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Antigenic differences between European and American isolates of porcine reproductive and respiratory syndrome virus (PRRSV) are encoded by the carboxyterminal portion of viral open reading frame 3

Jonathan B. Katz [a,*], Amy L. Shafer [a], Kenneth A. Eernisse [a], John G. Landgraf [a], Eric A. Nelson [b]

[a] *U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, National Veterinary Services Laboratories Ames, IA, USA*

[b] *Department of Veterinary Science, South Dakota State University Brookings, SD, USA*

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Abstract

Antigenic differences between European and American isolates of porcine reproductive and respiratory syndrome virus (PRRSV) were revealed by serologic analysis of a recombinant protein derived from PRRSV open reading frame 3 (ORF 3). The hydrophilic carboxyterminal 199 amino acids encoded by the ORF 3 of a European (Lelystad) isolate of PRRSV were expressed as a recombinant fusion protein (BP03-P) in a baculovirus gene expression system. Sera from gnotobiotic swine exposed to prototypic reference European and American isolates of PRRSV and sera from conventionally reared European and American swine convalescing from naturally acquired PRRSV infections were used to characterize the BP03-P protein. Sera from gnotobiotic and conventionally reared swine exposed to European isolates of PRRSV were significantly more reactive ($P<0.01$) with BP03-P than were the corresponding American PRRSV antisera using the indirect immunoperoxidase monolayer assay (IPMA). Prototypic European, but not American, PRRSV antisera also recognized BP03-P using western immunoblotting and radioimmunoprecipitation assay (RIPA) procedures. However, gnotobiotically derived antiserum to an atypical American-origin PRRSV was reactive with BP03-P by both IPMA and western immunoblot. Despite a predicted potential for N-linked glycosylation, studies with tunicamycin and peptide-N-glycosidase F (PNGase F) indicated that BP03-P was not N-glycosylated in either insect cell cultures or *Trichoplusia ni* larvae infected with the recombinant baculovirus. Sera from rabbits inoculated with BP03-P failed to neutralize both the European (Lelystad) and American (ATCC VR-2332) reference isolates of PRRSV and did not react by IPMA with PRRSV-infected cell cultures. Taken together, the data suggest that the carbox-

* Corresponding author. Diagnostic Virology Laboratory, National Veterinary Services Laboratories, P.O. Box 844, Ames, IA 50010, USA. Tel: (515) 239-8551; fax: (515) 239-8348

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yterminal portion of PRRSV ORF 3 encodes a non-neutralizing viral peptide that is partially responsible for the serologic differences noted between European and most American isolates of PRRSV.

Keywords: Antigen, virus; Arterivirus; Serodiagnosis; Porcine Reproductive and Respiratory Syndrome Virus; Recombinant protein

1. Introduction

In the few years since it was initially described (Keffaber, 1989; Lindhaus and Lindhaus, 1991; Paton et al., 1991), the disease complex now known as the porcine reproductive and respiratory syndrome (PRRS) has become an economically significant swine health problem throughout Europe and North America (Goyal, 1993). The viral etiology of PRRS was confirmed both in The Netherlands (Meulenberg et al., 1993; Terpstra et al., 1991) and in the United States (Collins et al., 1992) with the isolation of two previously unknown PRRS viruses (PRRSV), a prototypic European PRRSV (Lelystad isolate) and a prototypic American PRRSV (VR-2332 isolate). These 50 to 60 nm enveloped RNA viruses (Benfield et al., 1992; Wensvoort et al., 1992) share common antigenic determinants but may be differentiated using both polyclonal antisera (Wensvoort et al., 1992) and monoclonal antibodies (Nelson et al., 1993). Serologic analysis of European and American swine naturally exposed to PRRSV has confirmed that geographically based antigenic differences do exist between most North American and most European isolates of PRRSV; these differences are consistent with those first noted between the Lelystad and the VR-2332 isolates (Wensvoort et al., 1992; Bautista et al., 1993). There is some evidence, however, that antigenically intermediate PRRSV or possibly both prototypic antigenic types may exist within the North American swine population (Bautista et al., 1993). PRRSV has been tentatively classified as an arterivirus, along with equine arteritis virus (EAV) and two other proposed members of that genus: lactate dehydrogenase elevating virus (LDV) and simian hemorrhagic fever virus (SHFV) (Plagemann and Moennig, 1992).

