are
indications that a common ancestor may have preceded the currently identified
Arteriviruses (Meng et al., 1995a).

**Genome Organization and Replication**

PRRSV has a polyadenylated, positive-strand RNA genome of about 15 kb with an organization and replication mechanism characteristic of its family Nidoviridae (Figure 1) (Cavanagh, 1997). It had been generally accepted that there were 8 open reading frames (ORFs) in the PRRSV genome, but recently a ninth ORF of unknown significance was described (Conzelmann et al., 1993; Meulenberg et al., 1993a; Snijder et al., 1999). In LV, each of the ORFs overlap each other from just a 1 base overlap of ORFs 4 and 5 to a 253 base overlap between ORFs 3 and 4 (Conzelmann et al., 1993; Meulenberg et al., 1993a). For the U.S. PRRSV isolates, there is no overlap between ORFs 4 and 5, instead there is a 10 base non-coding region, while the remaining ORFs overlap (Meng et al., 1995b; Morozov et al., 1995). At the 5' end of the genome there is a region known as the leader sequence. This sequence is also located on the 5' end of every subgenomic mRNA (sgmRNA) produced by the PRRSV. ORFs 1a and 1b make up 80% of the viral genome and are believed to code for the replicase gene (Meulenberg et al., 1993a). ORF 1a is translated directly from the genomic RNA (gRNA), but a -1 translational frameshift is required for ORF 1b expression. The genome sequence contains both a slippery sequence and a downstream pseudoknot characteristic of a frameshift motif. ORF 1b is then expressed as part of the polyprotein ORF 1ab. Recently, four host proteins were reported to bind the negative strand RNA of SHFV at the 3' end, which is the leader sequence complement (Hwang and Brinton, 1998). These proteins could also interact with the same
Figure 1. Genome organization and subgenomic messenger RNAs of a typical PRRSV strain. The solid blocks at the 5' end of each RNA represent the leader sequence. ORFs 1a and 1b can be immediately translated from the positive-strand RNA genome. The genome also acts as a template for new genome copies and for subgenomic mRNA production. The subgenomic RNAs have a 3' coterminal end which includes all of the downstream ORFs, but only the 5' ORF of each subgenomic mRNA is believed to be translated.
region on LDV and EAV and are assumed to be important for initiating plus-strand synthesis of the RNA (Snijder and Meulenberg, 1998).

The order of viruses in which PRRSV resides, Nidovirales, is named for its hallmark replication mechanism which is the formation of a 3' co-terminal nested set of mRNAs. For PRRSV this nested set consists of 6-8 subgenomic mRNAs (Conzelmann et al., 1993; Meng et al., 1994; Meng et al., 1996b; Meulenberg et al., 1993a; Snijder et al., 1999). Each of these subgenomic mRNAs also has a common 5' leader sequence and the same 3' terminal sequence including the poly-(A) tail. The leader-mRNA junction sequence is conserved and shows a motif of UCAACC or another highly similar sequence (Meng et al., 1996a; Meulenberg et al., 1993b). Base-pairing between the leader mRNA and the RNA genome is required for production of the sgmRNAs (van Marle et al., 1999). These subgenomic mRNAs are polycistronic, but it is believed that only the 5' open reading frame of each sgmRNA is translated to a viral protein.

The genome of PRRSV is positive-strand RNA, and upon infection, it can be immediately translated. The ORF 1 lab polycistronic message at the 5' end contains the RNA-dependent RNA polymerase (RdRp). The polymerase protein is then used to transcribe the negative strand complement of the genome, which is able to act as a template for new genome copies. The mechanism used to produce the nested set of sgmRNAs has been debated for many years, and currently there are two favored models to explain the discontinuous transcription required to generate this unique nested set (Sawicki and Sawicki, 1998; van der Most and Spaan, 1995). The first model is referred to as leader-primed transcription, in which the positive-strand leader is synthesized from the
antigenome, dissociates, and then rejoins at a complementary intergenic region acting as a
primer for sgmRNA synthesis (Baric et al., 1985; Lai et al., 1984; Lai, 1989). In this
capacity the intergenic sequences would act as both a promoter and a transcription
initiation site. The second model is known as discontinuous extension of negative strands.
In this model, as the negative strand is being synthesized the RNA polymerase falls off of
the template when it pauses at an intergenic sequence and then joins the leader producing a
negative sgmRNA that serves as a template to produce positive strand sgmRNAs (Sawicki
and Sawicki, 1990; Sawicki and Sawicki, 1995; Sawicki and Sawicki, 1998). This
mechanism of site-specific RNA recombination during negative strand synthesis may be
guided by distinct RNA structures (van Marle et al., 1999). There is evidence that supports
both models and the two models are not mutually exclusive of one another, providing
mechanisms for leader regulation in cis and in trans (van der Most and Spaan, 1995).

**Viral Proteins**

After sequencing the 15.1 kb LV genome, it was predicted that open reading frames
(ORFs) 2-7 encoded structural proteins and that ORFs 1a and 1b encoded the viral RNA
replicase gene (Table 1) (Meulenberg et al., 1993a). The proteins have since been further
characterized and subdivided, with ORFs 5, 6, and 7 determined to be the major structural
proteins encoding the major envelope, matrix and nucleocapsid proteins respectively.

**Replicase gene**

The Arterivirus replicase consists of a two large polyproteins, in PRRSV the genes
encoding the replicase are about 80% of the genome or nearly 12 kb. ORF 1a is translated
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<th>Abbreviation</th>
<th>Predicted function</th>
<th>Amino Acid Length</th>
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<tr>
<td>1a</td>
<td>Nsp1</td>
<td>Two putative papain-like cysteine proteases</td>
<td>383-385</td>
</tr>
<tr>
<td></td>
<td>Nsp2</td>
<td>Chymotrypsin-like cysteine protease</td>
<td>834-980</td>
</tr>
<tr>
<td></td>
<td>Nsp3</td>
<td>Unknown</td>
<td>446-447</td>
</tr>
<tr>
<td></td>
<td>Nsp4</td>
<td>Chymotrypsin-like cysteine protease</td>
<td>203-204</td>
</tr>
<tr>
<td></td>
<td>Nsp5</td>
<td>Unknown</td>
<td>445-455</td>
</tr>
<tr>
<td>1b</td>
<td>RdRp</td>
<td>RNA dependent RNA polymerase</td>
<td>645-646</td>
</tr>
<tr>
<td></td>
<td>CP2</td>
<td>Zinc-finger and helicase domains</td>
<td>441-442</td>
</tr>
<tr>
<td></td>
<td>CP3</td>
<td>Unknown</td>
<td>223-224</td>
</tr>
<tr>
<td></td>
<td>CP4</td>
<td>Unknown</td>
<td>152-153</td>
</tr>
<tr>
<td>2</td>
<td>GP2</td>
<td>Minor glycosylated envelope protein</td>
<td>246-249</td>
</tr>
<tr>
<td>3</td>
<td>GP3</td>
<td>Highly glycosylated Structural protein (Europe)</td>
<td>254-265</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soluble, non-structural (Canada)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>GP4</td>
<td>Minor glycosylated envelope protein</td>
<td>178-183</td>
</tr>
<tr>
<td>5</td>
<td>GP5 (E)</td>
<td>Major glycosylated envelope protein</td>
<td>200-201</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>Membrane protein</td>
<td>173-174</td>
</tr>
<tr>
<td>7</td>
<td>N</td>
<td>Nucleocapsid protein</td>
<td>123-128</td>
</tr>
</tbody>
</table>
Directly from the gRNA, but a -1 translational frameshift is required for ORF 1b expression. ORF 1b is then expressed as part of the polyprotein ORF lab encodes. EAV and mouse hepatitis virus, a coronavirus, have the same arrangement of conserved domains in the replicase, which further separates the nidoviruses from the other positive strand RNA viruses. The proteolytic processing pathway has been determined for EAV, with ORF lab being cleaved 10 times by 3 different ORF 1a proteases (Snijder et al., 1994; van Dinten et al., 1999; Wassenaar et al., 1997). These cleavages and the translational frameshift result in 12 end products and a lot of intermediates, some of which have extended half-lives (van Dinten et al., 1999). One difference between PRRSV and EAV is that in PRRSV, as well as LDV, there is an additional papain-like cysteine protease at the 5' end of ORF 1a (Allende et al., 1999; den Boon et al., 1995; Godney et al., 1993; Meulenberg et al., 1993a). There is a highly variable region in the center of non-structural protein 2 (nsp2), which is encoded in ORF 1a. In this region there are major differences among arteriviruses, therefore, it has been speculated to be involved in species-specific functions (Allende et al., 1999; de Vries et al., 1997). There is only 32% amino acid identity and a 120 amino acid size difference in the nsp2 protein of the U.S. PRRSV and European LV (Allende et al., 1999). In U.S. isolate VR-2385, two independent deletions have been detected in the nsp2 protein. The predominant deletion results in a protein that is 145 amino acids shorter than the other U. S. isolates (Morozov unpublished data; Schommer et al., 2000). This variation may indicate that the function of this protein may involve something other than species specificity, because both U.S. and European PRRSV isolates are restricted to pigs. It is
unknown if differences in this region of the genome are related to differences in pathogenicity observed among PRRSV isolates.

**Minor envelope proteins GPs 2, 3 and 4**

The nucleocapsid, membrane, and major envelope proteins make up 90-95% of an arterivirus particle, so it has been very difficult to characterize the products of ORFs 2-4 (Snijder and Meulenberg, 1998). ORF 2 of LV was determined to be a 29.5 kDa typical class I integral membrane glycoprotein with 2 glycosylation sites, but has not been detected in a U.S. isolate (Meulenberg and Petersen-Den Bensten, 1996). A disulfide-bond form of the ORF 2 protein is retained in the endoplasmic reticulum, but no disulfide linked dimers were detected in lysates or extracellular virus, as had been detected with EAV (Meulenberg and Petersen-Den Bensten, 1996). The function of this protein is unknown. However, it has been determined that it is indispensable for virus replication because its deletion rendered the EAV cDNA clone non-infectious (van Dinten et al., 1997). Recently, an additional protein was described in EAV which utilizes a start site upstream of ORF 2. This ORF has been named ORF 2b and is found in all arteriviruses. In EAV, LDV, and SHFV the start site is located in the region between ORF 1b and ORF 2a, previously believed to be untranslated. However, in PRRSV the positions of ORF 2a and ORF 2b are reversed and the newly recognized start site is 2 nucleotides downstream of the ORF 2a initiation codon (Snijder et al., 1999).

Glycoproteins 3 (GP3) and 4 (GP4) were determined to be minor structural proteins in LV by identification with monoclonal antibodies (van Nieuwstadt et al., 1996; Wieczorek-Krohmer et al., 1996). However, a Canadian PRRSV study found that the
highly glycosylated ORF 3 protein was non-structural and released as a soluble form (Gonin et al., 1998; Mardassi et al., 1998). The ORF 3 protein's function is unknown, but the product has been found to be highly antigenic and monoclonal antibodies to the portions of the sequence that overlap with ORF 4 are neutralizing in vitro (Duran et al., 1997; Gonin et al., 1998; Katz et al., 1995; Meulenberg et al., 1997). In Europe, a GP3 deletion mutant has been recently identified and appears to be outcompeting the nondeleted strains without any changes in the PRRSV clinical effects. Perhaps analysis of these naturally occurring mutants will help elucidate the function of this protein (Oleksiewicz et al., 2000). Little is known about GP4 other than it can induce neutralizing antibody activity, further indicating that it is a structural protein and that part of it is exposed on the virion surface (Meulenberg et al., 1997; van Nieuwstadt et al., 1996).

Major structural proteins: GPs 5, 6 and 7

GP5 has been predicted to be a hydrophobic membrane-associated protein that may span the envelope 1 or 3 times, has 2 potential glycosylation sites, and a predicted 32-33 amino acid signal sequence at the N-terminus (Conzelmann et al., 1993; Meng et al., 1994; Meulenberg et al., 1993a; Snijder and Meulenberg, 1998). Two differently glycosylated forms of the protein are found in extracellular virions (Mardassi et al., 1996). Neutralizing linear epitopes found on GP5 were not affected by the absence of carbohydrate residues (Pirzadeh and Dea, 1997). GP5 demonstrates a great deal of antigenic diversity, a property that several groups are trying to use to characterize the isolates into serotypes and to map the epitopes on this protein (Pirzadeh et al., 1998). It has also been demonstrated that a
vaccinia virus recombinant ORF 5 protein was able to induce apoptosis in cell culture (Suarez et al., 1996a).

The ORF 6 protein is the membrane protein and is the most conserved structural protein. A short stretch of only 10-18 amino acids is believed to be exposed, probably explaining this conservation and its lack of immunogenicity. The ORF 6 protein accumulates in the ER until it forms disulfide linked heterodimers with the ORF 5 protein (Mardassi et al., 1996). This protein is predicted to play a role in virus assembly and budding (Snijder and Meulenberg, 1998).

ORF 7 encodes the nucleocapsid protein. This protein is found predominantly as a disulfide-linked homodimer when immunoprecipitated (Mardassi et al., 1996; Meulenberg and Petersen-Den Bensten, 1996). This protein has epitopes that are conserved between the U.S. and European PRRSV isolates and is very abundant in cells, making monoclonal antibodies to ORF 7 a common diagnostic tool. Currently several groups are mapping antigenic sites on this protein, and it is suggested that the C-terminus is important for maintaining protein conformation (Meulenberg et al., 1998b; Wootton et al., 1998). Two basic regions in the N-terminal half of the nucleocapsid have been putatively identified as containing the nuclear localization signal for the PRRSV (Rowland et al., 1999).

