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Development of an epitope-based marker system for use in categorizing porcine reproductive and respiratory syndrome viruses

Liuzhan Yang

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Development of an epitope-based marker system for use in categorizing porcine reproductive and respiratory syndrome viruses

by

Liuzhan Yang

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

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Major Professor: Kenneth B. Platt

Iowa State University

Ames, Iowa

2000

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Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Graduate College
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ABSTRACT

A marker system based on the presence or absence epitopes of the N, M, GP5 and GP3 proteins of porcine reproductive and respiratory syndrome (PRRS) virus was developed for use in epidemiological studies. Murine monoclonal antibodies (MAbs) were generated that represented: 7 continuous and 3 discontinuous epitopes of the N protein and were designated EpORF7-A through Hd; 3 discontinuous epitopes of the M protein designated EpORF6-Ad, Bd and Cd; 3 continuous epitopes of GP5 designated EpORF5-A, B and C; and 2 continuous epitopes of GP3 designated EpORF3-A and B. Monoclonal antibodies representing EpORF5-C and EpORF6-Ad and Bd had neutralizing activity. Epitope profiles were determined for 69 North American (NA) PRRS viruses and the Lelystad virus using a panel of MAbs that represented the 18 epitopes. The 69 NA viruses were categorized first into 5 major antigenic groups designated I_{15} through IV_{15} and V_{15} based on the N protein. These 5 groups represented 82.6, 11.6, 2.9, 1.4 and 1.4% of all NA viruses respectively. The European Lelystad virus constituted a separate antigenic group designated V_{15}. Groups I_{15}, II_{15}, and III_{15} were subsequently categorized into 9, 4 and 2 subgroups based on seroreactivity with MAbs representing specific epitopes of the M, GP3 and GP5 proteins. Groups IV_{15} and V_{15} each represented a single virus. The stability of each of the above 18 epitopes was evaluated for stability by serially passaging and maintaining 5 NA isolates in pigs in 3 separate experiments for periods ranging from 16 to 22 weeks. No differences were observed in the epitope profile of any of these 5 viruses following passage. Preliminary studies were also undertaken to identify the region of ORF 7 that encode specific epitopes. Analysis of the amino acid sequence of the N protein using the GCG package and blocking
assays suggested that EpORF7-B, C, and D are located close to one another between position 70 and 122 and that EpORF7-A and E are spatially distant from each other and EpORF7-B, C and D.
GENERAL INTRODUCTION

Introduction

The porcine reproductive and respiratory syndrome (PRRS) is an economically significant viral disease of swine. The disease is characterized by late-term abortions in sows, stillborn and weak piglets at birth, and increased mortality rates in weaned pigs (Goyal, 1993).

The PRRS virus is a single-stranded, positive-sense RNA virus and belongs to the family *Arteriviridae* (Cavanagh, 1997; Pringle, 1996). The genome of PRRS virus encodes 6 structural proteins with molecular masses of 15, 19, 25, 30, 31 and 45 kD that are designated N, M, GP2 through 5 (Conzelmann et al., 1993; Meulenberg and Petersen-Den Besten, 1996; Meulenberg et al., 1993a; 1995; Van Nieuwstadt et al., 1996). After PRRS viruses were isolated from pigs, antigenic variability was demonstrated between European and North American viruses (Wensvoort et al., 1992a). Since then, both genetic and antigenic differences have been repeatedly demonstrated between and among European and North American PRRS viruses by comparing nucleotide sequences (Allende et al., 1999; Andreyev et al., 1997, Drew et al., 1997; Kapur et al., 1996; Le Gall et al., 1998; Mardassi et al., 1995; Meng et al. 1995; Murtaugh et al., 1995; Suárez et al., 1996; Wesley et al., 1998) and sero-reactivity with monoclonal antibodies (Dea et al., 1996; Drew et al., 1995; Le Gall et al., 1997; Magar et al. 1997; Nelson et al., 1993; Pirzadeh and Dea, 1997; Rodriguez et al., 1997; Van Nieuwstadt et al., 1996; Wieczorek-Krohmer et al., 1996; Yoon et al., 1995; Zhang et al., 1998). The extent and dynamics of this variability in genetic and antigenic characteristics of PRRS viruses are of particular importance with respect to development of a universal
diagnostic test, effective vaccines and control procedures. For example, there is evidence that failure to prevent and control PRRS using management protocols with or without vaccine may be due to antigenic/genetic variability between field viruses (Mengeling et al., 1998). Accordingly, the primary objective of this study was to develop a virus marker system for epidemiological studies based on the presence or absence of epitopes. This marker system could be used to determine if a correlation exists between specific viruses and success or failure of specific management strategies, and monitor movement of PRRS virus within and between geographical regions. Consequently, a panel of MAbs specific for North American PRRS viruses was developed and the in vivo stability of the epitopes represented by these MAbs was evaluated. A secondary objective of this study was to identify the regions within open reading frames that encode specific epitopes.

Dissertation Organization

This dissertation is organized into 5 chapters. The first chapter contains the general introduction and the literature review. Three papers that represent the experimental studies are presented in the second, third and fourth chapters. The doctoral candidate is the principal investigator and senior author for all of the three papers. The first paper, "Antigenic and genetic variations of the 15 kD nucleocapsid protein of porcine reproductive and respiratory syndrome virus isolates," was published in the Archives of Virology, 1999, 144: 525-546. The second paper, "Categorization of North American porcine reproductive and respiratory syndrome viruses: Epitopic profiles of the N, M, GP5 and GP3 proteins and susceptibility to neutralization," is in press in the Archives of Virology. The third paper, "Stability of epitope profiles of North American porcine reproductive and respiratory syndrome viruses during
serial passage in pigs,” will be submitted to the Journal of General Virology. The last chapter contains the general conclusions of the research studies.

Literature Review

History of PRRS

The porcine reproductive and respiratory syndrome (PRRS) was first reported as devastating outbreaks of reproductive failure in North Carolina, Minnesota, and Iowa in the United States during 1987 and 1988 (Hill, 1990; Keffaber, 1989). In the years following, PRRS spread throughout the United States and Canada (Hill, 1990). In 1990, the disease was first recognized in Germany and soon afterwards in several other Western European countries (Wensvoort, 1993). Presently, PRRS is endemic in most swine-producing countries throughout the world.

The PRRS virus was first isolated in Europe by Wensvoort et al. (1991) using porcine alveolar macrophages (PAMs) and designated the Lelystad virus. Subsequently, Benfield et al. (1992) and Collins et al. (1992) isolated a PRRS virus from North American swine using the proprietary continuous cell line CL2621 and designated it ATCC VR-2332.

Before the PRRS virus was isolated and confirmed as the etiological agent of the syndrome, a number of different microbial organisms were implicated as the cause of PRRS. These agents included porcine parvovirus, pseudorabies virus, leptospira, porcine enteroviruses, encephalomyocarditis virus, hog cholera virus, mycoplasma, swine influenza virus, paramyxovirus, Chlamydia psittaci, and Streptococcus suis, all of which were isolated from suspected PRRS cases (Bilodeau et al., 1991; Christianson, 1992; Dea et al., 1992; Loula, 1991; Meredith, 1992; Paton et al., 1991; Wensvoort et al., 1991).
Initially, many names were used to describe the syndrome. These names included: mystery swine disease (MSD) (Wensvoort et al., 1991), blue eared pig disease (White, 1991), porcine epidemic abortion and respiratory syndrome (PEARS) (Pol et al., 1991; Terpstra et al., 1991), or swine infertility and respiratory syndrome (SIRS) (Benfield et al., 1992; Collins et al., 1992; Morrison et al, 1992; Yoon et al., 1992a; 1992b).

With the discovery of the PRRS virus, the official name of the syndrome was designated as the Porcine Reproductive and Respiratory Syndrome (PRRS). This nomenclature was officially presented at the First International Symposium on SIRS/PRRS held in St. Paul, Minnesota in 1992 (Meredith, 1993).

**Clinical manifestations**

The clinical signs of pigs infected with PRRS virus are highly variable and depend on a number of factors including age of the pigs, health status, management practices, immune status, and reproductive status. The onset of the disease in sows is often marked by depression, lethargy, anorexia and pyrexia. Periocular edema and conjunctivitis are seen in some pigs. Some sows show respiratory symptoms whereas others have slightly elevated body temperatures; however, reproductive failure occurs in all parities. Pregnant sows abort at late-term of gestation (days 107-112) or deliver high numbers of stillborn, mummified or weak piglets that die shortly after birth or grow poorly. The herd abortion rate ranges from 1 to 3% (Hopper et al., 1992; White 1992). The mortality rate of acutely ill sows ranges from 1 to 4% (Loula, 1991). Clinical signs of infected boars include anorexia, fever and inappetence. The semen quality of infected boars is also low (Benfield et al., 1999).
Respiratory disease is the major clinical manifestation of PRRS in weaned piglets in the nursery. Affected piglets show a short period of anorexia and mild fever. Piglets born to infected sows often develop severe respiratory disease within 4-8 weeks (Meredith, 1995). Secondary infections with swine influenza virus, paramyxovirus, *Chlamydia psittaci*, *Mycoplasma hyopneumoniae*, *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, *Hemophilus parasuis* and *Pasteurella multocida* are common.

A wide range of variation in the severity of PRRS infection is commonly observed in both breeding animals and in young pigs. While variations in severity may be influenced by management factors and coinfection with other swine pathogens, such as mycoplasmas (Thacker et al., 1999), variation in severity may also be due to strain differences among PRRS viruses as illustrated by the emergence of highly severe clinical manifestations of PRRS in 1996 (Epperson and Holler, 1997; Halbur and Bush, 1997; Hurd et al., 1998; Joo et al., 1998a; Mengeling et al., 1997; Rossow, 1997; Zimmerman et al., 1997). The apparent increase in severity of PRRS infection in these herds led to the use of the term “atypical” or “acute” PRRS to describe these severe clinical manifestations. These cases are defined by acute onset, persistence of clinical signs over a 2-4 week period, herd case mortality rates as high as 5% in sows and boars, and herd abortion rates as high as 10% in all parities and in all stages of gestation (Zimmerman et al., 1997).

Differences in virulence between PRRS viruses isolated from herds with both traditional and atypical manifestations have been demonstrated in controlled experiments. Mengeling et al. (1998) inoculated 2 pregnant gilts oronasally at 90 days of gestation with each of 4 different PRRS viruses. Each gilt received 10 ml of cell culture medium containing $10^5$ TCID$_{50}$/ml of the selected PRRS virus. These viruses were isolated from 4 separate
herds. Abortion occurred in 10 out of 200 sows in herd 1, in 200 out of 2000 sows in herd 2, and in 80, 130, 500 and 650 out of 2000 sows in 4 gestation buildings in herd 3. In herd 4 with 1100 sows, 700 sows aborted and 85-100 pregnant sows died. Although all of the 4 viruses caused reproductive failure in pregnant gilts, the virus that was isolated from herd 4 caused complete loss of fetuses and severe clinical disease. Each of the 4 viruses was also inoculated into 2 pregnant gilts at 45 days of gestation as mentioned above. Four mummified fetuses were delivered by the 2 gilts that were exposed to the virus from herd 4. No reproductive problems were observed in gilts that were exposed to the other 3 viruses.

The severity in virulence of atypical PRRS virus was also demonstrated by other investigators. Lager et al. (1998) inoculated 8 pregnant gilts oronasally at 85-89 days of gestation with "liver homogenate" derived from a sow with "atypical PRRS". All of the 8 gilts developed anorexia, pyrexia and depression. Two gilts aborted at 107 and 109 days of gestation whereas five gilts farrowed between 112 and 125 days of gestation. One gilt died at 16 days post-inoculation. A total 8 live piglets were delivered.

Halbur et al. (1998) inoculated seven 3-week-old cesarean-derived, colostrum-derived (CDCD) piglets oronasally with "liver homogenate" derived from a sow with "atypical PRRS". The piglets developed severe respiratory disease. Three out of 7 piglets developed icterus. The liver homogenate from these affected piglets was inoculated into an additional thirty-six 3-week-old CDCD piglets. The animals exhibited respiratory disease, persistent fever and lethargy. Severe lung and liver lesions and lymphadenopathy were observed at necropsy. These studies led to the conclusion by Mengeling et al. (1998) that highly virulent PRRS viruses circulate among U.S. herds.
Porcine reproductive and respiratory syndrome virus

The porcine reproductive and respiratory syndrome virus is a member of the family Arteriviridae of the order Nidovirales (Cavanagh, 1997; Pringle, 1996). Lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV) and simian haemorrhagic fever virus (SHFV) are also members of this family (Cavanagh, 1997; Pringle, 1996).

The porcine reproductive and respiratory syndrome virus is an enveloped virus. The virus is 45-83 nm in diameter and contains a cubical nucleocapsid core of 25-35 nm in diameter (Benfield et al., 1992; Bøtner et al., 1994; Kuwahara et al., 1994; Mardassi et al., 1994a; Onlinger et al., 1991; Pol and Wagenaar, 1992; Wensvoort, 1993; Wensvoort et al., 1991). The buoyant density of the virus is 1.13-1.19 g / ml in cesium chloride and 1.18-1.23 g / ml in sucrose (Bautista et al., 1996; Benfield et al., 1992; Christianson and Joo, 1994; Mardassi et al., 1994a; Meng et al., 1994; Wensvoort et al., 1991; 1992b).

The virus loses its infectivity when treated for 15-20 minutes at 56 °C, 10-24 hours at 37 °C, 6 days at 20 °C and over 1 month at 4 °C. Infectivity can be maintained for at least 18 months at -70 °C (Benfield et al., 1992; Yoon et al., 1992a). The virus infectivity titers are reduced over 90% at a pH less than 5 or greater than 7 (Benfield et al., 1992). In culture media, at pH 7.5, the half-life of the Lelysated virus was estimated to be 0.1, 3, 20 and 140 hours at 56, 37, 21, and 4 °C respectively (Bloemraad et al., 1994).

The virus does not hemagglutinate swine, sheep, goat, cattle, mouse, rat, rabbit, guinea pig, human type O, duck or chicken erythrocytes (Benfield et al., 1992; Meng et al., 1996; Wensvoort et al., 1991; 1992b). However, Jusa et al. (1996) observed that cell-free and cell-associated PRRS viruses hemagglutinated mouse erythrocytes. The
hemagglutination inhibition titers of pig sera showed a positive correlation with neutralization titers. Pretreatment of the virus with Tween 80 and ether greatly increased hemagglutination. Moreover, Jusa et al. (1997a) found that the hemagglutinin activity peaked at 1.17 g/cm$^3$ in cesium chloride gradient centrifugation. The hemagglutinin peak fraction was demonstrated in the SDS-PAGE to be composed of the N protein.

The porcine reproductive and respiratory syndrome virus can grow in PAMs (Wensvoort et al., 1991) and the African green monkey kidney cell line MARC-104 and a subpopulation of this cell line that has been designated MARC-145 cells (Kim et al., 1993). The proprietary cell lines CL2621 (Benfield et al., 1992) and CRL11171 (Meng et al., 1996) also support the virus growth. Voicu et al. (1994) reported that peripheral blood monocytes can be infected with PRRS virus. In addition, Molitor et al. (1997) and Thanawongnuwech et al. (1997) reported that spleen macrophages, brain microglial cells and porcine intravascular macrophages (PIMs) also supported replication of the virus in vitro. Sur et al. (1997) also reported that the virus replicated in testicular germ cells such as spermatids and spermatocytes in infected boars. Some viruses replicated in PAMs only or in CL2621 only but not in both cell types (Bautista et al., 1993a). A variety of other cell lines of animal origin such as PK-2, PK-15 and SK-6, were tested but none of them was permissive for virus replication (Benfield et al., 1992; Meng et al., 1996; Wensvoort et al., 1991).

The cell tropism may be determined by the presence or absence of receptors on the cell surface. Meulenberg et al. (1998) found that BHK-21 cells could support gene expression of PRRS virus when infectious cDNA was introduced by transfection. Either treatment of virus with heparin or of MARC-145 cells with heparinase blocked virus infection, suggesting that a heparin-like molecule is associated with the virus attachment
process (Jusa et al., 1997b). Duan et al. (1998) reported that a 210 kD membrane protein on
the surface of PAMs might serve a putative receptor for PRRS virus because MAbs specific
for this protein blocked virus attachment to PAMs. This protein is apparently not present on
MARC-145 cell membranes because the same panel of MAbs did not interfere with virus
attachment to MARC-145 cells. This observation suggests that one or more different
receptors are used for virus attachment to MARC-145 cells. Porcine reproductive and
respiratory syndrome virus enters permissive cells through receptor mediated endocytosis
and the completion of the penetration process is low pH dependent (Kreutz and Ackermann,
1996; Nauwynck et al., 1999).

The presence and extent of the cytopathic effect (CPE) depend on cell types and
viruses. The CPE in PAMs is observed 1-4 days after infection. Infected cells round up and
clump together (Wensvoort et al., 1991). The virus titer in PAMs ranges between $10^5$-$10^6.5$
TCID$_{50}$/ml (Bloemraad et al., 1994; Christianson and Joo, 1994; Meng et al., 1996;
Wensvoort et al., 1992b; Wensvoort, 1993). The CPE appears 2-6 days after infection in
CL2621 and MARC-145 cells, and is characterized by rounding and clumping of cells
followed by pyknosis and detachment (Benfield et al., 1992; Kim et al., 1993). The virus
titers in CL2621 and MARC-145 cells can reach to $10^7$ TCID$_{50}$/ml (Benfield et al., 1992) and
$10^8$ TCID$_{50}$/ml (Kim et al., 1993) in standard cell culture containers, respectively. In
contrast, some viruses are not highly cytopathic and do not form extensive CPE even if
incubation time is extended. A few viruses do not form visible CPE in PAMs or MARC-145
cells. The presence of these non-cytopathic viruses in cell cultures can be demonstrated by
the IFA test (Frey, 1999).
The genome of the arteriviruses is a single strand of positive sense RNA that varies from 12.7 to 15.7 kb in size (Snijder and Meulenberg, 1998). The genomic organization of the arteriviruses was first described for EAV by Den Boon et al. (1991) who identified the presence of a nested set of 8 open reading frames (ORFs). More recently, a region between ORF 1b and ORF 2 of the EAV genome that encodes a 67 amino acid structural protein has been identified (Snijder et al., 1999). This region of the EAV genome has been designated ORF 2a and the former ORF 2 has been renamed ORF 2b. The viruses in the *Arteriviridae* are similar with respect to genome organization, replication strategy, expression of proteins, establishment of persistent infection and preference for infection of macrophages both *in vitro* and *in vivo* (Snijder and Meulenberg, 1998).

The genome of the European Lelystad virus is a polyadenylated RNA molecule of about 15 kb and contains 8 open reading frames (ORFs). These ORFs are designated 1a, 1b, and 2 through 7 (Meulenberg et al., 1993a). An additional ORF equivalent to ORF 2a of EAV may also exist in the PRRS virus genome (Snijder et al., 1999). The complete nucleotide sequence of the European Lelystad virus was determined in 1993 (Meulenberg et al., 1993a). The ORFs 1a and 1b account for 75% of the whole genome and encode a viral replication-related enzyme. The other 6 ORFs account for 25% of the genome. The ORFs 2 to 5 encode 4 envelope-associated proteins with molecular masses of 30, 45, 31 and 25 kD respectively. These 4 envelope proteins are glycosylated and are designated GP2 through GP5 (Van Nieuwstadt et al., 1996). The GP2 and GP5 proteins are counterparts of the Gs and Gl proteins of EAV (De Vries et al., 1992; Snijder and Meulenberg, 1998). The ORF 6 encodes an unglycosylated, matrix (M) protein with a molecular mass of 19 kD. The ORF 7 encodes the nucleocapsid (N) protein with a molecular mass of 15 kD. The GP4 and GP5
proteins induce neutralizing antibodies (Gonin et al., 1999; Meulenberg et al., 1997; Persch et al., 1995; Pirzadeh and Dea, 1997; Van Nieuwstadt et al., 1996; Weiland et al., 1999; Zhang et al., 1998).

The ORFs 2 to 7 are overlapping and are expressed from 6 subgenomic RNAs (Conzelmann et al., 1993; Meulenberg et al., 1993a). These subgenomic RNAs form a 3' co-terminal nested set and are composed of a common leader sequence, which is derived from the 5' end of the viral genome (Conzelmann et al., 1993; Meulenberg et al., 1993b). The open reading frames (ORFs) 2 through 7 and encoded proteins of the Lelystad virus are depicted in Table 1.

Table 1. The open reading frames (ORFs) 2 through 7 and encoded proteins of the Lelystad virus

<table>
<thead>
<tr>
<th>ORF</th>
<th>Size (kb)</th>
<th>No. of amino acids</th>
<th>Calculated MW&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N-glycosylation sites&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Signal sequence&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Putative transmembrane region</th>
<th>Molecular weight&lt;sup&gt;d&lt;/sup&gt;</th>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
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<td>249</td>
<td>28.4</td>
<td>2</td>
<td>37-38</td>
<td>210-228</td>
<td>30</td>
<td>nd</td>
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<td>3</td>
<td>3.1</td>
<td>265</td>
<td>30.6</td>
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<td>183</td>
<td>20.0</td>
<td>4</td>
<td>nd</td>
<td>1-17/165-183</td>
<td>31</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.0</td>
<td>201</td>
<td>22.4</td>
<td>2</td>
<td>32-33</td>
<td>108-131</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>173</td>
<td>18.9</td>
<td>2</td>
<td>nd</td>
<td>17-88</td>
<td>18</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.9</td>
<td>128</td>
<td>13.8</td>
<td>1</td>
<td>nd</td>
<td>nd</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

(Modified from Conzelmann et al., 1993 and Meulenberg et al., 1995.)

<sup>a</sup> Calculated molecular weight (kD) on the basis of the amino acid sequence.

<sup>b</sup> Number of putative N-glycosylation sites.

<sup>c</sup> Amino acid position of the putative signal sequence cleavage site.

<sup>d</sup> MW (kD) on SDS-PAGE of the gene products of the ORFs expressed in <em>in vitro</em> and <em>in vivo</em>.

<sup>nd</sup> not determined.

**Genetic variability**

Analyses of the nucleotide sequences of PRRS viruses indicate that European and North American PRRS viruses represent 2 distinct genotypes that emerged from a common
precursor (Allende et al., 1999; Kapur et al., 1996; Kwang et al., 1994; Le Gall et al., 1998; Mardassi et al., 1994b, 1994c, 1995; Meng et al., 1994, 1995; Murtaugh et al., 1995). The differences in the number and percentage identity of nucleotides and deduced amino acids between representative PRRS viruses of European and North American origins are summarized in Tables 2 and 3.

