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Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of porcine reproductive and respiratory syndrome virus

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Abstract

We describe B-cell linear epitopes detected by Pepscan in the Nsp2 and all of the structural proteins of a US PRRSV strain, using sera of 15 experimentally infected pigs. The Nsp2 was found to contain the highest frequency of immunodominant epitopes ($n = 18$) when compared to structural proteins. Ten of these 18 Nsp2 peptides were reactive with 80 to 100% of the examined sera. In the structural proteins, epitopes consistently recognized by immune sera were located at gp2 ($n = 2$), gp3 ($n = 4$), gp5 ($n = 3$), M ($n = 2$) and N ($n = 2$). Overall, the highest degree of immunogenicity and conservation was exhibited by two epitopes identified in the C-terminal end of the M protein (ORF6). The antibodies recognizing the immunodominant epitopes of each protein were detected as early as days 7 to 15 pi and remained detectable until the end of the experiment (day 90 pi). These findings have direct implications for PRRSV differential diagnostics and eventual eradication as the identified epitopes may represent serologic marker candidates for differential (DIVA) PRRSV vaccines, derived from infectious cDNA clones.

Keywords: PRRSV; B-cell epitopes; Peptides; Infectious cDNA clone, Pepscan

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is considered to be one of the most economically important infectious diseases of swine causing late-term reproductive failure in pregnant sows and severe pneumonia in neonatal pigs (Snijder and Meulenberg, 2001). The disease was first reported in 1989, and the causative agent was isolated and characterized for the first time in Europe in 1991 and 1 year later in the United States (Collins et al., 1992).

PRRSV, the etiological agent of PRRS, is an enveloped single-stranded RNA virus belonging to the order Nidovirales, family Arteriviridae (Snijder and Meulenberg, 2001). Other members of Arteriviridae are lactate-dehydrogenase-elevating virus of mice (LDEV), equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV) (Snijder and Meulenberg, 2001). The PRRSV genome is approximately 15 kb in length and contains eight open reading frames (ORFs). ORFs 1a and 1b comprise 80% of the genome size and encode a polyprotein which is co- and post-translationally processed by autoproteolytic cleavage into 12 nonstructural polypeptides (Nsps). The other six ORFs (2 to 7) are translated into structural proteins (gp2, gp3, gp4, gp5, M and N). An additional protein encoded by ORF2b has also been recently characterized (Lee and Yoo, 2005; Wu et al., 2001).

Significant antigenic and genetic differences have been reported among North American and European strains of PRRSV (Allende et al., 1999; Wensvoort et al., 1992). Such diversities have led to the recognition of two distinct serotypes of PRRSV: European (type 1) and North American (type 2). However, the origin of PRRSV remains unknown especially since the European and North American PRRSV isolates cause similar clinical symptoms but represent two distinct viral genotypes with genomic divergences of approximately 40% (Nelsen et al., 1999).

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Vaccination against PRRSV infections is being carried out since 1995 in the US. The most commonly used vaccine is a modified-live virus, consisting of a wt PRRSV US strain attenuated by multiple passages in cell cultures. The efficacy of these attenuated vaccines currently in use is somewhat disputed and is generally acknowledged that significant latitude still exists for technical improvements on their safety and efficacy. One technical improvement which is central to the effective implementation of these PRRSV vaccines is endowing them with differential capability to make them compatible with elimination of the wt PRRSV infection from a herd. The serological differentiation between vaccinated and naturally infected animals has proved crucial for the success of eradication programs of important livestock diseases (van Oirschot, 1999).

Marker vaccines (also termed DIVA—Differentiating Infected from Vaccinated individuals) carry at least one antigenic protein less than the corresponding wild-type virus, i.e. has a "negative marker" (van Oirschot, 1999), which allows the serological tracing of wt strains (which obviously are "marker positive" instead) in vaccinated herds. Classical examples of modified-live vaccines carrying deletions of non-essential and immunogenic structural proteins have been produced for large DNA viruses such as pseudorabies virus (PRV) and bovine herpesvirus-1 (BHV-1) (Kaashoek et al., 1994; Moormann et al., 1990; van Oirschot, 1999). These first successful approaches to DIVA vaccines based on deletion of an entire glycoprotein from veterinary herpesvirus contributed to define two fundamental properties of an ideal serologic marker antigen: 1. the marker candidate has to be immunodominant and therefore recognized by the vast majority of a population infected with wt virus; 2. their deletion from the vaccine genetic make up would not alter the viability and/or protective immunogenicity of this vaccine. However, the applicability of such approach for small RNA viruses like PRRSV, which encode only a few proteins with essential functions, seems difficult (Welch et al., 2004; Wissink et al., 2005; Yoo et al., 2004). Therefore, one alternative to be considered for the selection of negative serological markers for RNA viruses would be the identification of immunogenic non-essential epitopes in viral proteins. The approach of epitope deletion has proved feasible for arteriviruses through deletion of a 46 amino acid immunodominant region from the ectodomain of the glycoprotein L (gL) of EAV without deleterious effects on the replication and immunogenicity of the virus (Castillo-Olivares et al., 2003). Furthermore, a peptide ELISA based on this particular domain enabled serological discrimination between vaccinated and wild-type virus-infected animals.

The presence of B-cell epitopes in nonstructural (mainly Nsp2) and structural proteins of an EU-type PRRSV strain have been previously demonstrated by using phage display libraries (Oleksiewicz et al., 2001, 2002). A few additional epitopes identified by means of PRRSV N monoclonal antibodies (Mabs) have also been reported (Meulenberg et al., 1998; Zhou et al., 2005). Nonetheless, data regarding functional epitopes on US-type PRRSV-encoded proteins are scarce. Likewise, there are no available data about immunodominant and conserved B-cell epitopes consistently recognized by the humoral immune response of US-type PRRSV-infected animals. This study reports a detailed and systematic investigation by Pepscan technology of the US-type PRRSV B-cell linear epitopes recognized by the convalescent sera from PRRSV-infected pigs. Our results identify several immunodominant epitopes within the Nsp2 as well as the structural proteins of PRRSV.