Characterization of PRRSV protein function has just begun. There are now monoclonal antibodies to each protein in the 3' end of the genome and an infectious clone for both EAV and LV. These tools should help advance this area of research greatly in the next few years.
Epidemiology

According to the current data, it appears that PRRSV entered the swine population relatively recently, with the earliest reported clinical outbreaks in 1987, and then spread rapidly throughout the world (Hill, 1990). In both North America and Europe there is serologic evidence that PRRSV existed before anyone had noticed the clinical signs of the syndrome. The earliest known positive serum samples were found during a retrospective study in Ontario, Canada. All sera tested from 1978 were negative, while in 1979 3.9% were positive and by 1980 15.7% of the herds were positive for PRRSV antibodies (Carman et al., 1995). A similar study performed on samples from Iowa showed that 0/1425 sera samples representing 118 herds were positive for PRRSV in 1980 and that 3.8% of a smaller sample size were positive in 1985, progressing to 63% of sampled herds testing positive in 1988 (Zimmerman et al., 1997b). The low number of PRRSV positive herds in 1985 and the fact that the earliest evidence of PRRSV in Minnesota was found in sera from 1986, suggests that entry of PRRSV into the U.S. pig population occurred at about that time (Yoon et al., 1992b; Zimmerman et al., 1997b). In Germany, serologic evidence for PRRSV dates back to 1988, although clinical signs of the syndrome now known as PRRS weren’t noted until 1990 (Benfield et al., 1999). Despite the fact that PRRS seems to have emerged at about the same time on two different continents, antigenic and genetic variation between the U.S. isolates and LV suggest the two viruses either arose from two separate sources or shared a common ancestor and diverged some time ago before being recognized (Benfield et al., 1999; Kapur et al., 1996). Since that time, PRRSV infection has become endemic in most swine producing areas.
The origin of PRRSV is unknown. The only animals reported to be naturally infected by PRRSV are feral and domestic swine. One study demonstrated that some avian species could be infected with PRRSV and were shown to shed the virus in their feces for 25 days post-infection (Zimmerman et al., 1997a). Mallard ducks were found to be able to transmit virus to each other and to swine, suggesting a potential involvement in PRRSV spread but it is probably not a major participant (Zimmerman et al., 1997a). Rodents, which are more commonly associated with pigs in their natural environment, were not able to be infected by the virus and no other vectors or animal reservoirs have been documented (Hooper et al., 1994).

PRRSV is highly infectious while being, relatively speaking, not highly contagious. Swine are readily infected by 10 or less infectious virus particles by both the intranasal and intramuscular routes, yet it may take weeks or months for all the pigs in a herd to become infected. (Benfield et al., 1999). Herds can remain infected after cessation of symptoms, making clinically normal carriers an important source of PRRSV infection, particularly when adding replacement stock. The predominant route of PRRSV transmission is direct contact, but in at least one experiment even direct contact did not facilitate infection with only 2 of 8 contact controls becoming positive for PRRSV (Christianson et al., 1992; Collins et al., 1992; Terpstra et al., 1991; Torremorell et al., 1997; Wensvoort et al., 1991). Aerosolized virus was originally believed to be a major source of infection since PRRSV is found in the upper respiratory tract. This mode of transmission has been very difficult to achieve experimentally, however, and even pigs only 40 inches away from infected pigs were much less likely to become infected than those pigs in direct contact (Wills, 1997;
Wills et al., 1994). Artificial insemination and semen have also been demonstrated to cause transmission of PRRSV to naïve animals (Shin et al., 1997; Swenson et al., 1994b; Yeager et al., 1993).

Potential routes of the excretion of PRRSV have been studied in experimentally-infected animals (Wills et al., 1997a). Urine and feces had been reported to contain virus, but other reports have shown that shedding in feces does not occur at all or may only intermittently occur (Rossow et al., 1994; Wills et al., 1997a; Yoon et al., 1993). PRRSV was isolated from saliva 42 days post-infection (DPI), urine 14 DPI, and semen 43 DPI (Swenson et al., 1994a; Wills et al., 1997a). Although PRRSV is fairly labile in the environment, LV was demonstrated to have an infectious half-life of 140 hours at 4 °C and a pH of 7.5, and virus could be isolated from city water after 11 days (Bloemraad et al., 1994; Pirtle and Beran, 1996). Therefore, slurry and contaminated water may serve as sources of PRRSV infection (Pirtle and Beran, 1996; Wills et al., 1997a).

Modified live virus vaccines (MLV) have been developed in an effort to control PRRSV. Live vaccines replicate in vivo and virus is shed from vaccinated animals. There is potential danger of recombination with field isolates, as well as danger of reversion of the vaccine virus to a more virulent form. Several studies in both the U.S. and Denmark have suggested that live PRRS vaccine virus can revert to virulence under field conditions (Boetner et al., 1997; Mengeling et al., 1999b; Storgaard et al., 1999). Due to the extreme antigenic and genetic sequence differences between U.S. and European PRRSV isolates, it has been relatively easy to follow the spread of a U.S. PRRSV vaccine in Denmark (Storgaard et al., 1999). Unfortunately this lack of antigenic relatedness may have
contributed to the unexpected spread of clinical disease following use of the vaccine in Denmark.

**Clinical Signs**

As the name porcine reproductive and respiratory syndrome implies, clinical signs fall into two main categories, reproductive signs and respiratory effects. Clinical signs of PRRS are extremely variable in both field and experimental conditions, with a transient fever and inappetence being the most consistent clinical change (Halbur et al., 1996b; Halbur et al., 1995b; Keffaber, 1989; Wensvoort, 1993; White, 1992). Reproductive signs of infection caused by PRRSV are characterized as late-term abortions, late-term in utero death, mummified fetuses, increased stillbirths, and weak-born piglets (Christianson et al., 1992; Mengeling et al., 1994; Plana et al., 1992; White, 1991). Signs of PRRSV infection may also be observed as decreased farrowing rates, delayed returns to estrus, and increased infertility increasing the amount of time sows are open and requiring repeat services (Keffaber, 1989; Loula, 1991). Reproductive problems may persist in a herd for 4-5 months, which is longer than an entire reproductive cycle, and the herds often become enzootic at that point (Done et al., 1996). Infected adult animals may present with anorexia, fever, lethargy, and sometimes transitory skin discoloration (Christianson, et al., 1992; Mengeling et al., 1994; Park et al., 1996; Plana et al., 1992; Terpstra et al., 1991; White, 1991). Occasionally there may be mortality of adult pigs with some herds experiencing a 3-4% loss of sows (Loula, 1991), recently a more virulent form of PRRS was described in which mortality rates were as high as 10% (Benfield et al., 1999; Loula,
The new, highly virulent PRRSV strains also caused a high prevalence of congenital infection and marked clinical illness in gilts in contrast to strains previously studied by the same research group (Mengeling et al., 1999a). Boars infected by PRRSV may show inappetence, lethargy, fever and respiratory signs (Loula, 1991). A decrease in the semen quality from infected boars may or may not contribute to the increased infertility on infected farms (Christopher-Hennings et al., 1997; Prieto et al., 1996b; Sur et al., 1997; Swenson et al., 1994a; Yeager et al., 1993).

Piglets born to infected sows are often weak and may show an abnormal doming of the head (Gordon, 1992). An increase in pre-weaning mortality, which may be as high as 60% usually accompanies PRRSV outbreaks (Benfield et al., 1999; Stevenson et al., 1993). Nearly all premature pigs die within hours of birth and surviving piglets are often considered “poor doers” and may exhibit splay legs, a rough hair coat, decreased appetite, and rapid abdominal breathing (Loula, 1991). Central nervous signs in neonatal pigs have also been reported including paddling and tremors (Keffaber, 1989; Loula, 1991; Rossow et al., 1999). Suckling and nursery pigs may exhibit lethargy, dyspnea, and hyperpnea. Weaned pigs may have a reduction in feed efficiency and an increase in secondary infections causing a large variation in size of age-matched groups of pigs (Keffaber, 1989; Loula, 1991; Stevenson et al., 1993; White, 1992). Respiratory signs may be accompanied by swelling of the eyelids and conjunctiva (Van Alstine et al., 1993; White, 1992). PRRS-associated diarrhea is common in Britain, but has rarely been associated with PRRS in other countries (Gordon, 1992; Goyal, 1993; Hopper et al., 1992; White, 1992). The severity of PRRS generally decreases with increased age of the pig and may go undetected
at any age unless accompanied by a secondary bacterial or concurrent viral infection (Goyal, 1993; Joo and Dee, 1993; Van Reeth, 1997). Secondary infections are commonly associated with PRRSV in the field and can contribute to the degree of clinical signs observed. PRRSV-induced pneumonia is significantly prolonged and more severe when there is a concurrent infection with *Mycoplasma hyopneumoniae* (Thacker et al., 1999). Severe respiratory disease has only been reproduced using U.S. isolates and there is considerable variability in the severity of respiratory disease induced by U.S. isolates (Halbur et al., 1995b; Rossow et al., 1995; Van Reeth, 1997).

**Gross and Microscopic Pathology**

Viral pathogenesis is based primarily on the cell type in which replication occurs. For PRRSV, cells of the monocyte/macrophage lineage are infected (Halbur et al., 1996a; Rossow et al., 1995; Voicu et al., 1994; Wensvoort et al., 1991). PRRSV antigen or RNA is detected in the macrophages of most tissues as well as in monocytes, endothelial cells, glial cells, and dendritic cells (Halbur et al., 1995a; Haynes et al., 1997; Larochelle and Magar, 1995; Magar et al., 1993; Pol et al., 1991; Rossow et al., 1996a; Sur et al., 1996). Most PRRSV replication is believed to occur in macrophages at the mucosal surface, with the virus being distributed to other tissues by the lymphoid system and subsequently found systemically (Rossow et al., 1995).

**Reproductive Pathology**

Several *in vivo* studies have been done to determine if time of gestation is important for the reproductive failure seen in PRRSV infection. Inoculation of gilts or sows has
resulted in transplacental infection from 30 - 95 days of gestation, with fetal lesions occurring only if infection is after 77 days of gestation (Christianson et al., 1992; Christianson et al., 1993; Dea et al., 1992; Lager and Ackermann, 1994; Mengeling et al., 1994; Plana et al., 1992; Terpstra et al., 1991; Wensvoort et al., 1991; Yoon et al., 1992b). It had been observed previously that infection at mid-gestation did not seem to have the same consequences, so a study was done to define when this change in manifestation took place (Christianson et al., 1993). Several methods of inoculation including intranasal inoculation of the sow, intravenous inoculation of the sow, and direct inoculation of the fetal amnion were used to determine if it was a change during fetal development or the placental barrier preventing fetal infection during early gestation (Lager and Mengeling, 1995). No decrease in fertility or increase in abortions was observed when gilts were given an intrauterine dose of PRRSV at or near conception (Lager and Halbur, 1996; Prieto et al., 1996a). In contrast, semen spiked with PRRSV and used to inseminate gilts had little effect on conception, but resulted in embryonic death prior to 20 days post-infection (Prieto et al., 1997). It was found that fetuses infected in the first half of gestation, up to 31 days, could replicate virus without severe pathological consequences and that fetuses at all stages of gestation could support PRRSV replication (Christianson et al., 1993; Lager and Mengeling, 1995). Directly inoculating the amnion with PRRSV in the second half of gestation could result in fetal death within days (Lager and Mengeling, 1995). Previous speculation of intrauterine spread was confirmed by directly infecting some fetuses in the uterus, while leaving others as uninfected controls. Some control fetuses became PRRSV positive, possibly due to an ability to cross the placental barrier in association with
maternal macrophages, but it was not possible to determine whether fetus-fetus or fetus-dam-fetus spread of the virus was taking place (Christianson et al., 1992; Lager and Mengeling, 1995). Using intranasal inoculation of the dam, it was found that the ability of PRRSV to spread transplacentally was also time dependent. At 30 days gestation there was no transplacental infection, limited infection at 50 and 70 days, and late term abortions were caused by infection at 90 days gestation (Lager and Mengeling, 1995). These studies suggest that although the fetus is capable of replicating virus at 31 days of gestation, isolate VR-2332 was incapable of crossing the placenta in order to cause infection at this time. This is likely due to the changes in the layers that separate fetal and maternal blood which are brought closer together later in gestation making for a more efficient exchange (Christianson et al., 1993).

It is often difficult to demonstrate the presence of PRRSV in the fetuses of infected sows, due to the instability of the virus in autolyzed tissue and depending on the gestation stage at infection (Mengeling et al., 1994). The most consistent gross abnormality observed in fetuses containing virus was a sticky meconium staining (Lager and Halbur, 1996). Stillbirths and weak-born piglets from infected sows had an abundance of clear liquid in the thoracic cavity (Lager and Halbur, 1996; Plana et al., 1992). Some placenta had brown-tan discoloration and other normal appearing placenta were separating from the uterus (Lager and Halbur, 1996). The only fetal lesion that has been reported consistently is perivascular hemorrhage of the umbilical cord (Done et al., 1996; Lager and Halbur, 1996). Many infected fetuses had this gross lesion which included segments of hemorrhagic areas 1-2 cm in length to full length involvement of the cord. Necrotizing arteritis and periarterial
hemorrhage may also be observed. Sometimes there was no hemorrhage, but the cord was edematous and enlarged 2-3 times normal size. All fetuses showing gross umbilical cord lesions were viremic, and it was postulated that blood flow disruption may cause hypoxia leading to death for some of the fetuses (Lager and Halbur, 1996).

Microscopic uterine and placental changes have been observed in some animals, but there is limited evidence for viral replication at these sites (Christianson et al., 1992; Stockofe-Zurwieden et al., 1993). Mild lymphoplasmacytic inflammation, endometritis and myometritis were observed in experimentally-infected gilts (Christianson et al., 1992; Lager and Halbur, 1996). In the fetus, microscopic examination sometimes reveals myocarditis, interstitial pneumonia, vasculitis, and encephalitis (Rossow et al., 1996b).

