Cross-reactive antibody responses to nsp1 and nsp2 of *Porcine reproductive and respiratory syndrome virus*

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Porcine reproductive and respiratory syndrome virus (PRRSV) non-structural proteins (nsps) play a key role in processing and maturation of the repertoire of structural and nsps of the virion, but little is known about the anti-nsp immune response. Here, it was hypothesized that pronounced antibody responses are generated to PRRSV nsp1 and nsp2, as they are present in infected cells and cytolysis of infection releases viral proteins into interstitial spaces. Accordingly, nsp1 and nsp2 were cloned and expressed, and antibody responses in the sera of infected and vaccinated pigs were determined. Pigs mounted significant cross-reactive antibody responses that appeared equivalent to or greater than the response to nucleocapsid (N). Antibody reactivity to nsp1 and N was highly dependent on refolding of denatured proteins, suggesting that the porcine antibody response is directed primarily to conformational epitopes. The proteins reacted with sera from pigs infected with other PRRSV strains, indicating that multiple epitopes are conserved. Antibody responses to nsp1 and nsp2 were much higher than those to nsp4, which is encoded on the same RNA molecule and is equivalent in predicted antigenicity. These findings suggest either that nsp1 and nsp2 are highly immunogenic or that they are expressed at higher levels than nsp4 in PRRSV-infected cells, or both. Strong antibody responses to nsp1 and nsp2 may benefit the host by limiting potentially pathological consequences of viral protease activities encoded in these proteins that are released from dying cells. The identification of strain-specific antibody responses to a highly variable region of nsp2 may also provide the basis for immunoassays that differentiate serological responses of vaccines from field isolates.

**INTRODUCTION**

A new viral disease of pigs, typified by late-term abortions and stillbirths in sows and interstitial pneumonia in nursery pigs, was detected in North America in 1987 (Hill, 1990; Keffaber, 1989) and in Europe in 1990 (Paton et al., 1991), and then characterized as porcine reproductive and respiratory syndrome (PRRS) caused by *Porcine reproductive and respiratory syndrome virus* (PRRSV) (Collins et al., 1992; Wensvoort et al., 1991). The causative agent is a small, enveloped, positive-stranded RNA virus. It is a member of the family *Arteriviridae*, which includes *Equine arteritis virus* (den Boon et al., 1991; Meulenberg et al., 1993), *Lactate dehydrogenase-elevating virus* of mice (Plagemann & Moennig, 1992) and *Simian hemorrhagic fever virus* (Zeng et al., 1995), in the order *Nidovirales* (Cavanagh, 1997). PRRSV predominantly infects macrophages (Cavanagh, 1997). PRRSV predominantly infects macrophages and establishes a persistent infection in resident macrophages of numerous lymphoid tissues (Lawson et al., 1997).

A complex immunological interaction exists between PRRSV and pigs that involves both induction and subversion of host defences (Murtaugh et al., 2002). Exposure to PRRSV induces an immune response that protects pigs against re-exposure to the same virus. However, pigs exposed to PRRSV also demonstrate prolonged viraemia and persistent infection, may continue to shed virus, can become re-infected and may suffer a repeat episode of the disease (Christopher-Hennings et al., 1997; Mavromatis et al., 1999; Mengeling et al., 1999; Nielsen et al., 1997; Rossow, 1998; van Woensel et al., 1998). We are interested in the development of antigen-specific antiviral immune responses whose characteristics might help to explain the ability of PRRSV to persist in swine.

The current study aimed to determine the humoral immune response to the viral proteins expressed early in infection and to develop tools for elucidation of the immune response to PRRSV. nsp1 is a multifunctional protein containing two papain-like cysteine proteases (PCPα and PCPβ) and a zinc-finger motif required for subgenomic mRNA transcription (den Boon et al., 1995; Oleksiewicz et al., 2004; Tijms & Snijder, 2003; Tijms et al., 2001). Intracellular concentrations of nsp1 may be higher than for other nsps, due to translation from heteroclite RNAs (Yuan et al., 2000, 2004). The nsp2 polypeptide contains a cysteine protease active site, although no viral or
cellular prototypes are known (Ziebuhr et al., 2000). These proteins are vital to the viral life cycle and their presence in cells is likely to be toxic, due to their protease activities. The proteases are encoded in the 5′ terminus of the first open reading frame (ORF) of the genomic RNA, whereas downstream ORFs are synthesized after formation of subgenomic nested mRNAs (Meng et al., 1996; Yuan et al., 2001b; Ziebuhr et al., 2000). Hence, nsp1 and nsp2 are available from the earliest time of infection for presentation to the immune system in the context of major histocompatibility complex (MHC) class I antigen-presentation pathways. As cytolysis also releases viral proteins into interstitial spaces, we hypothesized that a pronounced antibody response, equivalent to the immune response to structural proteins, would be generated to nsp1 and nsp2.