Results and discussion

Experimental inoculation

Inoculation of fifteen piglets with $10^{5.0}$ $TCID_{50}$ of a PRRS virus recovered from a NVSL 97-7895 strain full-length cDNA infectious clone by intranasal and intramuscular routes resulted in a slight increase in rectal temperature (≤1.5 °C) between days 2 and 6 post-infection (pi). Viremia titers ranging from $10^{3.7}$ to $10^{5.1}$ $TCID_{50}$/ml of serum were observed at 7 dpi in all infected animals indicating active viral replication (data not shown). Two serum samples collected at 7 dpi and all samples collected at days 15, 30, 45, 60 and 90 pi were positive for PRRSV-specific antibodies as assayed by a commercially available ELISA kit (Idexx Labs, Inc) (data not shown). The results of virus isolation from blood and ensuing positive serology demonstrated that productive virus replication and induction of a normal humoral response took place in all the experimentally infected piglets. Eight of the piglets were euthanized at 60 dpi, and the remaining animals (n = 7) were monitored serologically until day 90 pi.

Identification of US-type PRRSV-specific linear B-cell epitopes

For the identification of US-type PRRSV-specific B-cell linear epitopes, convalescent sera (60 dpi) collected from experimentally infected piglets were used for screening of the peptide-specific immune response against Nsp2 and all structural proteins. The serum samples were examined for antibodies that recognize synthetic peptides used individually as antigen in a peptide-based indirect ELISA as described in Materials and methods. The 213 synthetic peptides were designed based on the amino acid sequence of the North American strain of PRRSV NVSL 97-7895 (GenBank accession no. AY545985). Peptide scanning (Pepscan) for epitope mapping has become increasingly recognized as a method for identification of diagnostically relevant epitopes within viral proteins (He et al., 2004a, 2004b; Hohlich et al., 2003; Khudyakov et al., 1999; Lundkvist et al., 1995; Niikura et al., 2003).

We identified several B-cell linear epitopes along the amino acid sequence of all the studied proteins. The identity and location of the immunodominant epitopes identified in each protein are presented in Fig. 1 and in Table 1. In general, the antibodies recognizing the immunodominant epitopes appeared between day 7 and 15 pi, increased in titer with time and remained at fairly steady levels up to day 60 pi (Fig. 2). Nevertheless, the antibody response to the individual epitopes varied greatly among individual pigs as measured by the optical

density values of the peptide-based ELISAs (Fig. 3). All seven serum samples available at 90 dpi were also reactive against the immunodominant epitopes identified (data not shown).

B-cell linear epitopes are scattered along the Nsp2 amino acid sequence

The Nsp2 protein has been shown to be highly variable among arteriviruses, with similarities observed only in the amino- and carboxy-terminal domains whereas the central region of the protein varies in both length and amino acid composition (Allende et al., 1999). Interestingly, the Nsp2 was found to contain the highest frequency of immunogenic epitopes when compared to the structural proteins examined in this study. Among the 97 peptides spanning the entire amino acid sequence of Nsp2, 18 were found to be immunoreactive with more than 50% of the sera tested. Ten of these peptides were reactive with 80–100% of the sera examined (Table 1; Fig. 4A). Furthermore, the identified immunodominant B-cell epitopes were scattered along the protein sequence, and most of them were localized within predicted hydrophilic regions of the protein (Fig. 4B). These results were not unexpected since hydrophilic amino acid sequences are likely exposed on the surface of the protein and thus may be more easily recognized by B-lymphocytes. In addition, several other peptides were recognized by fewer serum samples. No antigenic reactivity was found within the region comprising peptides #84 and 97 located in the C-terminal end of the protein (Fig. 4). The lack of reactivity of peptides spanning this region might be attributed to the high level of conservation and hydrophobicity of this segment. A previous report has also demonstrated the occurrence of a cluster of B-cell epitopes in Nsp2 of an EU-type PRRSV isolate, 111/92 (Oleksiewicz et al., 2001). However, the six epitopes identified in that study were not recognized by antibodies from animals infected with US-type PRRSV, and no comparison could then be drawn with the findings of our experiment. In addition, the systematic Pepscan methodology used in our study allowed the identification of a higher number of B-cell epitopes in Nsp2 when compared to those reported for the European strain of PRRSV studies by phage display technology.

B-cell linear epitopes in the ORF2 protein

The 29–30 kDa glycoprotein 2 (gp2) and the glycoprotein 4 (gp4) are minor components of the PRRSV envelope (Snijder and Meulenberg, 2001). The antigenicity of the gp2 is largely unexplored, and there are no data available regarding the North American strains. In this study, two B-cell linear epitopes were found to be immunoreactive with 60% (9/15) of the sera (Fig. 5). The reactive peptides comprise regions at amino acid positions 41–55 and 121–135 within the ORF2 sequence (Table 1). Using phage-displayed peptides, Oleksiewicz et al. (2002) identified

Table 1
Immunodominant B-cell linear epitopes identified on Nsp2 and structural proteins of a North American strain of PRRSV

Peptide no./ protein	Amino acid sequence	Position aa[a]	No. of reactive sera[b]
3/Nsp2	ALPAREIQQAKKHED	21–35	10/15
4/Nsp2	KKHEDAGADKAVHLR	31–45	8/15
15/Nsp2	ECVQGCCEHKSGLGP	141–155	14/15
24/Nsp2	LCQVVEECCCHQNKT	231–245	14/15
44/Nsp2	PPPPPRVQPRKTKSV	431–445	14/15
45/Nsp2	KTKSVKSLPGNKPVP	441–455	15/15
48/Nsp2	PDGREDLTVGGPLDL	476–490	8/15
50/Nsp2	PMTPLSEPALMPALQ	496–510	10/15
54/Nsp2	VTPLSEPIFVSAPRH	536–550	10/15
55/Nsp2	SAPRHKFQQVEEANL	546–560	14/15
58/Nsp2	ASSQTEYEASPLTPL	576–590	9/15
59/Nsp2	PLTPLQNMGILEVGG	586–600	10/15
61/Nsp2	VLSEISDTLNDINPA	606–620	13/15
76/Nsp2	VPRILGKIENAGEMP	756–770	12/15
79/Nsp2	QPVKDSWMSSRGFDE	786–800	15/15
81/Nsp2	SAGTGGADLPTDLPP	806–820	15/15
82/Nsp2	TDLPPSDGLDADEWG	816–830	10/15
83/Nsp2	ADEWGPLRTVRKKAE	826–840	9/15
102/ORF2	LPSLAGWWSSASDWF	41–55	9/15
110/ORF2	KAGQAAWKQVVSEAT	121–135	9/15
129/ORF3	QAAAEVYEPGRSLWC	61–75	13/15
130/ORF3	RSLWCRIGHDRCSED	71–85	14/15
131/ORF3	RCSEDDHDDLGFMVP	81–95	15/15
132/ORF3	GFMVPPGLSSEGHLT	91–105	15/15
153/ORF4	SCLRHGDSSSQTIRK	51–65	5/15
165/ORF5	MLGRCLTAGCCSRLL	1–15	7/15
168/ORF5	ANSNSSSHLQLIYNL	31–45	8/15
184/ORF5	TPLTRVSAEQWGRL	187–200	13/15
200/ORF6	LKSLVLGGRKAVKQG	151–165	15/15
201/ORF6	AVKQGVVNLVKYAK	161–174	15/15
203/ORF7	KKRGNGQPVNQLCQM	11–25	14/15
206/ORF7	PGKKIKNKNPEKPHF	41–55	7/15