The European PRRS viruses are genetically variable. The identity of deduced amino acid sequences between the Lelystad virus and the European virus 10 varied from 99 to 100% among ORFs 2 through 7 (Conzelmann et al., 1993; Meulenberg, 1998b). Suárez et al. (1996) analyzed the nucleotide sequences of the complete ORF 5 of 15 European PRRS viruses including the Lelystad virus. The nucleotide identity of ORF 5 of these viruses ranged from 87.1 to 99.2%. Analysis of the partial nucleotide sequences of ORF 7 of these viruses revealed a higher identity, i.e., 94.1-99.6%. Drew et al. (1997) compared the nucleotide sequences and deduced amino acids of ORFs 3, 4 and 7 of 8 European PRRS viruses including the Lelystad virus. The average percent identity of nucleotide sequences of ORFs 3, 4 and 7 ranged from 96.4 to 99.7% and the percent identity of deduced amino acid sequences ranged from 92.5 to 99.2%. Le Gall et al. (1998) compared the nucleotide sequences of ORF 7 of 15 European PRRS viruses. The identity of deduced amino acid sequences varied from 97.7 to 100%. These results indicate that genetic variation exists among European PRRS viruses. However, this variation appears to be relatively low.
Table 2. The numbers of nucleotides and deduced amino acids of the European and North American porcine reproductive and respiratory syndrome viruses

<table>
<thead>
<tr>
<th>Open reading frame / protein</th>
<th>European viruses nucleotide / amino acid</th>
<th>North American viruses nucleotide / amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 / GP2</td>
<td>750 / 249</td>
<td>771 / 256</td>
</tr>
<tr>
<td>3 / GP3</td>
<td>798 / 265</td>
<td>765 / 254</td>
</tr>
<tr>
<td>4 / GP4</td>
<td>552 / 183</td>
<td>537 / 174</td>
</tr>
<tr>
<td>5 / GP5</td>
<td>606 / 201</td>
<td>603 / 200</td>
</tr>
<tr>
<td>6 / M</td>
<td>522 / 173</td>
<td>525 / 174</td>
</tr>
<tr>
<td>7 / N</td>
<td>387 / 128</td>
<td>372 / 123</td>
</tr>
</tbody>
</table>

Table 3. The percent identity of the deduced amino acid sequences of proteins encoded by open reading frames (ORFs) 2 through 7 of the Lelystad virus with the European PRRS virus 10 and 4 North American PRRS viruses

<table>
<thead>
<tr>
<th>Open reading frame / protein</th>
<th>PRRS virus 10\textsuperscript{a}</th>
<th>ATCC VR-2332\textsuperscript{b}</th>
<th>ATCC VR-2385\textsuperscript{c}</th>
<th>PRRS virus 16244B\textsuperscript{d}</th>
<th>PRRS virus IAF-exp91\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 / GP2</td>
<td>99</td>
<td>63</td>
<td>nd</td>
<td>62</td>
<td>nd</td>
</tr>
<tr>
<td>3 / GP3</td>
<td>99</td>
<td>58</td>
<td>nd</td>
<td>60</td>
<td>54</td>
</tr>
<tr>
<td>4 / GP4</td>
<td>99</td>
<td>68</td>
<td>nd</td>
<td>66</td>
<td>68</td>
</tr>
<tr>
<td>5 / GP5</td>
<td>99</td>
<td>59</td>
<td>54</td>
<td>58</td>
<td>52</td>
</tr>
<tr>
<td>6 / M</td>
<td>100</td>
<td>78</td>
<td>78</td>
<td>79</td>
<td>81</td>
</tr>
<tr>
<td>7 / N</td>
<td>100</td>
<td>65</td>
<td>58</td>
<td>60</td>
<td>59</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Conzelmann et al. (1993) and Meulenberg (1998b)
\textsuperscript{b} Murtaugh et al. (1995)
\textsuperscript{c} Meng et al. (1994)
\textsuperscript{d} Allende et al. (1999)
\textsuperscript{e} Mardassi et al. (1995)
nd, not determined
Genetic variability also exists among North American PRRS viruses. Meng et al. (1995) compared the nucleotide sequences of ORFs 6 and 7 of 8 North American PRRS viruses including ATCC-VR-2332 and ATCC VR-2385. Comparisons of both ORF 6 and 7 of these PRRS viruses revealed 96-100% amino acid identity. Kapur et al. (1996) analyzed genetic variability among 10 midwestern U. S. PRRS viruses. The average nucleotide identity of these viruses ranged varied from 92.1 to 97.5%. Andreyev et al. (1997) analyzed the nucleotide sequences and deduced amino acids of ORF 5 of 24 North American PRRS viruses including ATCC-VR-2332 and ATCC VR-2385. These viruses had 91-95% nucleotide and 89-94% deduced amino acid sequence identity respectively. Le Gall et al. (1998) demonstrated a 93.5 to 100% identity of the deduced amino acid sequences encoded by ORF 7 of 13 North American PRRS viruses including ATCC VR-2332 and IAF-exp91.

Unique genetic differences have also been demonstrated between North American and European PRRS viruses. The identity of deduced amino acid sequences between the Lelystad virus and 4 North American viruses (ATCC VR-2332, ATCC VR-2385, IAF-exp91, and 16244B) was low, i.e., 62-63% in ORF2, 54-60% in ORF3, 66-69% in ORF4, 52-59% in ORF5, 78-81% in ORF6, and 59-65% in ORF7 (Allende et al., 1999; Mardassi et al., 1995; Meng et al., 1994; Murtaugh et al., 1995).

Genetic variation among North American PRRS viruses and European PRRS viruses is characterized by random nucleotide substitutions. Genetic variation between North American and European viruses is characterized by nucleotide additions or deletions as illustrated in Table 2. According to Nelson et al. (1999) who compared the entire genomes of PRRS viruses ATCC VR-2332 and the Lelystad virus, North American and European viruses evolved independently from a common precursor. Nelson et al. (199) also speculated
that the simultaneous emergence of a similar disease on the two continents was due primarily
to changes in animal husbandry and management.

The genetic heterogeneity of PRRS viruses can be expected to increase with
international movement of swine and the use of modified-live vaccines. Such heterogeneity
can be the result of quasispecies (Holland et al., 1992) emerging in response to
immunological pressure of vaccine usage, or a result of natural mutations (Palese, 1993) and
recombination. For example, Le Gall et al. (1997) observed the loss and gain of a specific
epitope of GP3 in 2 different PRRS viruses during maintenance in pigs (see page 28 of this
review). Similarly, Rowland et al. (1999) demonstrated a unique nucleotide substitution in
the first 215 nucleotides of ORF 5 of ATCC VR-2332 following virus passage in 3 pigs that
were infected in utero. This nucleotide substitution resulted in a deduced amino acid change
from aspartic acid to asparagine. No changes were observed in the last 312 nucleotides of
ORF 4 and a 10 nucleotide untranslated region. These observations by Le Gall et al. (1997)
and Rowland et al. (1999) suggest that some areas of the PRRS virus genome are more
susceptible to mutation than others.

Recombination between PRRS viruses has been demonstrated in vitro. Yuan et al.
(1999) reported a recombination between the ORFs 3 and 4 of the modified-live vaccine
viruses RespPRRS and Prime Pac PRRS in co-infected MA-104 cells. Similar
recombination events involving other ORFs can be expected to occur in the field. For
example, Kapur et al. (1996) analyzed 611 polymorphic nucleotide sites in ORFs 2 through 7
of 10 midwestern U.S. PRRS viruses and suggested that intragenic recombination might
occur in all of these ORFs except ORF 6.
Antigenic variability

The first evidence of antigenic variation among and between North American and European PRRS viruses was provided by Wensvoort et al. (1992a). These investigators compared the titers of 11 swine sera representing 5 different European countries and 13 sera representing the United States and Canada. Titers were determined by the immunoperoxidase monolayer assay (IPMA) using 3 North American viruses and 4 European viruses as antigen. A similar observation was made when 214 swine sera from Canada were tested for the presence of PRRS virus antibody in the indirect fluorescence assay (IFA) using ATCC VR-2332 and Lelystad virus infected MA-104 cells as antigen (Frey et al., 1992). Bautista et al. (1993b) also conducted a study that revealed differences in sero-reactivity of the Lelystad virus and ATCC VR-2332 with swine sera. A subsequent study by Bautista et al. (1994) which utilized swine sera raised against ATCC VR-2332, MN-18 and the Lelystad virus further revealed the existence of antigenic variation among 20 PRRS viruses which included ATCC VR-2332 and the Lelystad virus. Varying susceptibility of field PRRS viruses to neutralization mediated by swine sera was also demonstrated by Yoon et al. (1994) and Yoon et al. (1997). Antigenic variation between and among North American and European PRRS viruses has also been demonstrated by using monoclonal antibodies (MAbs) specific for structural proteins as described below in this review.

N protein

The N protein is associated with the nucleocapsid surrounding the virus genome. The N protein is a basic protein as it does not contain hydrophobic amino acid sequences
(Meulenberg et al., 1995). As has been revealed by the appearance and multitude of antibody specific for this protein (Nelson et al., 1994; Yoon et al., 1995a), the N protein is strongly immunogenic. Hence, the majority of MAbs produced in mice by conventional hybridoma methodology are specific for this protein. Therefore, characteristics and antigenic variation of the N protein have been extensively studied. The N protein does not induce neutralizing activity.

Nelson et al. (1993) generated 3 MAbs (SDOW17, EP147 and VO17) by immunizing mice with North American PRRS viruses ATCC VR-2332 and SD1. They tested the sero-reactivity of these 3 MAbs with 63 North American PRRS viruses and 57 European viruses including ATCC VR-2332 and the Lelystad virus. Monoclonal antibody SDOW17 reacted with all North American and European viruses and MAbs EP147 and VO17 reacted with all North American viruses but not with any of the European viruses. Magar et al. (1995) observed that these 3 MAbs reacted with all of 20 North American PRRS viruses including ATCC VR-2332 but again MAbs EP147 and VO17 did not react with the Lelystad virus. Yoon et al. (1995a) reported that MAbs SDOW17 and EP147 reacted with 22 North American viruses but MAb EP147 did not react with 3 of these viruses.

Drew et al. (1995) generated 5 MAbs (WBE1, 3 through 6) by immunizing mice with the European virus H2 and tested sero-reactivity of these MAb with 25 European and 8 North American PRRS viruses. Monoclonal antibodies WBE1, 4, 5 and 6 reacted with the Lelystad virus and all of the other 24 European viruses. Monoclonal antibody WBE3 reacted with 4 of the European viruses but not with the Lelystad virus. None of these 5 MAbs reacted with any of the 8 North American viruses tested. Subsequently, Le Gall et al. (1997) observed that MAbs WBE1 and 4 through 6 reacted with 13 European viruses but only MAb WBE1
reacted with 3 North American viruses, indicating their difference in recognition of specific epitopes.

Van Nieuwstadt et al. (1996) prepared MAbs 126.9 and 122.17 by immunizing mice with the Lelystad virus. Both MAbs reacted with the Lelystad virus and ATCC VR-2332. Monoclonal antibody 126.9 also reacted with all of 15 European viruses tested but not with 3 North American viruses (Le Gall et al., 1997). Monoclonal antibody 122.17 was not tested against these viruses.

Monoclonal antibody P3/27 raised against the Dutch virus I10 or I2 (not specified in literature) reacted with all of 27 European viruses including the Lelystad virus and 15 North American viruses (Le Gall et al., 1997; Wieczorek-Krohmer et al., 1996). Monoclonal antibody IAFK8 raised against the North American virus IAF-Klop reacted with 2 European viruses including the Lelystad virus and 15 North American viruses including ATCC VR-2332 (Dea et al., 1996).

Rodriguez et al. (1997) reported that 7 MAbs (1CH5, 1DH10, 1BD11, 1EB9, 1DA4, 1AG11 and 1AC7) raised against the European virus Olot/91 reacted with the homologous virus and only MAbs 1DA4, 1AG11 and 1AC7 reacted with the North American virus Québec 94. Le Gall et al. (1997) further reported that MAb 1CH5 reacted with an additional 15 European viruses but not with 3 North American viruses.

Among the MAbs described above, MAbs SDOW17, EP147, VO17, 1CH5 and 1DH10 did not react with denatured antigen in Western immunoblotting, suggesting these MAbs may be specific for discontinuous epitopes (Nelson et al., 1993; Rodriguez et al., 1997). All other MAbs reacted with denatured antigen in Western immunoblotting, suggesting these MAbs may be specific for continuous epitopes.
In summary, at least 3 distinct continuous epitopes are associated with the N protein and may be represented by MAbs WBE1, 3, 4, 5 or 6. The epitopes represented by MAbs WBE3 and WBE 4, 5 or 6 are present in European viruses. The epitopes represented by MAbs WBE1 and P3/27 are present in both European and North American viruses. In addition, at least 4 discontinuous epitopes are represented by MAbs SDOW17, VO17, EP147 and possibly 1CH5. Monoclonal antibody SDOW17 reacts with both North American and European viruses including the Lelystad virus, MAbs VO17 and EP147 react with North American viruses only, and MAb 1CH5 apparently reacts only with European viruses.

Rodriguez et al. (1997) used Western immunoblotting to characterized the N protein into 3 antigenic sites (I, II and III). They cloned and expressed 6 overlapping segments of ORF 7 of the European virus Olot/91, designated A through F, in a baculovirus system. They subsequently tested the expressed products for reactivity with a panel of MAbs. Antigenic site I represented a discontinuous epitope that was recognized by MAbs 1CH5 and 1DH10. Antigenic site II represented a continuous epitope encoded by nucleotide segments A and B because the proteins expressed from these fragments was recognized by MAbs 1BD11 and 1EB9. Antigenic site III also represented a continuous epitope encoded by nucleotide segments E and F because the proteins expressed from these fragments were recognized by MAbs 1DA4, 1AG11 and 1AC7.

Nelson et al. (1997) reported that the substitution of the amino acid aspartic acid at position 61 by tyrosine caused failure of MAb SDOW17 to react with the modified-live vaccine PRIME PAC PRRS, suggesting the presence of an epitope around this area of the N protein. In addition, Wootton et al. (1998) generated a series of deletion mutants of the N protein and tested them for sero-reactivity with a panel of MAbs. Their results suggested that
the C-terminal of the N protein played an important role in the formation of discontinuous epitopes.

The monoclonal antibodies described here and their characteristics are summarized in Table 4. The sero-reactivity of these MAbs with the Lelystad virus and ATCC VR-2332 is indicated.

Table 4. Monoclonal antibodies specific for the N protein of PRRS viruses

<table>
<thead>
<tr>
<th>Reference</th>
<th>MAb</th>
<th>Epitope</th>
<th>LV</th>
<th>VR-2332</th>
<th>Other EUs</th>
<th>Other NAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nelson et al., 1993</td>
<td>SDOW17</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td>56/56</td>
<td>62/62</td>
</tr>
<tr>
<td></td>
<td>EP147, VO17</td>
<td>D</td>
<td>-</td>
<td>+</td>
<td>0/56</td>
<td>62/62</td>
</tr>
<tr>
<td>Magar et al., 1995</td>
<td>SDOW17</td>
<td>D</td>
<td>-</td>
<td>+</td>
<td>19/19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP147, VO17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yoon et al., 1995a</td>
<td>SDOW17, VO17</td>
<td>D</td>
<td>nt</td>
<td>nt</td>
<td>22/22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP147</td>
<td>D</td>
<td></td>
<td></td>
<td>19/22</td>
<td></td>
</tr>
<tr>
<td>Drew et al., 1995</td>
<td>WBE1, 4, 5, 6</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>24/24</td>
<td>0/8</td>
</tr>
<tr>
<td></td>
<td>WBE3</td>
<td>C</td>
<td>-</td>
<td>nt</td>
<td>4/24</td>
<td>0/8</td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>WBE1</td>
<td>C</td>
<td></td>
<td></td>
<td>13/13</td>
<td>3/3</td>
</tr>
<tr>
<td>Van Nieuwstadt et al., 1996</td>
<td>126.9, 122.17</td>
<td>C</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>126.9</td>
<td>C</td>
<td></td>
<td></td>
<td>15/15</td>
<td>0/3</td>
</tr>
<tr>
<td>Wieczorek-Krohmer et al., 1996</td>
<td>P3/27</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>16/16</td>
<td>12/12</td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>P3/27</td>
<td>C</td>
<td></td>
<td></td>
<td>10/10</td>
<td>3/3</td>
</tr>
<tr>
<td>Dea et al., 1996</td>
<td>IAFK8</td>
<td>C</td>
<td>+</td>
<td>+</td>
<td>1/1</td>
<td>15/15</td>
</tr>
<tr>
<td>Rodriguez et al., 1997</td>
<td>1CH5, 1DH10</td>
<td>D</td>
<td>nt</td>
<td>nt</td>
<td>1/1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>1BD11, 1EB9</td>
<td>C</td>
<td>nt</td>
<td>nt</td>
<td>1/1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>1DA4, 1AG11,</td>
<td>C</td>
<td>nt</td>
<td>nt</td>
<td>1/1</td>
<td>1/1</td>
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<td>1AC7</td>
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<td></td>
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<td>Le Gall et al., 1997</td>
<td>1CH5</td>
<td>D</td>
<td></td>
<td></td>
<td>15/15</td>
<td>0/3</td>
</tr>
</tbody>
</table>

D, discontinuous; C, continuous; nt, not tested; EUs, European viruses; NAs, North American viruses
**M protein**

The M protein is a non-glycosylated, envelope-associated protein. Results from genetic analysis suggest that the M protein of PRRS viruses of the same continent origin are the most conserved. However, a vast antigenic difference has been demonstrated between North American and European viruses. A total of 5 MAbs specific for the M protein have been generated. These MAbs represent 4 continuous epitopes as determined by their sero-reactivity patterns with PRRS viruses. The monoclonal antibodies 122.9 and 126.3 raised against the Lelystad virus (van Nieuwstadt et al., 1996) react with the Lelystad virus but only MAb 122.9 reacts with the North American virus ATCC VR-2332. The monoclonal antibodies 2C12 raised against North American virus LHVA-93-3 (Magar et al., 1997) and MAbs IAFK3 and 6 raised against the North American virus IAF-Klop (Dea et al., 1996) represent two separate epitopes that appear to be present only in North American viruses. Monoclonal antibody 2C12 reacted with ATCC VR-2332 but not with the Lelystad virus. Monoclonal antibodies IAF3 and 6 did not react with either ATCC VR-2332 or the Lelystad virus. No discontinuous epitopes of the M protein have been described.

The monoclonal antibodies described here and their characteristics are summarized in Table 5. The sero-reactivity of these MAbs with the Lelystad virus and ATCC VR-2332 is indicated.

**GP2 protein**

The GP2 protein is a minor structural envelope associated glycoprotein of the Lelystad virus (Meulenberg and Petersen-Den Besten, 1996). It has not been demonstrated in any of the North American PRRS viruses to date. No MAb specific for this protein has
been produced to date. The function and antigenic variation of the GP2 protein of PRRS viruses have not been determined.

Table 5. Monoclonal antibodies specific for the M protein of PRRS viruses

<table>
<thead>
<tr>
<th>Reference</th>
<th>MAb</th>
<th>Epitope</th>
<th>LV</th>
<th>VR-2332</th>
<th>Other EUs</th>
<th>Other NAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Nieuwstadt et al., 1996</td>
<td>126.3</td>
<td>C</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>122.9</td>
<td>C</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>122.9</td>
<td>C</td>
<td></td>
<td>15/15</td>
<td></td>
<td>2/3</td>
</tr>
<tr>
<td>Magaret et al., 1997</td>
<td>2C12</td>
<td>C</td>
<td>-</td>
<td>+</td>
<td>0/13</td>
<td>121/121</td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>2C12</td>
<td>C</td>
<td></td>
<td>0/15</td>
<td></td>
<td>2/3</td>
</tr>
<tr>
<td>Dea et al., 1996</td>
<td>IAFK3</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>0/2</td>
<td>10/15</td>
</tr>
<tr>
<td></td>
<td>IAFK6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C, continuous; EUs, European viruses; NAs, North American viruses

GP3 protein

The GP3 protein is envelope associated. It has been demonstrated in European viruses H2, Lelystad, I10 and I2 (Drew et al., 1995; van Nieuwstadt et al., 1996; Wieczorek-Krohmer et al., 1996) and is described by these investigators to be a minor structural component of the virion. Its molecular weight ranges from 45 to 50 kD (Van Nieuwstadt et al., 1996), or from 40 to 50 kD (Wieczorek-Krohmer et al., 1996) as determined by SDS-PAGE. A homologous protein has been demonstrated in the North American virus IAF-Klop (Gonin et al., 1998; Mardassi et al., 1998). These investigators believe that GP3 is not a structural component of mature virions. Antisera and MAbs specific for this protein do not have neutralizing activity. All MAbs generated to date for GP3 represent continuous epitopes.
At least 6 continuous epitopes have been associated with GP3 as determined by sero-reactivity patterns of MAbs with North American and European PRRS viruses. Five of these epitopes are represented by MAbs P7/a1-19, P9/a3-20, P9/b3-1, P7/a2-55 and P7/b2-14 that were prepared by Wieczorek-Krohmer et al. (1996) using the Dutch virus 110 or 12. These 5 MAbs represent distinct sero-reactivity patterns with 17 European viruses including the Lelystad virus and 12 North American viruses. The virus ATCC VR-2332 was not included in their study. A sixth epitope was represented by MAb WBE2 that was raised against the European PRRS virus H2 (Drew et al., 1995). This MAb reacted with 16 of 25 European viruses and not with any of 8 North American viruses. Three epitopes represented by MAbs P7/a1-19, P9/a3-20 and P9/b3-1 were present in both European and North American viruses while 3 other epitopes represented by MAbs P7/a2-55, P7/b2-14 and WBE2 were only detected in European viruses. All 6 epitopes were represented in the Lelystad virus.

A possible fourth epitope of GP3 common to European and North American viruses was represented by MAb122.14 that was prepared by Van Nieuwstadt et al. (1996) using the Lelystad virus. This MAb reacted with the Lelystad virus but not with ATCC VR-2332. Further comparisons by Le Gall et al. (1997) using 15 European and 3 North American viruses showed that MAbs P9/a3-20, 122.14 and WBE2 were distinct with respect to their sero-reactivity patterns.

Antigenic variation between European and North American viruses is apparently mediated by differences located at the C-terminal portion of the GP3 protein (Katz et al., 1995). Swine antisera raised against European viruses reacted in the IPMA test with a 199 amino acids located at the hydrophilic C-terminal of GP3 of the Lelystad virus that was expressed as a recombinant fusion protein (BP03-P) in a baculovirus system, but swine
antisera raised against North American viruses did not. Rabbit antisera raised against BP03-P did not neutralize European or North American viruses.

The monoclonal antibodies described here and their characteristics are summarized in Table 6. The sero-reactivity of these MAbs with the Lelystad virus and ATCC VR-2332 is indicated.