**Respiratory Pathology**

Gross pneumonia consisting of multifocal areas with irregular indistinct borders giving lungs a mottled tan or red appearance have been described in natural and experimental infection (Halbur et al., 1996b; Halbur et al., 1995a). In experimental studies, obvious differences in the severity of gross lung lesions generally correlated with the severity of clinical disease and usually peaked at 10-14 DPI, but can be seen as early as 4 DPI (Beyer et al., 1998; Halbur et al., 1995b). Generally speaking gross lung lesions caused by PRRSV are more severe in younger pigs, making it less likely that gross lesions will be observed in grower-finishers or adult pigs. Enlarged lymph nodes that are 3-10 times normal size, tan, and edematous with multiple cysts containing clear fluid are a consistent gross sign of PRRSV infection and can be observed in pigs of all ages (Halbur et al., 1995b; Rossow et al., 1994; Rossow et al., 1995).
The hallmark of PRRSV is a characteristic interstitial pneumonia. These microscopic lesions are usually present even when gross lesions are absent. Histological changes of mild, multifocal, interstitial pneumonia can be noticed as early as 2 DPI (Beyer et al., 1998; Pol et al., 1991). There are several hallmark characteristics of PRRSV interstitial pneumonia that are consistently observed in field cases and described after experimental infection of colostrum-deprived-cesarean-derived (CDCD) and gnotobiotic pigs (Halbur et al., 1995b; Rossow et al., 1995). The three most consistent lesions are marked septal thickening from infiltration by macrophages and lymphocytes, type-2 pneumocyte hypertrophy and hyperplasia, and alveolar lumina filled with inflammatory cells and necrotic debris (Halbur et al., 1995b; Rossow et al., 1995). Mild to moderate peribronchial and perivascular lymphohistiocytic cuffing has been described, with the unaffected bronchi and bronchiolar epithelia (Halbur et al., 1995a; Halbur et al., 1995b). These characteristics are observed in varying degrees in experimental infections with both high and low virulence isolates (Halbur et al., 1996b).

PRRSV antigen is widespread throughout the respiratory and lymphatic systems within 24-48 hours of infection. Immunohistochemistry has also confirmed PRRSV in the endothelial cells of the heart and intestine (Halbur et al., 1996a). PRRSV infected pigs may also show an accumulation of lymphocytes in nasal turbinates and lymphoid depletion in the lymphoid tissue including spleen, thymus, tonsil and lymph nodes (Done and Paton, 1995; Pol et al., 1991). Microscopically, lymph nodes are reactive, with follicular hyperplasia and hypertrophy, and have foci of necrosis in the center of lymphoid follicles (Halbur et al., 1995b; Rossow et al., 1995). In one study, polykaryocytes were observed in
the lymph nodes (Rossow et al., 1995), but this has not been described in infections with other PRRSV isolates. It has since been suggested that these pigs may have also been infected with porcine circovirus (Ellis et al., 1999; Rossow et al., 1995).

Less consistent lesions in several different tissues have also been described. Some researchers have reported rhinitis, encephalitis, and myocarditis, primarily in neonatal pigs (Halbur et al., 1996b; Rossow et al., 1994; Rossow et al., 1995). Histiocytic perivascular cuffing has been described in the brain (Halbur et al., 1995b). Severe lymphoplasmacytic interstitial nephritis with vasculitis has also been reported (Cooper et al., 1997; Stevenson et al., 1993).

**Immunology**

Early studies into the immunologic response to PRRSV focused on the humoral response which can be examined using relatively simple methodologies including indirect fluorescent antibody test (IFA), enzyme-linked immunosorbent assay (ELISA), immunoperoxidase monolayer assay (IPMA), or a serum virus neutralization test (SVN). PRRSV specific IgM antibody can be detected by 5 days post-infection (DPI), peaks at 14-21 DPI, and is undetectable by 35-42 DPI (Loemba et al., 1996; Venzia et al., 1996). Anti-PRRSV IgG antibodies develop by 7-10 DPI, with those reacting with the N protein appearing first and those antibodies to the GP5 and M protein appearing by 9-14 DPI (Albina et al., 1994; Christianson et al., 1992; Christianson et al., 1993; Nelson et al., 1994; Park et al., 1995; Stevenson et al., 1994; Yoon et al., 1992a; Yoon et al., 1994). PRRSV-specific antibodies decay by approximately 137 DPI by IFA and 356 DPI by SVN (Yoon et
al., 1995a). Neutralizing antibody can be detected 9-105 DPI and often exists concurrently with viremia (Christianson et al., 1992; Lager and Ackermann, 1994; Loemba et al., 1996; Stevenson et al., 1994; Yoon et al., 1995a). Monoclonal antibodies to ORFs 4 and 5 have been demonstrated to neutralize PRRSV, indicating that these two proteins may be important for viral infectivity (Meulenberg et al., 1997; Prizadeh and Dea, 1997). There is no correlation between neutralizing antibody response and viremia, suggesting that it is probably not an essential part of the immune response (Loemba et al., 1996). The presence of low levels of virus specific antibody has actually been demonstrated to enhance PRRSV infection (Yoon et al., 1994; Yoon et al., 1996). There is some protective immunity however, since previously infected sows don’t transmit virus to their fetuses or suffer reproductive failure when rechallenged late in gestation (Gorcyca et al., 1993; Lager and Ackermann, 1994).

PRRSV causes a degeneration of alveolar macrophages, not unexpectedly since this is the target cell of the virus. Molitor and others (1992) have reported that there is a startling decrease in the number of macrophages collected by lung lavage after PRRSV infection. In normal pigs 90% of the cells collected in this manner are alveolar macrophages, but in acutely infected pigs they make up only 50% of the cells, with a relative increase in lymphocytes and neutrophils (Molitor et al., 1992). Experimental infection with PRRSV isolate VR-2332 resulted in a decrease in blood monocytes and lymphocytes, especially in T-lymphocytes. The decrease in T-lymphocytes was first seen 3 days post-infection and had returned to normal by 14 days after infection (Christianson et al., 1992). Along with the destruction of alveolar macrophages, PRRSV induces a dramatic
increase in inflammatory cytokine expression and suppression of nonspecific bactericidal activity, such as the ability to release superoxide anion (Halbur et al., 1996a; Molitor et al., 1995; Thanawongnuwech et al., 1998). The inflammatory response can account for the increase number of mononuclear cells seen in the lung. The suppression of nonspecific bactericidal activity when combined with the transient decrease in T-lymphocytes, helps explain the perceived predisposition to secondary bacterial infections.

Since neutralizing antibody does not appear to be the major factor in controlling PRRSV infection, recent studies have examined the cellular immune response. Immunity to PRRSV can be transferred via colostrum, but antibody alone was not totally protective, further suggesting the role of cellular immunity (Molitor et al., 1997). Antigen specific lymphocyte proliferation was observed at 28 DPI, peaked at 49 DPI, and declined by 77 DPI (Bautista and Molitor, 1997). CD^+^ and CD^8+^ cells have been shown to increase in number after PRRSV infection (Albina et al., 1998; Bautista and Molitor, 1997; Shimizu et al., 1996). The proliferation of CD^8+^ cells was shown to coincide with the decrease in viremia observed at 3-4 weeks post-infection, indicating this cell type may play a role in clearing the virus, as they are the precursor of cytotoxic T-lymphocytes (Albina et al., 1998). T-cell responses are also boosted to proliferation upon second exposure to the virus and administration of antibodies to CD^4+^ and CD^8+^ cells resulted in a major inhibition of the proliferative response to PRRSV in vitro (Bautista and Molitor, 1997). ORFs 2, 4, 5, and 6 induced T-cell proliferation in vitro when proteins expressed in vaccinia virus were used as antigen (Bautista et al., 1999). Overall ORF 6 elicited the greatest response,
indicating that this protein may play a major role in cell-mediated immunity (Bautista et al., 1999).

There is some debate as to whether or not PRRSV causes immunosuppression. The observation that secondary infections are commonly associated with PRRSV in the field, combined with the fact that the virus infects cells of the immune system, led to speculation that suppression of the immune system may occur. Infections by *Streptococcus suis*, porcine respiratory coronavirus, and swine influenza virus are exacerbated by PRRSV infection, while the systemic humoral and cell-mediated response to pseudorabies virus and *Escherichia coli* was enhanced (Galina et al., 1994; Molitor et al., 1992). This has led to the speculation that PRRSV may cause transient local immune impairment, but not general immunosuppression (Wensvoort et al., 1992).

**Persistence**

Persistence of RNA viruses has been defined as the continued presence of virus within a host for extended periods of time after acute infection (Ahmed et al., 1996). Persistence in PRRSV infections is important at both a pig and herd level. Infectious virus, viral RNA, and viral antigen have been shown to be present in pigs for an extended period of time after serum antibodies have been detected and clinical signs have subsided (Christopher-Hennings et al., 1995; Swenson et al., 1994a; Wills et al., 1997b). Transmission by direct contact with susceptible animals has been demonstrated using sows infected 99 days prior to their exposure to the naïve animals (Zimmerman et al., 1992). Another study showed that animals stressed and given corticosteroids 22 weeks after
infection could transmit virus to naïve specific pathogen free (SPF) pigs, even though a similar unstressed group was unable to infect sentinel pigs at 13 weeks post-infection (Albina et al., 1994). The presence of viral RNA has been demonstrated, using PCR, in semen at 92 DPI, although serum from the same timepoint was negative (Christopher-Hennings et al., 1995). Previously, infectious virus was found to be present in serum for 43 days following PRRSV exposure (Swenson et al., 1994a). Oropharyngeal scrapings have been shown to harbor infectious virus the longest of any tissue studied so far, with PRRSV isolation at 157 DPI, 134 days after virus was last isolated from serum in that animal (Wills et al., 1997b). There does not appear to be any correlation between the pathogenicity of an isolate and its ability to persist, as several isolates including a vaccine strain have been shown to persist equally well (Halbur et al., 1995b; Mengeling et al., 1996).

Persistence within a herd is more difficult to study experimentally, therefore most of the information available is based on epidemiological studies. In one swine operation, a PRRSV strain isolated 4 months after the disappearance of clinical signs from that herd was found to remain highly virulent when used to inoculate naïve sows (Bilodeau et al., 1994). There are many theories as to how PRRSV is able to persist in an operation including: 1) incomplete infection of susceptible pigs during the acute phase of the disease, thus leaving susceptible animals; 2) introduction of susceptible pigs as replacements; 3) persistent infections in individual animals that may be induced to shed virus when stressed; 4) variable decreases in active and passive immunity (Albina et al., 1994). The two key components in maintaining PRRSV endemically appear to be the presence of clinically normal carriers and the continual introduction of susceptible animals (Wills et al., 1997b).
Diagnosis

Diagnosis of PRRSV infection can be made based on clinical signs, gross lesions, and histopathology, but these pathological examinations are rarely used alone for a definitive diagnosis. As previously discussed, many animals and herds infected with PRRSV have only subclinical signs, so the absence of signs and lesions does not necessarily mean that those animals are PRRSV-free. Many standard testing methods are now available for aid in PRRSV diagnosis.

Serological tests for antibody to PRRSV are often done using a commercial ELISA (Albina et al., 1992) that is available from IDEXX, but may also be done by IFA (Park et al., 1995; Yoon et al., 1992a), IPMA (Wensvoort et al., 1991; Wensvoort et al., 1992), or a SVN (Hill et al., 1993; Takikawa et al., 1997; Yoon et al., 1994). PRRSV antibodies can be detected as early as 5 days post-infection and reach maximal titers by 30-50 days post-infection, at which point antibody levels begin to decline (Albina et al., 1994; Christianson et al., 1993; Park et al., 1995; Yoon et al., 1992a; Yoon et al., 1994; Yoon et al., 1995a). The SVN test has been modified to identify neutralizing antibody earlier by adding 20% fresh swine serum, enabling detection as early as 9 DPI (Yoon et al., 1994). At this time, there is no serological test that is able to differentiate between an infection by a field strain or vaccination, and antibody alone does not confirm the presence of an acute infection since PRRS is endemic in the swine population. To help determine if an infection is acute, paired sera several weeks apart should be tested. In a diagnostic laboratory setting, the ELISA is most likely to be used since it can be automated and has a similar specificity, wider isolate detection range, and greater sensitivity than IFA or IPMA (Cho et al., 1996).
A blocking ELISA has also been developed that has decreased background and is more sensitive than IPMA or indirect ELISA (Houben et al., 1995; Sorensen et al., 1997). Despite the limitations, serological testing is the most efficient way to screen a herd for PRRSV, using a sample size of 30 animals will allow a 95% confidence of detecting antibodies if there is a seroprevalence of 10% or more (Dea et al., 1992).

Methods for detecting the presence of viral antigen in tissues include immunohistochemistry (IHC), commonly performed on fixed tissue, and IFA, commonly used for frozen tissues (Halbur et al., 1994; Lager and Ackermann, 1994; Larochelle and Magar, 1995; Yoon et al., 1992b). There are also tests that identify viral RNA such as in situ hybridization (ISH) and RT-PCR (Chueh et al., 1999; Haynes et al., 1997; Larochelle and Magar, 1997; Sur et al., 1996). IHC, IFA, and ISH all allow the localization of the virus to be visualized in tissues, which is helpful in correlating lesions in an infected animal to the agent causing the lesions. In situ hybridization has the added benefit of being able to differentiate between North American and European isolates (Larochelle and Magar, 1997). A disadvantage to IHC, IFA, and ISH is their dependence on technician skill in performing and interpreting the tests.

Virus isolation can be done from serum, lung lavage, or tissue samples to confirm the presence of an active infection of PRRSV. Different isolates have differing abilities to replicate in the various cell types used to propagate PRRS, in order to maximize isolation from positive animals both PAMs and a cell line should be used (Bautista et al., 1993b). Cocultivation of PAMs from infected pigs with MARC-145 cells has been shown to be a
sensitive method for PRRSV detection, with virus isolated at 63 DPI as compared to 28 DPI from serum (Mengeling, 1996).