The sequence of the immunoreactive synthetic peptides and the number of seropositive animals are indicated.
[a] Localization of the peptide within the amino acid sequence of the respective ORF.
[b] Number of reactive sera in the peptide ELISA. The reactivity of 15 sera was examined against each peptide.

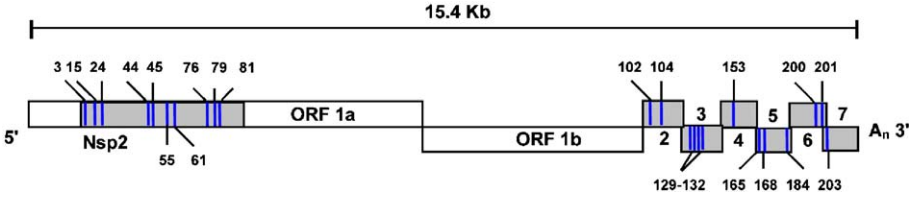

Fig. 1. Position of the immunodominant B-cell linear epitopes identified on the Nsp2 and structural proteins of PRRSV. The locations within the respective ORFs and identity number of the major synthetic peptides identified as B-cell epitopes in each protein are indicated. See Table 1 for the amino acid position of each identified epitope within the sequence of the respective ORF.

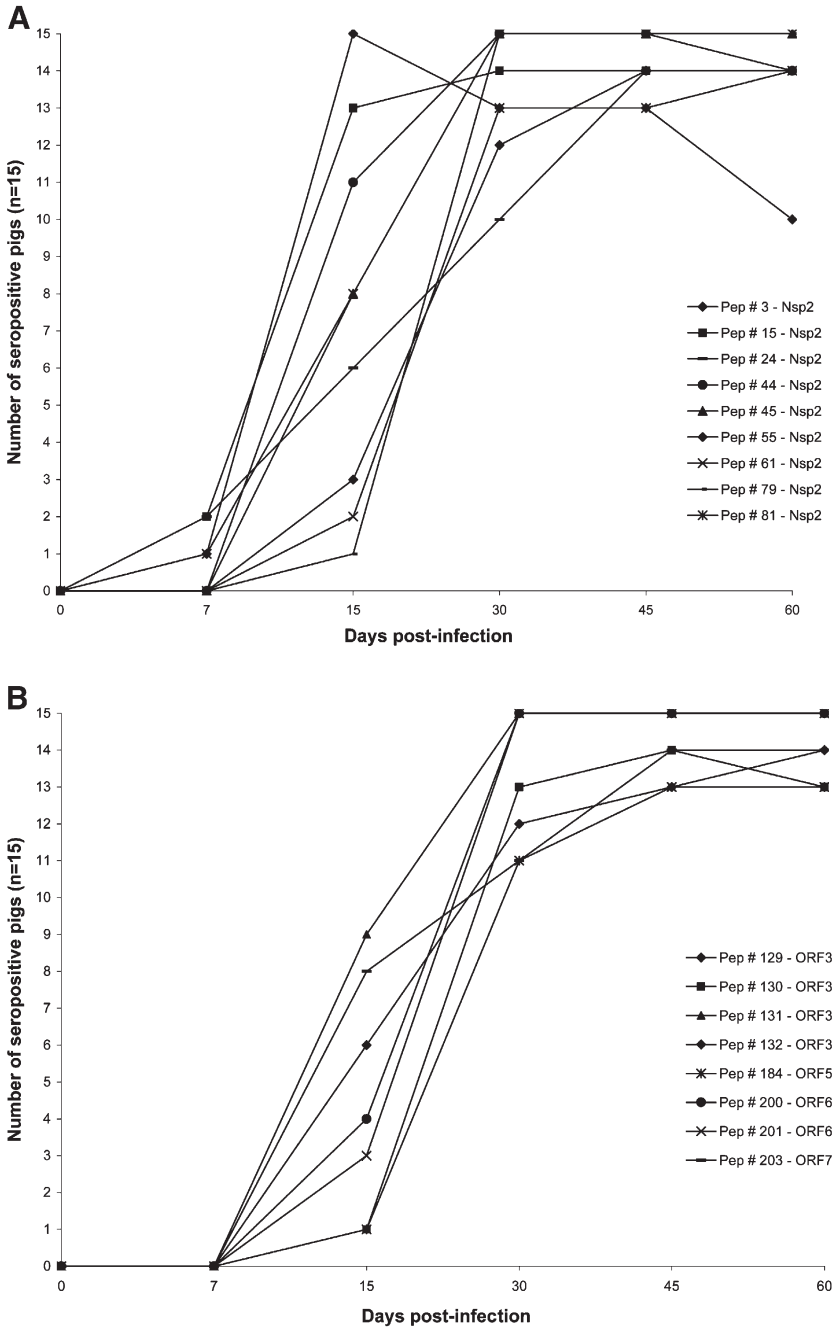

Fig. 2. Seroconversion kinetics of 15 experimentally infected pigs against PRRSV-specific B-cell epitopes identified on Nsp2 (A) and structural proteins (B) of a North American strain. Serum samples collected at 0, 7, 15, 30, 45 and 60 dpi were examined by peptide ELISA against all the immunodominant epitopes identified.

three weakly antigenic B-cell epitopes in the ORF2 at positions 36–51, 117–139 and 120–142 of an EU-type strain. Although the epitope mapping experiments were carried out using distinct approaches and different strains, the amino acid sequence G-^{123}QAAWKQVVXEAT^{135} localized in the predicted most hydrophilic domain of gp2 was identified in our study (peptide #110) as well as in that by Oleksiewicz et al. (2002). Thus, those residues might constitute the core of an epitope recognized by sera from pigs infected with EU- and US-type of PRRSV. However, it is important to consider that this region was recognized only by 1 out of 6 sera tested by Oleksiewicz et al. (2002) and by 60% of the 15 sera tested in our study, indicating a lesser immunodominance and diagnostic usefulness.