PRRSV contains a 12 to 14 Kb single strand positive polarity polyadenylated genome (Plagemann and Moennig, 1992) and utilizes a nested 3′ coterminal mRNA gene expression strategy (Meulenberg et al., 1993; Conzelmann et al., 1993). The PRRSV genome contains 8 open reading frames (ORFs), including the large 5′ terminal ORFs 1a and 1b encoding the putative viral polymerase and the 3′ terminal ORF 7 encoding the nucleocapsid protein (Meulenberg et al., 1993; Conzelmann et al., 1993). PRRSV virions are believed to contain two other structural, presumably envelope, proteins (Nelson et al., 1993). It has not been determined which of the ORFs 2 through 6 encode these proteins or the identities and functions of the other proteins encoded by these ORFs.

Recombinant DNA technology permits the expression of individual gene segments in order to better understand the antigenicity of their protein products and their roles in the host-virus relationship. We report here the expression and serologic analysis of a markedly hydrophilic portion of PRRSV ORF 3 (Lelystad isolate). This segment was of additional interest because comparison of LDV, EAV, and PRRSV ORFs 2 through 6 revealed that the ORF 3 protein products of these viruses would be the most divergent in size but potentially the most universally highly N-glycosylated of all the proteins encoded by those

ORFs (den Boon et al., 1991; Conzelmann et al., 1993; Godeny et al., 1993). These features suggested to us that PRRSV ORF 3 might encode a glycoprotein mediating virus-host cell interactions; therefore, it might be a target of host defense mechanisms subjected to selective evolutionary pressure. We hypothesized that it might account in part for the geographically oriented antigenic differences between the majority of European and North American PRRS viruses, and that was the basis for this study.

2. Materials and methods

2.1. PRRS viruses, cells, and antisera

The Lelystad and VR-2332 isolates of PRRSV were provided by C. Terpstra, Central Diergeneeskundic Institut, The Netherlands, and by D. Chladek, Boehringer Ingelheim Inc., USA, respectively. A British reference PRRSV isolate, Humberside 2, was a gift from S. Edwards, Central Veterinary Laboratory, United Kingdom. PRRSV isolates 46448 and 27199 were recovered in this laboratory from midwestern American swine tissues. Antisera to the 46448 isolate had been found uniformly cross-reactive with numerous North American PRRS virus isolates; isolate 27199 was of interest because it was completely fastidious for replication in swine pulmonary alveolar macrophages. All viruses except 27199 were propagated in cultures of MARC-145 cells, which are a PRRSV permissive cell line subcloned from MA-104 cells (Kim et al., 1993). The identity of all viruses was confirmed using PRRSV-specific monoclonal antibodies in an indirect immunoperoxidase monolayer assay (IPMA) format.

Gnotobiotic swine were used to prepare antisera to each PRRSV isolate. Each 10-day-old piglet was intranasally inoculated with 10^5 to 10^6 $TCID_{50}$ of one virus isolate, and the resultant convalescent antiserum was collected between 48 to 56 days post-inoculation.

Twenty field origin sera derived from clinical cases of PRRS in swine from northern Germany were generously provided by T. Blaha, Tierartzliche Hochschule, Hanover, Germany. Fifteen American field origin PRRSV seropositive swine sera were also obtained from D. Kinker, Iowa State University, Ames, IA, USA. Each serum originated from a separate swine herd experiencing PRRS, and these sera represented samples from herds located in 11 states in the eastern, southern, and midwestern United States.