Table 6. Monoclonal antibodies specific for the GP3 protein of PRRS viruses

<table>
<thead>
<tr>
<th>Reference</th>
<th>MAb</th>
<th>Epitope</th>
<th>LV</th>
<th>VR-2332</th>
<th>Other EUs</th>
<th>Other NAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wieczorek-Krohmer et al., 1996</td>
<td>P7/a1-19</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>16/16</td>
<td>12/12</td>
</tr>
<tr>
<td></td>
<td>P9/a3-20</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>12/16</td>
<td>12/12</td>
</tr>
<tr>
<td></td>
<td>P9/b3-1</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>13/16</td>
<td>12/12</td>
</tr>
<tr>
<td></td>
<td>P7/a2-55</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>13/16</td>
<td>0/12</td>
</tr>
<tr>
<td></td>
<td>P7/b2-14</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>12/16</td>
<td>0/12</td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>P9/a3-20</td>
<td>C</td>
<td></td>
<td></td>
<td>10/10</td>
<td>3/3</td>
</tr>
<tr>
<td>Drew et al., 1995</td>
<td>WBE2</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>16/25</td>
<td>0/8</td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>WBE2</td>
<td>C</td>
<td></td>
<td></td>
<td>8/13</td>
<td>0/3</td>
</tr>
<tr>
<td>Van Nieuwstadt et al., 1996</td>
<td>122.14</td>
<td>C</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>122.14</td>
<td>C</td>
<td></td>
<td></td>
<td>14/15</td>
<td>3/3</td>
</tr>
</tbody>
</table>

C, continuous; nt, not tested; EUs, European viruses; NAs, North American viruses

GP4 protein

The GP4 protein is a minor structural protein of PRRS viruses. Its molecular weight ranges from 31 to 35 kD (Van Nieuwstadt et al., 1996), or from 30 to 40 kD (Wieczorek-Krohmer et al., 1996) as determined by SDS-PAGE.

The GP4 protein is represented by at least 3 and as many as 5 continuous epitopes as determined by sero-reactivity patterns and neutralizing activity of MAbs with specific European viruses (Le Gall et al., 1997; Meulenberg et al., 1997; van Nieuwstadt et al., 1996;
Three epitopes were represented by MAbs P3/35, P7/b1-7 and P7/b1-2 that were raised against the Dutch virus I10 or I2 (Wieczorek-Krohmer et al., 1996; Weiland et al., 1999). Two of these epitopes are represented by neutralizing MAbs P3/35 and P7/b1-7. A third epitope is represented by the non-neutralizing MAb P7/b1-2.

An antigenic region spanning amino acids 40-79 of GP4 of the Lelystad virus was identified by using a panel of 15 MAbs including MAbs 122.68 and 121.4 (Meulenberg et al., 1997). These MAbs were raised against the Lelystad virus. Although both MAbs recognized this region only MAb 122.68 had neutralizing activity, indicating that the 2 MAbs represent different epitopes. In a comparative study by Le Gall et al. (1997), MAb 122.68 differed from the neutralizing MAb P3/35 with respect to sero-reactivity patterns with 1 North American and 2 European PRRS viruses. Comparative data is not available to determine if MAb 122.68 represents the same or a different epitope as MAb P7/b1-7.

Zhang et al. (1998) also described a group of 4 MAbs that were prepared by using the recombinant GP4 of the North American virus ATCC VR-2385 expressed in baculovirus system. These MAbs represented at least 1 discontinuous epitope present among 23 North American viruses as tested by using the ELISA.

The monoclonal antibodies described here and their characteristics are summarized in Table 7. The sero-reactivity of these MAbs with the Lelystad virus and ATCC VR-2332 is indicated.
### Table 7. Monoclonal antibodies specific for the GP4 protein of PRRS viruses

<table>
<thead>
<tr>
<th>Reference</th>
<th>MAb</th>
<th>Epitope</th>
<th>VN</th>
<th>LV</th>
<th>VR-2332</th>
<th>Other EUs</th>
<th>Other NAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>De Kluijver et al., 1995</td>
<td>13 MAbs (122.68 etc.)</td>
<td>nd</td>
<td>nt</td>
<td>+</td>
<td>-</td>
<td>4/19</td>
<td>0/4</td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>122.68</td>
<td>4/16</td>
<td>1/3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Van Nieuwstadt et al., 1996</td>
<td>122.1, 122.29, 126.1, 130.7</td>
<td>C</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1/2</td>
<td></td>
</tr>
<tr>
<td>Meulenberg et al., 1997</td>
<td>14 MAbs (122.1, 122.29, 122.68, 126.1, 130.7, etc.) 121.4</td>
<td>C</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wieczorek-Krohmer et al., 1996</td>
<td>P3/35 P7/a2-7 P7/b1-2 P7/b1-7</td>
<td>C</td>
<td>nt</td>
<td>+</td>
<td>nt</td>
<td>10/16</td>
<td>0/12</td>
</tr>
<tr>
<td>9 (P3/35, P7/a2-7 etc.)</td>
<td>C</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weiland et al., 1999</td>
<td>P7/b1-2, P7/b1-7</td>
<td>C</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Le Gall et al., 1997</td>
<td>P3/35</td>
<td>C</td>
<td>nt</td>
<td>nt</td>
<td>6/16</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Zhang et al., 1998</td>
<td>PP4bB3, PP4bC5, PP4cB11, PP4dG6</td>
<td>D</td>
<td>-</td>
<td>nt</td>
<td>nt</td>
<td>23/23</td>
<td></td>
</tr>
</tbody>
</table>

nd, not determined; nt, not tested; C, continuous; D, discontinuous; EUs, European viruses; NAs, North American viruses

### GP5 protein

The GP5 protein is a major envelope-associated glycoprotein and is a major target for neutralizing antibodies. Persch et al. (1995) demonstrated that GP5 induced neutralizing antibodies to PRRS virus by inoculating pigs with GP5 cut from PAGE gels. In addition, Pirzadeh and Dea (1998) reported that vaccination with a plasmid DNA containing ORF5 of PRRS virus IAF-Klop induced neutralizing antibodies in pigs. Neutralizing activity was not induced by GP5 expressed by *Escherichia coli* (*E. coli*). Evidence of neutralizing activity
induced by GP5 has been provided by Gonin et al. (1999). These investigators compared neutralizing antibody titers of convalescent sera from PRRS virus-infected pigs to ELISA antibody titers using \textit{E. coli}-recombinant N, M, GP3, GP4 and GP5 proteins as antigen. A highly significant correlation was observed between neutralizing titers and antibody specific for GP5 but not with antibodies specific for the N, M, GP3 or GP4.

At least 4 continuous and 2 discontinuous epitopes of GP5 have been identified by sero-reactivity patterns with specific MAbs. Pirzadeh and Dea (1997) prepared 5 MAbs by immunizing mice with an \textit{E. coli}-expressed ORF 5 gene product representing North American virus IAF-Klop. These MAbs represented at least 2 epitopes based on their ability to neutralize viruses IAF-Klop and ATCC VR-2332. Monoclonal antibodies IAF8A8 and 1B8 neutralized both viruses but MAbs 2A5, 3A12 and 1C10 only neutralized IAF-Klop. Neither group of MAbs sero-reacted with the Lelystad virus. A third continuous epitope that does not induce neutralizing antibody was detected by Zhang et al. (1998) in North American viruses. Zhang et al. (1998) also described 2 discontinuous epitopes present among North American viruses one of which induced neutralizing antibody.

A unique continuous epitope of the GP5 protein represented by MAb P10/b38 was only detected in a clone of the Dutch virus 110 but not in any of 12 American viruses or 5 European viruses including the Lelystad virus (Weiland et al., 1999). This epitope also induced neutralizing antibody.

The monoclonal antibodies described here and their characteristics are summarized in Table 7. The sero-reactivity of these MAbs with the Lelystad virus and ATCC VR-2332 is indicated.
Table 8. Monoclonal antibodies specific for the GP5 protein of PRRS viruses

<table>
<thead>
<tr>
<th>Reference</th>
<th>MAb</th>
<th>Epitope</th>
<th>VN</th>
<th>LV</th>
<th>VR-2332</th>
<th>Other EUs</th>
<th>Other NAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pirzadeh and Dea, 1997</td>
<td>IAF-8A8</td>
<td>C</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAF-1B8</td>
<td>C</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>1/1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAF-2A5</td>
<td>C</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAF-3A12</td>
<td>C</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAF-1C10</td>
<td>C</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zhang et al., 1998</td>
<td>PP5dH4</td>
<td>C</td>
<td>+</td>
<td>nt</td>
<td>nt</td>
<td>23/23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PP5dB4</td>
<td>D</td>
<td>-</td>
<td>nt</td>
<td>nt</td>
<td>23/23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PP5eA12</td>
<td>D</td>
<td>-</td>
<td>nt</td>
<td>nt</td>
<td>23/23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PP5cH5</td>
<td>D</td>
<td>-</td>
<td>nt</td>
<td>nt</td>
<td>23/23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PP5cG12</td>
<td>D</td>
<td>-</td>
<td>nt</td>
<td>nt</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Pl/b4</td>
<td>C</td>
<td>+</td>
<td>-</td>
<td>nt</td>
<td>1/6</td>
<td></td>
</tr>
</tbody>
</table>

C, continuous; D, discontinuous; nt, not tested; EUs, European viruses; NAs, North American viruses

Changes of epitopes during virus passage in pigs

Although a number of studies have repeatedly demonstrated the presence of antigenic variation of field PRRS viruses, little is known about how soon epitopes of proteins change during virus persistence in pigs. Le Gall et al. (1997) passaged 2 viruses (I and II) in pigs and compared sero-reactivity of recovered and parent viruses with a panel of 14 MAb specific for the N, M, GP3 and GP4 proteins. Virus I was maintained in pigs for 27 weeks. This period included 3 weeks in sows, 19 weeks in their offspring and 5 weeks in contact pigs. One virus recovered from a contact pig lost an epitope of the GP3 protein. Virus II was maintained in pigs for 5 weeks. One of the 5 recovered viruses gained the same epitope that was not present in virus II before passage. This epitope is present in GP3 of some European PRRS viruses tested and is represented by MAb WBE 2 (Drew et al., 1995). No other changes occurred in the epitope profiles of viruses I and II following passage in pigs.
Immune responses

Humoral Immune Response

The ontogeny of the humoral immune response to PRRS virus has been characterized by several investigators. Virus-specific IgM antibody was detected by the IFA test as early as one week post-infection (p.i.), reached maximum levels by 2 weeks p.i., and persisted for 5 weeks (Loemba et al., 1996; Vézina et al., 1996). Virus-specific IgG was initially detected from one to 3 weeks p.i., reached maximum levels by 3 to 6 weeks p.i., and persisted for over 20 weeks (Albina et al., 1992; Cho et al., 1996; Frey et al., 1992; Loemba et al. 1996; Morrison et al., 1992; Nelson et al., 1994; Vézina et al., 1996; Wensvoort et al., 1991; Yoon et al., 1992b, 1994, 1995b). Neutralizing antibody titers were initially detected approximately 1-2 months p.i. and remained for 9 months p.i. (Kwang et al., 1999; Nelson et al., 1994; Yoon et al., 1995b). When a modified SVN test using 20% fresh swine serum as a virus diluent was utilized, the neutralizing antibody was detected 9-11 days p.i. and reached maximum levels by 41-45 days p.i. (Yoon et al., 1994).

The ontogeny of the antibody response to structural proteins N, M and GP5 proteins of PRRS viruses is variable. Nelson et al. (1994) evaluated the humoral immune response of pigs infected with ATCC VR-2332 by Western immunoblotting (WI). These investigators found that antibodies specific for the N protein were detected as early as 7 days p.i., and for the M and GP5 proteins as early as 15-21 days p.i. Yoon et al. (1995b) reported similar findings in pigs exposed to the North American virus ATCC VR-2402 (ISU-P). Antibodies specific for the N protein were detected by WI at 7 days p.i. Antibodies specific for the M and GP5 proteins were detected by 9-28 days p.i. Bautista et al. (1994) reported that antibodies to the N, M and GP5 proteins of ATCC VR-2332 were detected by
radioimmunoprecipitation at 28, 7, and 21 days p.i., respectively. Loomba et al. (1996) detected antibodies by WI to the N and M proteins in naturally and experimentally infected pigs by 14 days p.i., and antibodies to GP5 by 7 days p.i.

**Cell-mediated immune response (CMI)**

Limited information is available on the development of cell-mediated immunity (CMI) in pigs to PRRS virus. Vézina et al. (1996) observed a significant enhancement of *in vitro* uptake of $[^3]H$-thymidine in peripheral blood lymphocytes that were collected from PRRS virus infected pigs at 7 or 14 days p.i. Bautista et al. (1999) demonstrated T-cell proliferation in addition to delayed-type hypersensitivity (DTH) responses in pigs. Lymphocyte proliferation following exposure to whole virus antigen was first detected at 4 weeks p.i., reached maximum values at 7 weeks and declined to undetectable levels by 11 weeks. Bautista et al. (1999) also compared the effectiveness of the N, M, GP2, GP4 and GP5 proteins as antigens in the lymphocyte proliferation assay. These antigens were either produced in a vaccinia virus expression system or obtained from PAGE gels. The M protein was the most efficient antigen, suggesting that it played a major role in inducing CMI. The DTH reaction that was observed in pigs exposed to PRRS virus was directly proportional to the dose used as antigen.

Both natural and experimental PRRS virus infection alters the T-cell subpopulations (CD4$^+$/CD8$^+$) in the peripheral blood of pigs. Shimizu et al. (1996) reported that the number of CD4$^+$ Th cells decreased for at least 14 days p.i. while CD8$^+$ Tc cells increased and reached maximum levels at 28-35 days p.i. In contrast, Zhou et al. (1992) reported that the CD4$^+$ subpopulation increased and the CD8$^+$ subpopulation decreased in infected pigs.
Similar variable responses have been reported in studies measuring the effect of PRRS virus infection on the function of alveolar macrophages and natural killer cells (Chiou et al., 1999; Lamontage et al., 1999; López-Fuertes et al., 1999; Nielsen et al., 1999; Samsom et al., 1999; 2000). Reasons for these variable responses include: variation in pathogenicity among PRRS viruses used in these experiments, difficulty in studying alveolar macrophages due to their heterogeneous populations, and the microbial status and susceptibility of experimental pigs (Zimmerman et al., 1998). These investigators were not able to show a correlation between these affects and the ontogeny of the immune response or the pathogenesis of PRRS virus infection.

The role of cytokines in regulating immune response to PRRS virus infection has not been extensively characterized to date. A major focus of studies to date has been on the role of interferon-γ (IFN-γ) (Meier et al., 1999). These investigators were not able to detect IFN-γ producing cells in pigs until 8-10 weeks p.i. The number of these cells then increased gradually to maximum levels by 9-10 months p.i. and remained at these levels for 300 to 690 days p.i. when the pigs were killed. These investigators also demonstrated in vitro that IL-12 enhanced the IFN-γ response and IL-10 suppressed the IFN-γ response. It was assumed that IFN-γ would play a role in controlling PRRS. However, no correlation was able to be demonstrated between the level and duration of IFN-γ in the host and the ontogeny of the immune response and status of persistent virus infection.

**Persistent infection**

Although pigs produce neutralizing antibodies and cell-mediated immunity to PRRS virus infection, a number of studies have shown that the virus can establish a persistent
infection as determined by virus isolation and detection of viral genomic materials.

Zimmerman et al. (1992) reported that the PRRS virus ISU-P spread from clinically healthy pigs to contact pigs 99 days after being infected. Albina et al. (1994) reported the spread of PRRS virus from 15-week-old pigs born to infected sows to contact pigs. Christopher-Hennings et al. (1995) reported the detection of viral RNA from semen samples of 2 boars at 92 days p.i. and the isolation of virus from the bulbourethral gland of a boar at 101 days p.i. Wills et al. (1997) recovered PRRS virus from oropharyngeal scrapings at 157 days p.i. Benfield et al. (1997) demonstrated that pigs born to sows infected during gestation became infected and shed the virus for periods up to 112 days after birth. Viral RNA was also detected in these pigs up to 210 days of age.

Lymph nodes and tonsils are sites of persistent PRRS virus infection (Benfield et al., 1997; Horter et al., 2000; Wills et al., 1997). Osorio (1998) recovered the PRRS virus from tonsils of pigs up to 150 days p.i. (unpublished data). Wills et al. (1999) detected viral RNA from tonsil biopsies of 4 pigs at 251 days p.i. The testes of boars are also a site that harbors the PRRS virus (Sur et al., 1997). The eye has also been suggested as a site of virus persistence (Shin and Molitor, 1998).

The mechanisms for the establishment and maintenance of persistence of PRRS virus infection are not known. Antigenic variation and subsequent evasion of the immune system is a possible mechanism (Ahmed et al., 1996).
Prevention and control

Prevention and control of PRRS have been a priority for the swine production industry since this disease emerged. Management protocols with and without vaccination programs have been used with variable success to control PRRS.

Management protocols

A variety of management protocols with and without concurrent use of vaccine have been used for prevention and control and for eradication of PRRS virus from herds. These methods include total depopulation, test and removal (Andreasen, 1999; Blanquefort and Benoit, 1999; Dee and Molitor, 1998; Dee et al., 1996a, 1996b, 1999; Plomgaard, 1998), medicated early weaning (MEW) and segregated early weaning (SEW) (Christianson et al., 1994; Donadeu et al., 1999; Gramer et al., 1999; Rajic et al., 1999), and MCREBEL (Management Changes to Reduce Exposure to Bacteria to Elimination Losses) (McCaw, 1995).

Identification of clinically normal but persistently infected pigs is critical for the success of eradication protocols since PRRS virus infection does not always cause clinical problems. These apparently healthy pigs are the most important source of infection. Serological tests including the IFA, SVN and ELISA, separately or in combination with the RT-PCR are used to detect the presence of inapparently infected pigs in herds (Dee, 1999; Dee and Philips, 1999; Donadeu et al., 1999).

Vaccination

Two modified-live vaccines (RespPRRS/Pro and Prime Pac PRRS) and 1 inactivated vaccine (PromisePRRS) were developed by Boehringer Ingelheim/NOBL Laboratories,
Schering-Plough Animal Health and Bayer Corporation, respectively (Bayer Corporation, 1999; Gorcyca et al., 1995; Hesse et al., 1996) and are currently available in the United States. Vaccine-induced immunity has been shown to prevent reproductive failure in endemic areas. However, their use and effectiveness are controversial (Gillespie, 1995; Gorcyca et al., 1995; Joo et al., 1998b; Sanford and Nuhn, 1996; Sornsøn et al., 1998a; 1998b; Trayer, 1995).

Experimental studies have shown that vaccinated pigs can spread modified-live vaccine viruses to non-vaccinated pigs. Christopher-Hennings et al. (1997) vaccinated boars with the modified-live vaccine RespPRRS. At 39 days post-vaccination the semen samples remained positive by RT-PCR testing. Mengeling et al. (1999a) investigated the potential of the modified-live vaccine RespPRRS for virus shedding, persistence and mutation during replication in sows. They recovered the attenuated virus at 14 days post-vaccination. In another study using the vaccine RespPRRS, Mengeling et al. (1999b) recovered the vaccine virus at 37 days post-vaccination and observed a partial protection against subsequent challenge. The transmission of the modified-live vaccine virus RespPRRS from vaccinated to non-vaccinated pigs has also been documented by other researchers (Dee et al., 1996a; Sornsøn et al., 1998b). Vaccination with RespPRRS/Repro in Danish herds caused severe outbreaks of PRRS in 1996 (Bøtner et al., 1997). The viruses isolated from affected pigs had identical nucleotide sequences to ATCC VR-2332, the progenitor of the vaccine virus (Madsen et al., 1998; Storgaard et al., 1999). These studies suggest that vaccination programs involving the modified-live vaccine virus should be implemented carefully in the field.
Osorio et al. (1998) compared 2 commercially available modified-live vaccines and 1 inactivated autogenous vaccine for their ability to induce protection against an acute PRRS virus disease. Parameters of this study included the ELISA and SVN antibody titers, cell-mediated immunity as measured by IFN-γ producing cells in peripheral blood mononuclear cells, viremia and viability of piglets. No correlation was observed between protection and humoral and cell-mediated immunity.

Modified-live vaccines are not recommended for immunization of gilts and sows during gestation. Attenuated virus can cross the placenta and infect fetuses (Mengeling et al., 1996).

Cross protection between and among European and North American viruses

Both homologous and heterologous protective immunity against reproductive failure has been demonstrated among wild type North American field viruses (Lager et al., 1999a; 1999b) and the vaccine virus RespPRRS (Hesse et al., 1996a; 1996b). However, heterologous protection was incomplete and persisted for a shorter period than homologous protection (Lager et al., 1999b). Similar conclusions were made by Labarque (1999) following cross protection studies using North American and European modified-live vaccines.

Strain differentiation

Both restriction fragment length polymorphisms (RFLPs) and epitope profiles have been used to differentiate vaccine viruses from wild type PRRS viruses. The epitope profiles of PRRS viruses have also been used to differentiate North American viruses from European viruses (Nelson et al., 1993; Drew et al., 1995; Le Gall et al., 1997; Magar et al., 1995,
For example, MAbs WBE4, 5 and 6 specific for the N protein have been shown to react with all European PRRS viruses tested including the Lelystad virus (Drew et al. 1995; Le Gall et al., 1997). In contrast, MAb VO17 specific for the N protein and MAb 2C12 specific for the M protein have been show to react with all North American PRRS viruses tested including ATCC VR-2332 (Magar et al., 1995, 1997; Nelson et al., 1993; Yoon et al., 1995a). Epitope profiles of PRRS viruses have also been used to monitor changes in PRRS virus populations in infected swine herds where killed autogenous vaccines are used (Nelson, unpublished data, 2000).

Wesley et al. (1998) reported that the modified-live vaccine (RespPRRS/Repro, Boehringer Ingelheim/NOBL Laboratories, Sioux Center, Iowa, U.S.A.) and field viruses differ in the RFLP patterns of the RT-PCR products of ORF 5. Viral ORF 5 was amplified by using the non-nested-set RT-PCR. The RT-PCR products were cut by 3 restriction enzymes (MluI, HincII and SacII) and numerical codes were assigned to represent specific restriction patterns. The vaccine virus and ATCC VR-2332 exhibited identical RFLP patterns (2-5-2) which were different from the restriction patterns of 22 wild type viruses. The 22 wild type viruses were represented by 10 different patterns. Additionally, 50 PRRS viruses from 3 states (45 from Iowa, 4 from Illinois, and 1 from Minnesota) were tested using this method. The restriction patterns of the vaccine virus and ATCC VR-2332 were different from the patterns of all of these 50 viruses.