Antibody responses to linear epitopes in nsp2 have been reported to appear within 1–4 weeks of infection in PRRSV nsps or on relative levels of anti-nsp antibodies. Antibody responses to linear epitopes in nsp2 have been reported to appear within 1–4 weeks of infection in PRRSV nsps or on relative levels of anti-nsp antibodies. These findings indicate that nsp1 and nsp2 are national epitopes may be important in the porcine immune response. These findings indicate that nsp1 and nsp2 are national epitopes may be important in the porcine immune response.

PCR amplification, cloning of DNA fragments and restriction analysis. Primers were designed to regions of the PRRSV strain VR2332 sequence (GenBank accession no. U87392) by using Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and obtained from Integrated DNA Technologies. PRRSV cDNA fragments for cloning were obtained by RT-PCR amplification of regions of VR2332 genomic RNA encoding nsp1α and -β and nsp2 (Table 1). Briefly, 50 μl PCR mixtures contained 10× buffer II (1× concentration), 1.5 mM MgCl₂, 200 μM each of dATP, dCTP, dGTP and dTTP; 0.2 μM each primer pair (Table 1), 1.0 U AmpliTaq Gold (Roche Molecular Systems) and 1–100 μg of the appropriate cDNA. Amplification was performed in a GeneAmp PCR system 2400 (Perkin Elmer) with one cycle of 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, then 72 °C for 7 min and a 4 °C hold. Amplified DNA was electrophoresed on an agarose gel. Bands corresponding to the predicted product sizes were gel-extracted (Qiagen) then purified further by using a PCR purification kit (Qiagen). The isolated products were cloned into the pGEM-T vector (Promega), transformed into Escherichia coli DH5α cells (Invitrogen) and spread on Luria–Bertani (LB) agar plates containing 100 μg ampicillin (Amp) ml⁻¹, 0.5 mM IPTG and 50 μg X-Gal ml⁻².

White colonies were grown and sequenced by using the standard T7 and SP6 primers (Advanced Genetic Analysis Center, University of Minnesota, St Paul, MN). Standard laboratory supplies, bacterial growth media and electrophoresis chemicals were from Sigma.

A modified vector based on pET 24b (Novagen) containing a myc tag 5′-leader sequence and a terminal 3′ His tag was engineered for high-efficiency protein expression and isolation. The plasmid, pET 24b myc His, contains a BamHI site immediately 3′ to the myc tag and an Xhol site preceding the terminal 6× His tag. The vector was prepared for insertion by digestion with BamHI and Xhol, followed by dephosphorylation with calf intestinal alkaline phosphatase (Promega). Inserts were prepared either by restriction digestion of the pGEM-T constructs or by PCR amplification followed by BamHI and Xhol digestion. Ligation was performed with 100 ng dephosphorylated vector, 20 ng insert, 1× ligation buffer and 400 U T4 DNA ligase (New England Biolabs) in a total volume of 10 μl for 16 h at 16 °C. Transformed DH5α colonies were selected on LB agar plates containing kanamycin (kan; 30 μg ml⁻¹). Purified plasmid (referred to as pET 24b myc nsPHis) integrity was confirmed by DNA sequencing.