RT-PCR can be used to differentiate different strains of virus and has a maximum sensitivity of less than 10 viral particles, depending on the exact sample and primers used (Christopher-Hennings et al., 1995; Guarino et al., 1999; Mardassi et al., 1994; Oleksiewicz et al., 1998b; Suarez et al., 1994; Van Woensel et al., 1994). This technique is especially useful for detecting PRRSV in samples that are not good for virus isolation such as semen and partially autolyzed fetal tissue (Christopher-Hennings et al., 1995). A modification of RT-PCR, restriction fragment length polymorphism (RFLP) has come into use as a method of not only identifying that PRRSV is present, but for differentiation of the isolates found (Gagnon and Dea, 1998; Wesley et al., 1998). This technique has quickly become the most common method for differentiating the RespPRRS/Repro™ vaccine strain from field strains (Wesley et al., 1998).

Clinical signs, gross lesions, and microscopic lesions are important in identifying that the disease being observed is actually caused by the virus. While this may not always be possible due to the often subclinical presentation of PRRS, without these observations it may indicate that another agent is actually responsible for the clinical disease observed. This is especially important when using serology, which may indicate exposure to a virus the animal has long since recovered from, and RT-PCR which is so sensitive it may identify animals not being affected by the virus.
Variation among PRRSV Isolates

Variations in Virulence

The clinical signs of PRRS vary significantly in both natural and experimental infections. This variation is due to a variety of factors related to the environment, the host, and the virus itself. Environmental factors are the most controllable factors for preventing a severe outbreak and include pig density, pig movement, air quality, and housing system (Dee, 1992). Pig age, breed, and genetics may contribute to pig-to-pig differences in susceptibility (Halbur et al., 1998; Thanawongnuwech et al., 1998). Herd health status, especially the presence of secondary infections, can influence the severity of clinical signs observed in a given herd (Dee, 1992; Van Reeth, 1997). The virus isolate that infects the animal plays a significant role in determining the clinical signs and their severity (Meng et al., 1995a; Meng et al., 1995b; Van Reeth, 1997; Yoon et al., 1999). Under experimental conditions, the dose of virus an animal is exposed to has also been demonstrated to influence disease severity (Yoon et al., 1999).

Variations in virulence due to differences in the PRRSV strain infecting the host have been well characterized. European and U. S. PRRSV isolates represent two genotypes and the differences in clinical manifestations caused by each reflect the genetic differences. In Europe, PRRS is commonly accompanied by scouring and a bluish discoloration of the ears or vulva, whereas these signs are rarely reported in the U.S. (Done and Paton, 1995). Differences in reproductive and respiratory pathogenicity have been demonstrated among U.S. PRRSV isolates and in comparison to those from Europe (Halbur et al., 1996b; Halbur et al., 1995b; Mengeling et al., 1996; Park et al., 1996). Gross lung lesions in CDCD pigs
provide an excellent parameter for comparing the virulence of isolates and their severity correlates well with clinical disease severity (Halbur et al., 1996b; Halbur et al., 1995b). The severity of the reproductive effects of a PRRSV isolate did not directly correlate with the severity of respiratory disease caused by the same isolates (Mengeling et al., 1996). Lymphadenopathy, viremia and viral persistence were observed consistently with all virus isolates despite their differences in virulence (Halbur et al., 1996b; Halbur et al., 1995b; Mengeling et al., 1996). The demonstrated differences in pathogenicity due to PRRSV isolate variation may help explain the variability of disease seen in the field (Halbur et al., 1996b; Halbur et al., 1995b; Mengeling et al., 1996).

Antigenic Variation

It has been demonstrated that despite the wide genetic diversity between U.S. and European isolates that all the isolates share some common antigenic epitopes, yet are antigenically distinct (Nelson et al., 1993; Wensvoort et al., 1992). This has been ascertained from the observation that while two monoclonal antibodies raised against the highly conserved nucleocapsid (N) protein of U.S. PRRSV isolates cross-reacted with the European PRRSV isolates, several others did not (Nelson et al., 1993). One of those cross-reactive monoclonal antibodies, SDOW17, reacts with an extensive number of U.S. and Canadian PRRSV isolates, as well as European isolates representing 8 countries (Magar et al., 1997; Nelson et al., 1993). However, at least 1 U.S. isolate was found to lack reactivity with the broadly reacting antibody (Yoon et al., 1995b).

Serum samples, submitted to a diagnostic laboratory from 18 U.S. states were tested for the presence of antibody that recognized U.S. and European PRRSV isolates. Of the
837 samples tested, 20% of the PRRSV sera reacted with only Lelystad virus, 44% reacted with VR-2332 only, and 36% reacted with both (Bautista et al., 1993a). This indicates that either some Lelystad virus is found in the United States or that there is so much variation in antigenicity among U.S. isolates that some isolates can’t react with VR-2332, but can react with the less closely related European isolate. Serum antibody response to homologous PRRSV isolates can be detected earlier after infection than antibody that cross-reacts with heterologous virus (Bautista et al., 1993a). When animals were infected with U.S. PRRSV isolate VR-2332, antibody that cross-reacted with Lelystad virus was not detected until 63 DPI (Bautista et al., 1993a). This serological survey may underestimate the percentage of PRRSV infected pigs producing antibody that reacts with both U.S. and European isolates, since most serum samples submitted to a diagnostic laboratory would be submitted during acute infection.

Recently an antibody mapping system was tested using monoclonal antibodies to the highly conserved nucleocapsid protein. Sixty-seven North American field isolates from prior to 1996, 2 modified live vaccine strains, and the Lelystad virus were analyzed for their reactivity to the monoclonal antibody panel. All the isolates were assigned to one of 5 antigenic groups, with Lelystad forming its own unique group. Surprisingly, less antigenic diversity was found among the field isolates than expected, with 84% of the isolates sorting into the same antigenic group (Yang et al., 1999). This may be due to the samples being nonrandom geographically, may indicate that after the initially diverse PRRSV population only some serotypes predominated by 1996, or may indicate that the nucleocapsid protein is too conserved to adequately separate the various isolates. North
American isolates may be more antigenically diverse than that study demonstrated, regardless of year of isolation, if antibodies to proteins other than the nucleocapsid are looked at, such as monoclonal antibodies to the membrane protein (Dea et al., 1996). A panel of monoclonal antibodies recognizing different PRRSV proteins can be used to further study antigenic variation and to differentiate PRRSV isolates (Jones et al., 1999; Magar et al., 1997).

**Genetic Variation**

PRRSV has an RNA genome, increasing the likelihood that the viral genome will be genetically diverse. Synthesis of RNA is error prone and the discontinuous nature of PRRSV replication makes this virus especially likely to undergo recombination (Lai, 1996; Yuan et al., 1999). Studies have clearly indicated that there are two distinct genotypes of PRRSV, the European and North American, and probably several minor genotypes within North America (Kapur et al., 1996; Meng et al., 1994; Meng et al., 1995a; Meulenberg et al., 1993a; Murtaugh et al., 1995). The currently described Asian PRRSV isolates are related to the North American genotype (Saito et al., 1996).

North American isolates are genetically distant from the European isolates, sharing only about 60% of their amino acid sequence in ORFs 2-7 (Conzelmann et al., 1993; Meng et al., 1995a; Meng et al., 1995b; Meulenberg et al., 1993a). There is also genetic diversity within each of the PRRSV genotypes. Eight U.S. isolates of varying pathogenicities were compared by sequencing the structural proteins, membrane (M) and nucleocapsid (N) genes were 96-100% identical among the isolates (Meng et al., 1995a). ORFs 2, 3, 4 and 5 were about 90% identical, overall ORFs 3 and 5 were the most variable and ORF 6 was the most
conserved (Kapur et al., 1996; Meng et al., 1995b). ORFs 5 and 7 were compared among European countries and it was found that there was 95-100% amino acid identity in ORF 7 and 88% in ORF 5 (Suarez et al., 1996b). The results of amino acid sequence comparisons within the North American genotype and between the North American and European genotypes are summarized in Table 2 (Andreyev et al., 1997; Kapur et al., 1996; Meng et al., 1995a; Meng et al., 1995b; Meulenberg et al., 1993a).

Due to the great deal of variation between the different strains of PRRSV, it has been difficult to identify particular amino acids or even regions of the genome that are responsible for the differences observed in pathogenicity. Basic studies on the functions of the different PRRS viral proteins have just begun to be reported. Previously most of the attention to PRRSV genetic variation has been related to the corresponding differences in

<table>
<thead>
<tr>
<th>Protein</th>
<th>North American isolates</th>
<th>Lelystad Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicase (1a)</td>
<td>88-99</td>
<td>32-62</td>
</tr>
<tr>
<td>Replicase (1b)</td>
<td>97-99</td>
<td>42-75</td>
</tr>
<tr>
<td>GP2</td>
<td>91-99</td>
<td>57-62</td>
</tr>
<tr>
<td>GP3</td>
<td>87-98</td>
<td>55-60</td>
</tr>
<tr>
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<td>Envelope (GP5)</td>
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</tr>
<tr>
<td>Membrane</td>
<td>96-100</td>
<td>78-81</td>
</tr>
<tr>
<td>Nucleocapsid</td>
<td>96-100</td>
<td>57-60</td>
</tr>
</tbody>
</table>
antigenicity that result from this variation, thus focusing on ORF 5 (Andreyev et al., 1997; Kapur et al., 1996). Since it appears that neutralizing antibody is not the main mechanism of PRRSV clearance (Christianson et al., 1992; Lager and Ackermann, 1994; Loemba et al., 1996; Stevenson et al., 1994; Yoon et al., 1995a), it is likely that there are other viral factors responsible for differences in viral virulence. The use of a single isolate of PRRSV at different cell passages, decreases the number of random background genetic mutations that would normally be seen when sequencing two completely different isolates. An infectious clone would then be useful in testing the potential virulence determinants of PRRSV. Currently an infectious clone exists for LV (Meulenberg et al., 1998a), but because of the extensive genetic diversity between the two genotypes of PRRSV it would be difficult to make meaningful corresponding mutations unless they occur in a highly conserved region.
Short Communication

Porcine reproductive and respiratory syndrome virus (PRRSV) was first isolated in Europe on primary porcine alveolar macrophages (PAM) and designated Lelystad virus. The U.S. PRRSV isolate was isolated on a continuous cell line ATCC CL2621. In addition to PAM cultures and the CL2621 cell line, the MARC 145 and ATCC CRL11171 cell lines have also been shown to support PRRSV replication. Both US and European PRRSV isolates replicate well in PAM cultures, but difficulties and expense in obtaining these cells limit their use. Therefore, a continuous cell line is most often used to isolate and propagate PRRSV.

Virus isolation is a commonly used method for the detection of PRRSV infection in many diagnostic laboratories. Clinical and diagnostic laboratories that maintain cell cultures for virus isolation can be affected by the cost and availability of fetal bovine serum (FBS). FBS carries the risk of contamination with bovine viruses, batch-to-batch variation, and increased expense making a serum replacement desirable, particularly in clinical and
diagnostic settings. Commercially available serum replacements are often used as a substitute for FBS.

Previous studies of serum replacements have shown variable results in their ability to support the growth of cell cultures and to replicate virus. This indicates that the adaptability of cell lines and their capability to propagate virus must be examined on an individual cell line basis.\[2,6\] The variation in PRRSV propagation on various cell types has been reported, but differences due to media or media supplement changes have not been reported.\[1,7\] This study compares the growth of PRRSV isolates in CRL1171 cell cultures maintained in either FBS or a serum replacement\[8].

In order to compare the replication of PRRSV in both media supplements, CRL1171 cells were split into two populations, one maintained in Dulbecco's modified Eagle media (DMEM) containing 10% FBS and the other in DMEM supplemented with 10% serum replacement. After 3 passages in the respective supplemented media, confluent monolayers of both cell populations were grown on 12-well plates. The cells showed no difference in growth rates or morphology regardless of the serum supplement used (data not shown). The CRL1171 monolayers were infected in triplicate with passage 11 VR-2385, a well-characterized virulent US PRRSV isolate, at 0.05 and 0.005 moi.\[4,5\] After 1 hour adsorption, the inoculum was removed and replaced with DMEM plus 2% of the appropriate serum. At 24, 48, 72, 96, and 120 hours post-infection, triplicate wells grown in each serum and at each moi were harvested by scraping and the cell/supernatant mixture was frozen overnight at \(-80^\circ\text{C}\). The virus stocks were thawed and clarified by centrifugation at 1100 x g for 10 minutes. The virus was then serially diluted at 10-fold
dilutions and 100 µl was used to infect monolayers of cells on 96-well plates in quadruplicate. After 1 hour adsorption, 100 µl of DMEM plus 2% FBS was added to each well and the plates were incubated for 48 hours. Fifty percent tissue culture infectivity dose (TCID₅₀) was then determined by IFA using a monoclonal antibody, B7ef₁₁⁵, which recognizes the nucleocapsid protein.¹⁰ The growth curves were then repeated using the same virus stock.

PRRS virus isolate VR-2385 replicated to a higher titer in cells supplemented with FBS, as compared to the cells grown in the serum replacement. The maximum titer of virus grown in serum replacement was 1.5-2 log₁₀ lower than that of the FBS grown virus. Figure 1 shows that VR-2385 consistently grows to at least 1 log₁₀ higher titer in FBS than in serum replacement from 24-120 hours post-infection. Using a standard t-test, the difference in viral titer of VR-2385 grown in FBS-supplemented media was statistically significant (P ≤ 0.05) at all timepoints and at each moi. This demonstrates that an increased viral titer in FBS-supplemented media is not time dependent. The difference in the VR-2385 titer in the two growth conditions is a little larger at most timepoints with the lower moi of 0.005, with an average 99.3% reduction in titer, as compared to a 97.8% reduction at a moi of 0.05 (Table 1). This suggests that the growth of PRRS virus in serum replacement-supplemented cells may be more affected by adverse growing conditions, such as low numbers of infectious virus.