Furthermore, Wesley et al. (1999) demonstrated that the RFLP patterns of 2 field viruses (2-5-2 SD and NADC-8) and a modified-live vaccine virus (RespPRRS/Repro) were stable during passage in pigs. Viruses were recovered from infected pigs 2-7 weeks p.i., from sentinel pigs at 3 weeks after contact with pigs that were inoculated 8 weeks previously,
and from 5-week-old piglets born of sows that were inoculated with PRRS viruses 8 weeks before farrowing. The RFLP patterns and sequences of ORF 5 of parent and recovered viruses were compared. Some nucleotide substitutions were identified. These substitutions included 1 nucleotide in viruses recovered at 2 weeks p.i., 2 to 4 nucleotides in viruses recovered at 7 weeks p.i. and 5 nucleotides in viruses recovered from 5 week-old piglets born of infected sows. However, these substitutions did not cause changes in the RFLP patterns of any of the viruses recovered after passage.

Umthum and Mengeling (1999) developed a nested-set RT-PCR for ORF 5 as an adjunct to the RFLP analysis described above. This type of PCR was 100 to 1000-fold more sensitive than the non-nested-set RT-PCR and could detect 1 infective unit of PRRS virus/ml in samples that included pulmonary lavage, serum and virus-infected MARC-145 cell lysates.

Gagnon and Dea (1998) found that the RFLP patterns of RT-PCR fragments containing both ORFs 6 and 7 could also be used to differentiate North American viruses from the modified-live vaccine virus (RespPRRS/Repro). The RT-PCR products were cut by endonucleases *Mspl*, *BsaJI*, *Alul* and *HaeIII*. Fourteen out of 16 field viruses had RFLP patterns that were different from this modified-live vaccine virus.

References


Osorio FA (1998) unpublished data, University of Nebraska at Lincoln.


ANTIGENIC AND GENETIC VARIATIONS OF THE 15 kD NUCLEOCAPSID PROTEIN OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ISOLATES

A paper published in the Archives of Virology

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M. L. Frey^2,7, K. M. Harmon^2,5 and K. B. Platt^2,4,8

Summary. The antigenic variability of the 15 kD nucleocapsid protein of porcine reproductive and respiratory syndrome (PRRS) virus was characterized with a panel of 24 monoclonal antibodies (MAbs) raised against the American PRRS virus isolate ISU-P. Five continuous epitopes designated EpORF7-A through E were revealed by the reactivity pattern of these MAbs with 67 American field isolates, two modified-live vaccine viruses, and the European Lelystad virus as determined by the indirect immunofluorescence assay and Western immunoblotting and confirmed by additivity and blocking enzyme-linked.

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immunosorbent assays. The reactivity pattern of isolates in the IFA permitted their subdivision into 4 American antigenic groups which represented 84.1, 11.6, 2.9 and 1.4% of viruses tested. The antigenic variation among isolates was correlated to single, group specific nucleotide substitutions and mediated by a combination of at least 4 of the 5 epitopes. EpORF7-A was conserved in all American isolates and the Lelystad virus which constituted a separate antigenic group. Consequently, monoclonal antibodies specific for EpORF7-A may prove useful as the antigenic basis for a universal diagnostic test for the PRRS virus. EpORF7-C, D and E were only present in the American isolates tested.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is a major disease of swine throughout the world. Clinical manifestations are characterized by reproductive failure in sows and gilts, pneumonia in grower/finisher pigs, and increased morbidity and mortality in preweanling pigs [11]. The syndrome was first reported as devastating outbreaks of reproductive failure in North Carolina, Minnesota, and Iowa in the United States during 1987 and 1988 [12, 14]. In the years following, PRRS spread throughout the United States and Canada [12]. In 1990, the disease was recognized in Germany and soon afterwards in other Western European countries [35]. Presently, PRRS is endemic in most swine producing countries throughout the world.

The etiologic agent responsible for the syndrome was first isolated by researchers in the Netherlands and identified as the Lelystad virus [33]. Soon after, a virus (ATCC VR-2332) was isolated in the United States using the proprietary cell line CL2621 and identified as the swine infertility and respiratory syndrome (SIRS) virus [1, 5]. Currently, the
International Office of Epizootics (O.I.E.) recognizes the terms Síndrome Disgenesico y Respiratorio del Cerdo (Spanish), Syndrome Dysgénésique et Respiratoire du Porc (French), and Porcine Reproductive and Respiratory Syndrome (English).

The PRRS virus is an enveloped RNA virus that is currently classified as a member of the family Arteriviridae [6, 22, 28]. The genome is a positive sense, 15 kb single strand of RNA consisting of 8 open reading frames (ORFs) which are expressed as a nested set of subgenomic RNAs. To date, 6 structural proteins with molecular masses of 15, 19, 25, 30, 31, and 45 kD and their corresponding genes have been reported for the European Lelystad PRRS virus isolate [23, 24, 31]. However, only 3 proteins with molecular masses of 15, 19, and 25 kD have been consistently detected in purified virions and lysates of cells infected with North American virus isolates [26, 27].

Previous studies have shown that field isolates of PRRS virus vary antigenically. Antigenic variation among PRRS virus isolates was first demonstrated between North American and European PRRS virus isolates by the immunoperoxidase monolayer assay using PRRS virus-specific polyclonal antibodies [34]. Similar antigenic variation between the European and North American isolates was also shown by the indirect fluorescent antibody test using monoclonal antibodies (MAbs) specific for the 15 kD nucleocapsid protein [8, 16, 19, 26]. The MAbs responsible for these distinctions represented at least one epitope that was unique to American isolates, one epitope unique to European isolates and at least 3 epitopes present in both American and European isolates. Genomic diversity of ORF 7 of European and American isolates has also been demonstrated by nucleotide sequence analysis [17, 21, 25, 30]. In the following study we describe the generation of a panel of MAbs to further characterize the antigenic nature of the 15 kD nucleocapsid protein and to
determine if a sufficient degree of antigenic variation exists in the 15 kD protein among field isolates that can provide the basis of a reliable virus marker system for use in epidemiological studies and for monitoring virus traffic in swine populations.

Materials and methods

Viruses, cells, and media

A total of 70 PRRS viruses were used in the present study. These viruses included isolate ISU-P, ATCC VR-2332, 2 modified-live vaccine viruses, PRIME PAC® PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA) and RespPRRS/ReproTM (NOBL Laboratories, Sioux Center, IA, USA), the Lelystad virus [33] and 65 field isolates that were isolated between 1989 and 1995 from Canada, Guatemala, and 13 states of the United States of America (Table 1). All PRRS virus isolates were propagated and passaged.

Table 1. The geographic origins of PRRS virus isolates and modified-live vaccine viruses tested and their antigenic groups as determined by sero-reactivity with a panel of 24 monoclonal antibodies in the indirect immunofluorescence antibody test

<table>
<thead>
<tr>
<th>Geographic origin</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southeastern U.S.</td>
<td>29</td>
<td>2</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Midwestern U.S.</td>
<td>27</td>
<td>5</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Southwestern U.S.</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guatemala</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The Netherlands</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1b</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a Includes PRRS viruses ISU-P, VR-2332 and the 2 modified-live vaccine viruses, PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA) and RespPRRS/Repro (NOBL Laboratories, Sioux Center, IA, USA)

b The Lelystad virus
at least one time after initial isolation in MARC-145 cells, a clone of an African green monkey kidney cell line [15]. The cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS).

**Antigen preparation for MAb production**

**Antigen for mouse immunization**

Semi-purified whole virus preparations sufficient to immunize 16 mice were prepared from eleven 75cm² cell culture flasks of MARC-145 cell monolayers which were inoculated with PRRS virus isolate ISU-P at a multiplicity of infection of approximately 5. Inoculated cells were incubated at 37°C in a humid atmosphere containing approximately 5% CO₂. Four days later virus was harvested by 3 cycles of freezing/thawing at -80°C/+37°C followed by clarification at 2,000 x g for 30 mins at 4°C. The supernatant was subsequently centrifuged at 90,000 x g in a Ti 60 rotor (Beckman Instruments, Inc., Palo Alto, CA, USA) for 2 hrs at 4°C. The resulting pellet was resuspended in approximately 1 ml of 0.01 M phosphate buffered saline (PBS), pH 7.4, overlaid onto a discontinuous sucrose gradient (30% and 60%), and centrifuged at 120,000 x g in a SW 28 rotor (Beckman Instruments, Inc., Palo Alto, CA, USA) for 70 mins at 4°C. The band at the interface of the 2 sucrose layers was collected. Two thirds of this material, which contained virus particles, was diluted in PBS to a total volume of 4 ml and emulsified in an equal volume of Freund’s incomplete adjuvant (GIBCO Laboratories, Grand Island, NY, USA). This preparation was stored at 4°C until used. The remaining material was diluted to 4 ml in PBS and stored at -70°C until used.
Antigen for Western immunoblotting and the enzyme-linked immunosorbent assay (ELISA)

MARC-145 cells were infected with PRRS virus ISU-P as described. Cells were harvested 4 days later, washed 2 times in PBS and pelleted by centrifugation at 500 x g for 15 mins at 4°C. Ten volumes of 0.25 M Tris/0.11 M tricine buffer, pH 8.6, containing 0.14 mM calcium lactate and 1% (V/V) Triton X-100 were added to one volume of packed cells, gently agitated overnight at 4°C and subsequently centrifuged at 2,000 x g for 20 mins at 4°C. The supernatant containing solubilized antigen was harvested and stored at -20°C until used.

MAb production

Monoclonal antibody secreting hybridomas were generated from 6 to 8 week-old specific pathogen free BALB/c mice. Each mouse was given two 0.25 ml injections of antigen with adjuvant subcutaneously 2 weeks apart. Two to 3 weeks later, mice were given a third 0.25 ml injection of antigen without adjuvant. Three days later, mouse spleens were removed. Fusions of mouse spleen cells with mouse SP2/O myeloma cells were carried out using the protocol described by Zola [38]. Hybridomas were screened for PRRS virus specific antibody secretion by the indirect ELISA. An indirect immunofluorescence assay (IFA) using normal and PRRS virus ISU-P infected MARC-145 cell monolayers as antigen [10] was used to confirm the identity of MAb secreting hybridomas. Antibody positive hybridoma populations were cloned 3 times with the EPIC 752 cell sorter (Coulter Corporation, Hialeah, FL, USA). Positive clones were identified by the IFA. The subclass and light chain type of individual MAbs were determined with a commercial mouse MAb isotyping kit (Boehringer Mannheim Corporation, Indianapolis, IN, USA).
Ascitic fluid containing specific MAbs was produced in 10 week-old BALB/c mice. All mice were primed with 0.5 ml pristane (2, 6, 10, 14-tetramethyl-pentadecane) (Sigma Chemical Company, St. Louis, MO, USA) given intraperitoneally 10 days before inoculation with specific hybridomas. Each mouse was injected with approximately 107 hybridoma cells in 0.5 ml PBS by the intraperitoneal route. Ascitic fluid was harvested from anesthetized mice between 7 and 10 days post inoculation. Monoclonal antibodies were extracted from ascitic fluid by protein A affinity chromatography using the protocol described by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA) and stored at -20°C.

**Indirect enzyme-linked immunosorbent assay (ELISA)**

Viral antigen and MARC-145 cell control antigen preparations were diluted to optimum concentrations in antigen coating buffer consisting of 0.02 M Na2CO3/NaHCO3, pH 9.6. One hundred ml of each preparation was added to individual wells in alternate rows of 96-well polystyrene microtiter plates (Immunlon I, Dynatech Laboratories, Inc., Chantilly, VA, USA) and incubated first at room temperature for 1 hr and subsequently overnight at 4°C. Excess viral and control antigen were removed and nonspecific binding sites in each well were blocked by adding PBS containing 10% FBS and incubating overnight at 4°C. The blocking solution was removed and the plates were stored in airtight plastic bags at -20°C until used.

Supernatants from hybridoma cultures were tested for antibody without dilution. Ascitic fluid was tested for antibody by first diluting it 1:100 in PBS containing 10% FBS. One hundred μl samples were added in duplicate to viral and cell antigen coated wells, incubated for 1 hr at 37°C and washed 4 times with washing buffer (PBS with 0.05% Tween
20). Subsequently 100 µl of goat anti-mouse IgA+IgG+IgM (H+L) labeled with horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) was added to each well and incubated for 1 hr at 37°C. The plates were washed 4 times and 100 µl ABTS peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) was added to each well. Reactions were allowed to develop for 20 mins at room temperature.

The optical density (OD) of each well was determined at 405 nm using a computerized Vmax microtiter plate reader (Molecular Devices Corporation, Palo Alto, CA, USA). A signal to noise ratio of 1.5 was considered positive in the screening test. Serological reactivity in the additivity and blocking ELISA was expressed as a corrected OD which was calculated by subtracting the mean OD generated from cell control antigen from the mean OD generated from viral antigen.

Additivity ELISA

The additivity ELISA was conducted using a modification of the methods originally described by Friguet et al. [9] and Levieux et al. [18]. In brief, MAbs representing each MAb group (Table 2) were diluted to concentrations that would consistently react with all available epitopes in antigen coated wells. Fifty µl of such an optimally diluted MAb was added to viral and cell antigen coated wells and incubated for 30 mins at 37°C. Subsequently 50 µl of the homologous MAb or heterologous MAb were added to the same wells and reacted for an additional hour at 37°C. Each reaction was conducted in duplicate. Antigen/antibody reactivity was visualized with horseradish peroxidase conjugate. Corrected ODs were determined at 405 nm for at least 3 replicates. Mean additivity values were calculated using the formula:
Table 2. Characterization of PRRS virus isolates and monoclonal antibodies specific for the 15 kD nucleocapsid protein as determined by their sero-reactivity in the indirect immunofluorescence antibody test

<table>
<thead>
<tr>
<th>PRRS virus</th>
<th>Monoclonal antibody group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (n=7)</td>
</tr>
<tr>
<td>Group</td>
<td>No. of isolates</td>
</tr>
<tr>
<td>I</td>
<td>58\textsuperscript{a}</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
</tr>
<tr>
<td>V</td>
<td>1\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Includes PRRS viruses ISU-P, VR-2332 and the 2 modified-live vaccine viruses, PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA) and RespPRRS/Repro (NOBL Laboratories, Sioux Center, IA, USA)

\textsuperscript{b} The Lelystad virus

\textsuperscript{c} Weak positive reaction

\[ \text{AV} = \left( \frac{\text{Corrected OD MAb } a + b}{\text{Corrected OD MAb } a + \text{Corrected OD MAb } b} \right) \times 100\% \]

where MAbs "a" and "b" are any 2 MAbs tested for additivity.

The additivity value (AV) of a homologous MAb combination should not be significantly greater than 50% whereas the AV resulting from a combination of 2 MAbs representing different and widely separated epitopes should approach 100%. Differences between homologous and heterologous MAb combinations were analyzed by Student's $t$-test.

**Blocking ELISA**

The blocking ELISA was performed essentially as described by Cecilia et al. [3]. In brief, affinity purified IgG preparations representing each of the 5 MAb groups summarized
in Table 2 were labeled with biotin hydrazide according to the manufacturer's instructions (Pierce, Rockford, IL, USA) and diluted to a concentration that would consistently react with all available epitopes in antigen coated wells. Purified unlabeled MAbs representing each MAb group were serially diluted 10-fold in PBS containing 10% FBS and used for blocking. Viral and cell antigen preparations were treated in duplicate with either blocking MAb or diluent and subsequently reacted with homologous or heterologous biotinylated MAbs. Antigen/antibody reactivity was visualized with peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Percent binding was calculated for each MAb interaction by comparing the corrected ODs generated in viral and cell antigen preparations treated with blocking MAb to the corrected ODs generated in antigen preparations that were treated with only diluent. The mean percent binding representing at least 3 replicates was calculated and plotted against dilution using the linear regression model. The one-way analysis of variance was used to test for differences at each dilution between homologous and heterologous interactions and among heterologous interactions mediated by individual blocking MAbs. Significant inhibition of binding by blocking antibodies was considered to be an indication of identity or interference by adjacent or overlapping epitopes.

Western immunoblotting

Western immunoblotting was done as previously described by Yoon et al. [37] with the exception that antigen preparations were mixed 1:1 with sample buffer containing 5% 2-mercaptoethanol.
**Virus neutralization test**

The microtiter serum virus neutralization test was used to assess the susceptibility of PRRS virus isolate ISU-P, the Lelystad virus and 4 individual American isolates representing virus antigenic groups I through IV to neutralization by MAbs representing groups A through E. Viruses were diluted in DMEM containing 10% FBS to yield approximate 200 TCID\textsubscript{50} in 50 $\mu$l. Ascitic fluid representing each MAb group was serially diluted two-fold in DMEM containing 10% FBS. Fifty $\mu$l aliquots of virus and MAb were added together in duplicate wells and incubated at 37°C for 1 hr. Subsequently 100 $\mu$l containing approximately $10^5$ MARC-145 cells were added to virus/MAb mixtures and incubated for 7 days and observed for cytopathic effect.

**RT-PCR**

Viral RNA was extracted from 16 group I, 8 group II, 2 group III and 1 group IV viruses using the protocol described by Boom et al. [2]. Individual viral RNA preparations were retrotranscribed using the protocol of the GeneAmp RNA PCR kit (Perkin Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA). Amplification of cDNA representing ORF 7 was mediated with the 2 oligonucleotide primers 5'-TAAATATGCCAAATAACAAC-3' and 5'-TAGGTGACTTAGAGGCACA-3'. These primers were originally designed by Mardassi et al. [20] and synthesized for use in this study by the Iowa State University Nucleic Acid Facility. The amplification was performed for 40 cycles by denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extending at 72°C for 1 min. The time of the final extension step was 8 mins. The PCR products were purified
using a PCR DNA purification kit (Promega Corporation, Madison, WI, USA) by using the instructions of the manufacturer.

**Sequencing and sequence analysis**

DNA sequencing was performed in triplicate on both strands with the Applied Biosystems 377 automated sequencer (Applied Biosystems, Foster City, CA, USA) using Taq polymerase, fluorescein labeled dNTPs and the 2 primers previously described. Sequence analysis was conducted using programs of the GCG package developed by Genetics Computer Group, Inc. of Madison, Wisconsin. Nucleotide sequences were compared using the BESTFIT program and translated to amino acid sequences by the TRANSLATE program. Multiple alignment of the sequences was done with the PILEUP program.

**Prediction of the nucleocapsid protein structure**

Unique structural features and characteristics of the nucleocapsid protein of all PRRS viruses representing antigenic groups I through V that were sequenced were predicted from the translated amino acid sequences using the PEPTIDESTRUCTURE program of the GCG package. Parameters that were used to compare amino acid sequences included hydrophilicity, surface probability, flexibility, Jameson-Wolf (JW) antigenic indices [13], Chou-Fasman (CF) turns [5], CF alpha helices, CF beta sheets, Garnier-Osguthorpe-Robson (GOR) turns, GOR alpha helices, and GOR beta sheets.
Results

Generation and characterization of MAbs

Twenty-four MAbs were generated. All were of the IgG1 subclass and possessed kappa light chains. These MAbs were specific for the 15 kD nucleocapsid protein of PRRS virus as determined by Western immunoblotting. All MAbs reacted with PRRS virus isolate ISU-P in the IFA and produced bright cytoplasmic fluorescence. None of the MAbs representing each group neutralized PRRS virus isolate ISU-P, the Lelystad virus or the 4 other American isolates representing antigenic groups I through IV.

Reactivity of MAbs with PRRS virus isolates

Five distinct groups of MAbs (A-E) and four distinct groups of American isolates (I-IV) and the Lelystad virus (group V) were revealed by sero-reactivity as determined by Western immunoblotting and the IFA (Table 2). Monoclonal antibodies in group A were denominated as A1 through A7, group B as B1 through B3, group C as C1 through C3, group D as D1, and group E as E1 through E10. Virus group I represented 84.1% (58/69) of all American isolates tested including the 2 vaccine viruses and isolate ISU-P. Each isolate in this group reacted with all 5 groups of MAbs. Virus groups II, III, and IV represented 11.6% (8/69), 2.9% (2/69) and 1.4% (1/69) of the American isolates respectively. Each of these virus groups was characterized by their reactivity with at least 4 of the 5 groups of MAbs. The Lelystad virus (group V) was only recognized by MAb groups A and B. Group A MAbs reacted strongly with Lelystad virus in the IFA as indicated by bright fluorescence. In contrast, group B MAbs reacted weakly with this virus in the IFA and not at all when
evaluated by Western immunoblotting, possibly due to partial identity. The geographic
origins of the PRRS viruses and their antigenic groups are summarized in Table 1.

**Epitopic analysis of the 15 kD nucleocapsid protein**

Results of additivity assays are summarized in Table 3. Additivity values for any 2
MAbs tested that equal or approach 50% indicate identity as revealed by the calculated mean
additivity value for all homologous reactions of 51.5 ± 0.4%. Conversely, additivity values
for any 2 MAbs tested that differ significantly from 50% and approach 100% are indications
that the 2 MAbs tested represent different epitopes. The mean additivity values for all

Table 3. Epitopic analysis of the 15 kD nucleocapsid protein of PRRS virus isolate ISU-P
by the additivity test using group representative MAbs A1, B1, C1, D1 and E1

<table>
<thead>
<tr>
<th>Added MAb</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.97*</td>
<td>0.65</td>
<td>1.34</td>
<td>0.89</td>
<td>0.71</td>
</tr>
<tr>
<td>A</td>
<td>50.5%</td>
<td>(0.98/1.94)*</td>
<td>51.3%</td>
<td>(1.27/1.62)</td>
<td>53.0%</td>
</tr>
<tr>
<td>B</td>
<td>78.4%</td>
<td>51.3%</td>
<td>(1.27/1.62)</td>
<td>(0.67/1.30)</td>
<td>85.4%</td>
</tr>
<tr>
<td>C</td>
<td>82.7%</td>
<td>51.3%</td>
<td>(1.27/1.62)</td>
<td>(0.67/1.30)</td>
<td>35.4%</td>
</tr>
<tr>
<td>D</td>
<td>84.4%</td>
<td>51.3%</td>
<td>(1.27/1.62)</td>
<td>(0.67/1.30)</td>
<td>84.3%</td>
</tr>
<tr>
<td>E</td>
<td>75.6%</td>
<td>51.3%</td>
<td>(1.27/1.62)</td>
<td>(0.67/1.30)</td>
<td>82.5%</td>
</tr>
</tbody>
</table>

* Optical density was measured at 405 nm.

Additivity values expressed as binding percent were calculated using the formula:

\[
AV = \frac{\text{Corrected OD MAb } a + b}{(\text{Corrected OD MAb } a) + (\text{Corrected OD MAb } b)} \times 100 \%
\]

where MAbs "a" and "b" are any 2 MAbs tested for additivity.

The measured OD values and theoretical sum of OD values of any 2 MAbs are indicated in
parenthesis. The mean additivity values for any 2 heterologous MAbs are significantly increased
(P < 0.001), as determined by Student's t-test.
heterologous comparisons was 81.3 ± 4.8% and ranged from 71.3% to 86.3%. These values were significantly different (p<0.001) from homologous additivity values indicating that MAbs representing groups A through E represent 5 different continuous epitopes hereafter referred to as EpORF7-A through E.

