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Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus

Brief Report

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Summary. The kinetics of appearance of antibodies directed to the major structural proteins N, M and E of porcine reproductive and respiratory syndrome virus (PRRSV) was followed in pigs naturally- and experimentally-exposed to the virus. Specific IgM antibody titers were first detected by indirect immunofluorescence (IIF) at the end of the first week of PRRSV infection, peaked by day 14 to 21 post-inoculation (p.i.), then rapidly decreased to undetectable levels by day 35 to 42 p.i. On the other hand, specific IgG antibody titers peaked by day 21 to 28 p.i. and remained unchanged to the end of the 6- or 9-week observation period; in addition, a persistent viremia was observed. Virus neutralizing (VN) antibody titers >8 were not detected until 3 to 4 weeks p.i. Taken together, the results obtained by Western blotting analyses using purified virus and *E. coli*-expressed ORFs 5 to 7 gene products, suggested that antibodies directed against the envelope E protein appear by day 7 p.i., whereas antibodies directed against the nucleocapsid N and membrane M proteins can only be detected by the end of the second week p.i. No correlation could be demonstrated between VN and IIF antibody titers, viremia, and viral protein specificities of circulating antibodies at various times p.i.

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The porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped RNA virus which has been found to be responsible for reproductive failure in sows of any parities (late-term abortions, increased numbers of stillborn, mummified and weakborn pigs) and respiratory problems affecting pigs of all ages [8, 20]. This new porcine virus has been provisionally classified

within the genus *Arterivirus*, according to its biochemical and morphological characteristics [2, 15]. The viral genome is a positive single-stranded RNA molecule approximately 15 kb in length that contains eight open reading frames (ORFs) similarly organized to that of the *Coronaviruses* [6, 15]. The virion contains three major structural proteins: a nucleocapsid protein N of 15 kDa, an unglycosylated membrane protein M (matrix protein) of 18–19 kDa and a glycosylated membrane protein E (envelope associated glycoprotein) of 25–26 kDa which are encoded by ORFs, 7, 6 and 5, respectively [16].

Clinical symptoms and production losses may vary widely among herds, but the vast majority of PRRSV infections are subclinical [1, 3]. Presently some field and experimental evidence exists that indicates persistent lifelong viremia during PRRSV infection [3, 23]. An antibody-dependent enhancement mechanism has been reported to explain the persistent infection of porcine alveolar macrophages and circulating monocytes [4, 8]. So far, little is known about the immunological status of PRRSV-infected pigs. Some investigators suggest that PRRSV causes immunosuppression because of the recrudescence of secondary bacterial infections among the infected pigs [8]. Previous data indicated that antibodies to PRRSV may be detected as soon as 1 to 2 weeks post-exposure using indirect immunofluorescence [18, 21] and the immunoperoxidase monolayer assay [20]. Virus neutralizing antibodies were reported to appear later during the infection, usually when clinical signs are resolved [18, 22].

The purpose of the present study was to evaluate the humoral immune response of PRRSV-infected pigs. The kinetics of the appearance of specific IgM and IgG antibody titers during PRRSV infection, in addition to viral structural protein specificities of the antibodies and duration of viremia have been investigated in pigs naturally- and experimentally-exposed to PRRSV.

The Québec cytopathic strain IAF-Klop of PRRSV [11] was propagated in MARC-145 cells [9], a highly permissive cell line to PRRSV kindly provided to us by J. Kwang, U. S. Meat Animal Research Center, Clay Center, Nebraska. Serological identification of the cell culture-adapted IAF-Klop strain was confirmed by indirect immunofluorescence (IIF) using monoclonal antibody (MAb) SDOW17 [17], kindly provided by D. A. Benfield and E. Nelson, Department of Veterinary Science, South Dakota State University. This MAb was found to be directed to an epitope of the N protein shared by both the North American and European isolates of PRRSV [10, 17]. Homologous hyperimmune sera to the IAF-Klop strain of PRRSV were produced in pigs and rabbits, as previously described [11].