To confirm that the differences in PRRS viral growth rate were not unique for isolate VR-2385, another isolate, ISU-79, was then used to infect CRL11171 cells at 0.05 moi. ISU-79 had approximately a 1.5 log₁₀ higher titer when grown in FBS-supplemented
cells than in serum replacement-supplemented cells at all 5 timepoints (Figure 2). This demonstrates that the difference in serum used in cell propagation has an effect on the growth of more than one US PRRSV isolate.

PRRSV is commonly propagated on continuous cell lines. Clinical and diagnostic laboratories may use serum replacements in the maintenance of these cell lines in order to decrease costs and variability in the cultures. The adaptability of cell lines and viruses to commercial serum replacements has been variable and must be examined on an individual basis. In this study, we looked at the growth of two PRRSV isolates in the CRL1171 cell line maintained in 2 different media supplements, FBS and serum replacement. The results of this study show that PRRSV titers were consistently higher when the virus was propagated in cells grown in FBS as compared to cells grown in a serum replacement. The enhancement of viral propagation seen in FBS may be greater at lower multiplicities of infection, as would commonly be seen in virus isolations for diagnosis of field cases. This increase in viral titers in FBS supplemented media, as compared to those supplemented with serum replacement, was not time dependent and was consistent for two isolates. The observations made in this study suggest that the use of serum supplement in PRRSV propagation can influence viral titers, which may have implications for PRRSV diagnosis by virus isolation.

Sources and manufacturers

a. JRH Biosciences, Lenexa, KS.

b. Produced in our lab
References


Figure 1. Growth curve of VR-2385 passage 11 in CRL 11171 cells supplemented with either FBS or serum replacement. The titer of VR-2385 is at least $1 \log_{10} \text{TCID}_{50}/\text{ml}$ higher at each timepoint when the virus is grown in FBS-supplemented cells. A. VR-2385 at .05 moi. B. VR-2385 at .005 moi.
Table 1. Relative log_{10} titer of PRRSV VR-2385 in serum replacement (SR) versus fetal bovine serum (FBS) supplemented media at various multiplicities of infection

<table>
<thead>
<tr>
<th>Hours Post-Infection</th>
<th>TCID_{50}/ml in FBS at .05 moi</th>
<th>TCID_{50}/ml in SR at .05 moi</th>
<th>% Reduction in titer at .05 moi</th>
<th>TCID_{50}/ml in FBS at .005 moi</th>
<th>TCID_{50}/ml in SR at .005 moi</th>
<th>% Reduction in titer at .005 moi</th>
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<td>24</td>
<td>4.46</td>
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<td>99.7</td>
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</tr>
<tr>
<td>120</td>
<td>4.67</td>
<td>2.83</td>
<td>99.3</td>
<td>5.96</td>
<td>3.71</td>
<td>99.1</td>
</tr>
<tr>
<td>Average</td>
<td>97.8</td>
<td></td>
<td></td>
<td>99.3</td>
<td></td>
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</table>
Figure 2. Growth curve of ISU-79 in CRL 11171 cells supplemented with either FBS or serum replacement. At 24 hours post-infection, the titer of ISU-79 was below the detectable level of $1.75 \log_{10} \text{TCID}_{50}/\text{ml}$, with no positive wells at $10^{-1}$ dilution. The titer of ISU-79 is about $1.5 \log_{10} \text{TCID}_{50}/\text{ml}$ higher at each timepoint when the virus is grown in FBS supplemented cells.
Abstract

The genome of a cell culture-attenuated strain was analyzed and compared it to its highly virulent parent strain, VR-2385. The overall genome sequence differed by less than 1%, despite a significant change in virulence between the two strains. The vast majority of the nucleotide changes resulted in amino acid changes, with a 3:1 ratio of nonsynonymous to synonymous changes in the structural genes. Many of the amino acid changes in VR-2385 p85 resulted in the high passage virus acquiring an amino acid that is the same as the other U. S. PRRSV isolates at that position. The sequence of VR-2385 p7 has many amino acid positions that are unique to that isolate, which may contribute to the high virulence of this isolate relative to other PRRSV isolates and should be considered as potential virulence determinants.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) was first recognized in the United States in 1987 and has since been reported throughout much of Western Europe and Asia [Hill, 1990 #5; Keffaber, 1989 #10; Done, 1996 #24]. The disease syndrome caused
by the PRRS virus (PRRSV) can vary greatly in severity. These differences in virulence observed in both experimental and natural infections depend on a complex of factors associated with host susceptibility and virus strain differences. Differences in virulence among naturally occurring PRRSV have been well demonstrated in regard to respiratory tract illness [Halbur, 1996 #25; Halbur, 1995 #2] as well as in their effects on the reproductive tract [Mengeling, 1996 #31]. Genetic differences among isolates of varying pathogenicities have also been well established, but the regions of the viral genome which contribute to these differences in virulence are unknown [Meng, 1995 #148; Meng, 1996 #153].

This study compared a serially passaged attenuated PRRSV isolate to its virulent parent strain, VR-2385 [Schommer, 2000 #238]. This paper compares the entire genome of both passages of VR-2385 to identify genetic regions which may play a role in PRRSV virulence. The results of this study contribute candidate sequences for further study with the cDNA infectious clone and provide the first full-length sequence comparison of a high and low virulence isolate from the same lineage.

Materials and Methods

Virus and Cells

VR-2385 was isolated from a 160-sow herd in southwest Iowa experiencing severe respiratory disease and high numbers of late-term abortions [Halbur, 1995 #2; Meng, 1996 #1]. The isolate was plaque purified three times and propagated on ATCC CRL11171 cells
as previously described [Meng, 1996 #1]. This virus was then serially passaged 84 times in the CRL1171 cell line to produce the high passage virus isolate used in this study.

**RNA isolation and RT-PCR**

VR-2385 p84 was used to inoculate CRL1171 cells at approximately 0.01 moi. After 24 hours, the media was removed and total RNA was isolated by the guanidinium isothiocyanate method, using a commercial kit (Stratagene). The total RNA was then used in RT-PCR reactions using primers that were designed based on the VR-2385 p7 sequence (Table 1) [Meng, 1994 #42; Meng, 1995 #148; Morozov, 1995 #151; Morozov, #239]. ORFs 2-7 were amplified using random hexamers for cDNA synthesis (Invitrogen) and PCR was performed for 30 cycles with Taq polymerase (GibcoBRL) with conditions optimized for each primer pair. ORFs 1a and 1b were amplified by RT-PCR using virus specific primers for both reverse transcription and second-strand DNA synthesis with a commercial kit (Promega). PCR products ranged in size from 0.4 - 1.8 kb. At least 3 RT-PCR reactions for each set of primers were pooled and purified using a Wizard prep column (Promega). Both strands of the pooled purified PCR products were then sequenced at the Iowa State University DNA Sequencing and Synthesis Facility on an Applied Biosystems Prism 377 DNA sequencer using the Applied Biosystems Prism BigDye terminator cycle sequencing kit.

**Sequence analysis**

The results of the automated sequencing reactions were assembled into a full length sequence and analyzed using MacVector (International Biotechnologies) and PROSITE. The complete sequence was then compared to the previously sequenced parent strain, VR-
Results/Discussion

Genome analysis

Overlapping RT-PCR products were directly sequenced and assembled into one contiguous sequence of 14,991 nucleotides. The genome of VR-2385 p7 was previously determined to consist of a major viral population of the same length, 14,991 nucleotides, and a minor population with a length of 15,100 nucleotides (nt) [Morozov, #239]. PRRSV strain VR-2385 p85 is 420 nt shorter than the previously reported U.S. isolates and is 97 nt shorter than Lelystad virus (LV) (Figures 1 and 2) [Allende, 1999 #165; Meulenberg, 1998 #227; Nelsen, 1999 #33]. The entire genome of the attenuated PRRSV VR-2385 p85 was then analyzed and compared to its virulent parent strain and found to differ by about 0.6% at the nucleotide level (Table 2). Since the two passages of VR-2385 are from the same original isolate, the strain-to-strain variation normally seen among PRRSV isolates is eliminated, facilitating the search for genes important in virulence.

5’ NTR

The 5’ non-translated region of the PRRSV genome, also known as the leader sequence, is important in the formation of the nested set of subgenomic mRNAs [van Marle, 1999 #236; Sawicki, 1998 #154; van der Most, 1995 #155; Lai, 1989 #156; Baric, 1985 #157]. In VR-2385, the leader is 190 nucleotides long. Comparison to the other available U.S. PRRSV sequences demonstrated that the leader is highly conserved among
U.S. isolates, with 98% or greater nucleotide identity. The leader sequences of U.S PRRSV isolates are distinct from the European sequence, although there are regions of significant homology at the 3’ end of the leader sequence which is believed to be critical for its binding to leader-body junction sites within the genome and at the 5’ end [Oleksiewicz, 1999 #228; Nelsen, 1999 #33]. The only change between strains VR-2385 p85 and p7 in the leader sequence is in the middle, with an adenine to guanine substitution at position 55 (data not shown). Interestingly, the same nucleotide change occurred between the two European leader sequences that have been reported, LV and 111/92 [Oleksiewicz, 1999 #228; Meulenberg, 1993 #44]. This high degree of conservation between the attenuated and virulent VR-2385, is similar to the level of nucleotide identity between VR-2332 and the vaccine strain, and may indicate that the 5’ untranslated region is not involved in attenuation [Oleksiewicz, 1999 #228].

**ORF1a analysis**

The ORF1a of VR-2385 p85 encodes 2358 amino acids, which is 145 residues shorter than the other U.S. PRRSV isolates and 38 amino acids shorter than LV. This was due to an internal deletion in nonstructural protein 2 (nsp2). Nsp2 is the most variable region among arteriviruses and has been speculated to be involved in species-specific functions [de Vries, 1997 #164; Allende, 1999 #165]. However, even among the North American and European PRRSV genotypes, which both are restricted to pigs, there is only 32% homology [Allende, 1999 #165].

There are six non-structural proteins that are predicted to result after ORF1a polyprotein cleavage, with nsp1 being further cleaved into nsp1α and nsp1β [de Vries,
PRRSV VR-2385 p85 had 27 amino acid changes in ORF1a, evenly distributed at a rate of about 1% amino acid identity change in each of the individual proteins. Within nsp1-3, however, the amino acid changes tended to be clustered. All of the predicted catalytic sites and cleavage sites were conserved in all of the U.S. isolates [Allende, 1999 #165].

Nsp2 is the most variable protein in the replicase region and was the protein that contained the deletion. The parent strain, VR-2385 p7, had been shown previously to contain two different deletion species in ORF1a, a major subpopulation that has the same deletion as the p85 strain, and an independent minor deletion that was lost during cell culture passage [Morozov, #239]. Sequence alignment of all available PRRSV sequences (Figure 2), showed that p85 and p7 VR-2385 were 97% identical and that these strains had 88% amino acid identity with PRRSV strains 16244B and VR-2332. There is less homology between VR-2385 strains and VR-2332 and 16244b in this protein than in the other replicase proteins, with most of the amino acid changes occurring in the area of the deletion. Despite low homology with LV, the deletion region identified in the VR-2385 strains overlapped with 76 amino acids that were deleted in LV. This may indicate that this region of ORF 1a is susceptible to and tolerant of deletions and insertions.

ORF 1b

The polyprotein encoded by PRRSV ORF 1b is believed to be cleaved by proteases from ORF1a of the genome and to produce 4 viral proteins. This protein is highly conserved, with the available U.S. isolates all having 98-99% nucleotide identity. VR-2385p7 and p85 have 99.5% nucleotide identity in ORF 1b. The changes in the cell culture
attenuated VR-2385 p85 were confined to the 5’ end where the RNA dependent RNA polymerase (RdRp) and cleavage protein 2 are located. All of the predicted cleavage sites were intact.

**ORFs 2-7**

The 3’ end of the PRRSV genome codes for the structural proteins and this region of the genome has been sequenced for a large number of PRRSV isolates. These include another cell culture-attenuated PRRSV, the MLV vaccine RespPRRS, and its parent strain, VR-2332 [Madsen, 1998 #191]. Among isolates characterized thus far, the most variable ORFs in this region are ORFs 3 and 5. Similarly we also identified the greatest percentage of nucleotide changes in these two open reading frames. These changes will be characterized in more detail below.

The proteins encoded in ORFs2 and 4 are believed to be minor structural proteins of PRRSV and are referred to as glycoprotein 2 (GP2) and glycoprotein 4 (GP4) [Meulenberg, 1996 #169; Meulenberg, 1995 #185]. The only notable difference between VR-2385 p7 and p85 in these proteins is a 10 amino acid truncation of GP2 due to a single nucleotide substitution that resulted in a premature stop codon. The C-terminus of GP2 is hydrophobic and has been predicted to be a membrane anchor [Meulenberg, 1996 #169]. The truncation of this protein results in the loss of a portion of the hydrophobic tail, which may decrease the stability of this protein in the membrane, but the biologic relevance of this truncation is not known.

**ORF6** encodes the non-glycosylated membrane protein, M, which is the most conserved structural protein [Kapur, 1996 #29; Meng, 1994 #42]. This corresponds to our
results of 98.9% amino acid identity, with three amino acid changes, with all three changes occurring in the N-terminal 25 amino acids. The only amino acid changes in the RespPRRS MLV strain, as compared to its parent strain, were also at the N-terminus although these changes are not in the same position as any of the changes observed here. Two of the changes observed in the attenuated, high passage VR-2385 actually match those amino acids already seen in the other U. S. PRRSV isolates, suggesting that it is the highly virulent low passage VR-2385 that is unique.