Results of blocking assays that are summarized in Table 4 and Figure 1 also support the existence of at least 5 epitopes on the 15 kD protein. The mean percent binding for all homologous interactions at the lowest MAb dilution was 32.6 ± 2.7 and ranged from 28.0 ± 2.3 to 34.3 ± 1.1. All homologous interactions were significantly different from heterologous interactions (p < 0.001). No significant differences were detected among heterologous interactions when MAb representing EpORF7-A, B, C and D were used for blocking with the exception that the MAbs representing EpORF7-B and C interfered (p<0.05) with subsequent reactivity by the MAb representing EpORF7-E. Similar interference of reactivity of MAbs representing EpORF7-B, C and D was observed when MAb representing EpORF7-E was

Table 4. Epitopic analysis of the 15 kD nucleocapsid protein of PRRS virus isolate ISU-P by the blocking ELISA using group representative MAbs A1, B1, C1, D1 and E1

<table>
<thead>
<tr>
<th>Biotinylated MAb</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>-&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>±&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
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</tr>
<tr>
<td>E</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant (p < 0.001) inhibition
<sup>b</sup> No inhibition
<sup>c</sup> Partial inhibition as indicated by significant blocking (p < 0.05) at a magnitude of 1.8 - 2.0 less than that which was observed in homologous interactions.
Figure 1. Competitive binding curves of 5 representative biotinylated MAbs A (O), B (V), C (▼), D (□), and E (■) in the presence of blocking MAbs A through E.
used for blocking (p<0.05). In both instances the magnitude of interference as demonstrated by percent binding among heterologous interactions was less than that observed among homologous interactions and ranged from 58.5 ± 2.4 to 64.2 ± 1.8. These observations suggest that EpORF7- A, B, C, and D are spatially separate from each other while EpORF7- E overlaps with or is adjacent to EpORF7- B, C and D. The postulated spatial relationship of these epitopes is presented in Figure 2.

Figure 2. The spatial relationship of 5 continuous epitopes (EpORF7-A through E) of the 15 kD nucleocapsid protein of PRRS virus isolate ISU-P. Group representative MAbs A1, B1, C1, D1, and E1 were used. Circles that touch indicate that the epitopes are adjacent or overlap.
Comparison of ORF 7 of representative viruses of the 5 antigenic groups

The nucleotide sequences of ORF 7 of 15, 8, 2, and 1 representative viruses of antigenic groups I, II, III, and IV respectively have been deposited in the GenBank with the accession numbers AF043949-AF043974. The nucleotide sequence of ORF 7 of RespPRRS/ReproTM vaccine virus (NOBL Laboratories) has been deposited in the GenBank with the accession number AF054970. These nucleotide sequences are presented in Figure 3. The deduced amino acid sequences of these viruses are summarized in Figure 4. The nucleotide and deduced amino acid sequences of ORF 7 of Lelystad virus and ATCC VR-2332 [22, 25] are presented for the purpose of comparison. The ORF 7 of all American PRRS virus isolates analyzed consisted of 372 nucleotides which encode 123 amino acids. In contrast, ORF 7 of Lelystad virus was 387 nucleotides long and hence 128 amino acids. Furthermore, 2 nucleotide segments corresponding to positions 38-51 and 372-387 in ORF 7 of the Lelystad virus were not present in American viruses. Similarly, 2 nucleotide segments corresponding to positions 117-127 and 357-361 in ORF 7 of American viruses were not present in the Lelystad virus.

Analysis of ORF 7 of the American isolates by the GCG package program revealed single, group specific nucleotide substitutions relative to virus group I that could account for unique antigenic differences. These nucleotide substitutions occurred at positions 358, 364 and 209 in virus groups II, III, and IV, respectively, and are indicated in Figure 4. In groups II and III, thymidine at positions 120 and 122 respectively was replaced by cytidine which changed the encoded amino acid from serine to proline. In group IV, guanine at position 70 was by adenine which changed the encoded amino acid from serine to asparagine.
Figure 3. Comparison of the nucleotide sequences of ORF 7 of representative PRRS viruses of antigenic groups I-V. The 15 nucleotides at the 5' end of ORF 7 are primer-derived. Dots represent bases identical to the consensus sequence, dashes indicate gaps introduced into the sequences, and the bold nucleotides are group-specific substitutions. The Lelystad virus and VR-2332 sequences used in this alignment were obtained from the GenBank [22, 25]. *The modified-live vaccine virus RespPRRS/Repro, manufactured by NOBL Laboratories, Sioux Center, IA, USA. **The modified-live vaccine virus PRIME PACPRRS, manufactured by Schering-Plough Animal Health Corporation, Omaha, NE, USA. ***A Canadian PRRS virus isolate.
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Figure 4. Alignment of deduced amino acid sequences encoded by ORF 7 of representative PRRS viruses of antigenic groups I-V. The 5 amino acids at the amino terminal of the 15 kD nucleocapsid protein are primer-derived. Dots represent residues identical to the consensus sequence, dashes indicate gaps introduced into the sequences, and the bold amino acids are group specific substitutions. ^The modified-live vaccine virus RespPRRS/Repro, manufactured by NOBL Laboratories, Sioux Center, IA, USA. "The modified-live vaccine virus PRIME PACPRRS, manufactured by Schering-Plough Animal Health Corporation, Omaha, NE, USA. A Canadian PRRS virus isolate.
The locations of these amino acid substitutions at positions 120, 122 and 70 in antigenic groups II, III and IV respectively are correlated to changes in the secondary structure of the protein that are predicted by Chou-Fasman (CF) turns (Figure 5).

Specifically, the carboxy terminal CF turn representing group II viruses is broader at the base and its apex is flatter relative to the homologous CF turn that represents group I viruses. No turn is present in this location for group III viruses and the height of the CF turn representing the group IV virus in this location is markedly shorter than its counterparts in groups I and II. The shoulder in the peak representing the CF turn at position 70 in the group IV virus is
Figure 5. Chou-Fasman turns shown for each antigenic group are representative of all PRRS virus isolates that were sequenced. Note that group specific changes marked with arrows are located in the carboxy terminal CF turns for antigenic groups II and III, and in the 6th CF turn for antigenic group IV.

noticeably narrower than the shoulder of the peaks representing the homologous CF turn in groups I, II, and III. No distinct group specific differences were observed between the 4 American antigenic groups when the deduced amino acid sequences were analyzed by the other parameters of the GCG package as specified in the Materials and Methods section.
American antigenic groups when the deduced amino acid sequences were analyzed by the other parameters of the GCG package as specified in the Materials and Methods section.

**Discussion**

The principal objectives of the current study were to further characterize the antigenic nature of the highly conserved 15 kD nucleocapsid protein of PRRS virus and to determine if a sufficient degree of antigenic diversity exists in this protein that could provide the basis of a marker system for PRRS viruses. Published data prior to the present study suggest that at least 5 epitopes in various combinations are associated with the 15 kD nucleocapsid protein of American and European PRRS virus isolates. Three of these epitopes are most likely discontinuous because of their inability to be recognized by MAbs in Western immunoblotting. These epitopes are represented by MAbs SDOW 17, VO 17 [7, 26], and ICH5 [29]. The MAbs SDOW 17 and VO 17 represent 2 distinctly different conserved epitopes. The original characterization of MAbs SDOW 17 and VO 17 by Nelson et al. [26] demonstrated that MAb SDOW 17 reacted with all 63 American and 57 European isolates tested. In contrast, MAb VO 17 only recognized the American isolates. The MAb ICH5 appears to be different from MAb SDOW 17 because it failed to react in the IFA with 3 American isolates [16]. The 2 continuous epitopes are represented by MAbs WBE 1 and WBE 4 respectively [8]. Their distinctness was demonstrated by their reactivity pattern with a combined total of 44 European and 11 American PRRS virus isolates [8, 16]. Both MAbs reacted with all European isolates tested but while MAb WBE 4 did not react with any of the 11 American isolates, MAb WBE 1 reacted with 3.
In addition to the 5 epitopes described above, a sixth epitope specific for the 15 kD protein may also exist. It is continuous and is represented by MAb P3/27 which was originally shown to react with all 14 European and 12 American isolates tested [36]. In a different study [16], MAb P3/27 was shown to have the same IFA reactivity pattern as MAb WBE 1. In that study, both MAbs reacted with all 21 European and 3 American isolates tested. There are no published accounts that clearly indicate whether or not MAb P3/27 reacts with the 8 American isolates that were not recognized by WBE 1 [8] or that WBE 1 reacts with the 12 American isolates recognized by P3/27 [36]. Consequently, it remains to be clarified whether or not MAbs P3/27 and WBE 1 represent the same or different epitopes. Nevertheless, the two published accounts which indicate that MAb P3/27 reacted with 15 of 15 American isolates tested [16, 36] and that MAb WBE 1 did not react with 8 different American isolates [8] suggest that 2 MAbs represent different epitopes.

In the present study, 5 distinct continuous epitopes designated EpORF7-A through E were revealed among American isolates further contributing to our knowledge of the antigenic nature of the 15 kD protein of PRRS virus. The uniqueness of the individual epitopes was confirmed by the results of blocking and additivity ELISA assays. In the blocking assay (Table 4), heterologous MAbs failed to inhibit binding of MAbs representing each of the 5 different epitopes (p < 0.001). While in the additivity assay (Table 3) optical density measured following treatment of antigen preparations with MAb representing each epitope was significantly enhanced (p < 0.001) after additional treatment with heterologous MAbs but not with homologous MAbs. At least 4 of the 5 continuous epitopes were present in all viruses tested with the exception of the Lelystad virus which contained only EpORF7-A as determined by both IFA and Western immunoblotting (Table 2). Consequently, it is
possible that EpORF7-A may represent the same epitope as P3/27. Analysis of a broad panel of European PRRS virus isolates will be necessary to determine which if any of the remaining 4 continuous epitopes are present in European isolates.

In an effort to determine the approximate locations of EpORF7-A through E in the 15 kD protein the deduced amino acid sequences were computer analyzed using the GCG package. None of the parameters as described in the Materials and Methods section could be correlated to antigenic sites with the possible exception of Chou-Fasman (CF) profiles, as indicated by number, location, shape and size of CF turns. Individual CF turns are a reflection of secondary structure which can influence antigenicity. Unique differences in the CF profiles of antigenic groups II, III and IV relative to the CF profile of group I were observed at locations encompassing amino acids 120, 122 and 70 respectively (Figure 5). These amino acids represent unique group specific substitutions which appear to be correlated to the absence of EpORF7-B, C and D in antigenic groups II, III and IV respectively. This observation and the demonstration of inhibition mediated by MAbs representing EpORF7-E with MAbs representing EpORF7-B, C and D (Figure 2) suggest that the amino acid sequences representing EpORF7-B, C and D are relatively close to one another at the carboxy terminal end of the 15 kD protein. The absence of reciprocal or one-way inhibition between MAbs representing EpORF7-A and EpORF7-B, C, D and E in the blocking assay (Table 4) suggests that EpORF7-A is spatially distant from these epitopes.

The available data is not sufficient to predict the probable location of EpORF7-A or E. However, a recent study by Rodriguez et al. [29] demonstrated the presence of a continuous epitope in the central region of the 15 kD protein in both a European (Olot/91) and an American (Quebec 807/94) PRRS virus isolate by reacting a panel of MAbs with
fragments of recombinant 15 kD protein by Western immunoblotting. They further demonstrated that a highly conserved amino acid sequence extended from amino acid 50-90 in 3 American and 2 European isolates which included the Lelystad virus. Amino acids 50-66 were associated with the common epitope. These observations suggest that the common epitope described by Rodriguez et al. could be identical to EpORF7-A. Competitive assays between MAbs representing EpORF7-A and the common epitope described by Rodriguez et al. may answer this question.

The degree of antigenic diversity demonstrated among American isolates in the present study was less than expected. The distribution of the 5 epitopes associated with American PRRS isolates permitted their subdivision into 4 antigenic groups that were distinctly different from the European Lelystad virus. However, antigenic groups I and II represented 84.1% and 11.6% of all isolates tested while groups III and IV represented the remaining 4.3% of the isolates. This limited antigenic diversity among American PRRS viruses as revealed by the panel of MAbs specific for the 15 kD protein indicates that a useful marker system will require a broader panel of MAbs representing epitopes of other PRRS virus proteins. Alternatively, more sensitive MAb based methodologies could be used such as the signature analysis that was originally described by Wands et al. [32]. This technique uses a panel of MAbs to generate individual antigen binding curves that reflect both the affinity by which individual MAbs bind to an epitope and the density of epitopes on the surface of the virus.

The reliable use of MAbs to identify PRRS virus isolates will also depend on the stability of the epitopes that they represent. Previous work by Le Gall et al. [16, 17] failed to demonstrate any antigenic change in the 15 kD protein following one or two passages of 6
virus isolates through pigs. However, they did observe antigenic change in an epitope of glycoprotein 3 in 2 of the 6 isolates. Consequently, the relative stability of individual epitopes that form the basis of a virus identification system must be thoroughly evaluated.

Acknowledgments

We are grateful to Dr. L. Tsao and Ms. L. Wu for statistical analysis, and to Dr. K. Harkins for providing SP2/O myeloma cells and for cloning hybridoma cells. Financial support for this study was provided by the Iowa Livestock Health Advisory Council, the National Pork Producers Council and the American Veterinary Medical Association. This study represents a portion of a dissertation submitted by the senior author to the Graduate College of Iowa State University in partial fulfillment of the requirements for the Ph.D. degree.

References


CATEGORIZATION OF NORTH AMERICAN PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUSES: EPITOPEC PROFILES OF THE N, M, GP5 AND GP3 PROTEINS AND SUSCEPTIBILITY TO NEUTRALIZATION

A paper in press in the Archives of Virology

L. Yang, M.L. Frey, K.-J. Yoon, J.J. Zimmerman and K.B. Platt

Summary. Eleven epitopes were identified by murine monoclonal antibodies (MAbs) that represented the N, M, GP5 and GP3 proteins of the North American (NA) porcine reproductive and respiratory syndrome (PRRS) virus, KY 35 (NVSL 46907). Three discontinuous epitopes of the N and M proteins were designated EpORF7-Fd through Hd and EpORF6-Ad through Cd. Five continuous epitopes of the GP5 and GP3 proteins were designated EpORF5-A through C and EpORF3-A and B. The MAbs representing EpORF5-C and EpORF6-A and B had neutralizing activity. The MAbs representing the above epitopes, except EpORF7-Gd and Hd, expanded the virus marker system described in a previous study in which a panel of 69 NA viruses and the Lelystad virus were categorized into 5 antigenic groups, I_{15} through V_{15} based on the presence or absence of 5 continuous epitopes of the N protein. Antigenic groups I_{15} and II_{15}, which represented 84.7 and 11.6% of all viruses tested, were categorized further into 9 and 4 subgroups, respectively. The remaining NA viruses and the Lelystad virus were distributed among 4 groups, one of which was represented by 2 subgroups. Significant (P<0.05) differences in sensitivity to neutralization of 28 viruses representing 6 antigenic groups by the 3 neutralizing MAbs suggested that sensitivity to neutralization may also be of value in categorizing PRRS viruses.
Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus is the cause of an economically significant disease of swine worldwide. The virus was initially isolated from European swine in 1990 [41] and designated the Lelystad virus. Subsequently, the PRRS virus ATCC VR-2332 was isolated from North American swine [2, 6]. Effective control of this disease, both with and without the use of vaccines, is dependent on understanding the ecology of PRRS virus. Consequently, a virus marker system was developed for this purpose in a previous study [43]. This marker system was based on the presence or absence of 5 continuous epitopes of the 15 kD nucleocapsid protein in individual viruses. It permitted the categorization of a panel of 69 North American (NA) PRRS viruses into 4 antigenic groups designated I\textsubscript{15} through IV\textsubscript{15}. However, 84.7% of the isolates were represented by antigenic group I\textsubscript{15} and 11.6% were represented by antigenic group II\textsubscript{15}. This unequal distribution of the majority of field viruses into 2 antigenic groups limited the usefulness of the marker system and clearly indicated that a system based on a broader distribution of epitopes was required.

The PRRS virus is a member of the Arteriviridae [4, 34]. Other viruses of this family include lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV) [4, 34]. The genome of the arteriviruses is a single strand of positive sense RNA that varies from 12.7 to 15.7 kb in size [36]. The genomic organization of the arteriviruses was first described for EAV by Den Boon et al. [9] who identified the presence of a nested set of 8 open reading frames (ORFs). More recently, a region between ORF 1b and ORF 2 of the EAV genome that encodes a 67 amino acid structural protein has been identified [37]. This region of the EAV genome has been
designated ORF 2a and the former ORF 2 has been renamed ORF 2b. The genome of PRRS virus is 15 kb in size. Eight ORFs have been identified and are designated 1a, 1b, and 2 through 7 [25]. An additional ORF equivalent to ORF 2a of EAV may also exist in the PRRS virus genome [37].

The ORFs 1a and 1b of PRRS virus encode nonstructural proteins associated with viral replicase activity [25]. The ORFs 2 through 7 encode structural proteins with molecular masses of 30, 45, 31, 25, 19 and 15 kD respectively, which are designated GP2, GP3, GP4, GP5, M and N respectively [7, 25, 26, 27, 39]. The N protein is associated with the nucleocapsid. The GP2, GP3, GP4, GP5 and M proteins are associated with the viral envelope. The GP2 and GP5 proteins are counterparts of the Gs and Gl proteins of EAV [10, 36]. The GP5 protein of PRRS virus is the major envelope protein and is a primary target of neutralizing antibody [32, 33, 40]. The GP4 protein is a minor envelope protein and is also a target of neutralizing antibody [28, 39, 40]. The GP2 protein of European viruses has not been demonstrated in NA viruses to date.

Both genetic and antigenic differences have been demonstrated between and among European and NA PRRS viruses. Murtaugh et al. [30] compared deduced amino acid sequences of ORFs 2 through 7 of the NA virus ATCC VR-2332 to the Lelystad virus. The identity between ORFs 2 through 7 of these 2 viruses ranged from 58% to 78%. Genetic variability between and among European and NA PRRS viruses has been observed by other investigators [1, 12, 16, 19, 21, 22, 24, 38, 43]. These genetic differences are also reflected by the distribution of protein-specific epitopes among viruses.

At least 5 and as many as 7 distinct continuous epitopes are associated with the N protein. Five of these epitopes, designated EpORF7-A through E, are distributed among NA
viruses [43]. Two of these epitopes, EpORF7-A and B are also present on the Lelystad virus. Monoclonal antibodies WBE 1 [11] and P3/27 [42] serologically react with viruses of both North American and European origin and may represent 2 additional continuous epitopes. In addition, at least 2 discontinuous epitopes are represented by MAb SDOW17 which reacts with both NA and European viruses including the Lelystad virus, and MAbs VO17 and EP147 which react with NA viruses but not with the Lelystad virus [31]. An additional discontinuous epitope may be represented by MAb 1CH5 that has only been shown to react with European viruses [35].

Four continuous epitopes have been identified for the M protein based on seroreactivity patterns of MAbs. Two of these epitopes are represented by MAbs 122.9 and 126.3 [39]. Both MAbs react with Lelystad virus but only 122.9 reacts with the NA virus, ATCC VR-2332. The other 2 epitopes are represented by MAb 2C12 [20] and by MAb IAFK3 or 6 [8]. Neither of these 2 epitopes is present on the Lelystad virus. No discontinuous epitopes of this protein have been described.

At least 4 continuous epitopes of the GP5 protein have been identified. Two epitopes described by Pirzadeh and Dea [33] were identified by the sero-reactivity patterns of 2 different groups of neutralizing MAbs. The first group was represented by MAbs 2A5, 3A12 and 1C10. The second group was represented by MAbs IAF8A8 and 1B8. Both MAb groups sero-reacted with the same panel of NA PRRS viruses with the exception that the NA virus, ATCC VR-2332 was only recognized by the second group. Neither group of MAbs recognized the Lelystad virus. A third continuous epitope that did not induce neutralizing antibody was detected by Zhang et al. [47] in NA viruses. These investigators also described
2 discontinuous epitopes among NA viruses, one of which induced neutralizing antibody. A fourth continuous epitope of the GP5 protein has been described by Weiland et al. [40]. This epitope was shown to be a target of neutralizing antibody and was only detected in a clone of the Dutch isolate I10, but not in any of 12 NA viruses tested or the Lelystad virus.

At least 3 and as many as 5 continuous epitopes have been associated with the GP4 protein of European viruses as determined by sero-reactivity patterns of MAbs and the presence or absence of neutralizing activity of these MAbs. Two epitopes were represented by the neutralizing MAbs P3/35, and P7/b1-7 [40, 42]. A third epitope was represented by the non-neutralizing MAb, P7/b1-2 [40, 42]. Monoclonal antibodies 122.68 and 121.4 described by Meulenberg et al. [28] may represent additional continuous epitopes [18, 28]. In addition, Zhang et al. [47] described a group of 4 MAbs that represented at least 1 discontinuous epitope of NA viruses.

At least 6 continuous epitopes have been associated with the GP3 protein. Three epitopes are represented by MAb WBE 2 [11], and MAbs P7/a2-55 and P7/b2-14 [42]. All 3 epitopes appear to be restricted to European viruses including the Lelystad virus. In 2 separate comparative studies, MAb WBE 2 did not react with any of 11 NA viruses tested, and MAbs P7/a2-55 and P7/b2-14 did not react with any of 12 NA viruses tested [11, 18]. The remaining 3 epitopes are represented by MAbs P7/a1-19, P9/a3-20, and P9/b3-1 and are associated with both NA and European viruses [42]. All 3 MAbs reacted with both the Lelystad virus and the NA viruses tested. A fourth epitope associated with both the Lelystad virus and NA viruses may be represented by the MAb designated 122.14 by van Nieuwstadt et al. [18, 39]. No discontinuous epitopes of the GP3 protein have been reported.
In the present study a panel of MAbs was developed to further characterize the antigenic nature of the N, M, GP3 and GP5 proteins of NA PRRS viruses and to identify specific epitopes that serve as targets for neutralizing antibody. These MAbs were then used to determine if the usefulness of the virus marker system previously described could be enhanced.

**Materials and methods**

**Viruses and cells**

Sixty-nine PRRS virus isolates of North American origin and the Lelystad virus of European origin used in the present study were also used in an earlier study [43]. The NA PRRS viruses included ATCC VR-2332, ISU-P (ATCC VR-2402), 2 modified-live vaccine viruses, RespPRRS/Repro (Boehringer Ingelheim/NOBL Laboratories, Sioux Center, Iowa, USA) and PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, Nebraska, USA). All field isolates were isolated and propagated at least one time in MARC-145 cells [17]. Vaccine viruses used in the present study were propagated one time in MARC-145 cells. Virus inoculated and uninoculated MARC-145 cells were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% and 2% fetal bovine serum respectively at 37 °C in a 5% carbon dioxide atmosphere.