2.2. Serologic assays

The IPMA (Holm-Jensen, 1981) was used to evaluate serial 4-fold serum dilutions for reactivity against both the Lelystad and the 46448 PRRSV isolates. These viruses had been inoculated 48 h previously onto separate MARC-145 cell monolayers. Immunodetection was accomplished using protein G-horseradish peroxidase (HRP) conjugate and aminoethylcarbazole substrate (Zymed, So. San Francisco, CA, USA). A PRRSV serum neutralization (SN) procedure was used to evaluate selected sera as recently described (Yoon et al., 1994).

Recombinant ORF 3 antigen, cesium chloride purified PRSSV (Nelson et al., 1993), and ultrafiltered concentrates of cell cultures infected with the Lelystad isolate were used as

antigens in western immunoblotting following denaturing polyacrylamide gel electrophoresis in 10% to 15% or 8% to 25% gradient gels (Laemmli, 1970; Burnett, 1981). Immunodetection was again accomplished with protein G-HRP conjugate and an insoluble tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry Inc., Gaithersburg, MD, USA). A radioimmunoprecipitation assay (RIPA) recently described for PRRSV (Nelson et al., 1993) was used with the same antigens to evaluate sera from the gnotobiotic swine inoculated with PRRSV isolates.

2.3. Recombinant expression vector construction

A hydrophobicity/hydrophilicity plot (Kyte and Doolittle, 1982) of the putative ORF 3 protein revealed a markedly hydrophobic amino terminus followed by the remaining predominantly hydrophilic 75% (199 of 265 amino acids) of the protein (Fig. 1). We postulated that this latter portion might be externalized and, therefore, available to interact with host cell receptors or antibodies and that the amino terminus perhaps represented a biosynthetically cleaved leader peptide or otherwise cryptic segment not directly interactive with the host. To focus our examination on the hydrophilic body of the protein, we excluded the hydrophobic segment through a subunit cloning strategy.

Total RNA was extracted from a 25 cm^2 cell monolayer infected with the Lelystad isolate of PRRSV. A 20-mer oligonucleotide complementary to the viral RNA sequence (Meulenberg et al., 1993) 58 nucleotides downstream from the ORF 3 termination codon (positions 13,249 to 13,230 inclusive) was used to prime viral cDNA synthesis in a 20 μl reaction (Maniatis et al., 1982). A 655 base pair (bp) cDNA segment was then amplified through 20 cycles of the polymerase chain reaction (PCR) (Saiki et al., 1988) using a 5′ primer located between positions 12,559 to 12,578 and a 3′ primer (13,213 to 13,194) to which a Pst I recognition sequence had been appended (Fig. 1). Following Xho I and Pst I digestion and gel purification, the PCR product was directionally ligated into a baculovirus polyhedrin gene transfer plasmid, pAcSG-HIS-NT (Pharmingen, Inc., San Diego, CA, USA). The resulting plasmid contained the 199 carboxyterminal amino acid-encoding portion of ORF 3 fused in frame to a 5′ vector sequence encoding 27 amino acids including a polyhistidine domain. The resulting fusion polypeptide had a calculated mass of 25,984 daltons (26 kDa). Following DNA sequence verification, recombinant plasmid DNA (2 ug) was cotransfected with Bsu 36I-digested AcNPV baculoviral DNA (0.5 ug) (Pharmingen, Inc.) into a *Spodoptera frugiperda* SF-9 cell culture using a liposomal transfection reagent (DOTAP, Boehringer Mannheim, Indianapolis, IN, USA). Clonally purified baculoviruses expressing PRRS virus-immunoreactive material were identified by IPMA. Separate PCRs of cell culture fluids from monolayers infected by these cloned viruses were used to confirm the presence of the ORF 3 gene sequence and the absence of the nonrecombinant baculovirus polyhedrin gene sequence. The latter PCR employed primers spanning the polyhedrin gene insertion site (Invitrogen, Inc., San Diego, CA, USA). One rDNA baculovirus, BP03, was used for recombinant protein production.