B-cell linear epitopes in the ORF3 protein

The highly glycosylated ORF3-encoded protein is the second most variable PRRSV protein, showing approximately 54 to 60% aa identity between the North American and European genotypes (Dea et al., 2000). In our investigation, four overlapping consecutive peptides (pep #129–132) were strongly immunoreactive with 85–100% of the tested sera

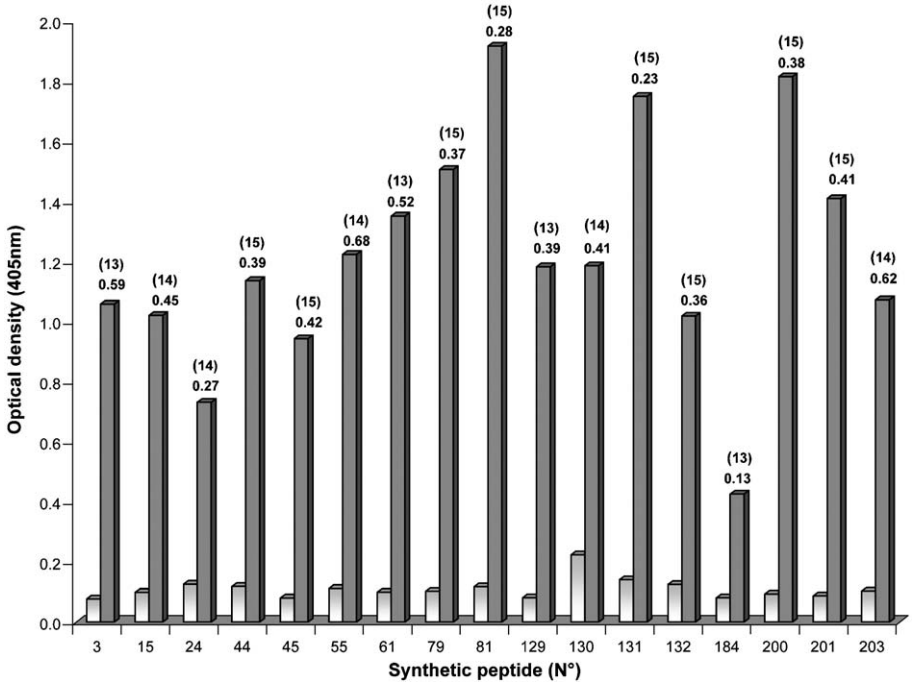

Fig. 3. Mean optical density (OD) scored by ELISA test using synthetic peptides recognized by the majority of the sera at 0 (open bars) and 60 dpi (darkened bars). The two decimal values located above the bars indicate the standard deviations of the seropositive samples, while the number in parentheses correspond to the number of reactive sera with each peptide (total of 15 sera examined). Peptides #3 to 81 (Nsp2); #102 and 110 (ORF2); #129 to 132 (ORF3); #184 (ORF5); #200 and 201 (ORF6) and # 203 (ORF7). Refer to Table 1 for exact location of the peptides.

(Table 1 and Fig. 5). Those peptides cover a region comprising amino acids 61–105, which is predicted to be located in the most hydrophilic region within the ORF3 sequence. These data suggest that this region might be considered as one important immunodominant domain of the gp3 of North American strains of PRRSV. Our findings are supported by results of a recent analysis of the antigenic structure of gp3 encoded by a Chinese isolate (US-type) of PRRSV (Zhou et al., 2005). After sequential deletion of amino acid residues from each peptide, these authors found that the minimal epitopes recognized by the MAbs were located at residues Y^{67}EPGRSLW^{74} and W-^{74}CRIGHDRCGED^{85}. Interestingly, except for a serine instead of a glycine at position 83, identical sequences recognized by MAbs were found in the peptides #129 and #130 which were reactive with 86.7 (13/15) and 93.3% (14/15) of the swine sera examined in our study. Most importantly, a high degree of sequence conservation within a segment comprising residues 69–78 and 90–99 was observed among North American isolates and reference strains of PRRSV (Fig. 6). Furthermore, in spite of the sequence variability observed among North American and European strains in this segment of gp3, Oleksiewicz et al. (2002) observed strong reactivity within a region comprising the amino acids 60–87 of an EU-type isolate of PRRSV.

B-cell linear epitopes in the ORF4 protein

The glycoprotein 4 (gp4) is a typical class I membrane protein, and it is a minor constituent of the viral envelope (Meulenberg, 2000). In the present study, only a small fraction (33.3% and 26.6%) of the tested sera were found to be reactive with peptides #153 and #158 comprising amino acid residues 51–65 and 101–115 within the ORF4 protein (Fig. 5). The core of a neutralization domain of the glycoprotein encoded by ORF4 of Lelystad virus and recognized by MAbs consists of amino acids 59 to 67 and is located at the most variable region of the protein (Meulenberg et al., 1997). However, further studies are necessary to demonstrate whether the linear epitope identified in our study (aa 51–65) is recognized by neutralizing antibodies. In addition, a single linear epitope in gp4 (aa 59–71) encoded by a European strain was found to be immunodominant in pigs, and a putative decoy function for this region has been suggested (Oleksiewicz et al., 2001, 2005). However, we did not detect immunodominant sequences in this region (peptide #153) since it was recognized by only 5 out of 15 tested sera.