**Antigen preparations**

Two antigen preparations were produced as previously described [43] for use in Western immunoblotting (WI) and the indirect enzyme-linked immunosorbent assay (ELISA). A detergent solubilized lysate of MARC-145 cells infected with PRRS virus, KY
35 was used in both WI and the ELISA. Semi-purified whole virus preparations derived from MARC-145 cells infected with virus KY35 were used only in WI.

Antigen for radioimmunoprecipitation (RIP) was prepared as described by Burleson et al. [3]. Briefly, MARC-145 cell monolayers in 25 cm² flasks were inoculated with PRRS virus KY 35 at a multiplicity of infection (MOI) of 5 or left as uninoculated controls. Medium was removed from virus infected and uninfected cells 36 h after virus or sham inoculation. The cell monolayers were washed 3 times with serum-free and methionine-free DMEM and subsequently replenished with the same medium at the rate of 2 ml per flask. After 1.5 h of incubation at 37 °C the medium was again replaced with 2 ml of serum-free DMEM supplemented with 40 µCi of 35S-methionine per ml (ICN Biomedical, Inc., Costa Mesa, CA, USA) and incubated for an additional 5 h. The medium was removed and cell monolayers were washed once with lysis buffer (0.25 M Tris/0.11 M Tricine buffer, pH 8.6, containing 0.14 mM calcium lactate and 1% (V/V) TritonX-100). Virus-infected and uninfected cells were treated with 10 volumes of cold lysis buffer for 15 min at 4 °C. Cell lysates were centrifuged for 4 min at 10,000 x g. The supernatant fluids containing 35S-methionine labeled viral and cell control antigen were collected and stored at -80 °C until used.

Antigen for mouse inoculation was prepared from MARC-145 cells singly infected at a MOI of 5 with PRRS viruses ATCC VR-2332 or KY 35. Four days later, virus infected cells were frozen and thawed 2 times at -80 °C and +37 °C. The suspension was centrifuged at 2,000 x g for 30 min at 4 °C. Supernatant fluid containing virus was collected and stored at -80 °C until used.
Western immunoblotting

Virus and cell control antigens were mixed 1:1 with sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 100 μg bromphenol blue) and boiled for 3 min. Individual proteins were separated by SDS-PAGE on 14% acrylamide gels. The separated proteins were electroblotted onto nitrocellulose membranes. The nitrocellulose strips were immersed overnight in 0.01 M phosphate buffered saline (PBS) containing 5% (W/V) skim milk to block non-specific binding by antibody. Subsequently, nitrocellulose strips were incubated 2 h at 37 °C with supernatant fluids of hybridoma cell cultures or ascitic fluids containing specific MAbs. The nitrocellulose strips were then washed 4 times in PBS containing 0.05% Tween 20 and treated for 1.5 h at 37 °C with horseradish peroxidase conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) diluted 1:1000 in PBS. Serological reactivity between MAbs and specific protein bands was visualized by adding the substrate 3, 3', 5, 5'-tetramethylbenzidine (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) to the strips after a final washing.

Radioimmunoprecipitation (RIP)

Eighty μl of virus and cell control antigens were separately incubated on a roller apparatus overnight with 4 μl of ascitic fluid containing specific MAbs. The antigen-antibody complexes were collected by adding 50 μl of protein A-Sepharose CL-4B beads (Sigma Company, St. Louis, MO, USA) to the mixtures and incubating at 4 °C for an additional hour. The precipitates were washed 3 times in lysis buffer, 3 times in PBS, resuspended in an equal volume of the sample buffer described above and boiled for 3 min. Individual ³⁵S-methionine labeled proteins were separated by SDS-PAGE in 14% acrylamide
gels. Electrophoresed gels were impregnated with 2, 5-diphenyloxazole scintillate in dimethyl sulfoxide, washed 3 times with deionized water, dried under vacuum and exposed to XAR5 film (Eastman Kodak Co, Rochester, NY) at -80 °C for 3 to 5 days.

**Additivity and blocking assays**

The additivity assay and blocking ELISA were used to confirm the epitope specificity of individual MAbs with unique sero-reactivity patterns. The additivity assay originally described by Friguet et al. [14] was conducted as modified by Yang et al. [43] using affinity purified MAb from ascitic fluid. Serological reactivity between MAbs of unknown epitope specificity and antigen, and homologous MAbs and antigen was expressed as a corrected optical density (OD). Mean additivity values (AVs) for MAb interactions were calculated by the formula:

\[
AV = \frac{\text{Corrected OD MAb } a + b}{(\text{Corrected OD MAb } a) + (\text{Corrected OD MAb } b)} \times 100% 
\]

where MAb "a" and "b" are any 2 MAbs tested for additivity. The theoretical AVs of homologous and heterologous MAb interactions should approach 50 and 100% respectively. Differences between AVs were analyzed for significance by Student's t-test.

The blocking ELISA originally described by Cecilia et al. [5] and modified by Yang et al. [43] was conducted using affinity purified MAbs from ascitic fluid. Percent binding was calculated for each MAb interaction by comparing the corrected ODs generated in viral and cell antigen preparations treated with blocking MAb to the corrected ODs generated following treatment of antigen and cell controls with diluent. Differences between mean
percent bindings representing at least 3 replications for individual MAbs were compared for significance by the one-way analysis of variance. Significant (P < 0.05) inhibition of binding by blocking MAbs was considered to be an indication of identity or interference by adjacent or overlapping epitopes.

**Virus neutralization**

The constant virus varying antibody protocol was used to detect neutralizing activity of MAbs in ascitic fluids as previously described [43] with the exception that PRRS virus KY 35 was used as antigen. Neutralization titers were expressed as the reciprocal of the highest dilutions of MAbs that completely inhibited CPE formation.

The constant antibody varying virus protocol was used to detect susceptibility of viruses to neutralization by polyclonal swine serum and neutralizing MAbs. Individual viruses were serially diluted 10-fold in DMEM supplemented with 2% FBS and antibiotics. Fifty µl aliquots of each virus dilution and equal quantities of swine antiserum or ascitic fluid were added together in duplicate wells of 96-well microtiter plates and incubated at 37 °C for 1 h. One hundred µl of a MARC-145 cell suspension diluted to contain 10^5 cells was added to each well including wells representing virus and cell controls. Microtiter plates were incubated for 5 days at 37 °C and observed for CPE. Titers of individual viruses in the presence and absence of specific neutralizing MAbs were expressed as TCID_{50} and neutralization indices were calculated. Assays were replicated 4 times.

**MAb production**

Monoclonal antibodies were generated as previously described [43] with the exception that a protocol to establish partial immunotolerance [23] to the N protein of PRRS
virus was adapted. PRRS viruses ATCC VR-2332 and KY 35 were selected from 34 NA viruses. These 2 viruses were the most susceptible and the most resistant, respectively to neutralization by polyclonal swine serum raised against ATCC VR-2332. However, all 5 continuous epitopes of the N protein [43] were present in both viruses. Consequently, ATCC VR-2332 was used to partially immunotolerize mice to the N protein and KY 35 was subsequently used in the generation of MAbs.

Eight-week-old BALB/c mice were injected intraperitoneally with 0.5 ml of virus suspension diluted to contain $10^6$ TCID$_{50}$ of PRRS virus ATCC VR-2332. Ten minutes later, mice were injected intraperitoneally with cyclophosphamide at the rate of 100 mg/kg (Sigma Chemical Company, St. Louis, MO, USA) prepared in PBS. Mice were injected a second and third time with cyclophosphamide at 24 h and 48 h after the first injection. This treatment was repeated 2 times at 2 week intervals. Three weeks after the last tolerizing treatment, mice were injected intraperitoneally with 0.5 ml of adjuvanted viral antigen prepared by adding together equal volumes of aluminum hydroxide adjuvant (Armour Pharmaceutical Company, IL, USA) and virus suspension containing $10^{6.3}$ TCID$_{50}$/ml of PRRS virus KY 35. Mice were injected with adjuvanted antigen a second time 2 weeks later and maintained for 2 to 3 weeks before being used for hybridoma cell production. Mice were injected intraperitoneally with 0.5 ml of KY 35 virus suspension without adjuvant 3 days before harvesting spleens.

Characterization of PRRS viruses

The sero-reactivity of the 69 NA PRRS viruses and the Lelystad virus was determined for each MAb using the indirect immunofluorescence assay as described by Frey
et al. [13]. Individual viruses with common epitope profiles were subsequently categorized into subgroups within each antigenic group.

Twenty eight NA viruses representing antigenic groups IV₁₅ and VI₁₅, and 15 subgroups representing antigenic groups I₁₅, II₁₅ and III₁₅ were evaluated for their relative sensitivity to neutralization by MAbs ISU19A1, 19B1 and 25C1 using the constant antibody varying virus method. Differences between mean NIs of individual viruses were analyzed for significance by the one-way analysis of variance.

Results

Generation of MAbs and identification of epitopes

Twenty-one antibody-secreting hybridoma cells were generated from 5 fusions. These MAbs represented the N, M, GP3 and GP5 proteins of PRRS virus, respectively.

Identification of epitopes

N protein

Twelve (57%) of the 21 MAbs were specific for the N protein. Two of the 12 MAbs represented the continuous epitopes EpORF7-A and B that were previously described [43]. Their identity was determined by sero-reactivity in WI and their sero-reactivity with the panel of 70 PRRS viruses (Table 1). The remaining 10 MAbs reacted with native antigen in RIP (Fig.1) but not with denatured antigen in WI, indicating that these 10 MAbs recognized discontinuous epitopes. Sero-reactivity with the panel of 70 PRRS viruses (Table 1) indicated that these 10 MAbs represented 3 discontinuous epitopes designated EpORF7-Fd, Gd and Hd and were represented by MAbs ISU15Fd1 through Fd3, ISU15Gd1 through Gd4
Table 1. Categorization of 70 PRRS viruses by sero-reactivity with monoclonal antibodies representing 5 continuous and 3 discontinuous epitopes of the N protein as determined by the indirect immunofluorescence antibody test

<table>
<thead>
<tr>
<th>Antigenic group</th>
<th>No. of PRRS viruses</th>
<th>Monoclonal antibody group&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>I&lt;sub&gt;15&lt;/sub&gt;</td>
<td>57&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
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<td>+</td>
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<tr>
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<tr>
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<tr>
<td>VI&lt;sub&gt;15&lt;/sub&gt;</td>
<td>1</td>
<td>+</td>
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</tbody>
</table>

<sup>a</sup> Monoclonal antibody groups A through Hd represent specific epitopes of the N protein EpORF7-A through Hd, respectively.

<sup>b</sup> The lowercase "d" indicates that the epitopes represented by these 3 groups of monoclonal antibodies are discontinuous.

<sup>c</sup> Includes PRRS viruses KY 35 (NVSL 46907), ATCC VR-2332, ISU-P (ATCC VR-2402, the prototype virus used for the inactivated vaccine PRRomiSe by Bayer Corporation, Shawnee Mission, KS, USA) and the 2 modified-live vaccine virus strains, PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA), and RespPRRS/Repro (Boehringer Ingelheim/NOBL Laboratories, Sioux Center, IA, USA).

<sup>d</sup> The Lelystad virus.

<sup>e</sup> Weak positive reaction.

M protein

Three of the 21 MAbs represented the M protein. They were designated MAbs ISU19A1, B1 and C1 and were shown to represent 3 discontinuous epitopes designated EpORF6-Ad, Bd and Cd, respectively. The uniqueness of EpORF6-Cd from EpORF6-Ad and Bd was demonstrated by sero-reactivity patterns of the 3 MAbs with the panel of 70 PRRS viruses (Table 2). The sero-reactivity patterns of MAbs ISU19A1 and B1 with this panel of viruses were the same. However, these 2 MAbs were shown to represent different epitopes by the Veterinary Diagnostic Laboratory at South Dakota State University (Data not shown). Monoclonal antibody ISU19A1 sero-reacted with PRRS viruses 92-23224 and 98-
Fig. 1. Radioimmunoprecipitation. A, B, C and D represent treatments with monoclonal antibodies ISU45A1 through B1, ISU25A1 through C1, ISU19Ad1 through Cd1, and ISU15A1 through Hd1, respectively. The left lane of each panel represents virus antigen derived from MARC-145 cells infected with PRRS virus KY 35 (NVSL 46907). The right lane of each panel represents cell control antigen. Molecular weight markers (in kilodalton) are indicated in the left margin.
Table 2. Characterization of 70 PRRS viruses by sero-reactivity with monoclonal antibodies specific for the N, M, GP5 and GP3 proteins as determined by the indirect immunofluorescence antibody test

<table>
<thead>
<tr>
<th>Antigenic group</th>
<th>Subgroup</th>
<th>No. of PRRS viruses</th>
<th>ISU19A1 and B1</th>
<th>ISU19C1</th>
<th>ISU25A1</th>
<th>ISU25B1</th>
<th>ISU25C1</th>
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<th>ISU45B1</th>
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</table>

<sup>a</sup> Based on the distribution of continuous and discontinuous epitopes of the N protein

<sup>b</sup> Monoclonal antibody ISU19B1 is considered to be different from ISU19A1 because it did not react with PRRS viruses 92-23224 and 98-15995 when tested at South Dakota State University, Brookings, USA

<sup>c</sup> Includes PRRS virus KY 35 (NVSL 46907)

<sup>d</sup> Includes ATCC VR-2332 and the modified-live vaccine virus strain RespPRRS/Repro (Boehringer Ingelheim/NOBL Laboratories, Sioux Center, IA, USA)

<sup>e</sup> The virus ISU-P (ATCC VR-2402), the prototype virus used for the inactivated vaccine PPRoMiSe (Bayer Corporation, Shawnee, KS, USA)

<sup>f</sup> The modified-live vaccine virus strain PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA)

<sup>g</sup> The Lelystad virus
15995 but MAb ISU19B1 did not. The discontinuous nature of these 3 epitopes prevented further confirmation of their uniqueness by the blocking and additivity assays. All 3 of these MAbs were of the IgG1 subclass and had kappa light chains.

**GP5 protein**

Three of the 21 MAbs represented the GP5 protein as determined by both WI and RIP. These MAbs were designated ISU25A1, B1 and C1 and were shown by their seroreactivity patterns with the panel of PRRS viruses to represent 3 continuous epitopes that were designated EpORF5-A, B and C (Table 2). The distinctness of each epitope was further demonstrated by both additivity and blocking assays (Tables 3 and 4). The differences

<table>
<thead>
<tr>
<th>Added MAb</th>
<th>ISU25A1</th>
<th>ISU25B1</th>
<th>ISU25C1</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52</td>
<td>0.41</td>
</tr>
<tr>
<td>ISU25A1</td>
<td>54.3% (0.63/1.16)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.8% (0.57/1.04)</td>
<td>92.5% (0.86/0.93)</td>
</tr>
<tr>
<td>ISU25B1</td>
<td>86.4% (0.95/1.10)</td>
<td>92.5% (0.86/0.93)</td>
<td>52.4% (0.43/0.82)</td>
</tr>
<tr>
<td>ISU25C1</td>
<td>86.2% (0.94/1.09)</td>
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</table>

<sup>a</sup> Optical density was measured at 405 nm.

<sup>b</sup> Additivity values (AVs) expressed as binding percent were calculated using the formula:

\[
AV = \frac{\text{Corrected OD MAb } a + b}{\text{(Corrected OD MAb } a) + (\text{Corrected OD MAb } b)} \times 100\%
\]

where MAbs "a" and "b" are any 2 MAbs tested for additivity.

The measured OD values and theoretical sum of OD values of any 2 MAbs are indicated in parenthesis. AVs ≤ 50% indicate identity.
Table 4. Epitopic analysis of the GP5 protein of PRRS virus KY 35 (NVSL 46907) by the blocking ELISA using 3 monoclonal antibodies (MAbs) ISU25A1, B1 and C1

<table>
<thead>
<tr>
<th>Biotinylated MAb</th>
<th>Blocking MAb</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>ISU25A1</td>
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<tr>
<td>ISU25A1</td>
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<tr>
<td>ISU25B1</td>
<td>-</td>
</tr>
<tr>
<td>ISU25C1</td>
<td>-</td>
</tr>
</tbody>
</table>

* Significant (P < 0.001) inhibition by blocking MAbs. The mean percent binding for homologous MAb interactions at the lowest dilution (1:10) of blocking MAbs was 33.5 ± 2.6 and ranged from 30.5 ± 3.2 to 35.6 ± 2.1.

b No inhibition by blocking MAbs. The mean percent binding for heterologous interactions at the lowest dilution (1:10) of blocking MAbs was 86.1 ± 2.4 and ranged from 82.8 ± 1.9 to 88.9 ± 1.5.

between homologous and heterologous interactions in both assays were significant (P < 0.001). The mean AVs of the 3 homologous MAb interactions in additivity assays was 53.8 ± 1.4% and ranged from 52.4 to 54.8%, indicating identity. The mean AVs of the 3 heterologous interactions was 88.4 ± 2.8% and ranged from 86.8 to 92.5 %, indicating nonidentity. Similarly, the mean percent binding in homologous interactions was 33.5 ± 2.6% and ranged from 30.5% to 35.6%, indicating identity. The mean percent binding in heterologous interactions was 86.1 ± 2.4% and ranged from 82.8% to 88.9%, indicating nonidentity. All 3 MAbs were of the IgG1 subclass with lambda light chains.

**GP3 protein**

Three of the 21 MAbs represented the GP3 protein as determined by both WI and RIP. These MAbs were designated ISU45A1, A2 and B1 and were shown by their seroreactivity patterns with the panel of 70 PRRS viruses to represent 2 continuous epitopes that
were designated EpORF3-A and B (Table 2). The distinctness of the 2 epitopes was defined further by both additivity and blocking assays. The mean AV observed following interaction of MAbs ISU45A1 and A2 was 58.6 ± 1.5% indicating identity (Table 5). This AV was significantly (P < 0.001) different from the AV observed following the interaction of MAbs ISU45A1 or A2 with MAb ISU45B1. These values were 92.3 ± 2.0% and 94.9 ± 1.8% indicating nonidentity. Similarly, percent binding generated by the reciprocal interactions between MAbs ISUA1 and A2 was 30.5 ± 3.0 and 31.7 ± 1.8 % which indicated that significant (P<0.001) blocking occurred (Table 6). These percent binding values were significantly different (P < 0.001) from those that were observed following reciprocal

Table 5. Epitopic analysis of the GP3 protein of PRRS virus KY 35 (NVSL 46907) by the additivity test using 3 monoclonal antibodies (MAbs) ISU45A1, B1 and C1

<table>
<thead>
<tr>
<th>Added MAb</th>
<th>Test MAb</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ISU45A1</td>
<td>ISU45A2</td>
<td>ISU45B1</td>
</tr>
<tr>
<td>None</td>
<td>0.52*</td>
<td>0.59</td>
<td>0.45</td>
</tr>
<tr>
<td>ISU45A1</td>
<td>52.9%</td>
<td>(0.55/1.04)b</td>
<td></td>
</tr>
<tr>
<td>ISU45A2</td>
<td>58.6%</td>
<td>57.6%</td>
<td>(0.65/1.11)</td>
</tr>
<tr>
<td>ISU45B1</td>
<td>94.9%</td>
<td>92.3%</td>
<td>56.7%</td>
</tr>
<tr>
<td></td>
<td>(0.92/0.97)</td>
<td>(0.96/1.04)</td>
<td>(0.51/0.90)</td>
</tr>
</tbody>
</table>

a Optical density was measured at 405 nm.

b Additivity values (AVs) expressed as binding percent were calculated using the formula:

\[
\text{AV} = \frac{\text{Corrected OD MAb } a + b}{(\text{Corrected OD MAb } a) + (\text{Corrected OD MAb } b)} \times 100\%
\]

where MAbs “a” and “b” are any 2 MAbs tested for additivity.

The measured OD values and theoretical sum of OD values of any 2 MAbs are indicated in parenthesis. AVs = 50% indicate identity.
Table 6. Epitopic analysis of the GP3 protein of PRRS virus KY 35 (NVSL 46907) by the blocking ELISA using 3 monoclonal antibodies (MAbs) ISU45A1, A2 and B1

<table>
<thead>
<tr>
<th>Biotinylated MAb</th>
<th>ISU45A1</th>
<th>ISU45A2</th>
<th>ISU45B1</th>
</tr>
</thead>
</table>
| ISU45A1          | +
| ISU45A2          | +       | +       | -       |
| ISU45B1          | -       | -       | +       |

* Significant (P < 0.001) inhibition by blocking MAbs. The mean percent binding for homologous MAb interactions at the lowest dilution (1:10) of blocking MAbs was 32.9 ± 2.4 and ranged from 31.9 ± 2.5 to 34.6 ± 2.4. The mean percent bindings for interactions of MAbs ISU45A1 and A2 were 30.5 ± 3.0 and 31.7 ± 1.8.

* No inhibition by blocking MAbs. The mean percent bindings for interactions of biotinylated MAbs ISU45A1 and A2 with blocking MAb ISU45B1 were 85.6 ± 1.6 and 84.8 ± 2.7. The mean percent bindings for interactions of biotinylated MAb ISU45B1 with blocking MAbs ISU45A1 and A2 were 89.6 ± 2.0 and 90.5 ± 2.3.

Interactions between MAbs ISU45A1 and A2 with MAb ISU45B1. The mean percent binding for interactions of biotinylated MAbs ISU45A1 and A2 with blocking MAb ISU45B1 was 85.6 ± 1.6% and 84.8 ± 2.7%, indicating nonidentity. The mean percent binding for interactions of biotinylated MAb ISU45B1 with blocking MAbs ISU45A1 and A2 was 89.6 ± 2.0% and 90.5 ± 2.3%. These observations indicated that the epitope represented by MAbs ISU45A1 and A2 was different from the epitope represented by MAb ISU45B1. All of the GP3 specific MAbs were of the IgG1 subclass with kappa light chains.

Categorization of NA PRRS viruses

Monoclonal antibodies that represented 9 of the 11 epitopes identified for the NA PRRS virus, KY 35 expanded the virus marker system that was initially based on the distribution of 5 continuous epitopes of the N protein among viruses. Antigen group VI15
was created when EpORF7-Fd was not detected in a virus formerly of group I$_{15}$ and as a result increased the number of antigenic groups representing NA viruses from 4 to 5 (Table 1). The use of the remaining 8 MAbs further subdivided antigenic groups into subgroups (Table 2). Antigenic group I$_{15}$ was represented by 9 subgroups. Subgroups 1, 2 and 3 of I$_{15}$ were represented by 17 (24.6%), 30 (43.5%) and 4 (5.8%) of all NA PRRS viruses tested. The remaining 6 subgroups of I$_{15}$ were represented by single viruses. Antigenic group II$_{15}$ was represented by 4 subgroups. Subgroup 1 of this group was represented by 5 viruses and the remaining 3 subgroups were represented by single viruses. Antigenic group III$_{15}$ was represented by 2 subgroups, each containing single viruses. Antigenic group IV$_{15}$ was represented by a single NA virus while antigenic group V$_{15}$ was represented by the Lelystad virus. This categorization of NA PRRS viruses by epitopic profiles also permitted differentiation of vaccine viruses from field isolates. North American PRRS viruses were also distinguished from the Lelystad virus which shared only 2 epitopes, EpORF7-A and Hd, in common with NA viruses used in this study (Table 7).