2.4. Protein production, processing, and utilization

Recombinant protein (BP03-P) was produced by infecting both SF-9 cell cultures and 8-day-old *Trichoplusia ni* larvae according to standard procedures (O'Reilly et al., 1992).

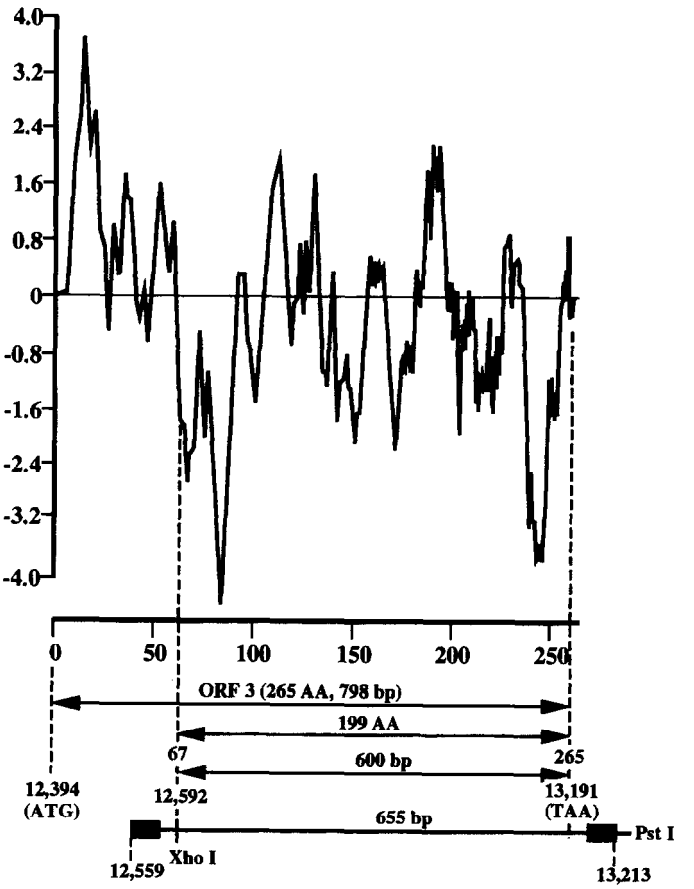

Fig. 1. Hydropathicity analysis and carboxyterminal subunit cloning strategy for LV ORF 3. Units on the vertical axis are hydropathy values calculated with a serial amino acid span setting of 7 (Kyte and Doolittle, 1982). Positive values indicate net hydrophobic regions; negative values indicate hydrophilic regions. Positions of the 265 amino acids comprising the ORF 3 protein are shown on the horizontal axis. The recombinant protein incorporated amino acids 67 to 265 inclusive, corresponding to LV nucleotides 12,592 to 13,188. A 3′ *Pst*I extension was added to facilitate directional cloning of the PCR-amplified gene segment.

Some cell cultures were treated daily with tunicamycin (15 ug/ml) (Boehringer Mannheim, Inc.) to inhibit potential N-linked glycosylation of BP03-P. Aliquots of purified BP03-P were also incubated for 18 h with peptide-N-glycosidase F [PNGase F, EC 3.5.1.52, peptide N^4-(N-acetyl-β-glucosaminyl)] asparagine amidase (Boehringer Mannheim, Inc.) in a further effort to evaluate the N-glycosylation status of the protein. The polyhistidine sequence within BP03-P enabled efficient affinity purification of both cell culture and larval origin BP03-P using immobilized nickel ion chromatography as previously described (Janknecht et al., 1991). Purified larval and cell culture origin BP03-P was adjuvanted (Fatunmbi et al., 1992) with avridine (Pfizer, Inc., Groton, CT, USA) and used to immunize four rabbits each on four biweekly occasions. Sera from these animals were then evaluated by IPMA, SN, western blotting, and RIPA for anti-PRRSV reactivity.