B-cell linear epitopes in the ORF5 protein

Glycoprotein 5 (gp5) is one of the major structural proteins encoded by PRRSV and forms disulfide-linked heterodimers with M protein in the viral envelope (Snijder and Meulenberg, 2001). Specific IgG antibodies to gp5 are detected at the end of the first week after infection and at around 14 dpi to M protein (Dea et al., 2000). A neutralizing epitope (epitope B) in the ectodomain of gp5 has been previously described (Ostrowski et al., 2002). The core sequence of this neutralizing epitope "B" (H^{38}, Q^{40}, I^{42}, Y^{43} and N^{44}) is present in peptide #168 that was found to be reactive with 8 out of 15 sera in our experiments. However, since neutralizing activity was observed in all of the

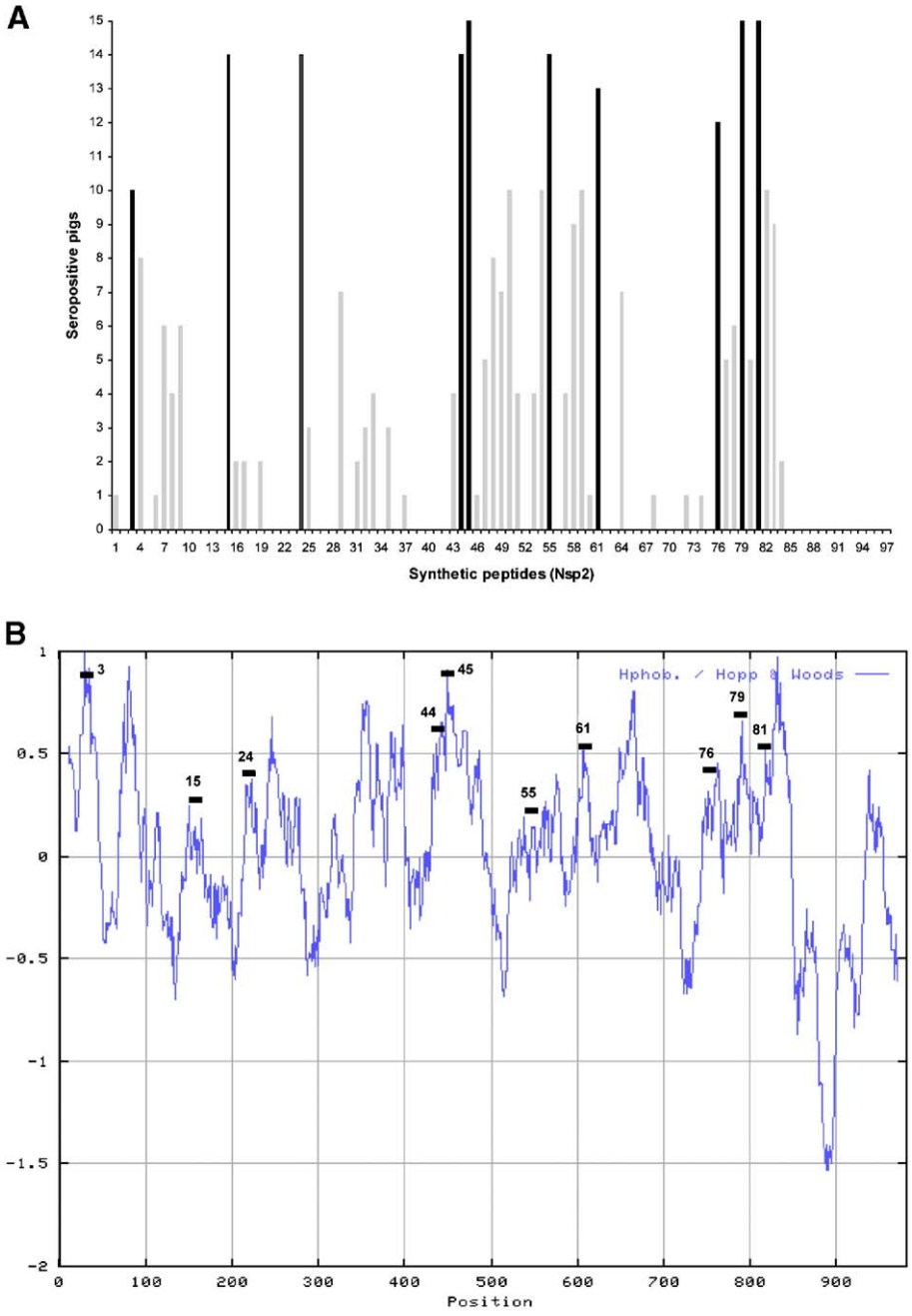

Fig. 4. (A) B-cell linear epitopes on the Nsp2 amino acid sequence of a North American strain of PRRSV identified by Pepscan analysis. Black bars represent the immunodominant epitopes identified. Sera were considered positive when the OD values were above the cutoff point (the mean OD of absorbance at 405 nm of the negative sera plus 3 standard deviations). (B). Hydropathic profile of the protein which was generated by the ProtScale program (http://us.expasy.org) with window size of 21 and using parameters defined previously (Hoop and Woods, 1981).

15 sera samples used in our experiments (including the seven sera which did not react with peptide #168, data not shown), one might speculate that, besides epitope B, there may be other neutralizing epitopes present on PRRSV that would contribute to the total PRRSV-neutralizing activity in the serum. In addition, the peptide comprising the residues 187–200 (pep #184) located in the 3′ endodomain of the protein was recognized by 13/15 of the examined sera. The aa sequence P^{188}LTR(V/T)SAEQW^{197} was also found to be reactive with sera raised against an European PRRSV strain (Oleksiewicz et al., 2002). Surprisingly, peptide #184 exhibited the lowest value of the mean optical densities obtained among all the immunodominant epitopes identified (Fig. 3). Despite some amino acid changes within this region and the low immunogenicity observed, this decapeptide is relatively well conserved among North American strains (Fig. 6). Most importantly, this peptide was recognized by the majority of the animals used in our experiment. This inverse correlation between immunogenicity and level of sequence conservation has been previously described (Oleksiewicz et al., 2001).