Table 1. PRRSV recombinant protein and polypeptide expression

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Nucleotide sequence*</th>
<th>Calculated molecular mass (Da)†</th>
<th>Expression yield [mg (l culture)⁻¹]</th>
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<tr>
<td></td>
<td></td>
<td>Total expressed Ni–NTA-purified After refolding</td>
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<tr>
<td>nsp1 (PCPs/β)</td>
<td>190–1341</td>
<td>43 020</td>
<td>20.0</td>
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<tr>
<td>nsp2 (unusual cysteine protease)</td>
<td>1339–4923</td>
<td>129 356</td>
<td>1.0</td>
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<tr>
<td>nsp2 P (cysteine protease fragment)</td>
<td>1339–3495</td>
<td>78 232</td>
<td>20.0</td>
</tr>
<tr>
<td>nsp2 HP (cysteine protease hypervariable region)</td>
<td>2731–3207</td>
<td>16 467</td>
<td>30.0</td>
</tr>
<tr>
<td>nsp2 HP S1 (small peptide 1)</td>
<td>1372–1473</td>
<td>3 638</td>
<td>25.0</td>
</tr>
<tr>
<td>nsp2 HP S2 (small peptide 2)</td>
<td>2098–2205</td>
<td>4 177</td>
<td>25.0</td>
</tr>
<tr>
<td>N (nucleocapsid)</td>
<td>14889–15257</td>
<td>13 576</td>
<td>40.0</td>
</tr>
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</table>

*Relative to GenBank accession no. U87392.
†Molecular mass (MM) does not include the amino-terminal 20 aa myc tag with linker (MEQKLISEEDLNRSMNSSGKS), MM=2255 Da, or the carboxyl-terminal 6× His tag (HHHHHHH), MM=841 Da, which are present in all of the polypeptides.
and T7 terminator primers for the pET 24b plasmid. Ten positive colonies were grown overnight at 30 °C in 2 ml 2 × YT medium (BD Diagnostic Systems) with antibiotics. Two hundred microlitres of each of the overnight cultures was used to inoculate ten temperature-equilibrated (30 °C) 10 ml aliquots of 2 × YT (kan, 30 μg ml⁻¹). These cultures were grown at 30 °C to an OD_{600} of 0.4, 200 μl was removed for SDS-PAGE analysis and IPTG was added to a final concentration of 1.0 mM. The induced samples were allowed to grow at 30 °C for 4 h, then 200 μl was removed for SDS-PAGE analysis.

Large-scale protein expression and purification. Protein was purified by using a modification of the Qiagen Ni–NTA agarose-affinity isolation procedure for native His-tagged proteins. Briefly, 11 induced bacterial cells was centrifuged at 4000 g for 20 min at 4 °C and supernatant was decanted. The pellet was resuspended in 30 ml 100 mM NaH₂PO₄, 10 mM Tris/HCl, 8 M urea (pH8.0), rotated at 200 r.p.m. at room temperature for 30 min and centrifuged for 30 min at 4 °C at 10 000 g to pellet the cellular debris. The supernatant containing recombinant protein was decanted into 6 ml 50 % Ni–NTA slurry and rotated gently at 200 r.p.m. for 1 h at 4 °C. The mixture was then poured into a 1.5 × 30 cm column and allowed to drain. The column was washed twice with 20 ml of a solution containing 100 mM NaH₂PO₄, 10 mM Tris/HCl, 8 M urea (pH 6.3). The protein was then eluted with 100 mM NaH₂PO₄, 10 mM Tris/HCl, 8 M urea (pH 5.9). Purified proteins were concentrated by either a tangential-flow filtration cassette (Pellicon XL Ultracel PLC 5 kD; Millipore) or a YM-3 Amicon Centricon centrifugal filter device (Millipore), followed by dialysis (Spectra/Por MWCO 8000; Spectrum Laboratories) against 20 mM sodium phosphate (pH 7.5). Protein concentrations were determined using Bio-Rad DC protein assay kit, A₂₈₀ measurement and Coomassie blue staining of SDS–acylamide gels that contained standard amounts of BSA and lysozyme. Purified protein solutions were stored at −80 °C.