**ORF7**

ORF7 encodes for the nucleocapsid, a 15 kDa highly basic protein that is predominant in the virion [Meulenberg, 1995 #185]. Antibody to this protein is abundant in convalescent pig sera and is suitable for diagnostic testing, but this antibody is not PRRSV-neutralizing [Meulenberg, 1995 #185; Denac, 1997 #187; Rodriguez, 1997 #188; Loemba, 1996 #186]. All 3 changes between high and low passage VR-2385 in this region are non-synonymous. Two potential nuclear localization sequences have been identified in this protein and none of the changes occur in these regions [Rowland, 1999 #183].

**ORF 3**

The protein encoded by ORF3 has been characterized as a highly glycosylated protein that may or may not be part of the PRRSV virion [Gonin, 1998 #173; Mardassi, 1998 #174; Meulenberg, 1995 #185]. The ORF3 protein of Lelystad virus has been demonstrated to be part of the structural virion, whereas the ORF3 of two other arteriviruses, lactate-dehydrogenase virus and equine arteritis virus has not been characterized as a structural protein [Meulenberg, 1995 #185; Faaberg, 1997 #189; de Vries, 1992 #190]. GP3 does not have a typical class 1 membrane protein hydrophobic
anchor, but there is a putative N-terminal signal sequence that may indicate its ability to pass through the membrane [Drew, 1997 #229].

The ORF 3 protein had the greatest percentage of amino acid changes for both cell culture-attenuated PRRSV strains (Table 3 and Figure 3). This protein is predicted to play a role in cellular immunity [Duran, 1997 #175], which may be especially important for clearing PRRSV infections since neutralizing antibody does not appear to play a significant role [Yoon, 1995 #133; Loemba, 1996 #186]. Recently there was a report of ORF 3 deletion mutants, with deletions as large as 8 amino [Oleksiewicz, 2000 #177]. These deletions were near the 3' end in the ORF 3/4 overlapping region and correspond to the region that is recognized by neutralizing antibody. These deletion mutants are outcompeting non-deleted viruses in the field in Europe, suggesting that they may be emerging as a result of immunological pressure [Oleksiewicz, 2000 #177]. None of the amino acid changes identified in VR-2385 p85 were at the 3' terminus. There is increasing evidence for the role of this protein in the host immune response to arteriviruses, but it is unknown if regions other than the 3' end play a role [Katz, 1995 #54; Duran, 1997 #175; Gonin, 1998 #173; Hedges, 1999 #240]. The host immune response may be an important contributor to PRRSV virulence, as this virus has been shown to induce apoptosis in vivo and in vitro, with many more cells undergoing apoptosis than are infected by virus [Sirinarumitr, 1998 #241][Sur, 1998 #242][Suarez, 1996 #180]. There are no differences in glycosylation sites between the VR-2385 p7 and p85 viruses, but there are additional potential enzymatic cleavage sites in the VR-2385 p85 strain. These cleavage sites could
a study comparing the MLV RespPRRS/Repro™ to its parent strain [47]. This mutation is in a highly conserved region of GP5 that is predicted to be exposed on the virion surface [2, 18, 27, 47]. While the same mutation is not present in this study, there is a mutation in the same region, at position 158 (Figure 3b). The high passage VR-2385 isolate has a serine substituted for a proline, which could affect the conformation of the membrane surface. This ectodomain may be involved in receptor binding, linkage to ORF6 or virus neutralization.

Overall, there are two main features of the changes in ORFs 2-7 in high passage VR-2385 that stand out. First, the vast majority of the nucleotide changes result in amino acid changes (Table 3). This suggests that the virus was under selective pressure to change during cell culture passage. Second, nearly 50% of the amino acid changes in VR-2385 p85 result in the high passage virus acquiring an amino acid that is the same at that position for the other U.S. PRRSV isolates. VR-2385 p7 has many amino acid positions that are unique to that isolate, which may contribute to the high virulence of this PRRSV isolate [13-15, 27].

Overall genomic differences

In this study, we have analyzed the genome of a cell culture attenuated strain and compared it to its highly virulent parent strain, VR-2385. The overall genome sequence differs by less than 1%, despite the drastic change in virulence [45]. By comparing these sequence changes to the ones reported in the attenuated vaccine strain, RespPRRS, we have identified several regions that should be looked at further as having a potential role in PRRSV virulence. In that study, there were only 11 nucleotide changes in ORFs 2-7,
resulting in 7 amino acid changes [23] (Table 3). Cell culture-attenuation of VR-2385 after 85 cell culture passages resulted in 28 amino acid changes as compared to the parent strain. A possible reason for having four times as many amino acid changes is that the parent isolates of the attenuated strains differ greatly in their virulence. The parent strain of RespPRRS, VR-2332, caused interstitial pneumonia, but did not cause gross lung lesions or clinical respiratory disease [4], while PRRSV isolate VR-2385 is commonly used as a challenge isolate because reproducible clinical respiratory disease develops in young pigs and gross lung lesions are often near 50% at 10-14 days post-infection [13, 14].

Nsp2, is the most variable protein within arteriviruses as well as within the PRRSV genotypes [6]. The presence of two different deletions in this region in VR-2385 may account for the difference in virulence observed between the high and low passages strains or cell culture passage may have selected for the smaller genome. It was previously suggested that ORF1a and 1b may be responsible for the attenuation of VR-2332 into the vaccine virus RespPRRS, since there were so few changes in the ORF2-7 sequence [23]. Further investigation into the function of this gene is required and a comparison of the RespPRRS attenuated strain to VR-2332 may be useful in determining if nsp2 plays a role in virulence.

ORFs 3 and 5 were also identified as having a potential role in virulence. These proteins have been identified as being important for the immune response to PRRSV infection [10, 19, 38-40]. GP3 and GP5 are both glycosylated, but this modification does not appear to contribute to the difference in virulence observed between the two passages of VR-2385.
There were amino acid changes in virtually every protein when comparing the attenuated VR-2385 to its parent strain, so we can not discount the possibility that virulence differences may be determined by other regions of the genome. This study provides identification of several regions that should be examined as having a potential role in PRRSV virulence.

References


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68


34. Morozov unpublished data


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Table 1: Summary of the primers used for VR-2385 p85 amplification and sequencing

<table>
<thead>
<tr>
<th>Oligonucleotide (5'→3')</th>
<th>Location</th>
</tr>
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<tr>
<td>+CAGCTTCTTTATCGGCTCTTC -CAAAGAACCTGGAAGATGACGCAGG</td>
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<td>+CGGCTGGCTGGAGCTGC -CAAATCTAGGGCCACGTCC</td>
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Table 2: Summary of the genetic differences between VR-2385 p7 and p85

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<td>123</td>
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<tr>
<td>3' UTR</td>
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<td>151</td>
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</table>

\* = based on comparison with the same deletion in both passages of VR-2385

\# = single nucleotide change results in premature stop, counted as 1 AA change, but results in a 10 AA truncation
Table 3: Synonymous versus nonsynonymous amino acid changes in cell culture attenuated PRRSV isolates

<table>
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<th>ORF</th>
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<th>VR-2385 Nonsynonymous</th>
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<td>Total</td>
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<td>27</td>
<td>4</td>
<td>7</td>
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* = Based on information from Madsen, et. al. [23]
Figure 1. Dot Matrix Plots of the genomes of selected PRRSV isolates. U. S. isolates analyzed at 95% stringency (A and B). VR-2385 p85 and LV analyzed at 65% stringency (C).
Figure 2. Amino acid comparison of Nsp2 among all the PRRSV sequences available. Dots (.) represent sites of amino acid identity, dashes (-) represent areas of deletions. VR-2385 pTM represents the major deletion species detected, VR-2385 p7m represents the minor deletion species detected.
Figure 2. (continued).
Figure 3. A. Amino acid comparison of ORF3 protein (GP3) sequence for VR-2385 p8S and p7. B. Amino acid comparison of ORF5 protein (GP5) sequence for VR-2385 p8S and p7. Dots (.) represent sites of amino acid identity.
BIOLOGICAL CHARACTERIZATION OF A CELL CULTURE-ATTENUATED PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) ISOLATE

A paper to be submitted to the journal Virus Research

Susan Schommer, Patrick Halbur, Ryan Royer, Susan Carpenter, Prem Paul

Abstract

A highly virulent porcine reproductive and respiratory syndrome virus (PRRSV) isolate, VR-2385, was serially passaged 84 times in a continuous cell line to produce an attenuated strain. Naïve pigs were inoculated with VR-2385LP (passage 8) or VR-2385HP (passage 85) to determine if cell culture passage resulted in decreased virulence as compared to the parent isolate. The pigs inoculated with VR-2385 LP showed moderate clinical respiratory signs and had elevated temperatures 5-11 days post-infection (DPI), whereas the pigs inoculated with VR-2385 HP had only mild respiratory signs and no fever. Gross lung lesions, microscopic lung lesions, and microscopic lymph node lesions were all significantly (P ≤ 0.05) more severe in the VR-2385LP-inoculated pigs at 10 and 31 DPI. At 42 DPI, however, interstitial pneumonia was moderate in both inoculated groups. The results of virus isolation and titration from sera indicated that VR-2385LP-inoculated pigs had higher virus loads at early timepoints post-infection than did pigs infected with high passage virus. However in cell culture, VR-2385HP was able to replicate to a higher titer in both a continuous cell line and primary cells. Despite the differences in virulence and serum virus titers between the two strains of VR-2385, both passages of the virus were able to persist equally well. This model of high and low virulence PRRSV strains from the
same original field isolate was established to examine the biologic basis for strain differences in viral virulence, as well as to look for a biologic marker that could be used to distinguish virulent and avirulent PRRSV strains.

I. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is economically one of the most important diseases facing the swine industry today. PRRS was first recognized in the United States in 1987 and has since been reported throughout much of Western Europe and Asia (Done et al., 1996; Hill, 1990; Keffaber, 1989). The disease syndrome caused by PRRS virus (PRRSV) varies greatly in severity. These differences in virulence observed in both experimental and natural infections depend on a complex of factors associated with host susceptibility and virus strain. Differences in virulence among naturally occurring PRRSV have been well demonstrated in regard to respiratory tract illness (Halbur et al., 1995; Halbur et al., 1996) as well as in their effects on the reproductive tract (Halbur et al., 1996; Halbur et al., 1995; Mengeling et al., 1996).

To date there is no known biological marker of virulence, therefore virulence can only be determined by infecting naïve pigs which is costly, time-consuming, and impractical. Genetic differences among isolates of varying pathogenicities have been well established, but the regions of the viral genome which contribute to these differences in virulence are unknown (Halbur et al., 1996; Meng et al., 1995; Mengeling et al., 1996). One mechanism proposed to account for virulence differences is that more virulent isolates are better able to replicate in pigs (Mengeling et al., 1999a). This led to the hypothesis that
porcine alveolar macrophages (PAMs) would tend to select for virulent field strains \textit{in vitro} (Mengeling et al., 1999a), but that hypothesis was not tested. Plaque size differences have also been suggested as a biological marker, but has not been demonstrated to be a predictor of virulence for other PRRSV isolates (Park et al., 1996).

The restriction fragment length polymorphism (RFLP) test has become a well-established method for typing PRRSV isolates (Wesley et al., 1998). This test is very effective at differentiating field strains from the RespPRRS/Repro\textsuperscript{TM} (Boehringer Ingelheim) vaccine strain, but no specific digestion pattern or patterns have been identified as an indicator of virulence. Indeed, isolates of varying virulence often type with common RFLP patterns. As more pigs are exposed to the MLV vaccines, it becomes more and more likely that field strains will contain the “unique” restriction enzyme site found in the vaccine (Wesley et al., 1999). Monoclonal antibody typing is under investigation as a virus typing method, but currently is not used to differentiate virulent from avirulent field strains (Benfield et al., 1999; Yang et al., 1999).

In this study, we compared a serially passaged PRRSV isolate, VR-2385\textsubscript{HP}, to its parent strain to determine if a highly virulent PRRSV strain could be attenuated by cell culture passage. The use of two strains from the same genetic lineage decreases the genetic variation normally observed between PRRSV isolates. This model was established to examine the biologic basis for strain differences in viral virulence, as well as to identify a biologic marker that could be used to distinguish virulent and avirulent PRRSV field strains.
2. Materials and Methods

2.1 Virus and Cells

VR-2385 was isolated from a 160-sow herd in southwest Iowa experiencing severe respiratory disease and high numbers of late-term abortions (Halbur et al., 1995; Meng et al., 1996). The isolate was plaque purified three times and propagated on ATCC CRL11171 cells as previously described (Meng et al., 1996). This virus was then serially passaged 84 times in the CRL11171 cell line to produce the high passage virus isolate used in this study. In these experiments passage 84 and 85 are termed high passage isolates (VR-2385<sub>HP</sub>), whereas passages 12 and under were used as low passage isolates (VR-2385<sub>LP</sub>).

2.2 Experimental infection

Forty-nine specific pathogen free (SPF) pigs were purchased at 13-17 days of age. The pigs were randomly divided into three separate animal isolation rooms, two rooms containing 18 pigs and the third containing 13 pigs. Virus inoculum was prepared by infecting confluent monolayers of ATCC CRL11171 cells with either p7 or p84 VR-2385, then freezing the flasks at -70 °C when 50-70% cytopathic effect (CPE) was observed. Frozen material was thawed and clarified, titrated, and a final inoculation dose of 10<sup>5</sup> fifty percent tissue culture infectivity dose (TCID<sub>50</sub>) per 5 ml dose was used for challenge. Cell culture control inoculum consisted of cell culture media that was used to mock-infect CRL11171 cells and then processed identically to the virus fluids. Control inoculum was also administered at 5 ml/ dose. After 48 hours of acclimation, 18 pigs were inoculated
intranasally with VR-2385LP, 18 pigs were inoculated intranasally with VR-2385HP, and 13 pigs were administered the cell culture control inoculum intranasally.