**Susceptibility of viruses to neutralization by MAbs**

Monoclonal antibodies ISU19A1, 19B1 and ISU25C1 which neutralized PRRS virus KY 35 were used to compare the relative susceptibility of NA PRRS viruses to neutralization. The mean neutralization titer of the ascitic fluid representing these 3 MAbs was 4, 8 and 4 respectively. The variation in sensitivity to neutralization of the 28 viruses that represented antigenic groups IV$_{15}$ and VI$_{15}$ and the 15 subgroups of antigenic groups I$_{15}$, II$_{15}$ and III$_{15}$ was represented by continuums (Figure 2). The differences between the mean neutralization indices of specific groups of viruses at the ends these continuums were
Table 7. Epitope profiles of selected PRRS field viruses and vaccine strains as determined by a panel of monoclonal antibodies specific for the GP3, GP5, M and N proteins

<table>
<thead>
<tr>
<th>PRRS virus</th>
<th>Antigenic group/subgroup</th>
<th>EpORF3-*</th>
<th>EpORF5-</th>
<th>EpORF6-</th>
<th>EpORF7-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A  B</td>
<td>A  B  C</td>
<td>Ad  Bd  Cd</td>
<td>A  B  C  D  E  Fd  Gd  Hd</td>
</tr>
<tr>
<td>KY 35</td>
<td>I₁₅ / 1</td>
<td>+ + + + +</td>
<td>+ + + A  B  C  D  E  Fd  Gd  Hd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISU-P&lt;sup&gt;c&lt;/sup&gt;</td>
<td>I₁₅ / 4</td>
<td>- - + + + + + - - + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VR-2332</td>
<td>I₁₅ / 2</td>
<td>- - + + + + + + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccine&lt;sup&gt;d&lt;/sup&gt;</td>
<td>I₁₅ / 2</td>
<td>- - + + + + + + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccine 2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>I₁₅ / 5</td>
<td>- - + + + - - - - + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV&lt;sup&gt;f&lt;/sup&gt;</td>
<td>V₁₅ / 1</td>
<td>- - - - - - - - - - + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> EpORF3-A and B, EpORF5-A, B, and C, EpORF6-Ad, Bd and Cd, and EpORF7-A through Hd represent epitopes of the GP3, GP5, M and N proteins, respectively

<sup>b</sup> The lowercase "d" indicates a discontinuous epitope

<sup>c</sup> The virus ISU-P (ATCC VR-2402) is the prototype virus used for the inactivated vaccine PRRomiSe (Bayer Corporation, Shawnee Mission, KS, USA)

<sup>d</sup> The modified-live vaccine virus RespPRRS/Repro (Boehringer Ingelheim/NOBL Laboratories, Sioux Center, IA, USA)

<sup>e</sup> The modified-live vaccine virus PRIME PAC PRRS (Schering-Plough Animal Health Corporation, Omaha, NE, USA)

<sup>f</sup> The Lelystad virus

significant (P<0.05) and ranged from 1.78 for viruses treated with MAb ISU19B1, to 0.85 and 0.75 for viruses treated with MAbs ISU19A1 and 25C1 respectively. Viruses IA-D21 of group III₁₅/subgroup 2 and NVSL 22332 of group I₁₅/subgroup 2 were among the most resistant to neutralization by all 3 MAbs. In contrast, viruses ATCC VR-2332 and NVSL 46448 of group I₁₅/subgroup 2, NVSL 9257 of group I₁₅/subgroup 3, and NVSL 6725 of group IV₁₅ were among the most susceptible to neutralization.
Fig. 2. Susceptibility of PRRS viruses to neutralization mediated by monoclonal antibodies (MAbs) ISU19A1, B1 and 25C1 using constant concentrations of MAbs and varying virus dilutions. No significant differences exist among viruses underlined by overlapping segments of lines as determined by the one-way analysis of variance. Least significant difference does not exceed 0.5797 for MAb ISU19A1, 0.7933 for MAb ISU19B1, and 0.5344 for MAb ISU25C1. Viruses without prefixes were obtained from National Veterinary Services Laboratories, United States Department of Agriculture, Ames, Iowa, USA.
Discussion

In the present study MAbs were generated and used to further characterize the antigenic nature of PRRS virus proteins, identify epitopes associated with neutralization and to increase the effectiveness of the virus marker system described in a previous study [43]. In that study only MAbs specific for the 5 continuous epitopes of the N protein were produced. No MAbs were generated to other structural proteins including envelope-associated proteins which are of particular interest with respect to their role in mediating susceptibility of PRRS virus to both antibody mediated neutralization and enhancement of virus infection [45]. The lack of representation of other proteins by MAbs in that study was attributed to the highly immunodominant nature of the N protein. Consequently, the immunotolerizing procedure described by Matthew and Sandrock [23] was adapted in the present study to establish partial immunotolerance in mice to the N protein to increase the likelihood of generating MAbs that represented different proteins. This strategy was successful because only 12 of the 21 (57%) MAbs that were produced in the present study represented the N protein and 9 (43%) MAbs represented the envelope-associated M, GP3 and GP5 proteins. These 21 MAbs represented 3 discontinuous epitopes of both the N and M proteins, 3 continuous epitopes of the GP5 protein and 2 continuous epitopes of the GP3 protein.

The identification of 3 discontinuous epitopes of the N protein in the present study increased the number of known discontinuous epitopes distributed among NA viruses from 2 to 4. The MAbs representing these 4 epitopes include ISU15Fd, Gd and Hd described in the present study, and MAb SDOW17 described by Nelson et al. [31]. The epitopes represented by MAbs ISU15Fd, Gd and Hd are present on all group I15 viruses which include PRRS.
viruses KY 35, ATCC VR-2332, ISU-P and the vaccine viruses RespPRRS / Repro and PRIME PAC PRRS (Table 1). The epitope represented by SDOW17 is present on all of these viruses except the vaccine virus PRIME PAC PRRS, which demonstrates its uniqueness [44]. Additional discontinuous epitopes may be represented by MAbs EP147 and VO17. These MAbs have a different epitope specificity from MAb SDOW17 [31]. However, additional comparison of sero-reactivity patterns of MAbs ISU15Fd and Gd with the sero-reactivity patterns of EP147 and VO17 are required to determine whether or not MAbs EP147 and VO17 represent additional discontinuous epitopes among NA viruses.

The 3 discontinuous epitopes of the M protein that were described in this study are the only discontinuous epitopes that have been reported for this protein to date. The identification of EpORF6A and B as targets for neutralizing antibody suggests that the domain of these epitopes is associated with the 10 to 18 amino acid portion of the M protein that is believed to be located on the surface of the virion and involved in receptor binding [36].

The identification of 3 continuous epitopes of the GP5 protein of isolate KY 35 increased the known number of continuous epitopes associated with this protein among NA viruses from 3 to at least 4. These epitopes include those represented by MAbs ISU25A1 and B1 in addition to the epitopes represented by the 2 groups of neutralizing MAbs described by Pirzadeh and Dea [33], i.e., MAbs IAFA8A8 and 1B8 and MAbs 2A5, 3A12 and 1C10. A non-neutralizing MAb described by Zhang et al. [47] may represent a fifth epitope distributed among NA viruses or represent the same epitope as either MAb ISU25A1 or 25B1. The neutralizing MAb ISU25C1 may represent a sixth epitope of the GP5 protein or
represent an epitope that is detected by MAbs IAF-8A8 and IAF-1B8. All 3 of these neutralizing MAbs react with ATCC VR-2332 but not with the Lelystad virus.

The identification of 2 continuous epitopes of the GP3 protein represented by MAbs ISU45A1 and B1 increased the known number of continuous epitopes associated with this protein among NA viruses from 3 to at least 5. The non-reactivity of these 2 MAbs with both ATCC VR-2332 and the Lelystad virus clearly demonstrates that the epitopes which they represent are distinct from epitopes represented by MAbs P7/a1-19, P9/a3-20, and P9/b3-1 [42] that are present in both NA viruses and the Lelystad virus. The epitopes represented by MAbs ISU45A1 and B1 are also different from the epitope represented by MAb 122.14. This epitope was detected in the Lelystad virus and 3 NA viruses [18, 39]. Additional comparisons are required to determine whether MAb 122.14 is different from MAbs P7/a1-19, P9/a3-20, and P9/b3-1.

The identification of EpORF6-A and B as targets for neutralizing MAbs in the present study is the first report that the M protein plays a role in the susceptibility of PRRS virus to neutralization. Prior to the current study only the GP4 and GP5 proteins had been identified as targets for neutralizing antibody. The role of the GP5 protein as a neutralizing antibody-inducing protein was first demonstrated by Persch et al. [32] who raised neutralizing antibody in pigs inoculated with native GP5 protein purified by PAGE. The role of the GP4 protein as a target of neutralization was demonstrated by van Nieuwstadt et al. [39] and Meulenberg et al. [28] who generated neutralizing MAbs that represented at least one distinct epitope of this protein and more recently by Weiland et al. [40] who described 2 MAbs (P11a/60 and P11/d26) which may or may not represent distinct epitopes.
The relative importance of the GP4 and GP5 proteins with respect to their ability to induce neutralizing antibody has been demonstrated by Gonin et al. [15]. These investigators compared neutralizing antibody titers of convalescent sera from PRRS virus-infected pigs to ELISA antibody titers using as antigen, recombinant N, GP3, GP4 and GP5 proteins that were expressed in *Escherichia coli*. A highly significant correlation was observed between neutralizing titers and antibody specific for the GP5 protein. No correlation was observed between neutralizing antibody and antibody specific for the N protein or the envelope associated GP3 or GP4 proteins suggesting that these proteins played a minor role in the induction of neutralizing antibody. The M protein was not evaluated in that study. However, in the present study ascitic fluid containing MAbs ISU19A1 and B1 had neutralizing titers of 4 and 8. These relatively low titers could be an indication that the M protein like the GP4 protein plays a minor role in the susceptibility of PRRS virus to neutralization. This possibility is reflected in the study by Weiland et al. [40] who generated MAb preparations under similar conditions with neutralizing titers ranging from 80 to 1280 for MAbs representing the GP5 protein and from 2 to 64 for MAbs representing the GP4 protein.

The effectiveness of the marker system described in an earlier study was markedly increased by the use of all of the MAbs that represented the M, GP3 and GP5 proteins and MAb ISU15Fd1 that represented one of the 3 discontinuous epitopes of the N protein (Table 2). However, while 13 of the 15 antigenic subgroups represented 1 to 5 of the 69 NA PRRS viruses each, subgroups 1 and 2 of antigenic group I15 represented 17 (24.6%) and 30 (43.5%) viruses, respectively. This disproportionately large representation of NA viruses by 2 antigenic subgroups indicates that additional MAbs will be needed if antigenic characterization is to be a useful virus marker.
Alternatively, additional markers such as relative sensitivity to neutralization may prove useful adjuncts to further categorize individual viruses within specific antigenic groups. The relatively wide spectrum of sensitivity of PRRS viruses to neutralization by MAbs ISU25C1, 19A1 and 19B1 in which significant differences (P ≤ 0.05) were shown demonstrates this point (Fig. 2). This variation in susceptibility to neutralization was not unexpected. A previous study by Yoon et al. [46] also demonstrated a wide range of susceptibility to neutralization when PRRS viruses were treated with polyclonal swine serum. Variation in susceptibility to neutralization could be a reflection in distribution and number and / or accessibility of specific epitopes to neutralizing antibodies. Consequently, signature analysis described by Monath et al. [29] for dengue virus may also be of value in categorizing PRRS viruses.

Acknowledgements

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References


STABILITY OF EPITOPE PROFILES OF NORTH AMERICAN PORCINE
REPRODUCTIVE AND RESPIRATORY SYNDROME
VIRUSES DURING SERIAL PASSAGE IN PIGS

A paper to be submitted to the Journal of General Virology

Liuzhan Yang, Rossana Allende, Fernando A. Osorio and Kenneth B. Platt

Abstract

The stability of 18 epitopes distributed among the N, M, GP5 and GP3 proteins of porcine reproductive and respiratory syndrome (PRRS) virus was evaluated by serially passaging 5 North American viruses in pigs in 3 separate experiments. In experiment 1, 10 pigs were inoculated intranasally with the PRRS virus 16244B and housed separately. The PRRS virus was isolated from tonsil biopsies of 5 pigs at 12 weeks post-inoculation (p.i.), and from homogenates of tonsil, lung, lung lymph nodes when 2 of these 5 pigs were killed at 22 weeks p.i.. In experiment 2, four groups of three pigs each were inoculated intranasally with PRRS viruses NVSL 6725, 14620, 46489 and 46907, respectively. Each group of 3 pigs was housed separately. These groups of pigs were killed at 6, 7, 4 and 4 weeks p.i., respectively. The PRRS virus was isolated from homogenates of tonsil, lung, lung lymph node and spleen tissues prepared from individual pigs of each group. A tissue homogenate from one randomly selected pig of each group was used to inoculate a second group of 3 pigs. This protocol was repeated again for a third group of 3 pigs. Consequently, NVSL 6725, 14620, 46489 and 46907 were serially passaged through 3 pigs for 20, 21, 16 and 19 weeks, respectively. In experiment 3, the viruses NVSL 6725 and 14620 were separately
serially passaged through 3 pigs essentially as described in experiment 2 with the exception that PRRS virus was isolated from tissue homogenates in MARC-145 cell cultures between pig passages. The two viruses were each serially passaged for a total of 19 weeks. No changes in the sero-reactivity with the panel of 18 MAbs were observed in all 5 viruses following in vivo passage in individual pigs. The stability of the 18 epitopes during maintenance and passage in pigs justifies their inclusion in an epitope-based virus marker system for PRRS viruses.

Porcine reproductive and respiratory syndrome (PRRS) virus is a single-stranded, positive-sense RNA virus of the Arteriviridae (Cavanagh, 1997). The genome of PRRS virus is approximately 15 kb and consists of 8 overlapping open reading frames (ORFs) (Meulenberg et al., 1993). The ORFs la and lb encode nonstructural proteins associated with viral replicase activity (Meulenberg et al., 1993). The ORFs 2 through 7 encode 6 structural proteins designated GP2 through GP5, M and N, respectively (Conzelmann et al., 1993; Meulenberg et al., 1993; Meulenberg et al., 1995; Meulenberg JJM, Petersen-Den Besten, 1996; Van Nieuwstadt et al., 1996). The PRRS virus was first isolated by European investigators in 1991 and named the Lelystad virus (Wensvoort et al., 1991). A similar virus ATCC VR-2332 was subsequently isolated from North American swine in 1992 (Benfield et al., 1992; Collins et al., 1992). The virus continues to be a significant economic problem for the swine industry. Efforts to control PRRS by vaccination and other management strategies have not met with consistent success.

To better understand the epidemiology of PRRS virus, a marker system based on 18 epitopes distributed among the N, M, GP5 and GP3 proteins has been developed and used to
characterize PRRS viruses (Yang et al., 1999; Yang et al., 2000a, 2000b). Currently North American PRRS viruses can be categorized into 5 major antigenic groups representing 17 subgroups. It is anticipated that this marker system will be expanded as additional epitopes are characterized. The effective and reliability of this system is dependent on the stability of individual epitopes of the PRRS virus in swine population.

Recent work by Le Gall et al. (1997) suggests that an epitope can be lost or gained during several passages of the PRRS virus in pigs. These investigators serially passaged the European PRRS viruses designated I and II in pigs in 2 separate experiments. In the first experiment, 5 pigs were infected with virus I. Five weeks later, virus I was isolated from each pig. The epitopic profile of virus I before and after passage was compared using a panel of 14 monoclonal antibodies (MAbs) representing the N, M, GP3 and GP4 proteins. No differences were observed in the epitopic profile of the uncloned population of virus I isolated from 4 of the 5 pigs. However, the uncloned population of virus I that was isolated from the fifth pig was serologically recognized by MAb WBE2 (Drew et al., 1995). This MAb recognized a continuous epitope of GP3 that was not present on virus I before passage. In a subsequent experiment Le Gall et al. inoculated sows at 90 days of gestation with virus II that is serologically recognized by MAb WBE2. The sows farrowed and 4-5 weeks later their pigs developed clinical signs consistent with PRRS. When these infected pigs were 17 weeks old they were commingled with 5 contact pigs. Seven weeks later PRRS virus II was isolated from the serum of one of these contact pigs. No differences were observed in the epitopic profile of virus II following passage in pigs except the epitope represented by MAb WBE2 was lost. The apparent acquisition and loss of the epitope represented by MAb WBE2 demonstrate that changes in epitopic profile can occur within a relatively short
maintenance period in pigs. The loss and gain of a specific epitope suggest that mutations that affect epitopes can occur in some regions of the virus genome more frequently than in other regions.

This possibility is supported by the observations of Rowland et al. (1999). These investigators extracted and sequenced viral RNA from tissues of 20 pigs ranging in age from 0 to 132 days. These pigs were farrowed from 3 sows that were infected with the PRRS virus ATCC VR-2332 at 90 days of gestation. The last 312 nucleotides of ORF 4, a 10 nucleotide untranslated region and the first 215 nucleotides of ORF 5 of the parent virus was compared to the homologous sequences of extracted viral RNA. A consistent nucleotide substitution was observed in the 215 nucleotide segment of ORF 5 in viral RNA extracted from lymph nodes of five 8-132 days-old pigs, thymus of one 63 days-old pig, and tonsil of one 10 days-old pig. No other nucleotide substitutions were found in viral RNA extracted from the remaining 13 pigs.

The following study was undertaken to determine the relative stability of 18 epitopes representing the N, M, GP5 and GP3 proteins of 5 North American PRRS viruses. Viruses NVSL 6725, 14620, 46489 and 46907 (KY 35) were isolated between 1989-1993 by the National Veterinary Services Laboratory, United States Department of Agriculture (Ames, Iowa). Virus NE 16244B was isolated from a pig in 1997 by the Diagnostic Laboratory at the University of Nebraska at Lincoln (Allende et al., 1999). These 5 viruses represented 4 epitopic profiles as determined by their reactivity to a panel of 18 MAbs specific for the N, M, GP5 and GP3 proteins of PRRS virus (Yang et al., 2000a, 2000b). The epitopic profiles of these 5 viruses are presented in Table 1. The MARC-145 cell line (Kim et al., 1993) was used to propagate viruses for pig inoculation and virus isolation from tissue homogenates.
The stability of individual epitopes represented by a panel of 18 MAbs (Table 2) was evaluated by comparing the epitopic profiles of each of the uncloned population of the 5 viruses before and after passage through one or more pigs in 3 separate experiments (Table 3). The sero-reactivity of individual viruses with the panel of MAbs was determined by the indirect fluorescent antibody (IFA) test as described by Frey et al. (1992). In experiment 1, 10 pigs were inoculated intranasally with $10^6$ TCID$_{50}$ of virus 16244B and housed separately for 22 weeks. Tonsil biopsies were collected at 12 weeks and samples of tonsil, lung and lung lymph nodes were collected when the pigs were killed at 22 weeks p.i. Virus was isolated from tonsil biopsies of all 5 pigs. Virus was also isolated from tissue homogenates of 2 of these 5 pigs when they were killed.

In experiment 2, four groups of 3 pigs each were inoculated intranasally with PRRS viruses NVSL 6725, 14620, 46489 and 46907, respectively. Each group of 3 pigs was housed separately. These groups of pigs were killed at 6, 7, 4 and 4 weeks p.i., respectively. The PRRS virus was isolated from homogenates prepared from a pool of tonsil, lung, lung lymph node and spleen tissues collected from individual pigs of each group. A tissue homogenate from one pig of each group was used to inoculate a second group of 3 pigs. This protocol was repeated for a third group of 3 pigs. Consequently, viruses NVSL 6725, 14620, 46489 and 46907 were maintained in pigs for a total of 20, 21, 16 and 19 weeks, respectively.
Table 1. The epitopic profiles of porcine reproductive and respiratory syndrome (PRRS) viruses used in the present study

<table>
<thead>
<tr>
<th>PRRS virus</th>
<th>Antigenic group/subgroup</th>
<th>EpORF7</th>
<th>EpORF6</th>
<th>EpORF5</th>
<th>EpORF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVSL 46907</td>
<td>I15/1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NVSL 14620</td>
<td>I15/9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NVSL 46489</td>
<td>II15/1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NVSL 6725</td>
<td>III15/1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>16244B</td>
<td>I15/1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Antigenic group/subgroup of PRRS viruses are determined in the indirect immunofluorescent assay using a panel of monoclonal antibodies specific for the N, M, GP5 and GP3 proteins (Yang et al., 2000b). I15, II15 and III15 represent antigenic groups that are determined based on the presence or absence of epitopes in the N protein. The numbers 1 and 9 represent antigenic subgroups that are further determined based on the presence or absence of epitopes in the M, GP5 and GP3 proteins.
Table 2. Characteristics of monoclonal antibodies used in the present study

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Isotype</th>
<th>Epitope</th>
<th>Protein specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISU15A1</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>N</td>
<td>Yang et al. (1999)</td>
</tr>
<tr>
<td>ISU15A2</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>N</td>
<td>Yang et al. (1999)</td>
</tr>
<tr>
<td>ISU15B1</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>N</td>
<td>Yang et al. (1999)</td>
</tr>
<tr>
<td>ISU15B2</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>N</td>
<td>Yang et al. (1999)</td>
</tr>
<tr>
<td>ISU15C1</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>N</td>
<td>Yang et al. (1999)</td>
</tr>
<tr>
<td>ISU15D1</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>N</td>
<td></td>
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<td>ISU15E1</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>ISU15Fd1</td>
<td>IgG1/κ</td>
<td>discontinuous</td>
<td>N</td>
<td>Yang et al. (2000b)</td>
</tr>
<tr>
<td>ISU15Gd1</td>
<td>IgG1/κ</td>
<td>discontinuous</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>ISU15Hd1</td>
<td>IgG1/κ</td>
<td>discontinuous</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>ISU19A1</td>
<td>IgG1/κ</td>
<td>discontinuous</td>
<td>M</td>
<td>Yang et al. (2000b)</td>
</tr>
<tr>
<td>ISU19B1</td>
<td>IgG1/κ</td>
<td>discontinuous</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>ISU19C1</td>
<td>IgG1/κ</td>
<td>discontinuous</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>ISU25A1</td>
<td>IgG1/λ</td>
<td>continuous</td>
<td>GP5</td>
<td>Yang et al. (2000b)</td>
</tr>
<tr>
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<td>IgG1/λ</td>
<td>continuous</td>
<td>GP5</td>
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<td>ISU25C1</td>
<td>IgG1/λ</td>
<td>continuous</td>
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</tr>
<tr>
<td>ISU45A1</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>GP3</td>
<td>Yang et al. (2000b)</td>
</tr>
<tr>
<td>ISU45B1</td>
<td>IgG1/κ</td>
<td>continuous</td>
<td>GP3</td>
<td></td>
</tr>
</tbody>
</table>

In experiment 3, viruses NVSL 6725 and 14620 were separately passaged through 3 groups of 3 pigs as described in experiment 2 with the exception that PRRS virus was isolated from tissue homogenates on MARC-145 cell cultures between pig passages. These 2 viruses were maintained in pigs for a total of 19 weeks.