Protein refolding. Refolding of the denatured recombinant proteins was performed essentially as described (Büchner & Kiefhaber, 2004; Büchner et al., 1992; Clark, 1998). Briefly, denatured protein solutions were dialysed (Spectra/Por MWCO 8000; Spectrum Laboratories) into 0.1 M Tris/HCl (pH 8.0), 6 M guanidine hydrochloride and 2 mM EDTA. Protein concentration was adjusted to 3 mg ml⁻¹ and dithiothreitol was added to 300 mM. The resulting 5 ml solution was stirred at room temperature for 2 h, followed by filtration using a 0.45 μm filter (Syringe Filter; Fisher Scientific). The reduced protein solution was diluted 1/100 by rapid addition at 4 °C with moderate stirring into 500 ml refolding buffer [100 mM Tris/HCl (pH 8.0), 0.5 M l-arginine, 8 mM oxidized glutathione, 2 mM EDTA, 10 μM pepstatin A, 10 μM leupeptin, 1 mM PMSF]. The resulting solution was filtered through a 0.22 μm membrane (Steritop; Millipore) to remove particulates and stirred overnight. Purified protein was concentrated by tangential-flow filtration (Pellicon XL Ultracel PLC 5 kD; Millipore) to a volume of 10 ml, followed by dialysis (Spectra/Por MWCO 8000) against 20 mM sodium phosphate (pH 7.5).

Gel electrophoresis and immunoblotting. Bacterial lysates, purification fractions and purified proteins were analysed by SDS-PAGE. Protein bands were visualized by staining with 0.025 % Coomassie blue. For immunoblotting, gels were electroblotted onto supported nitrocellulose membranes (MSI Separations). Membranes were incubated with anti-myc monoclonal antibody 9E10 for 1 h at room temperature, detected with alkaline phosphatase-conjugated goat anti-mouse IgG and visualized with the ECL Western blotting system (Amersham Biosciences).

ELISA determinations. ELISA plates were coated with individual PRRSV proteins in 100 μl carbonate buffer [15 mM Na₂CO₃, 35 mM NaHCO₃ (pH 9.6)] or buffer alone, left overnight and washed six times with 0.05% Tween 20 in PBS (PBS-Tween). Two hundred microlitres of PBS-Tween containing 2.5 % non-fat dried milk was added for 1 h at room temperature to block previously unbound sites, and the plates were washed five times. One hundred microlitres of pig serum at various dilutions was added in duplicate for 2 h at room temperature, and plates were washed four times with PBS-Tween. Levels of specific antibody were determined by incubation of wells with horseradish peroxidase-conjugated goat anti-swine IgG (heavy + light chains) (KPL) diluted 1/5000 for 1 h. Wells were washed five times and colour was developed with 100 μl TMB substrate (KPL). Reactions were stopped after 15 min with 100 μl 1 M phosphoric acid and A₄₅₀ was read (ThermoMax plate reader; Molecular Devices). End-point titration values (titres) were calculated by using a non-linear least-squares-four-parameter fit with background subtraction and a 0.1 absorbance unit cut-off (Kemeny, 1991).

RESULTS

Recombinant nsp fragment cloning, expression and purification

DNA fragments corresponding to all or portions of nsp1 and nsp2 were cloned into pET24b-mycHis and single, high-expressing clones were picked, grown, purified and sequenced. The results showed that the predicted ORFs for each of the constructs encoded the expected polypeptide.

A variety of E. coli strains and growth conditions were evaluated, and we obtained consistent expression of recombinant nsp1, nsp2 P (cysteine protease fragment), nsp2 HP (cysteine protease hypervariable region), nsp2 HP S1 (small peptide 1) and nsp2 HP S2 (small peptide 2) at concentrations of 20–25 mg l⁻¹ in shake flasks under the described conditions using E. coli strain BL21 (DE3)-RP in rich medium (2 × YT), at moderate temperature (30 °C) and short induction times (4 h). Approximately 50 % of the protein was recovered following affinity chromatography and refolding (Table 1). The purified and refolded proteins were homogeneous and contained fragment sizes consistent with predicted molecular masses (Fig. 1). As shown in Fig. 1(b), the nsp1 preparation consisted of intact 46 kDa polypeptide and two fragments cleaved autoproteolytically into PCP1α (20 kDa) and PCP1β (26 kDa). Immobilized metal-affinity chromatography bound the full-length polypeptide and the carboxyl-terminal PCP1β (Fig. 1b, lane 1). After storage in PBS, the preparation was entirely cleaved, so that immunoblotting with anti-myc antibody revealed only the amino-terminal PCP1α (Fig. 1b, lanes 2 and 3).