3. Results

3.1. Molecular analysis of BP03-P and homologous antisera

BP03-P expression was detected in the cytoplasm of infected SF-9 cells using gnotobiotically-derived Lelystad isolate antiserum in an IPMA format (Fig. 2). BP03-P expression was noted within 72 h post-infection (hpi) and persisted at least to 120 hpi. Comparison of BP03 infected and uninfected cell culture proteins by SDS-PAGE revealed the presence of a new protein in the infected cell cultures (Fig. 3A). The size of this protein was consistent with the 26 kDa mass predicted from the amino acid sequence of the fusion protein encoded by BP03. The PRRSV-specific identity of this protein was confirmed by western immunoblotting (Fig. 3C). BP03-infected insect larvae contained a similarly sized immunoreactive protein within 96 hpi (Figs. 3B, 3C). Protracted incubation of affinity purified larval BP03-P with PNGase F did not result in a detectable reduction in apparent BP03-P molecular mass (Fig. 3C). There was also no detectable decrease in the molecular mass of BP03-P from tunicamycin treated cell cultures relative to untreated controls (Fig. 3C). The IPMA, SN, RIPA, and western immunoblot procedures were used to evaluate rabbit antisera developed against both larval and cell culture origin BP03-P. None of these sera exhibited

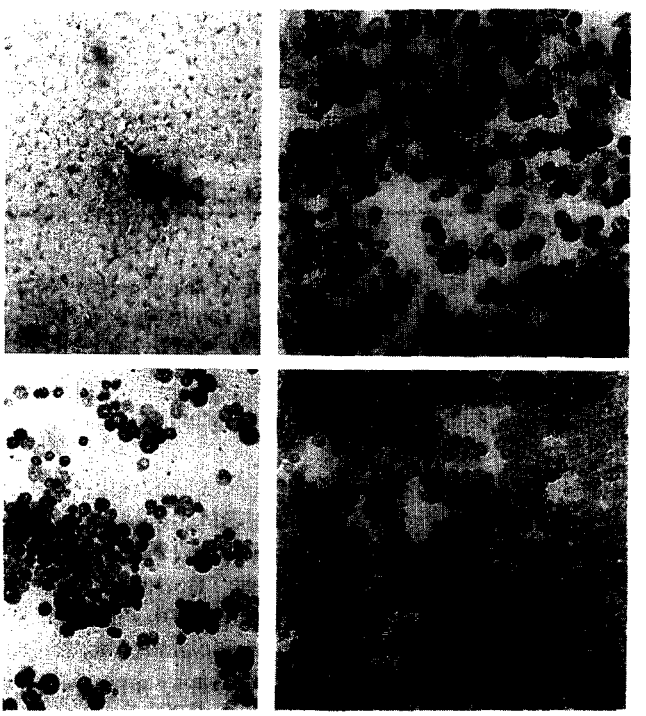

Fig. 2. IPMA visualization of LV-infected MARC-145 cells infected with the Lelystad isolate of PRRSV (A) and recombinant BP03 baculovirus-infected SF-9 cells [72 hpi (B), 120 hpi (C)]. Uninfected SF-9 cell culture control (D). Gnotobiotic anti-Lelystad PRRSV swine serum used at 1:20 dilution. Note cytoplasmic accumulation of BP03-P protein in (B) and (C). Magnifications: 200×.