In the first experiment, seven SPF piglets were placed in a clinically PRRSV-infected herd, whereas two similar piglets were introduced in a clinically-healthy neighboring barn [3]. The SPF pigs were selected from a farm free of atrophic rhinitis, *Mycoplasma hyopneumoniae*, Transmissible gastroenteritis virus, Swine influenza virus, encephalomyocarditis virus, *S. hyodysenteriae*, sarcoptic mange, salmonellosis, *A. pleuropneumoniae* and PRRSV. In the second experiment, four five-week old SPF piglets were intranasally inoculated with 10^5 $TCID_{50}$ of the IAF-Klop strain of PRRSV. Clinical signs of both the above piglets and

a simultaneously mock-infected group of three pigs were monitored daily. Blood samples were collected at days 3 and 6 p.i., then weekly during 42-day and 63-day observation periods for sentinel piglets and experimentally-infected pigs, respectively.

Among the principal clinical signs observed in both naturally- and experimentally-infected pigs were intermittent raising of body temperature (40–41 °C), anorexia, lethargy, periocular oedema, conjunctivitis and blue discoloration of the ears. Coughing and dyspnea (abdominal thumping) were usually noticed by the end of the first week of exposure [3, 19]. No symptoms were observed in control piglets. For each group, one representative animal was necropsied at the end of the first week p.i. Macroscopic and histopathological lesions of non-suppurative interstitial pneumonitis were only demonstrated in the two infected pigs [3, 19]. At the end of the 63-day observation period, no significant macroscopic and histologic lesions could be observed in pigs that had been infected experimentally. However, naturally-exposed sentinel pigs still manifested mild to moderate macroscopic and histologic lung lesions 42 days p.i., as previously described [3].

To attempt virus isolation, MARC-145 cell monolayers were inoculated with sera samples collected from PRRSV-infected pigs and monitored daily for the appearance of a cytopathic effect (CPE) or the presence of specific fluorescent foci [9]. For all pigs tested (naturally-exposed and experimentally-infected) the virus could be isolated from sera samples collected by the end of the first week of exposure till the end of the observation periods (data not shown). At no time, could PRRSV be recovered from serum samples collected from the control pigs.

The IIF test was carried out using acetone fixed PRRSV-infected MARC-145 cells, and fluorescein-conjugated anti-pig IgM (Kirkegaard & Perry Laboratories Inc.) or anti-pig IgG (Sigma), as previously described [21]. The IIF antibody titers were expressed as the reciprocal of the highest serum dilution giving specific cytoplasmic fluorescence (Table 1). In both naturally- and experimentally- infected pigs, IgM antibodies to PRRSV were detected as early as 6 days p.i. (IIF titers of 16 to 256) and maximal titers were reached by day 14 p.i. (IIF titers of 1024). Then, specific IgM antibody titers drastically decreased until day 35 or day 42 p.i. Specific IgG antibodies were also detectable by the end of the first week of exposure, maximal titers being obtained around day 21 to 28 p.i. (IIF titers of 1024 to 2048). Thereafter, there was generally little change in IgG antibody titers to PRRSV of sera collected till the end of the 6- or 9-week observation period.

The modified procedure described by Yoon et al. [21] was used for in vitro detection of virus neutralizing (VN) antibodies to PRRSV. The VN antibody titers were expressed as the reciprocal of the highest dilution of sera that neutralized CPE induced in MARC-145 cell monolayers by a constant dose of 100 $TCID_{50}$ of virus. Specific VN antibodies to PRRSV could be detected in sera samples from only half of the infected piglets (data not shown). Antibody titers >8 were not observed until 3–4 weeks p.i., with maximal titers ranging between

Table 1. Kinetics of specific IgM and IgG antibody titers in pigs naturally- and experimentally-exposed to PRRSV