Microtitre-plate wells coated with induced bacterial lysates from clones expressing nsp2 reacted strongly, specifically and in a dilution-dependent fashion with sera from PRRSV-infected pigs (data not shown). However, the protein was not evident on Coomassie blue-stained gels of whole-cell lysates and was observed by Western blot detection only with anti-myc antibody (Fig. 1c). Digestion of pET 24b myc nspHis with Xhol and subsequent religation produced a truncated nsp2 P that lacked a hydrophobic region. The resulting 81 kDa polypeptide was expressed at high levels in shake flasks (Table 1) and was confirmed by
immunoblotting with anti-myc antibody (Fig. 1c). nsp2 fragments HP, HP S1 and HP S2 also were cloned, expressed and confirmed by SDS-PAGE and Western blot (Table 1; Fig. 1c).

Effect of protein refolding on ELISA reactivity

We observed previously that the immunoreactivity of recombinant nucleocapsid (N) appeared to vary depending on the conditions of purification and refolding. Therefore, we evaluated the immunoreactivity of various cloned polypeptides before and after refolding to determine the effects on ELISA detection.

Non-refolded nsp1 was essentially non-reactive to immune serum, whereas refolded nsp1 revealed the presence of a pronounced antibody response with a mean peak titre of >25 000 at 21 days in this experiment (Fig. 2a). N also showed a marked enhancement of immunoreactivity to PRRSV-positive pig serum after refolding (Fig. 2a). These results were obtained with recombinant proteins that were initially denatured in 8 M urea. To further confirm the role of refolding in immunoreactivity independent of the denaturing conditions, N, nsp1 and nsp2P were denatured in 6 M guanidine hydrochloride and dialysed into PBS directly or after redox refolding, as described in Methods. The proteins were then applied to the microtitre plate in varying amounts and tested for reactivity. As shown in Fig. 2(b), the immunoreactivity of N and nsp1 was nearly completely dependent on refolding, and the reactivity of nsp2P was enhanced by about twofold. Loss of immunoreactivity was also observed when guanidine-denatured protein was dialysed into 8 M urea alone or followed by dialysis into PBS prior to coating on microtitre plates (data not shown).

The enhancing effect of refolding on immunoreactivity of the recombinant polypeptides was specific for both nsp1 and nsp2. We evaluated the immunoreactivity of nsp2 in serum samples at the peak of the humoral response by using an amino-terminally truncated nsp2 P fragment, lacking a hydrophobic region, that was expressed more readily in our bacterial system than was full-length nsp2. Refolding increased the immunoreactivity substantially of nsp2 P to sera of pigs infected with PRRSV VR2332, the strain from which the recombinant polypeptides were cloned (Fig. 2c). A similar enhancing effect was observed by using sera of pigs infected with a different viral strain, JA142, which shares 84% amino acid sequence identity in the same nsp2 P polypeptide, demonstrating the presence of conserved B-cell epitopes in nsp2 P (Fig. 2c). Sera from pigs not challenged with PRRSV showed no change in ELISA
reactivity (Fig. 2d) and sera from affected pigs showed no reactivity to irrelevant proteins refolded and coated on ELISA plates in the same manner (data not shown).

The antibody-reactivity data in Fig. 2(b–d) were determined by the absorbance values at a single serum dilution. We compared single-point dilution values to end-point titrations, a standard method of estimating antibody concentrations, to verify its use as a surrogate measure of titre, as the single serum-dilution approach simplifies the analysis of large sample sets generated in longitudinal infection studies. Absorbance determinations at a 1/2000 dilution of ten serum samples from the experiment shown in Fig. 2(a) were correlated highly with titre across a wide range of values for both nsp1 and N (Pearson $r^2=0.98$). Titres of N ranged from 1/2800 to 1/52 000 and those of nsp1 from 1/1500 to 1/30 000.

**Induction and duration of antibody responses to PRRSV nsps**

Antibody responses to nsp1 and nsp2 P were evident at 14 days after exposure of 4–6-week-old nursery pigs to PRRSV and reached a peak at 28–35 days after infection. As
shown in Fig. 3(a, b), pigs exposed to a wide variety of genetically distinct viral isolates based on ORF 5 sequence (Johnson et al., 2004) showed a robust antibody response to nsp1 and nsp2. The induction of the anti-nsp2 P response, in particular, was similar to, but more pronounced than, the response to N, the most prominent viral protein in infected cells (Johnson et al., 2004). Peak or near-peak antibody levels were maintained throughout the trial period. The mean responses shown in the figure were characteristic of all animals within a group. The kinetics of antibody response among all animals in a group were consistent, but the individual variation in magnitude was substantial, as shown in Fig. 3(c, d) for nsp1 and nsp2 P, respectively.