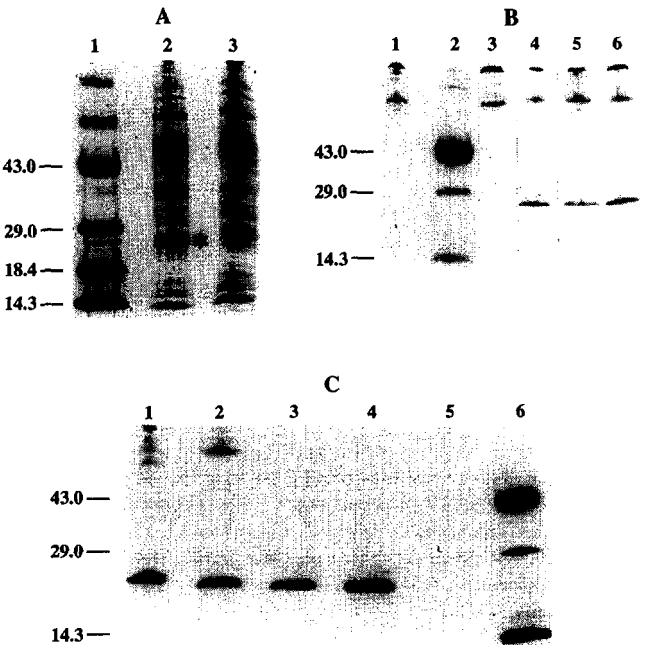

Fig. 3. Panel A: Profile of BP03-infected (lane 2) and uninfected (lane 3) SF-9 cell proteins following SDS-PAGE and Coomassie Blue R-250 staining. Asterisk (*) denotes 26 kDa BP03-P protein. Panel B: BP03-P protein produced in larvae and immunoblotted with 1:100 dilutions of gnotobiotic VR-2332, 46448, Lelystad, Humberside 2, and 27199 virus antisera in lanes 1, 3, 4, 5, 6, respectively. Lane 2: Molecular mass markers (kDa). Panel C: Immunoblot of affinity purified larval BP03-P protein with and without prior digestion with PNGase F (lanes 1 and 2, respectively). Immunoblot of SF-9 cell culture origin BP03-P protein produced in the presence or absence of tunicamycin (lanes 3 and 4, respectively). Lane 5: Cell culture control infected with recombinant baculovirus containing vector-only transfer plasmid sequences.

PRRSV SN activity at a 1:8 dilution. All sera were IPMA-reactive at $\geq$ 1:270 dilution using BP03-infected SF-9 cells but failed to react at a 1:20 dilution with 46448 isolate-infected MARC-145 cells. These sera also did not react at the 1:20 dilution with Lelystad isolate-infected cell monolayers fixed between 8 and 48 hpi. Two of the rabbit antipeptide sera were reproducibly reactive by western immunoblot with a diffuse (40 to 45 kDa) band of antigen found in homogenates of MARC-145 cells infected 16 h previously with the Lelystad isolate (Fig. 4). Repeated RIPA and western blot attempts using these sera were unsuccessful in identifying a virus-specific protein in purified virion preparations.

3.2. Serologic characterization of BP03-P

Antisera to gnotobiotic Lelystad, Humberside 2, and 27199 virus isolates reacted specifically at 1:100 dilutions with nitrocellulose blotted bp03-p, while antisera to vr-2322 and 46448 virus isolates were totally nonreactive at the same or lower dilutions (Fig. 3B). RIPA results were consistent with these findings. These sera were also evaluated by IPMA against Lelystad and 46448 infected Marc-145 cells and BP03-infected SF-9 cells (Table 1). Antisera to the Lelystad and Humberside 2 isolates were differentially reactive with cells