Animal group	Piglet no.	IIF antibody titers at day post-exposure								
		0	3	6	14	21	28	35	42	63
Mock-infected	8978	<16[a]	<16	<16	<16	<16	<16	<16	<16	<16
		(<16)[b]	(<16)	(<16)	(<16)	(<16)	(<16)	(<16)	(<16)	(<16)
	8980	<16	<16	<16	<16	<16	<16	<16	<16	<16
		(<16)	(<16)	(16)	(<16)	(<16)	(<16)	(<16)	(<16)	(<16)
	8982	<16	<16	<16	<16	<16	<16	<16	<16	<16
		(<16)	(<16)	(<16)	(<16)	(<16)	(<16)	(<16)	(<16)	(<16)
Naturally-exposed[c]	22	<16	<16	32	512	2048	1024	1024	1024	ND
		(<16)	(<16)	(16)	(1024)	(1024)	(256)	(64)	(<16)	ND
	33	<16	<16	<16	128	2048	1024	1024	512	ND
		(<16)	(<16)	(32)	(1024)	(1024)	(256)	(64)	(<16)	ND
Experimentally-exposed[d]	8979	<16	<16	32	256	256	2048	2048	2048	2048
		(<16)	(<16)	(256)	(1024)	(256)	(256)	(64)	(32)	(<16)
	8983	<16	<16	64	256	2048	2048	2048	2048	2048
		(<16)	(<16)	(256)	(1024)	(256)	(128)	(64)	(32)	(<16)
	8984	<16	<16	64	256	2048	2048	2048	2048	2048
		(<16)	(<16)	(64)	(1024)	(256)	(128)	(64)	(32)	(<16)

[a] Specific IgG antibody titers to PRRSV as determined by IIF test

[b] Numbers within parentheses indicate specific IgM antibody titers to PRRSV as determined by IIF test

[c] Pigs that have been introduced in a clinically PRRSV-infected herd

[d] Pigs infected intranassally with 10^5 $TCID_{50}$ of the Quebec PRRSV strain IAF-Klop

32 and 64. The VN antibodies to PRRSV persisted till the end of the 42- or 63-day observation period. Control pigs, however, remained serologically negative by IIF and VN to PRRSV till the end of the observation periods.

To study the reactivity of pig sera to PRRSV specific proteins, immunoblotting experiments were performed using sucrose-gradient purified virus, as previously described [11]. Briefly, replicas of viral proteins, separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes, were incubated for 1 h at 37 °C in the presence of 1:50 dilution of the tested porcine sera. After washing in a 0.05 M Tris-buffered saline (TBS) solution containing 0.05% Tween 20, the membranes were further incubated in the presence of 1:2000 dilution of an alkaline phosphatase-conjugated anti-porcine IgG (Sigma). The immune complexes were revealed using a commercial alkaline phosphatase conjugate substrate kit (BioRad, Mississauga, Ont.), containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in TBS buffer. Under reducing conditions, the three major PRRSV structural proteins N (15 kDa), M (18 kDa) and E (24.5 kDa) were consistently identified with the tissue culture-adapted IAF-Klop strain of PRRSV using homologous porcine hyperimmune serum [11]. In the immunoblotting experiments with sera from both naturally- and experimentally-infected pigs (Fig. 1), seroconversion to the M and N proteins of PRRSV could not be demonstrated before the end of the first two weeks of infection. On the other hand, seroconversion to the E glycoprotein was generally obtained by the end of the third or fourth week p.i.

Viral protein specificity of the humoral immune response of infected pigs was further confirmed by Western immunoblotting analyses using *E.coli*-expressed ORFs 5 to 7 gene products. Genomic RNA was extracted from purified virus by the one-step guanidinium isothiocyanate-acid phenol method [5], then respective viral genes were amplified by RT-PCR, as previously described [12, 13]. Six oligonucleotide primers, containing *Eco*RI (sense primers) and *Bam*HI (antisense primers) restriction sites at their 5′-end, were designed according to the sequence of the IAF-exp.91 strain of PRRSV (EMBL/GeneBank accession number L40898) [13]. The PCR amplified products were purified using the Geneclean II nucleic acid purification kit (BIO 101, La Jolla, CA) and cloned either in the pMAL^{C}-2 (New England Biolabs) or pGEX-4T1 (Pharmacia) plasmid vectors, according to the manufacturer's directions. Protein expression was induced by subsequent addition of 0.1 mM IPTG (Boehringer Manheim, Laval QC) into the culture medium of the recombinant bacteria. The resulting fusion proteins *maltose binding protein* (*MBP*)*-E or MBP-N*, and *glutathion sulfotransferase* (*GST*)*-M*, were purified either by electroelution or by affinity chromatography onto amylose resine or glutathione sepharose 4B columns, according also to the manufacturer's instructions. The recombinant proteins were analysed by SDS-PAGE and their serological identification was confirmed by Western immunoblotting using the porcine anti-PRRSV hyperimmune serum. The estimated M_{rs} of the recombinant proteins were 65 kDa for MBP-E, 56 kDa for MBP-N and 45,6 kDa for the GST-M proteins, in agreement with the M_{rs} determined previously from the amino acid sequences of the IAF-Klop strain ORFs 5 to