The antibody response to nsp1 was similar to the response to nsp2 P, but with a less distinct peak, followed by maintenance at a steady-state level over time (Fig. 3a, b). A similar response was observed in an independent experiment in 4-month-old gilts, shown in Fig. 2(a), in which the anti-nsp1 titre was approximately 1/23,000 at 14 days after infection and peaked 30 days after infection at approximately 1/31,000. Antibody levels declined gradually from 30 to 120 days after infection. The early response to nsp1 and nsp2 indicates that the proteins are being displayed on the surface of infected cells for presentation to B cells and are detected following release from dead and dying cells.

Cross-reactivity of anti-nsp antibodies to VR2332 nsp recombinant protein

As PRRSV shows extensive genetic variation that might result from immunological-escape selection, we were interested in the cross-reactivity of antibodies raised in pigs against heterologous viral strains. The data in Fig. 3(a, b) show that, within a target polypeptide and irrespective of the infecting PRRSV strain (including attenuated forms of virulent isolates), the response pattern is fundamentally the same. Group differences in magnitude of response are apparent and may be due to differences in viral load in the pig, which was previously shown to affect the antibody response under the same conditions of inoculation dose and route (Johnson et al., 2004). Differences may also be due to antigenic variation in the viral proteins such that the ELISA assay, in which VR2332 polypeptides were coated on the plate, did not detect all antibody species produced against cognate polypeptides of other strains. Nevertheless, as major antigenic epitopes appear to be conserved, infection with a wide range of viral isolates elicited antibody responses that could be detected readily.

Fig. 3. Antibody responses to nsp polypeptides in pigs exposed to a range of PRRSV isolates. Two- to three-week-old pigs were obtained from a PRRS-free commercial herd and divided randomly by weight into 10 groups with 10 pigs per group. On day 0, each of the eight PRRSV isolates and the PRRSV pool were inoculated intranasally. Control groups received only culture medium (Johnson et al., 2004). Values are mean absorbance of serum samples diluted 1/2000 from ten pigs for nsp1 (a) and nsp2 P (b). Bars in (a) represent 1 SEM for the group with the highest mean response. (c, d) Individual variation in antibody responses to nsp1 (c) and nsp2 P (d) for the ten pigs infected with VR2332.

Legend to (a) and (b)
- VR 2332
- Ingelvac MLV vaccine
- JA 142
- Ingelvac ATP vaccine
- SDSU 73
- ABST-1
- MN 184
- 17198-6
- All isolates
- No treatment
We produced additional nsp2 fragments, nsp2 HP S1, nsp2 HP S2 and nsp2 HP, encoding regions whose amino acid sequences were highly variable among strains (Fig. 4, peptides A, B and C, respectively) and that were predicted to be highly antigenic (Fig. 5), to determine whether strain-specific antibodies were produced. The nsp2 HP polypeptide, a large, hypervariable region (Fig. 4, peptide C), bound antibodies produced in pigs exposed to various strains, but with the greatest reactivity to pigs given Ingelvac MLV (Boehringer Ingelheim Vetmedica), the attenuated vaccine derivative of VR2332 (Fig. 6a). The difference was quantified by comparing the ELISA absorbance of anti-nsp2 HP test sera to the mean anti-nsp2 HP value from seven Ingelvac MLV-exposed pigs, normalized to anti-nsp2 P antibody, by using the formula 

\[ \frac{100}{\text{mean}} \times (1 - \frac{\text{OD}_{nsp2\ HP}}{\text{OD}_{nsp2\ P}}) \]

for Ingelvac MLV. Antibodies elicited in pigs by heterologous PRRSV strains reacted substantially less to VR2332 nsp2 HP than did antibodies elicited against Ingelvac MLV, ranging from 45.6 ± 12.0 to 57.1 ± 11.3 % (mean ± 1SD), depending on the infecting strain (P<0.001). The relative strain specificity of nsp2 HP for Ingelvac MLV was observed in all 42 pigs tested (Fig. 6b). The two smaller hypervariable-region peptides, nsp2 HP S1 and nsp2 HP S2, which are also predicted to be highly antigenic (Fig. 5b), showed lower absorbance values overall (Fig. 6a) and greater variation among animals (Fig. 6c, d).