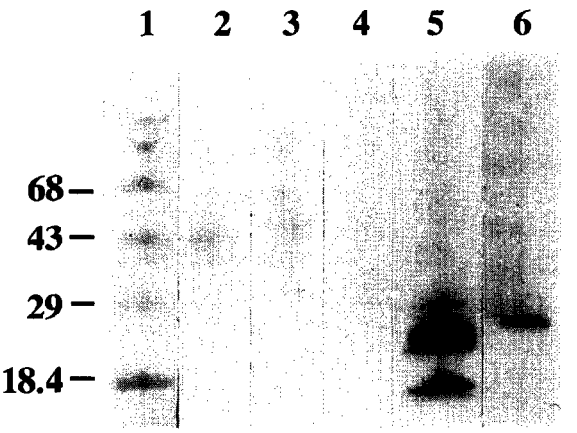

Fig. 4. Western immunoblot analysis of sera from two rabbits inoculated with BP03-P. Lane 1: Molecular mass markers (kDa). Lanes 2 and 3: Blots of rabbit anti-(BP03-P) sera, showing reactivity against 40 to 45 kDa proteins found in cells infected with PRRSV (Lelystad isolate) 16 hours previously. Lane 4: Blot of rabbit serum (same as used in Lane 3) against uninfected cell antigens. Lanes 5 and 6: Blots of hyperimmune anti-BPO3-P rabbit serum and gnotobiotic anti-PRRSV (Lelystad) swine serum, respectively, against the BP03-P immunogen. Hyperimmune rabbit serum also detects small (18–20 kDa) breakdown products of BPO3-P resulting from its catabolism in infected insect cells.

Table 1
Serologic characterization of Lelystad virus ORF 3 recombinant fusion protein using gnotobiotic and field origin PRRS virus antisera

Sera	IPMA titer[a]			Western immunoblot
	Lelystad virus	46448 virus	BP03 virus	BP03-P antigen
Gnotobiotic sera				
Lelystad	≥1280	20	320	+
Humberside 2	≥1280	80	320	+
27199	320	1280	320	+
46448	80	≥1280	<20	−
VR-2332	80	≥1280	<20	−
Field Origin sera				
German[b]	1194[c]	98	300[c]	ND[e]
($n=20$)	(320–1280)	(20–1280)	(80–1280)	
American[b]	32	735[d]	<20	
($n=15$)	(20–80)	(320–1280)	(<20–20)	ND

[a]IPMA titers determined using 3 different infecting viruses.
[b]Geometric mean titers (GMT) are presented (ranges in parentheses).
[c]German sera significantly more reactive ($P<0.01$) with Lelystad and BP03 viral antigens than the corresponding American sera and significantly ($P<0.01$) less reactive with 46448 antigen than with the other 2 viral antigens.
[d]American sera significantly more reactive ($P<0.01$) with 46448 viral antigen than the corresponding German sera and significantly ($P<0.01$) more reactive with 46448 antigen than with the Lelystad and BP03 viral antigens.
[e]Not determined.

infected with the Lelystad isolate and also recognized BP03-p in infected SF-9 cells. Antisera to the VR-2332 and 46448 isolates were differentially reactive with isolate 46448 infected cells. Antiserum to the 27199 isolate was nearly equally reactive with cell cultures infected with either of the PRRSV and with the BP03 virus (Table 1).

Convalescent sera from field origin PRRSV-infected German and American swine were differentially and antithetically reactive by IPMA. A two-way analysis of variance was used to assess the serologic differences. The geometric mean titer (gmt) of the German sera was significantly greater ($P < 0.01$) against Lelystad and BP03 viral antigens than against the 46448 viral antigen. Conversely, American sera were significantly ($P < 0.01$) more reactive with 46448 viral antigens than either of the other two antigens (Table 1).

4. Discussion

Taken together, the data demonstrate that immunoreactive epitopes within the carboxy-terminal portion of the protein encoded by Lelystad PRRSV ORF 3 are expressed during the course of European PRRSV infections, and that this polypeptide sequence is at least partially responsible for the serologic differences observed between European and American PRRSV isolates (Wensvoort et al., 1992; Bautista et al., 1993).

Antiserum to the macrophage-fastidious and antigenically intermediate 27199 isolate was reactive with BP03-P, further suggesting the importance of the ORF 3 protein in the serologic differentiation of PRRSV isolates. The similarity between the RIPA and western immunoblot reactivity patterns of BPO3-P using European and American PRSSV antigens and gnotobiotically derived convalescent swine antisera suggests that perhaps both linear and conformational ORF 3 epitopes may be involved in the antigenic differentiation of European and American PRRS viruses. Our data support the hypothesis (Bautista et al., 1993) that coexisting high Lelystad and ATCC VR-2332 PRRS virus antibody responses found in some American swine may result from infections with a virus of mixed antigenicity as opposed to dual infections with prototypic American and European-like PRRSV isolates.