No changes in the epitopic profiles were observed in uncloned populations of the 5 viruses following passage and maintenance in pigs. These results suggest that the epitopes represented by the 18 MAbs are encoded by relatively stable regions of the PRRS virus genome and are suitable for use in the virus marker system.
Table 3. The 3 experiments for porcine reproductive and respiratory syndrome (PRRS) virus passages in pigs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PRRS virus</th>
<th>Passage 1</th>
<th>Passage 2</th>
<th>Passage 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of pigs</td>
<td>Duration</td>
<td>Virus recovery</td>
<td>No. of pigs</td>
</tr>
<tr>
<td>1</td>
<td>16244B</td>
<td>3</td>
<td>12 weeks</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16244B</td>
<td>3</td>
<td>12 weeks</td>
</tr>
<tr>
<td>2</td>
<td>NVSL 14620</td>
<td>3</td>
<td>7 weeks</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>NVSL 6725</td>
<td>3</td>
<td>6 weeks</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>NVSL 46489</td>
<td>3</td>
<td>4 weeks</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>NVSL 46907</td>
<td>3</td>
<td>4 weeks</td>
<td>3/3</td>
</tr>
<tr>
<td>3</td>
<td>NVSL 14620</td>
<td>3</td>
<td>5 weeks</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>NVSL 6725</td>
<td>3</td>
<td>6 weeks</td>
<td>3/3</td>
</tr>
</tbody>
</table>

The 5 PRRS viruses were passaged in 3 separate experiments. In experiment 1, the virus 16244B was inoculated into 5 pigs. Tonsil biopsies were collected at 12 weeks post-infection from these 10 pigs. Tissue samples of tonsils, lungs, lung lymph nodes were collected from these pigs when they were killed at 22 weeks post-infection. In experiment 2, each of viruses (NVSL 6725, 14620, 46489 and 46907) was inoculated into 3 pigs. Pigs were killed at 4-7 weeks post-infection. Homogenates of tonsil, lung, lung lymph node and spleen from one pig at the end of each passage was inoculated into 3 additional pigs. These 4 viruses were consecutively passaged 3 times in pigs for a total of 16-21 weeks. In experiment 3, 2 viruses (NVSL 6725 and 14620) were passaged similar experiment 2 with the except that the viruses were propagated once on MARC-145 cells before next passage. Two ml of propagated viruses was inoculated into 3 additional pigs.

Viruses were recovered from pooled homogenates of tonsil, lung and lung lymph node in experiment 1, and from pooled homogenates of tonsil, lung, lung lymph node and spleen in experiments 2 and 3.
Acknowledgement

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References


GENERAL CONCLUSIONS

General Summary

This research focused on the development of an epitope-based marker system for use in categorizing porcine reproductive and respiratory syndrome (PRRS) viruses. Three papers are presented as Chapters 2, 3 and 4 in this dissertation. Chapter 2 described the generation and characterization of a panel of 24 murine monoclonal antibodies specific for the N protein of the North American virus ISU-P (ATCC VR-2402), and the categorization of PRRS viruses. Five continuous epitopes designated EpORF7-A through E were revealed in the N protein by the sero-reactivity pattern of these MAbs with 67 North American field viruses, two modified-live vaccine viruses, and the European Lelystad virus as determined by the indirect immunofluorescence assay and Western immunoblotting and confirmed by additivity and blocking enzyme-linked immunosorbent assays. The sero-reactivity pattern of North American viruses in the IFA enabled their subdivision into 4 antigenic groups which represented 84.1, 11.6, 2.9 and 1.4% of viruses tested. The antigenic variation among these viruses was correlated to single, group-specific nucleotide substitutions and mediated by a combination of at least 4 of the 5 epitopes. EpORF7-A was conserved in all North American viruses and the Lelystad virus which constituted a separate antigenic group. Consequently, monoclonal antibodies specific for EpORF7-A may prove useful as the antigenic basis for a universal diagnostic test for the PRRS virus. EpORF7-C, D and E were only present in the North American viruses tested.

Chapter 3 described the expansion of the MAb panel and further categorization of PRRS viruses. Eleven epitopes were identified by murine MAbs that represented the N, M,
GP5 and GP3 proteins of the North American PRRS virus, KY 35 (NVSL 46907). Three discontinuous epitopes of the N and M proteins were designated EpORF7-Fd through Hd and EpORF6-Ad through Cd. Five continuous epitopes of the GP5 and GP3 proteins were designated EpORF5-A through C and EpORF3-A and B. The MAbs representing EpORF5-C and EpORF6-A and B had neutralizing activity. The MAbs representing the above epitopes, except EpORF7-Gd and Hd, expanded the virus marker system described in Chapter 2 in which a panel of 69 North American viruses and the Lelystad virus were categorized into 5 antigenic groups, I_{15} through V_{15} based on the presence or absence of 5 continuous epitopes of the N protein. Antigenic groups I_{15} and II_{15}, which represented 84.7 and 11.6% of all viruses tested, were categorized further into 9 and 4 subgroups, respectively. The remaining North American viruses and the Lelystad virus were distributed among 4 groups, one of which was represented by 2 subgroups. Significant (P < 0.05) differences in sensitivity to neutralization of 28 viruses representing 6 antigenic groups by the 3 neutralizing MAbs suggested that sensitivity to neutralization may also be of value in categorizing PRRS viruses.

Chapter 4 described the evaluation of the stability of epitopic profiles collectively representing 18 epitopes distributed among the N, M, GP5 and GP3 proteins of PRRS viruses by serially passaging 5 North American viruses in pigs in 3 separate experiments. In experiment 1, 5 pigs were inoculated intranasally with the PRRS virus NE 16244B and housed separately. The PRRS virus was isolated from tonsil tissues from 3 pigs that were killed at 12 weeks post-inoculation (p.i.), and from tonsil biopsies of 2 of these 5 pigs at 12 weeks p.i. and again from tonsil tissues when these pigs were killed at 22 weeks p.i.. In experiment 2, four groups of three pigs each were separately inoculated intranasaly with
PRRS viruses NVSL 6725, 14620, 46489 and 46907. Each group of 3 pigs was housed separately. Individual groups of pigs were killed at 6, 7, 4 and 4 weeks p.i., respectively. The PRRS virus was isolated from homogenates consisting of tonsil, lymph node, lung and spleen tissues prepared from individual pigs of each group. A tissue homogenate from one pig of each group was used to inoculate a second group of 3 pigs. This protocol was repeated again for a third group of 3 pigs. Consequently, viruses NVSL 6725, 14620, 46489 and 46907 were maintained in pigs for 20, 21, 16 and 19 weeks, respectively. In experiment 3, the viruses NVSL 6725 and 14620 were serially passaged separately through 3 pigs essentially as described in experiment 2, with the exception that the PRRS virus was isolated from tissue homogenates in MARC-145 cell cultures between pig passages. The two viruses were each maintained in pigs for a total of 19 weeks. No changes were observed in the seroreactivity of these 5 viruses with the panel of 18 MAbs following maintenance in individual pigs for periods up to 22 weeks in experiment 1, 16-21 weeks in experiment 2, and 19 weeks in experiment 3. The stability of seroreactivity patterns of these viruses demonstrates that an epitopic profile-based virus marker system to characterize PRRS viruses can be used with reliability in epidemiological studies.

**General Discussion**

**Development of a virus marker system**

The principal objectives of the current study were to characterize the antigenic nature of PRRS virus proteins, to identify epitopes associated with neutralization activity and to develop a marker system for epidemiological studies of PRRS viruses. In the study described in Chapter 2, only MAbs specific for the 5 continuous epitopes of the N protein
were produced. No MAbs were generated to other structural proteins including envelope-associated proteins which are of particular interest with respect to their role in mediating susceptibility of PRRS virus to both antibody mediated neutralization and enhancement of virus infection. The lack of representation of other proteins by MAbs in that study was attributed to the highly immunodominant nature of the N protein. Consequently, the immunotolerizing procedure described by Matthew and Sandrock (1987) was used as described in Chapter 3 to establish partial immunotolerance in mice to the N protein to increase the likelihood of generating MAbs that represented different proteins. This strategy was successful because only 12 of the 21 (57%) MAbs that were produced represented the N protein and 9 (43%) MAbs represented the envelope-associated M, GP3 and GP5 proteins. These 21 MAbs represented 3 discontinuous epitopes of both the N and M proteins, 3 continuous epitopes of the GP5 protein and 2 continuous epitopes of the GP3 protein. The generation of a large panel of MAbs specific for continuous and discontinuous epitopes on the N, M GP5 and GP3 proteins has made it possible to develop a virus marker system to categorize PRRS viruses into unique antigenic groups.

**N protein**

Published data prior to the present study suggest that at least 5 epitopes in various combinations are associated with the N protein of North American and European PRRS viruses. Three of these epitopes are most likely discontinuous because of their inability to be recognized by these MAbs in Western immunoblotting. These epitopes are represented by MAbs SDOW 17, VO 17 (Nelson et al., 1993) and ICH5 (Rodriguez et al., 1997). The monoclonal antibodies SDOW 17 and VO 17 represent 2 distinctly different conserved
epitopes. The original characterization of MAbs SDOW 17 and VO 17 by Nelson et al. (1993) demonstrated that MAb SDOW 17 reacted with all 63 North American and 57 European viruses tested. In contrast, MAb VO 17 only recognized North American viruses. The monoclonal antibody ICH5 appears to be different from MAb SDOW 17 because it failed to react in the IFA with 3 North American viruses (Le Gall et al., 1997). The 2 continuous epitopes are represented by MAbs WBE 1 and WBE 4 respectively (Drew et al., 1995). Their distinctness was demonstrated by their reactivity pattern with a combined total of 44 European and 11 North American PRRS viruses (Drew et al., 1995; Le Gall et al., 1997). Both MAbs reacted with all European viruses tested but while MAb WBE 4 did not react with any of the 11 North American viruses, MAb WBE 1 reacted with 3.

In addition to the 5 epitopes described above, a sixth epitope specific for the N protein may also exist. It is continuous and is represented by MAb P3/27 that was originally shown to react with all 14 European and 12 North American viruses tested (Wieczorek-Krohmer et al., 1996). In another study (Le Gall et al., 1997), MAb P3/27 was shown to have the same IFA reactivity pattern as MAb WBE 1. In that study, both MAbs reacted with all 21 European and 3 North American viruses tested. There are no published accounts that clearly indicate whether or not MAb P3/27 reacts with the 8 North American viruses that were not recognized by WBE 1 (Drew et al., 1995) or that WBE 1 reacts with the 12 North American viruses recognized by P3/27 (Wieczorek-Krohmer et al., 1996). Consequently, it remains to be clarified whether or not MAbs P3/27 and WBE 1 represent the same or different epitopes. Nevertheless, the two published accounts which indicate that MAb P3/27 reacted with 15 of 15 North American viruses tested (Le Gall et al., 1997; Wieczorek-
Krohmer et al., 1996) and that MAb WBE 1 did not react with 8 different North American viruses (Drew et al., 1995) suggest that the 2 MAbs represent different epitopes.

In the present study, 5 distinct continuous epitopes designated EpORF7-A through E were revealed among North American viruses further contributing to our knowledge of the antigenic nature of the N protein of PRRS virus. The uniqueness of the individual epitopes was confirmed by the results of blocking and additivity ELISA assays. In the blocking assay (see Chapter 2, Table 4), heterologous MAbs failed to inhibit binding of MAbs representing each of the 5 different epitopes ($p < 0.001$). While in the additivity assay (see Chapter 2, Table 3) the optical density measured following treatment of antigen preparations with MAb representing each epitope was significantly enhanced ($p < 0.001$) after additional treatment with heterologous MAbs but not with homologous MAbs. At least 4 of the 5 continuous epitopes were present in all viruses tested with the exception of the Lelystad virus that contained only EpORF7-A as determined by both IFA and Western immunoblotting (see Chapter 2, Table 2). Consequently, it is possible that EpORF7-A may represent the same epitope as P3/27. Analysis of a broad panel of European PRRS viruses will be necessary to determine which if any of the remaining 4 continuous epitopes are present in European viruses.

The identification of 3 discontinuous epitopes of the N protein in the present study increased the number of known discontinuous epitopes distributed among North American viruses from 2 to 4. The monoclonal antibodies representing these 4 discontinuous epitopes include ISU15Fd, Gd and Hd described in the present study, and MAb SDOW17 described by Nelson et al. (1993). The epitopes represented by MAbs ISU15Fd, Gd and Hd are present on all group I viruses which include PRRS viruses KY 35, ATCC VR-2332, ISU-P and the
vaccine viruses RespPRRS/Repro and PRIME PAC PRRS (see Chapter 3, Table 1). The epitope represented by SDOW17 is present on all of these viruses except the vaccine virus PRIME PAC PRRS, which demonstrates its uniqueness (unpublished data, Yang & Platt, 2000). Additional discontinuous epitopes may be represented by MAbs EP147 and VO17. These MAbs have a different epitope specificity from MAb SDOW17 (Nelson et al., 1993). However, additional comparisons of sero-reactivity patterns of MAbs ISU15Fd and Gd with the sero-reactivity patterns of EP147 and VO17 are required to determine whether or not MAbs EP147 and VO17 represent additional discontinuous epitopes among North American viruses.

The degree of antigenic diversity demonstrated among North American viruses in the present study was less than expected. The distribution of the 5 continuous and 3 discontinuous epitopes associated with the N protein of North American PRRS viruses permitted their subdivision into 5 antigenic groups that were distinctly different from the European Lelystad virus. However, antigenic groups I and II represented 82.6% and 11.6% of all viruses tested while groups III, IV and VI represented the remaining 5.8% of the viruses. This limited antigenic diversity among North American PRRS viruses as revealed by the panel of MAbs specific for the N protein indicated that a useful marker system would require a broader panel of MAbs representing epitopes of other PRRS virus proteins. Consequently, MAbs representing the M, GP3 and GP5 proteins were generated and incorporated into the marker system.
M protein

The 3 discontinuous epitopes of the M protein that were described in this study are the only discontinuous epitopes that have been reported for this protein to date. The identification of EpORF6Ad and Bd as targets for neutralizing antibody suggests that the domain of these epitopes is associated with the 10 to 18 amino acid portion of the M protein that is believed to be located on the surface of the virion and involved in receptor binding (Snijder and Meulenberg, 1998).

GP5 protein

The identification of 3 continuous epitopes of the GP5 protein of the virus KY 35 increased the known number of continuous epitopes associated with this protein among North American viruses from 3 to at least 4. These epitopes include those represented by MAbs ISU25A1 and B1 in addition to the epitopes represented by the 2 groups of neutralizing MAbs described by Pirzadeh and Dea (1996), i.e. MAbs IAF8A8 and 1B8 and MAbs 2A5, 3A12 and 1C10. A non-neutralizing MAb described by Zhang et al. (1998) may represent a fifth epitope distributed among North American viruses or represent the same epitope as either MAb ISU25A1 or 25B1. The neutralizing MAb ISU25C1 may represent a sixth epitope of the GP5 protein or represent an epitope that is detected by MAbs IAF-8A8 and IAF-1B8. All 3 of these neutralizing MAbs react with ATCC VR-2332 but not with the Lelystad virus.

GP3 protein

The identification of 2 continuous epitopes of the GP3 protein represented by MAbs ISU45A1 and B1 increased the known number of continuous epitopes associated with this
protein among North American viruses from 3 to at least 5. The non-reactivity of these 2 MAbS with both ATCC VR-2332 and the Lelystad virus clearly demonstrates that the epitopes which they represent are distinct from epitopes represented by MAbS P7/a1-19, P9/a3-20, and P9/b3-1 (Wieczorek-Krohmer et al., 1996) that are present in both North American viruses and the Lelystad virus. The epitopes represented by MAbS ISU45A1 and B1 are also different from the epitope represented by MAb 122.14. This epitope was detected in the Lelystad virus and 3 North American viruses (Le Gall et al., 1997; Van Nieuwstadt et al., 1996). Additional comparisons are required to determine whether MAb 122.14 is different from MAbS P7/a1-19, P9/a3-20, and P9/b3-1.

The effectiveness of the marker system described in the Chapters 2 and 3 was markedly increased by the use of all of the MAbS that represented the N, M, GP3 and GP5 proteins. Sixty-nine North American PRRS viruses could be categorized into 5 antigenic groups which represented 17 subgroups (Chapter 3, Table 2). However, while 13 of the 15 antigenic subgroups represented 1 to 5 of the 69 North American PRRS viruses each, subgroups 1 and 2 of antigenic group I_{15} represented 17 (24.6%) and 30 (43.5%) viruses, respectively. This disproportionately large representation of North American viruses by 2 antigenic subgroups indicates that additional MAbS are needed to increase the efficiency of this marker system. In addition, it may be possible to further differentiate subgroups of these viruses by their susceptibility to neutralization. The potential usefulness of susceptibility of virus to neutralization to further characterize viruses was demonstrated in the present study (see Chapter 3, Figure 2). Significant (P<0.05) differences in sensitivity to neutralization of 28 viruses representing 6 antigenic groups were observed when viruses were treated with MAbS ISU19A1, B1 and ISU25C1.
Stability of epitopic profiles during passage in pigs

The effectiveness and reliability of the virus marker system developed in this study is dependent on the stability of individual epitopes of the PRRS virus in swine population. Recent work by Le Gall et al. (1997) suggests that an epitope can be lost or gained during several passages of the PRRS virus in pigs. These investigators serially passaged the European PRRS viruses designated I and II in pigs in 2 separate experiments. They found that virus I gained an epitope in GP3 that was represented by MAb WBE2 (Drew et al., 1995) while virus II lost the same epitope following maintenance in pigs for periods of 5 and 27 weeks, respectively. The loss and gain of a specific epitope suggest that mutations that affect epitopes can occur in some regions of the virus genome more frequently than in other regions. This possibility is supported by the observations of Rowland et al. (1999). These investigators observed a consistent nucleotide substitution at position 100 in the first 215 nucleotide segment of ORF 5 in viral RNA from tissues of 20 pigs ranging in age from 0 to 132 days. These pigs were farrowed from 3 sows that were infected 3 sows with the PRRS virus ATCC VR-2332 at 90 days of gestation. No other nucleotide substitutions were found in the last 312 nucleotides of ORF 4 and a 10 nucleotide untranslated region in viral RNA extracted from the remaining 13 pigs.

The study presented in Chapter 4 demonstrated that 5 North American PRRS viruses maintained their epitopic profiles following several passages in pigs for periods up to 22 weeks. These 2 experiments simulated field conditions. In experiment 3, virus propagation in MARC-145 cells between pig passages had no effect on the epitopes of 2 viruses (NVSL 6725 and 14620). The maintenance period of epitopic profiles included viremic and
persistent phases. Results obtained from the present study also justify the incorporation of the epitopes represented by the 18 MAbs in the virus marker system.

**Identification of nucleotide sequences that encode specific epitopes of PRRS virus proteins**

In an effort to determine the approximate locations of EpORF7-A through E in the N protein, the deduced amino acid sequences were computer analyzed using the GCG package. None of the parameters as described in the Materials and Methods section of Chapter 2 could be correlated to antigenic sites with the possible exception of Chou-Fasman (CF) profiles, as indicated by number, location, shape and size of CF turns. Individual CF turns are a reflection of secondary structure which can influence antigenicity. Unique differences in the CF profiles of antigenic groups II, III and IV relative to the CF profile of group I were observed at locations encompassing amino acids 120, 122 and 70 respectively (see Chapter 2, Figure 5). These amino acids represent unique group specific substitutions that appear to be correlated to the absence of EpORF7-B, C and D in antigenic groups II, III and IV, respectively. This observation and the demonstration of inhibition mediated by MAbs representing EpORF7-E with MAbs representing EpORF7-B, C and D (see Chapter 2, Figure 2) suggest that the amino acid sequences representing EpORF7-B, C and D are relatively close to one another at the carboxyl terminal end of the N protein. The absence of reciprocal or one-way inhibition between MAbs representing EpORF7-A and EpORF7-B, C, D and E in the blocking assay (see Chapter 2, Table 4) suggests that EpORF7-A is spatially distant from these epitopes.
The available data are not sufficient to predict the probable location of EpORF7-A or E. However, a recent study by Rodriguez et al. (1997) demonstrated the presence of a continuous epitope in the central region of the N protein in both a European (Olot/91) and a North American (Quebec 807/94) PRRS virus by reacting a panel of MAb by reacting a panel of MAb with fragments of recombinant N protein by Western immunoblotting. They further demonstrated that a highly conserved amino acid sequence extended from amino acid 50-90 in the N protein of 3 North American and 2 European viruses that included the Lelystad virus. Amino acids 50-66 were associated with the common epitope. These observations suggest that the common epitope described by Rodriguez et al. could be identical to EpORF7-A. Competitive assays between MAb representing EpORF7-A and the common epitope described by Rodriguez et al. (1997) may answer this question.

The inability to conclusively identify the location of specific epitopes by comparing amino acid substitutions of different viruses in the present study suggests that the approach used by Rodriguez et al. (1997) for the purpose has been more productive. Site-directed mutagenesis or synthesis of a series of protein fragments could be possible alternative methods.

**Future Studies**

In the present study a virus marker system was developed based on the presence and absence of 18 epitopes. As a result, North American PRRS viruses could be categorized into 5 antigenic groups representing 17 subgroups. However, 68.1% of all viruses tested were represented by only 2 subgroups. Consequently, there is a need to develop additional MAb, especially to the M, GP2 through GP5 proteins to further differentiate these viruses. The use
of additional markers such as susceptibility to neutralization and signature analysis (Monath et al., 1986) should be included for this purpose.

This marker system will be used in epidemiological studies of PRRS viruses to determine if success or failure of control programs can be correlated with specific antigenic groups of PRRS viruses. In addition, the MAbs characterized in this study should also be used to identify epitopes in the N, M, GP3 and GP5 proteins associated with pathogenesis, antibody-dependent enhancement of PRRS virus the infection and induction of protective immunity. The generation of this information will be valuable in the development of more effective vaccines.

References


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