**DISCUSSION**

nsp1 and nsp2 are among the first viral proteins synthesized in cells infected with PRRSV. These proteins are assumed to be essential to the viral life cycle and their presence in cells is likely to be toxic, due to their protease activities. As these proteins are expressed early in the viral life cycle, they are available from the earliest time of infection.

![Fig. 4. Sequence alignment of the nsp2 region of multiple PRRSV strains. Grey boxes highlight nsp2 HP S1 (A), nsp2 HP S2 (B) and nsp2 HP (C).](image-url)
infection to the macrophage proteosome machinery for degradation and presentation to the immune system in the context of MHC class I and II. For these reasons, it is reasonable to predict that antibodies directed against nsp1 and nsp2 might appear early in the course of PRRSV infection at a level dependent on their immunogenicity and abundance. Alternatively, the polypeptides encoded by ORF 1a might induce weak antibody responses, due to substantially lower expression levels compared with the major structural proteins encoded by ORFs 5, 6 and 7, (Meulenberg, 2000; Snijder & Meulenberg, 1998). Here, we observed strong and sustained antibody responses to both nsps that were within the same range or greater than the antibody responses to N, the most abundant structural protein. The levels of anti-nsp1 and anti-nsp2 were maintained at high levels for at least 70 days, in contrast to antibodies to N, which tended to decrease over time (Johnson et al., 2004).

The predicted antigenicity of nsp1 and nsp2 is equivalent to or lower than that of N, as shown in Fig. 5. Thus, the robust antibody responses suggest that the amount of the polypeptides produced in cells is higher than anticipated. The discovery of abundant heteroclite RNA molecules, so named because they deviate from the common form of arteriviral subgenomic mRNA, encoding nsp1 and the amino-terminal portion of nsp2 raises the possibility that non-structural, protease-containing polypeptides encoded in nsp1 and nsp2 may also be expressed abundantly (Yuan et al., 2000, 2004). This possibility would account for the robust antibody responses that are equivalent to the response to the highly antigenic and highly expressed N. A third polypeptide containing a protease active site, nsp4, encoded by ORF 1a, is translated in equimolar concentrations with nsp1 and nsp2 from genomic RNA, but is not thought to be encoded by heteroclite RNA (Yuan et al., 2000). Whilst its predicted antigenicity is similar to that of

**Fig. 5.** Jameson–Wolf protein antigenicity profiles for (a) nsp1; (b) nsp2; (c) N; (d) nsp4. x-axis values are amino acid residues for each protein; the y-axis represents the antigenic index (Jameson & Wolf, 1988). Positions of nsp2 polypeptide fragments that were produced in the study are shown in (b).
nsp1 and nsp2, as shown in Fig. 5, the porcine antibody response to nsp4 is very low (Johnson et al., 2004). Taken together, these observations are consistent with nsp1 and nsp2 being expressed more highly, possibly from heteroclite RNA, and thus providing a greater antigenic stimulation to the pig immune system.

A protein-refolding treatment appeared to be essential for immunoreactivity in the case of nsp1 and N, indicating that the dominant epitopes recognized by the pig are conformational, not linear. By contrast, Meulenbergh et al. (1998) identified four B-cell epitopes in N of Lelystad virus, a European PRRSV. Three of the epitopes were linear, based on reactivity of peptides to a panel of murine mAbs. An et al. (2005) also identified a linear epitope in N recognized by murine mAbs. By contrast, screening of immune pig serum with peptides or phage libraries revealed one linear epitope in nsp1 and no or two linear epitopes in N (Oleksiewicz et al., 2001b; de Lima et al., 2006). In summary, the results suggest that, in pigs, the B-cell response to N, as well as to nsp1, is directed primarily to conformational epitopes. For nsp2 P, reactivity was observed in the absence of refolding, but was increased substantially after refolding. Linear epitopes were previously reported in nsp2 (de Lima et al., 2006; Oleksiewicz et al., 2001b), indicating that the response to nsp2 is directed to both linear and conformational epitopes.