Like the homologous predicted LDV and EAV ORF 3 proteins, the PRRSV ORF 3 protein contains the greatest number of sites for potential N-glycosylation of all the proteins encoded by ORFs 2 through 6 (den Boon et al., 1991; Conzelmann et al., 1993; Godeny et al., 1993). N-linked glycosidic moieties have frequently been found important in mediating viral infectivity, in directing soluble and cell-associated host immune responses, in protecting critical viral protein epitopes from immune attack, and in interacting with viral polypeptide components to ensure correct viral glycoprotein conformational structure (Rademacher et al., 1988; Li et al., 1993; Grigera et al., 1991). Attempts to produce observably deglycosylated and nonglycosylated forms of BP03-P using both tunicamycin inhibition and enzymatic deglycosylation approaches indicated that the recombinant protein did not contain N-linked glycosidic modifications. These findings must be interpreted cautiously. Despite the theoretical potential for a high degree of N-linked glycosylation, the carboxyterminal portion of the native ORF 3 protein may in fact not be glycosylated. Perhaps more likely however, the aminoterminal peptide sequence omitted during BP03 construction may have represented a signal sequence required to direct nascent ORF 3 into the ER-Golgi complex for glycosylation during insect larval and SF-9 cell infections. Although insect

cells and tissues are generally assumed to be glycosylation-competent, differences between mammalian and insect glycosylation patterns have been observed (Rademacher et al., 1988; Shimokawa and Smith, 1992; O'Reilly et al., 1992) and could also account for the data. The addition or removal of very small N-linked glycosides to the BP03-P protein might have been undetectable given the molecular size resolution of the western immunoblotting, but this is unlikely because viral N-linked glycosidic moieties are usually substantial in apparent molecular mass (Rademacher et al., 1988). Regardless of the foregoing considerations, the ORF 3 of the Lelystad isolate clearly encodes epitopes recognized immunologically by the swine host during the course of both experimental and natural infections. Monospecific BP03-P rabbit antipeptide antisera reacted by immunoblot but not by RIPA with a diffuse band of antigen (40–45 kDa) found repeatedly in virus-infected cell cultures. This material was specific to virus infected cells, but was apparently not of virus structural protein origin, because it was not detected by either RIPA or immunoblot using purified virus preparations. The difference between the RIPA and the western immunoblot performance of the rabbit antipeptide antisera using unpurified infected cell culture material may be due to the presentation of the relevant reactive linearized nonstructural virus protein epitopes under the conditions of the denaturing immunoblot but not under the milder conditions of the RIPA.

Monospecific BP03-P antisera were not neutralizing, but the functional significance of this finding is unclear because other workers have suggested that antibody responses to PRRSV may not necessarily be protective and might even participate in antibody-dependent enhancement of infection (Choi et al., 1992). The failure of monospecific rabbit BP03-P anti-peptide antisera to react with PRRSV-infected cells is consistent with the ideas that the natural ORF 3 translation product may be conformationally (epitopically) different from the recombinant BP03-P fusion protein, or that the native protein may be a nonstructural, perhaps soluble, virus gene product. Lactate dehydrogenase elevating virus, EAV, SHFV, and apparently PRRSV all possess three structural proteins including a nucleocapsid protein encoded by the 3′ terminal ORF (Nelson et al., 1993). The other two presumed PRRSV structural proteins are 19 kDa and 26 kDa in apparent molecular mass (Nelson et al., 1993) and are thus far removed from the 40–45 kDa band identified here. Although the precise identity and functional role of the PRRSV ORF 3 gene product thus remain enigmatic, a differential virus serotype-specific serologic response is clearly directed against it by swine exposed to PRRSV.

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