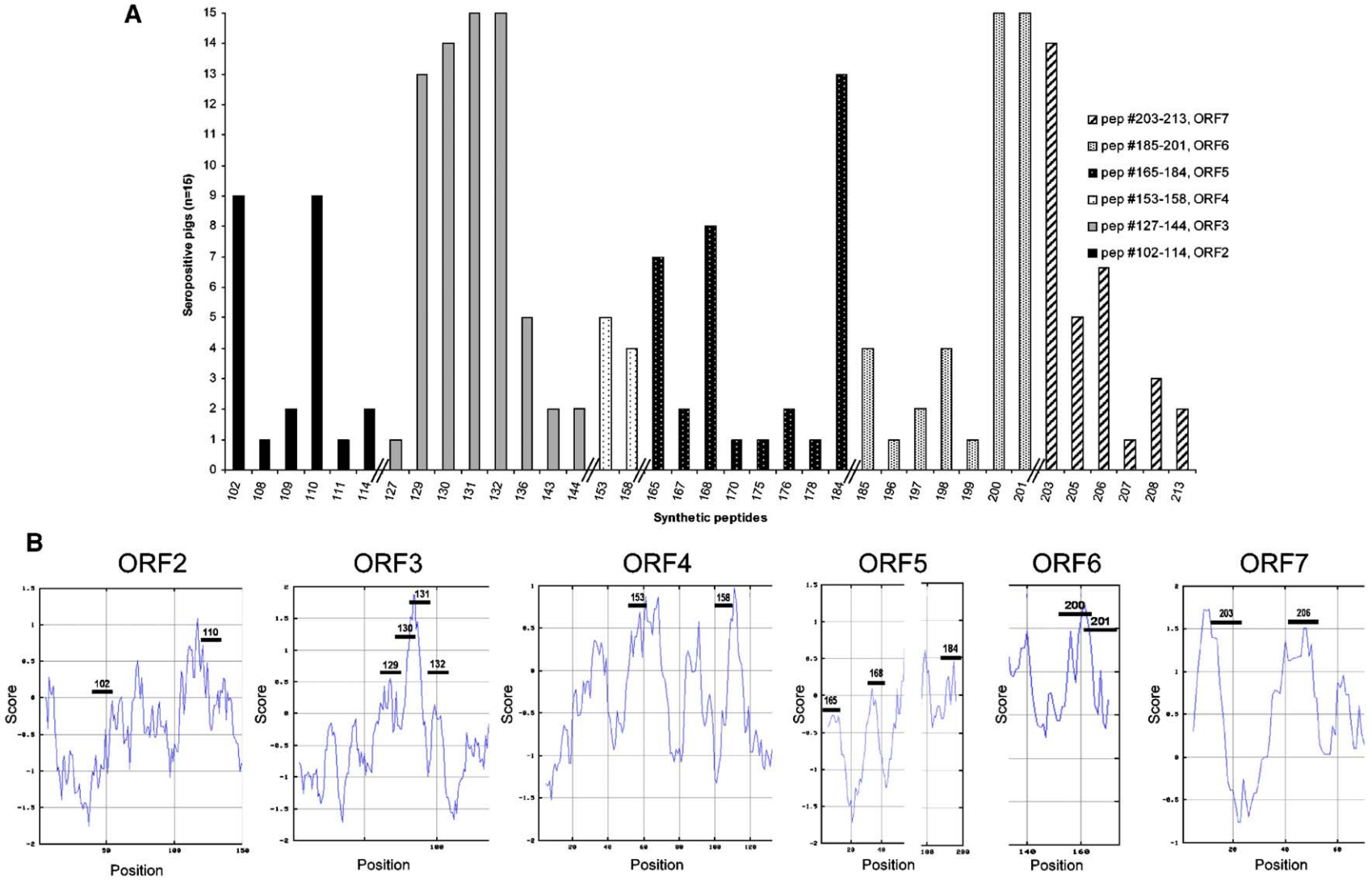

Fig. 5. (A) B-cell linear epitopes identified along the structural proteins (ORFs 2–7) of a North American strain of PRRSV by Pepscan analysis. The numbers of the corresponding peptides in each ORF are indicated. Sera were considered positive when the OD values were above the cutoff point (the mean OD of absorbance at 405 nm of the negative sera plus 3 standard deviations). (B) Hydropathic profiles of the proteins which were generated by the ProtScale program (http://us.expasy.org) with window size of 9 and using parameters defined previously (Hoop and Woods, 1981). The localization of the immunodominant epitopes is indicated.