Whilst it is not known whether the refolding treatment restored full biological activity to nsp1, nsp2 P and N, refolding reactions as performed here are used routinely to restore the native properties of bacterially expressed recombinant proteins (Swietnicki, 2006; Tsumoto et al., 2004). In addition, we observed that N was insoluble without, but soluble with, refolding and that nsp1 appeared to be catalytically active after refolding (Fig. 1). Although the Zn$^{2+}$ ion was not provided, the protein may have acquired Ni$^{2+}$ from the affinity resin used for purification (Oleksiewicz et al., 2004). These observations further support the conclusion that protein-refolding treatment changes the immunoreactive properties of nsp1, nsp2 and N to increase antibody titre in immune pig serum.

Fig. 6. nsp2 polypeptide fragments distinguish strain-specific humoral immunity. (a) Effect of viral strain on ELISA reactivity to VR2332-derived nsp2 P, nsp2 HP, nsp2 HP S1 and nsp2 HP S2. Seven serum samples per group at 28 days of infection with each of the strains indicated (from the study in Fig. 3) were tested in duplicate at a 1/2000 dilution. Mean responses are shown. (b–d) Relative percentage difference in background-subtracted absorbance values of individual serum samples in (a) reacted with VR2332 nsp2 P minus the value from reaction with VR2332 nsp2 HP (b), nsp2 HP S1 (c) or nsp2 HP S2 (d). The relative percentage difference was calculated by the formula $(1 - \frac{\text{OD}_{\text{HP fragment}}}{\text{OD}_{\text{nsp2 P}}}) \times 100\% - \text{mean} (1 - \frac{\text{OD}_{\text{HP fragment}}}{\text{OD}_{\text{nsp2 P}}}) \times 100\%$ for MLV.
Antigenic and genetic studies demonstrate a high level of genetic variation within and among PRRSV isolates that exist in Europe and North America (Allende et al., 2000; Kapur et al., 1996; Mardassi et al., 1994; Meng et al., 1995; Meulenberg et al., 1993; Murtaugh et al., 1995; Nelsen et al., 1999; Suarez et al., 1996; Wensvoort et al., 1992; Yuan et al., 2001a). The cross-reactivity of the humoral response to both nsp1 and nsp2 of pigs inoculated with a diverse set of North American PRRSV isolates indicates that many antigenic determinants are conserved, despite extensive genetic diversity. In nsp1 and nsp2, and perhaps other PRRSV proteins, immunological selection does not appear to be the principal driving force for genetic change. Thus, despite extensive evolutionary radiation of the virus, major antigenic determinants have been conserved.

We also identified regions of marked sequence variation within nsp2 that elicited strain-specific antibody responses. The nsp2 HP region is antigenic, but the VR2332-expressed sequence only detected antibodies in swine exposed to VR2332 and its attenuated form, Ingelvac MLV. By normalizing the anti-nsp2 HP response to a larger fragment of nsp2, nsp2 P, it was possible both to identify animals exposed to a PRRSV and to differentiate between a response to vaccine (Ingelvac MLV) and a field virus.

Our findings of high and sustained antibody responses to nsp1 and nsp2, commensurate with the response to N, the most abundant structural protein, suggest that these nsp2 are expressed at high levels that exceed the levels of other nsp2 encoded by the same RNA (Meulenberg, 2000). The presence of heteroclite RNA in PRRSV-infected cells provides a mechanism for overexpression of nsp1, but only the amino-terminal portion of nsp2 is encoded in a heteroclite RNA (Faaberg et al., 2001; Yuan et al., 2000, 2001b, 2004). The robust humoral immune response to these proteins may indicate a need to remove inappropriate protease activities from the environment of lysed cells. nsp1 and nsp2 might also provide important T-cell epitopes for cellular immunity, but this hypothesis has not been explored. Experiments measuring gamma interferon secretion as an index of PRRSV-specific T-cell activation use whole virus, in which nsp2 are thought to be absent (Xiao et al., 2004). In addition, antibody responses to nsp1 and nsp2 may be useful for serological diagnosis of animals exposed to PRRSV infection.

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REFERENCES


Mutations in the genome of porcine reproductive and respiratory syndrome virus responsible for the attenuation phenotype. Arch Virol 145, 1149–1161.


Examination of virus shedding in semen from