2.3 Clinical, pathological, and virological evaluation

Rectal temperatures and clinical respiratory disease scores were recorded daily from 0 days post-infection (DPI) to 14 DPI and then 3 times weekly until 28 DPI. The clinical respiratory disease score was assigned based on the degree of dyspnea or tachypnea observed for each pig on a scale of 0-6, where 0 = normal, 1 = mild dyspnea and/or tachypnea when stressed, 2 = mild dyspnea and/or tachypnea at rest, 3 = moderate dyspnea and/or tachypnea when stressed, 4 = moderate dyspnea and/or tachypnea at rest, 5 = severe dyspnea and/or tachypnea when stressed and 6 = severe dyspnea and/or tachypnea at rest, as described previously (Halbur et al., 1995). Clinical signs other than respiratory distress were recorded, but were not used to determine the respiratory score.

Necropsies were performed on all pigs. Pigs were equally divided among each timepoint, with 6 from the VR-2385LP and VR-2385HP necropsied at 10, 31 and 42 DPI. The control group consisted of 4 pigs at 10 and 42 DPI, and 5 pigs at 31 DPI. Gross lung lesion scores were assigned as an estimate of the percent of the lung exhibiting grossly visible pneumonia, as described previously (Halbur et al., 1995).

Sections were taken from all lungs, brain, heart, small intestine, spleen, tonsil, and lymph nodes for histopathologic examination. At the 10 and 31 DPI necropsies, lungs were inflated by infusion of 10% neutral buffered formalin in the trachea, then clamped and submerged in fixative. All tissues were fixed in 10% neutral buffered formalin for 1-5 days and routinely processed.
All tissue sections were blindly examined and any abnormal observations were noted. Lung sections were scored on a scale from 0-6, based on the severity of the interstitial pneumonia present; as 0 = no microscopic lesions, 1 = mild pneumonia, 2 = mild diffuse pneumonia, 3 = moderate multifocal pneumonia, 4 = moderate diffuse pneumonia, 5 = severe multifocal pneumonia, and 6 = severe diffuse pneumonia (Halbur et al., 1995). Cranial sternal and medial iliac lymph node sections were scored 0-3, based on the size and number of follicles. A score of 0 = 4-8 normally defined follicles per low power field (lpf), 1 = 9-14 well-defined follicles per lpf, 2 = 15-20 well-defined follicles per lpf with some coalescing follicles evident, and 3 = 21 or more well defined follicles per lpf and common coalescing of hypercellular and reactive follicles (Rotto et al., 1997).

Virus isolation from serum was attempted by adding 0.1 ml of experimental pig serum to the cell maintenance media on a confluent monolayer of CRL 11171 cells in a 6-well plate. The plates were incubated overnight at 37 °C and 5% CO2, then the media was removed and replaced with DMEM containing 2% FBS and 1% antibiotic/antimycotic (10,000 units/ml penicillin G, 10,000 mg/ml streptomycin, 25 mg/ml amphotericin B). The plates were monitored daily for cytopathic effect (CPE) for up to 7 days, if no CPE was observed the cultures were frozen, thawed and blindly passed 2 additional times before being called negative. CPE positive wells were confirmed to contain PRRSV by IFA (Yoon et al., 1992). Four pigs were randomly selected from each of the 3 experimental groups and 10-fold serial dilutions of serum were used to determine the serum PRRSV titer at 10, 28 and 35 DPI. The dilutions were performed in quadruplicate and the titrations were repeated independently.
2.4 Virus growth curves

Growth curves of VR-2385<sub>LP</sub> and VR-2385<sub>HP</sub> virus were conducted in both the continuous cell line, ATCC CRL 11171, and in primary cells, monocyte derived macrophages (MDMs). In both growth curves, a multiplicity of infection (moi) of 0.1 was used to infect 6-well plates. For the growth curve in CRL 11171 cells, 6-well plates were inoculated with 0.1 moi of either p11 or p84 VR-2385 virus and incubated at 37 °C for 1 hour. After 1 hour adsorption, the virus was removed and the plates were rinsed with fresh DMEM. New DMEM containing 2% FBS and 1% antibiotic/antimycotic was added to each well and incubated at 37 °C. At each timepoint three wells were scraped, the cells and media were frozen at -70 °C overnight, clarified the next day, and returned to -70 °C. When all the timepoints had been completed, each was titrated using 10-fold serial dilutions in quadruplicate. The growth curve was repeated.

For the growth curve in MDMs, blood was collected from 3 pigs and a density gradient was used to isolate peripheral blood monocytes (PBMCs) from each pig as previously described (Thacker et al., 1998). Each pig’s MDMs were resuspended in RPMI-1640 with 10% FBS and 1% antibiotic/antimycotic and evenly distributed on 6-well plates at a density of 8 x 10^4 per well. After 24 hours, nonadherent cells were removed and L929 cultured fibroblast supernatant was added as previously described (Genovesi et al., 1989; Thacker et al., 1998). Each well was infected with 0.1 moi of the appropriate virus passage and at each timepoint, 0.1 ml of supernatant was removed from 3 randomly selected wells for each pig and each passage of the virus isolate and stored at -70 °C until all timepoints
were collected. When all timepoints were completed, they were titrated on 96-well plates using 10-fold serial dilutions in quadruplicate to determine TCID$_{50}$.

2.5 **Assessment of Plaque Size**

Three-day-old monolayers of CRL 11171 cells in 6-well plates were infected in duplicate with 10-fold dilutions of VR-2385 p11 and p85. The virus was allowed to absorb for 1 hour at 37 °C, the virus was then removed and 3 ml of CM cellulose and Sea Plaque agar in 2X DMEM were added. The plates were incubated inverted at 37 °C for 3 days, after which an overlay of 2 ml of Sea Plaque agarose, 2X DMEM and neutral red was added and the plates were incubated for an additional 24 hours. At this time, 100 plaques per isolate passage were measured using a caliper.

3.0 **Results**

3.1 **Clinical Disease**

*In vitro* passage of PRRSV isolate VR-2385 in cell culture significantly reduced the severity of clinical signs associated with PRRSV infection. The VR-2385$_{LP}$-inoculated group had significantly (P ≤ 0.05) more severe respiratory signs than both the control and VR-2385$_{HP}$-inoculated groups by 2 DPI and continuing through 17 DPI (Figure 1). Clinical disease was most severe in the VR-2385$_{LP}$ group at 6 DPI, with a peak mean score of 2.5 on the 0-6 scale. Average daily rectal temperatures were also significantly elevated in the VR-2385$_{LP}$-inoculated group, as compared to the control and VR-2385$_{HP}$-infected pigs from 5-11 DPI (P≤ 0.05). The VR-2385$_{HP}$-inoculated pigs did not have an elevated temperature at any timepoint (data not shown), however, long-term cell culture passage did not completely
attenuate viral virulence. The VR-2385<sub>HP</sub>-inoculated pigs exhibited mild respiratory
disease that did not appear until 4 DPI, but persisted as long as the clinical disease observed
in VR-2385<sub>LP</sub>-inoculated pigs. Clinical respiratory disease scores were significantly (P ≤ 
0.05) higher in VR-2385<sub>HP</sub>-inoculated pigs than the controls from 4 - 13 DPI, as well as at
17 and 21 DPI. Control pigs remained normal throughout the study, while clinical
respiratory disease was observed in both the VR-2385<sub>HP</sub> and VR-2385<sub>LP</sub>-inoculated groups.
Therefore, cell culture passage significantly reduced the clinical disease caused by VR-
2385<sub>HP</sub> as compared to its parent strain, but the clinical signs of PRRSV were not
completely eliminated in this group of animals.

3.2 Gross and Microscopic Lesions

Pigs were necropsied at 10, 31 and 42 DPI, at which time gross lung lesions were
evaluated and scored based on the percentage of the lung affected. As previously observed
(Halbur et al., 1996; Halbur et al., 1995), the VR-2385<sub>LP</sub>-inoculated group had extensive
pneumonia at 10 DPI, which decreased in severity at 31 DPI (Table 1). Surprisingly, there
were still gross lung lesions present in this group at 42 DPI. The VR-2385<sub>HP</sub>-infected pigs
had significantly (P ≤ 0.01) reduced gross lung lesion scores as compared to the pigs
infected with VR-2385<sub>LP</sub> at all timepoints; however, the lesions were more severe (P ≤ 0.05)
than the control pigs at 10 and 31 DPI. By 42 DPI, lung lesions in the VR-2385<sub>HP</sub>-infected
group had resolved and were no longer significantly different than the controls. A single
pig in the control group had a gross lung lesion score of 1% at 31 DPI, no other control pigs
had any gross lesions at any timepoint.
All tissues collected at necropsy were examined microscopically, with the predominant lesions consisting of interstitial pneumonia and lymph node hypertrophy and hyperplasia. Interstitial pneumonia scores were reflective of clinical disease and gross lung lesion scores at 10 and 31 DPI (Table 2). VR-2385_LP-infected pigs had moderate multifocal pneumonia at all three necropsy timepoints (Figure 2). Unexpectedly, the VR-2385_HP-infected pigs had no microscopic lung lesions at 10 DPI; however, the animals showed significant interstitial pneumonia lesions at 42 DPI. Thus the VR-2385_HP-infected pigs had delayed onset of microscopic lung lesions, but lesions persisted similar to the lesions in VR-2385_LP-infected pigs.

Lymphadenopathy was observed in both the VR-2385_LP and VR-2385_HP-infected groups at all three timepoints with lymph nodes enlarged 2-4 times that of pigs in the control group (data not shown). Histopathologically, the lymph nodes of VR-2385_LP-infected pigs had significantly (P ≤ 0.05) higher follicular definition scores at all 3 timepoints than the control and VR-2385_HP-infected pigs (Figure 3). At no timepoint were the lymph nodes of the VR-2385_HP-infected pigs more reactive than those of the control pigs.

3.3 Virus Isolation and Titration

Clinical respiratory disease signs and microscopic lung evaluation of VR-2385_HP-inoculated pigs indicated that these animals had a more mild form of PRRSV respiratory disease that was delayed in onset but persisted equally well as compared to the disease in VR-2385_LP. In order to determine if these differences in disease course were associated with differences in the presence of virus, sera collected at sequential timepoints were
assayed for PRRSV (Figure 3). All of the pigs inoculated with VR-2385_{LP} were viremic from 3 -21 DPI. At 28 and 35 DPI, 42% and 83% of the of VR-2385_{LP} -inoculated pigs were viremic, respectively. By 42 DPI no virus could be detected in the serum of any pigs of this group. The number of viremic pigs in the VR-2385_{HP} -inoculated group was more variable, ranging from 33-67% throughout the experiment, with 1 pig remaining positive at 42 DPI. All of the control pigs remained negative throughout the study.

To determine if there was any difference in virus titer in the pig sera, selected pig sera were titrated, and the results are summarized in Figure 4. At 10 DPI, the pigs infected with VR-2385_{LP} had $10^{1.5-2.0}$ TCID$_{50}$/ml more virus in their serum than the VR-2385_{HP} -infected pigs. At 28 and 35 DPI there was very little difference among the virus titers between the groups and from one timepoint to another. The results of virus isolation and titration from sera indicate that VR-2385_{LP} replicated better \textit{in vivo} at early timepoints post-infection than the VR-2385_{HP} virus. Although the high passage isolate did not reach as high a titer in the pigs, it was able to persist equally well as the virulent low passage virus.

3.4 \textit{In vitro} growth characteristics

The \textit{in vivo} studies demonstrated that the major pathological differences between high and low passage VR-2384 were associated with differences in viral replication early in infection. To determine if differing replication rates of the two passages of VR-2385 could be used as an \textit{in vitro} marker for the changes in pathogenicity, growth curves were conducted in both cell line CRL 11171 and in MDMs. In both cell types, VR-2385_{HP} replicated to a significantly higher titer (p<0.01) than VR-2385_{LP}. In the continuous cell line, the peak viral titer of VR-2385_{HP} was reached at $10^{6.67}$ TCID$_{50}$/ml at 30 hours post-
infection (PI), whereas isolate VR-2385_{LP} peaked at 42 hours PI with a titer of $10^{5.92}$ TCID$_{50}$/ml (Figure 5). Since VR-2385 was attenuated by serial passage on this cell line, it was not surprising that VR-2385$_{HP}$ was able to reach a higher peak titer and to reach this peak more quickly. In the MDMs VR-2385$_{HP}$ replicated to a significantly ($p \leq 0.001$) higher titer at all timepoints post-infection than the low passage virus, in contrast to the in vivo replication abilities of these strains (Figure 6). Overall, both viruses grew to higher titers in CRL 11171 cells than in MDMs, which was not surprising since VR-2385 was isolated and plaque purified on CRL 11171 cells.

Cytopathogenicity, as measured by plaque size has been suggested as an in vitro virulence marker for PRRSV (Park et al., 1996). The plaque size determined for VR-2385$_{HP}$ was $1.57 \pm 0.35$ mm, while the plaque size of VR-2385$_{LP}$ was $1.48 \pm 0.29$ mm. The two passages of virus do not have significantly different plaque sizes, despite their significant differences in pathogenicity, indicating that this test may not be a good indicator of relative virulence among PRRSV isolates.

4.0 Discussion

Variation in PRRSV virulence due to differences between virus isolates has been well established (Halbur et al., 1996; Halbur et al., 1995; Meng et al., 1995; Mengeling et al., 1996). To date there are no known genetic or biologic markers of virulence for PRRSV isolates. In this study a highly virulent PRRSV isolate, VR-2385, was attenuated by cell culture passage to establish a model of high and low virulence strains from the same lineage. By using a high and low cell culture passage of the same isolate, reduces
background variability facilitating the identification of *in vitro* genetic and biologic predictors of virulence. In this study, differences in clinical signs and pathological lesions in the animals were reflective of the serum virus titer differences observed in the VR-2385<sub>LP</sub> and VR-2385<sub>HP</sub>-infected animals.