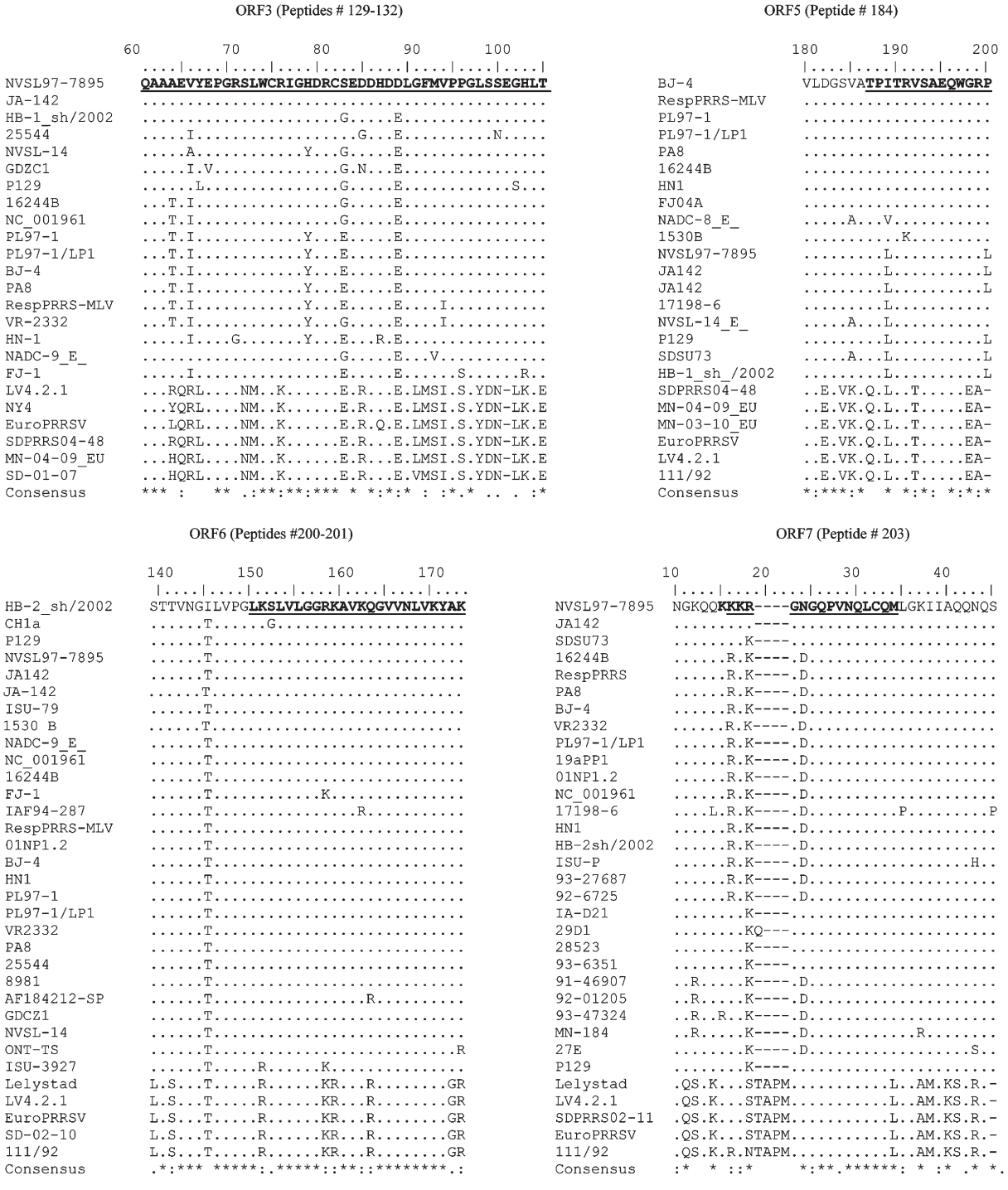

Fig. 6. Multiple alignment of ORFs 3, 5, 6 and 7 among North American- and European-type PRRSV isolates. The amino acid sequences of the immunodominant epitopes identified in each ORF are underlined. Alignments of the amino acid sequences were made using ClustalW (Thompson et al., 1994), and then the results were retrieved and analyzed by Bio-Edit sequence alignment editor v. 7.0.5.

B-cell epitopes in the ORF6 protein

The nonglycosylated M protein (16–20 kDa) is the most conserved structural protein of arteriviruses. Abundant molecules of M protein are present in the virion associated with gp5, and its N-terminal half presumably traverses the envelope membrane three times (Dea et al., 2000; Snijder and Meulenberg, 2001). The protein has only a short stretch of 10 to 18 residues exposed at the virion surface and a large endodomain (Dea et al., 2000; Snijder and Meulenberg, 2001). In this study, we demonstrated that peptides #200–201 constitute a unique combination of sequence conservation and antigenicity (Figs. 5 and 6). These peptides were found to be reactive with 100% of sera examined. These immunoreactive peptides are located

within the region at position 151–174 which corresponds to the C-terminus end of the endodomain of M protein. Eventually, the 5 aa (A^{161}VKQG^{165}) could be considered the minimal sequence recognized by antibodies since this segment corresponds to the overlapping residues of peptides #200 and #201. Oleksiewicz et al. (2002) also reported the identification of one phage-displayed epitope localized in the large putative endodomain of the M protein (aa 138–159) of a European-type strain. In their experiment, this epitope was recognized by a reduced number of sera collected very late in infection. In the present study, the overlapping peptides were reactive with 100% of the sera examined. The identified epitopes were found to be highly immunogenic and conserved among isolates and reference strains of both PRRSV genotypes (Fig. 6). In order to confirm the results demonstrated by the sequence alignment, we further tested the reactivity of both synthetic peptides with sera from 21 pigs experimentally infected with the homologous (NVSL 97-7895) PRRSV strain and antisera raised against four heterologous US strains. As expected, all sera samples were confirmed positive (data not shown). Reactivity of these two synthetic peptides with a sizable number of field sera was also observed (data not shown). In addition, antiserum against Lelystad virus (European prototype strain of PRRSV) was also reactive with peptide #201. These results demonstrate that the peptide containing the residues A^{161}VKQGVVNLVKYAK^{174} can be particularly useful for diagnostic purposes and one attractive candidate to be evaluated as a negative serological marker in a PRRSV vaccine derived from infectious cDNA clones, if proved to be dispensable from the ORF6 without affecting the viability of the vaccine strain and/or protective immunity induced by it.

B-cell epitopes in the ORF7 protein

After infection, most PRRSV-specific IgG antibodies are primarily directed against the nucleocapsid (N) protein and are detectable as early as 7 dpi. Thus, these antibodies may be useful for diagnostic purposes (Dea et al., 2000). A commonly used commercial PRRSV ELISA kit contains N protein as the single antigen for serological diagnosis of PRRSV infections. Our epitope mapping has demonstrated that, among the 12 peptides derived from the N protein amino acid sequence, six were found to be immunoreactive. Two out of these six peptides (pep #203, aa 11–25 and #206, aa 41–55) located at predicted hydrophilic domains of the protein were reactive with 14/15 and 7/15 of the sera, respectively (Fig. 5). Furthermore, we observed that residues G^{18}(N/D)GQPVNQ^{25} contained in peptide #203, which was recognized by 93.3% of the animals, were well conserved among 33 isolates and reference strains from both PRRSV genotypes (Fig. 6). Recently, An et al. (2005) using a phage-displayed peptide library, identified a well conserved B-cell epitope with an anti-N protein MAb. The core sequence recognized by this MAb comprised the residues I^{79}QTAFNQGA^{87} in the context of the N protein. No reactivity was observed within that region probably because no peptide examined in our study contained the minimal sequence previously identified as a B-cell epitope.

Antibodies recognizing the immunodominant epitopes of Nsp2 and structural proteins appear between day 7 and 15 pi and remain detectable until at least 90 dpi

Serum samples collected at different time points after experimental infection were used to study the seroconversion kinetics to the immunodominant epitopes identified in Nsp2 and structural proteins of the NVSL 97-7895 strain. Animals seroconverted to the immunogenic B-cell epitopes at different times post-infection ranging from 7 to 45 dpi. Seroconversion kinetics revealed that peptide-specific antibodies started appearing generally between days 7 and 15 post-infection, increased in titer with time and remained at fairly steady and high levels up to day 60 pi (Fig. 2). In addition, reactivity was also detected against all the identified epitopes with sera collected at 90 dpi, although a slight decrease in the OD values could be observed when compared to those recorded at 60 dpi (not shown). Furthermore, the antibody response to some Nsp2 epitopes seemed to appear slightly earlier in some animals when compared to the response against the structural proteins. However, the seroconversion kinetics to the immunodominant epitopes was not able to discriminate between the serological response to Nsp2 and structural proteins.

Significance of the epitope information

The feasibility of a new strategy for designing marker vaccines based on the deletion of immunodominant epitopes has been recently demonstrated for RNA viruses (Castillo-Olivares et al., 2003; Mebatsion et al., 2002). However, the possibility of using such approach for the development of PRRSV vaccines remains to be explored yet. Likewise, information regarding immunogenicity and presence of B-cell epitopes, which could be used as serological marker candidates, in different US-type PRRSV proteins is scarce. Fang et al. (2004) identified natural deletions within Nsp2 gene of European-like PRRSV isolated in the United States, suggesting that this protein could represent an ideal target for the development of marker vaccines. The data presented in our paper indicate the presence of several B-cell epitopes distributed along the amino acid sequences of Nsp2 and structural proteins of the North American strain NVSL 97-7895, which served as basis for the construction of an infectious full-length cDNA clone of PRRSV (Truong et al., 2004). Additionally, several epitopes (especially those found in ORF6) were found to be highly immunogenic, consistently recognized by the 15 PRRSV-infected pigs and well conserved among North American and European strains of PRRSV. To our knowledge, this is the first report demonstrating the presence of B-cell linear epitopes consistently recognized by immune serum from pigs experimentally infected with US-type PRRSV.

The detailed and systematic methodology employed in this study by using overlapping synthetic peptides enabled us to identify a higher frequency of B-cell linear epitopes (mainly in Nsp2) in comparison to previous findings reported for EU-type PRRSV strains. The identification of conserved and antigenic peptides corresponding to B-cell epitopes consistently

recognized by PRRSV-infected animals may have major practical significance by providing the molecular basis for development of improved diagnostic tests as the identified epitopes may be considered serological marker candidates for differential (infection vs. vaccination) marker PRRSV vaccines derived from infectious cDNA clones.

Finally, it must be born in mind that some of the immunodominant B-cell epitopes we identified on the PRRSV proteins may have a role in PRRSV-neutralizing activity and therefore on PRRSV protective immunity. It will be interesting to investigate if additional epitopes other than "B" epitope on GP5 and perhaps in other glycoproteins and M protein have a role in neutralization. Such quality, although important for protection and vaccine design, could recommend against their use as deletable serologic differential markers.

Materials and methods

Cells and virus

Infectious PRRSV virus (US-type) recovered from MARC-145 cells transfected with in vitro produced transcripts of the full-length cDNA clone (FL12) of PRRSV NVSL 97-7895 (Truong et al., 2004) was used for animal inoculation and antiserum production. The cells were propagated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 units/ml of penicillin, 20 μg/ml of streptomycin and 20 μg/ml of kanamycin). These cells were used for electroporation of RNA, viral infection and growth and for virus titration.

Animal inoculation

Fifteen mixed-breed (Landrace×Large White) piglets averaging 3 weeks of age were obtained from a PRRSV-free farm. The animals were allocated in three BL-2 isolation rooms and inoculated with a total dose of $10^{5.0}$ $TCID_{50}$/3 ml of PRRSV FL12 by intranasal (1 ml in each nostril) and intramuscular (1 ml) routes. The animals were clinically monitored on a daily basis, and their rectal temperatures were recorded from 2 days pre-inoculation to day 15 post-infection (pi). Sequential blood samples were collected from all animals at days 0 (zero), 7, 15, 30, 45, 60 and 90 pi and tested for PRRSV-specific antibodies by using a commercially available ELISA kit (Idexx Labs, Inc).

Synthetic peptides

A set of 213 overlapping 15-mer synthetic peptides, which overlapped each other by 5 aa, spanning the entire amino acid sequence of the nonstructural protein Nsp2 (n = 97) and all structural proteins (ORF2, n = 25; ORF3, n = 25; ORF4, n = 17; ORF5, n = 20; ORF6, n = 17 and ORF7, n = 12) of the North American strain of PRRSV (NVSL 97-7895), were used individually in a peptide-based enzyme-linked immunosorbent assay. Peptides were synthesized using Fmoc solid-phase chemistry by Open Biosystems, Inc, Huntsville, AL.

Peptide ELISA

Serum samples collected at day 60 pi from the 15 piglets experimentally infected with FL-12 strain were used for screening of the peptide-specific antibody response by ELISA. Briefly, Immulon 2HB flat bottom microtiter 96 well plates (Thermo Electron, Milford, MA) were coated with 100 μl of a peptide solution (10 μg/ml) in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After blocking with 250 μl of a 10 wt.% nonfat dry milk solution for 4 h at room temperature on a plate shaker, the plates were washed three times with PBS containing 0.1% Tween 20 (PBST-20). Unbound reagents were further removed by striking the plates repeatedly, bottom up, on a stack of absorbent paper towel. Then, 100 μl of pig sera (1:20) diluted in 5 wt.% nonfat dry milk in PBST-20 was added per well and plates were incubated on the shaker for 1 h at room temperature. After washing five times with PBST-20, each well received and was incubated with 100 μl of the affinity purified antibody peroxidase labeled goat anti-swine IgG (KPL, Gaithersburg, MD) diluted 1:2000 in PBST-20 with 5 wt.% nonfat dry milk for 30 min at room temperature. Following a final wash, 100 μl of ABTS (KPL) peroxidase substrate was added for 15 min at 37 °C and the reaction was stopped by adding 100 μl of SDS 1%. A 12-mer synthetic peptide (YKNTHLDLIYNA) which has been shown to be recognized by PRRSV neutralizing antibodies (Ostrowski et al., 2002) served as a positive peptide control. Serum samples collected at day 0 (zero) were used as negative control. Serum was considered positive when the OD value was above the cutoff point (the mean OD absorbance at 405 nm of the negative sera plus 3 standard deviations). The same experimental conditions were applied to ELISAs conducted with each one of the 213 synthetic peptides used in this study.

Seroconversion kinetics

The seroconversion kinetics to the immunonodominat epitopes identified in Nsp2, gp3 (ORF3), gp5 (ORF5), M protein (ORF6) and N protein (ORF7) was examined by using sequential serum samples collected from the infected piglets at days 0, 7, 15, 30, 45 and 60 dpi in the corresponding peptide-based ELISA. The serum was considered positive when the OD value was above the cutoff point.

Bioinformatics analysis

Hydropathic profiles were produced by the ProtScale program (http://us.expasy.org) using parameters defined previously (Hoop and Woods, 1981). Window sizes of 9 and 21 were used for all the structural proteins and for Nsp2, respectively. Multiple alignments of amino acid sequences were made using ClustalW (Thompson et al., 1994). Alignments were retrieved and analyzed by Bio-Edit sequence alignment editor v. 7.0.5.

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