At 10 and 31 DPI, clinical disease, gross lung lesion scores and interstitial pneumonia scores were consistent with moderate to severe disease for VR-2385<sub>LP</sub>-inoculated pigs and with mild respiratory disease for VR-2385<sub>HP</sub>-infected pigs. Recent studies with a commercial MLV PRRSV vaccine demonstrated clinical and pathological observations similar to the cell culture-attenuated isolate used in this study (Thanawongnuwech, 2000). Surprisingly, at 42 DPI the VR-2385<sub>LP</sub>-inoculated pigs still had 10% of the lung grossly affected by interstitial pneumonia. At 42 DPI the VR-2385<sub>HP</sub>-inoculated pigs had interstitial pneumonia scores that had become more severe and were no longer significantly different from those of the pigs infected with VR-2385<sub>LP</sub>. This indicated that the onset of pneumonia was delayed, rather than eliminated in the VR-2385<sub>HP</sub>-inoculated pigs. Most PRRSV pathogenicity studies have focused on the degree of lesions observed at peak clinical disease and have not observed the long-term effects of PRRSV infection. Based on the delayed onset of interstitial pneumonia observed when infecting pigs with this cell-culture attenuated isolate, and the identification of both natural and experimental examples of PRRSV MLV reversion to virulence (Boetner et al., 1997; Mengeling et al., 1999b), further investigation in this area is needed.

It was somewhat unusual that PRRSV-induced gross lesions remained 42 days after VR-2385<sub>LP</sub> infection. Dual infection studies with PRRSV and *Mycoplasma*
M. hyopneumoniae have demonstrated that PRRSV-induced pneumonia is significantly prolonged and increased in severity by the presence of M. hyopneumoniae (Thacker et al., 1999). To rule out the possibility that these lesions were due to the presence of a concurrent M. hyopneumoniae infection, ELISA was performed on all 42 DPI sera. ELISA results indicated the pigs in all three groups were negative for anti-M. hyopneumoniae antibodies at the end of the experiment. There also was no indication of M. hyopneumoniae-induced microscopic lesions in any of the pigs. The possibility of a break in biosecurity was also investigated, by sequencing selected PRRSV isolates obtained from the 35 DPI serum virus isolation results. The sequencing results indicated that there were not other PRRSV isolates present, from either within the experiment or from neighboring rooms. In a previous study utilizing VR-2385, gross lesions of 43.6% remained at 28 DPI in one trial, while they were completely resolved at 28 DPI in another trial (Halbur et al., 1996). It is also possible that the extended presence of gross lesions may be related to differences in host susceptibility (Halbur et al., 1998).

It has been previously suggested that the virulence of a particular isolate may be closely related to its ability to replicate in pigs (Haynes et al., 1997; Mengeling et al., 1999a). The increased viral load observed in VR-2385LP-infected pigs in this study, supports the theory that virus load early after infection may be associated with viral virulence. To investigate if in vitro growth characteristics could be used as an in vitro correlate of in vivo virulence, growth curves were performed in both a continuous cell line and primary cells. In vitro, the high passage VR-2385 was able to replicate better even when the cell type used was pig macrophages, thus indicating that a decreased ability to
replicate in primary porcine MDMs, at least in vitro, does not correlate with the attenuation of this isolate. There has been some debate as to whether or not macrophages derived from peripheral blood are permissive to PRRSV infection (Duan et al., 1997; Thacker et al., 1998). The technique used to isolate and infect MDMs in this study was previously demonstrated to produce numbers of VR-2385 infected cells equal to those of porcine alveolar macrophages (PAMs) infected with the same isolate, although viral titers were not determined (Thacker et al., 1998). An advantage to using MDMs over PAMs is the ability to use the same pigs for replicating and standardizing a test. Although it can not be ruled out that the in vitro growth results may be different if the growth curves were conducted in PAMs, pig-to-pig variation in PAM infectivity would make it difficult to develop a test that was dependent on detecting differences in viral titers. Therefore, the results of this study indicate that in vitro replication ability would not be a good predictor of virulence.

Despite differences in viral load and virulence, both virus passages of VR-2385 were equally able to establish persistent infections. This result correlates with previous findings that while the total numbers and distribution of PRRSV positive cells may differ among isolates of varying pathogenicities, several isolates including a vaccine strain, persist equally well (Halbur et al., 1995; Haynes et al., 1997; Mengeling et al., 1996). The ability of both high and low virulence isolates to persist has important implications for the use of modified live vaccines in herds, especially when taking into consideration that there is evidence for the evolution of different quasispecies during infection with VR-2332, a MLV parent strain (Rowland et al., 1999). Outbreaks of disease in endemically infected or vaccinated herds may be caused by changes in the viral population during persistence that
result in a mutation that results in clinical disease (Boetner et al., 1997; Mengeling et al., 1999b).

Isolate VR-2385 has been well established as a model for reproduction of gross and microscopic lesions in the respiratory system as a result of PRRSV infection (Halbur et al., 1996; Halbur et al., 1995; Thacker et al., 1999; Thanawongnuwech et al., 1998). The work in this paper demonstrates that attenuation of a highly virulent PRRSV isolate can be achieved by serial cell culture passage, thus providing a good model for identifying biological and genetic virulence determinants by allowing comparison of a virulent isolate and an attenuated strain from the same lineage. This model permits comparison of an attenuated strain and its parent isolate that differ in virulence to a greater extent than the MLV vaccine RespPRRS/Repro™ differs in virulence from its parent isolate, VR-2332 (Benfield et al., 1992; Benfield et al., 1999). Although no in vitro correlates of in vivo virulence were identified in this study, potential genetic markers of virulence may be identified by comparing the genomes of VR-2385LP and VR-2385HP, which share greater than 99% nucleotide identity (Schommer et al., 2000).

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Table 1: Mean (± SEM) gross lung lesion scores* at necropsy

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>DPI 10</th>
<th>DPI 31</th>
<th>DPI 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture control</td>
<td>0 ± 0*</td>
<td>0.2 ± 0.4*</td>
<td>0 ± 0*</td>
</tr>
<tr>
<td>HP (p85)</td>
<td>2.2 ± 1.5b</td>
<td>5.0 ± 4.6b</td>
<td>0 ± 0a</td>
</tr>
<tr>
<td>LP (p8)</td>
<td>45.7 ± 13.40c</td>
<td>17.7 ± 7.60c</td>
<td>10.8 ± 2.50b</td>
</tr>
</tbody>
</table>

* Estimate of the percentage of entire lung affected by pneumonia
+ Days post-inoculation, when pigs were necropsied
a, b, c Different superscripts represent statistically significant (P ≤ 0.05) differences within a column

Table 2: Mean (± SEM) microscopic lung lesion scores*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>DPI 10</th>
<th>DPI 31</th>
<th>DPI 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture control</td>
<td>0 ± 0a</td>
<td>0 ± 0a</td>
<td>1.0 ± 0a</td>
</tr>
<tr>
<td>VR-2385 p85</td>
<td>0 ± 0a</td>
<td>0.5 ± 0.5a</td>
<td>2.7 ± 1.0b</td>
</tr>
<tr>
<td>VR-2385 p8</td>
<td>3.5 ± 1.2b</td>
<td>2.7 ± 0.5b</td>
<td>3.3 ± 0.5b</td>
</tr>
</tbody>
</table>

* See methods for description of scoring system
+ Days post-inoculation, when pigs were necropsied
a, b Different superscripts represent statistically significant (P ≤ 0.05) differences within a column
Table 3: Mean (± SEM) microscopic lymph node scores*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>10</th>
<th>31</th>
<th>42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture control</td>
<td>0 ± 0a</td>
<td>0.5 ± 0.5a</td>
<td>1.3 ± 0.5a</td>
</tr>
<tr>
<td>VR-2385 p85</td>
<td>0 ± 0a</td>
<td>1.0 ± 0.6a</td>
<td>1.8 ± 0.8a</td>
</tr>
<tr>
<td>VR-2385 p8</td>
<td>1.3 ± 0.8b</td>
<td>2.2 ± 0.4b</td>
<td>2.8 ± 0.4b</td>
</tr>
</tbody>
</table>

* See methods for description of scoring system
+ Days post-inoculation, when pigs were necropsied
a, b Different superscripts represent statistically significant (P ≤ 0.05) differences within a column.

Figure 1. Average daily clinical respiratory score for each of the treatment groups based on the 0-6 scoring system as described in the methods.

- a = significantly (P ≤ 0.05) different than controls,
- b = significantly (P ≤ 0.05) different from VR-2385 HP-infected group
Figure 2. A. Microscopic section of lung from a control group pig. B. Microscopic section of lung from a pig infected 31 days previously with PRRSV strain VR-2385 hp. There is mild multifocal proliferative and lymphohistiocytic interstitial pneumonia. C. Microscopic section of lung from a pig infected 31 days previously with PRRSV strain VR-2385 LP. There is moderate diffuse proliferative and lymphohistiocytic interstitial pneumonia.
Figure 3. Percentage of VR-2385<sub>LP</sub> and VR-2385<sub>HP</sub>-infected pigs that were PRRSV positive by virus isolation from serum.
Figure 4. Serum virus titers (log_{10} TCID_{50}/ml) in VR-2385_{LP} and VR-2385_{HP}-infected pigs at selected timepoints post-infection. Each point represents the viral titer of an individual pig, based on the average of two independent titrations. The lowest detectable titer is 1.0 log_{10} TCID_{50}/ml.
Figure 5. Growth curve of p12 and p85 VR-2385 inoculated at 0.1 moi on a continuous cell line, ATCC CRL11171. VR-2385 p85 reaches a higher overall titer, $10^{7.08}$ TCID$_{50}$/ml versus $10^{5.92}$ TCID$_{50}$/ml for p12, and reaches the peak titer 12 hours earlier.
Figure 6. Growth curve of p12 and p85 VR-2385 inoculated at 0.1 moi on a primary cell line, monocyte derived macrophages (MDMs). The PRRSV strain VR-2385 p85 consistently grew 1 log_{10} TCID_{50}/ml higher at all timepoints than the VR-2385 p12 strain.
GENERAL CONCLUSIONS

My colleagues and I have achieved our overall objective of establishing a model of high and low virulence PRRSV strains from the same original field isolate, thus decreasing the amount of genetic heterogeneity that is normally associated with different isolates of a RNA virus. Differences in virulence were demonstrated in vivo, by infecting young pigs and documenting the amount of clinical and pathological respiratory disease. Biological characterization was also done in cell culture, comparing replication ability and plaque formation. Cell culture adapted virus showed phenotypical change as hypothesized, with changes in virulence and in replication ability. The complete genome of both passages of PRRSV strain VR-2385 were compared after sequencing of VR-2385 passage 85 was completed and genotypic change at a rate of about 1% at the nucleotide level was observed. However, the direct relationship of genotypic changes to their corresponding phenotypic changes still need to be elucidated.

In the first study, growth of PRRSV was optimized in our continuous cell line by determining if the use of an artificial serum replacement resulted in a similar virus replication rate in cell culture. It was discovered that the titers of two different PRRSV isolates were reduced by $1 \log_{10} TCID_{50}$ when the cells were cultured in media supplemented with the serum replacement instead of fetal bovine serum. This effect was observed to be even more severe when the titer of the viral inoculum was low, as would be found when isolating virus from diagnostic samples.
Genetic markers of virulence were searched for by sequencing the entire genome of p85 VR-2385 and comparing it to the previously sequenced p7 VR-2385 (Meng et al., 1994; Meng et al., 1995a; Meng et al., 1995b; Morozov et al., 1995). Overall, genetic differences were found to be less than 1% at the nucleotide level, with the polymerase being slightly more conserved than the 3’ end of the genome. Most of the nucleotide changes resulted in nonsynonymous amino acid sequence, indicating that selection was taking place during cell culture passage. The nsp2 protein, encoded in ORF1a was determined to have a 145 amino acid deletion in VR-2385 p85 as compared to the other two U.S. PRRSV isolates that have been sequenced in their entirety. The p7 VR-2385 strain had predominantly this deletion, but there was also a subpopulation that contained only a 103 amino acid deletion. The p85 VR-2385 strain only contained the 145 amino acid deletion. The nsp2 protein as well as the ORF3 protein, GP3, were determined to be the most likely regions of genetic changes that result in differences in viral virulence. Only 2 proteins were 100% conserved at the amino acid level for the two passages of VR-2385, so it can not be ruled out that changes in other regions may also contribute a change in pathogenicity.

Biologic characterization of the two passages of VR-2385 was completed. Pigs were inoculated with $10^5$ TCID$_{50}$/ml intranasally using either passage 8 VR-2385 or passage 85 VR-2385. Cell culture passage is a common method for producing attenuated strains of viruses and our study resulted in the attenuation of p85 VR-2385. Clinically the pigs inoculated with p85 VR-2385 did not have an elevated temperature and showed only mild respiratory signs, consistent with a mild form of PRRS and comparable to other low virulence isolates and the RespPRRS/Repro vaccine (Halbur et al., 1996;
Thanawongnuwech et al., 1998). Pigs inoculated with p8 VR-2385 had a significant temperature for 6 days and respiratory signs that were observed earlier and were significantly more severe than those in the p85 group.

Virus replication was decreased in vivo for VR-2385 p85 as compared to the parent strain. This was demonstrated by both a decrease in serum viral titer and a decrease in viremia. In vitro, however, the VR-2385 p85 isolate grew to a higher titer.

This study provides a good model of high and low pathogenicity isolates for determination of viral virulence markers. Complete genome sequencing provides identification of regions potentially important for these changes in pathogenicity. There is currently a cDNA infectious clone for LV, the European PRRSV isolate, but due to the low amount of homology between U. S. and European PRRSV, a U. S. infectious clone is needed to further study the effect of the genetic changes observed in our study. Further studies need to be done to determine the role each of the viral proteins plays in order to better understand viral replication mechanisms and how they relate to virulence. Although there are several PRRSV vaccines available, in many cases they have contributed to the lack control over PRRSV spread rather than eliminating it. Research into the basic biology of the viral proteins and their role in inducing immunity as well as the pathologic effects of the virus is needed.
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