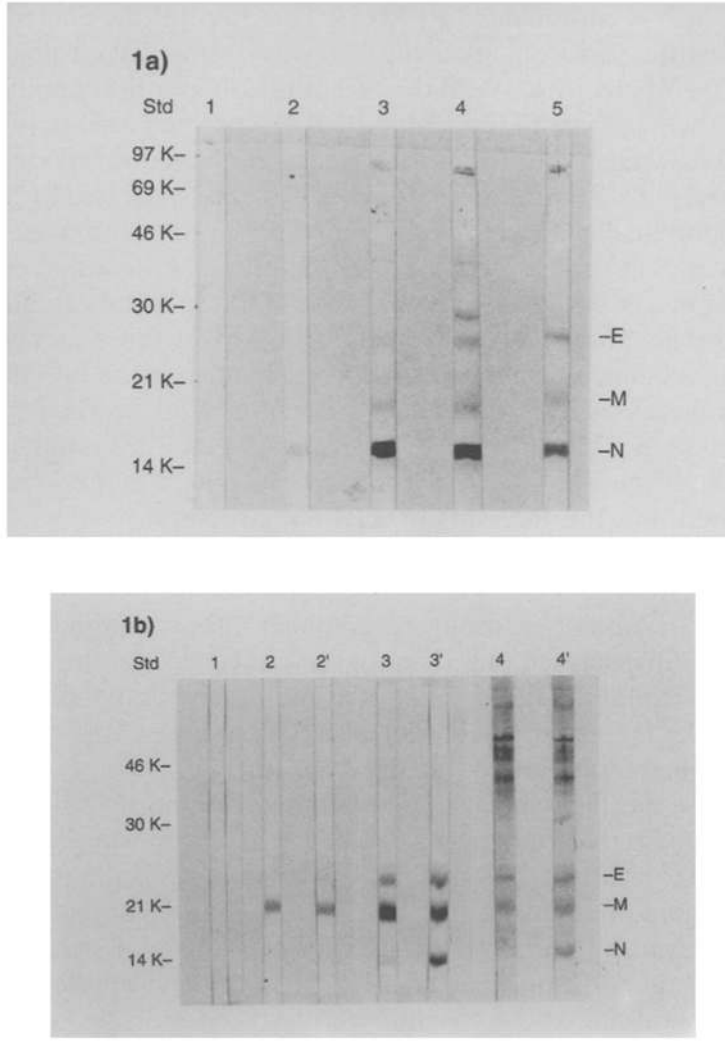

Fig. 1. Detection by Western immunoblotting of antibodies towards PRRSV major structural proteins in sera of naturally (**a**) and experimentally (**b**) infected pigs. The purified tissue culture-adapted IAF-Klop strain of PRRSV was electrophoresed in 12% polyacrylamide gels. The viral proteins were electrophoretically transferred to nitrocellulose membranes and incubated with sera samples collected from the infected pigs. The immune complexes were revealed by the use of an alkaline phosphatase anti-porcine IgG conjugate. **a** Serum samples were collected from pig no. 22 prior exposure to the PRRSV-infected barn (*1*) and at days 7 (*2*), 14 (*3*), 28 (*4*) and 35 (*5*) post-exposure. **b** Serum samples were collected from experimentally-infected pig no. 8979 and 8983 at day 0 (*1*) and at days 14 (*2*, *2'*), 28 (*3*, *3'*) and 35 (*4*, *4'*) p.i., respectively. Protein markers are indicated on the left

7 products [13]. The homologous rabbit and porcine hyperimmune serums reacted positively with the three recombinant proteins (MBP-E, MBP-N, GST-M), and failed to recognize the GST or the MBP protein alone (Fig. 2, lanes A1, B1, D1).

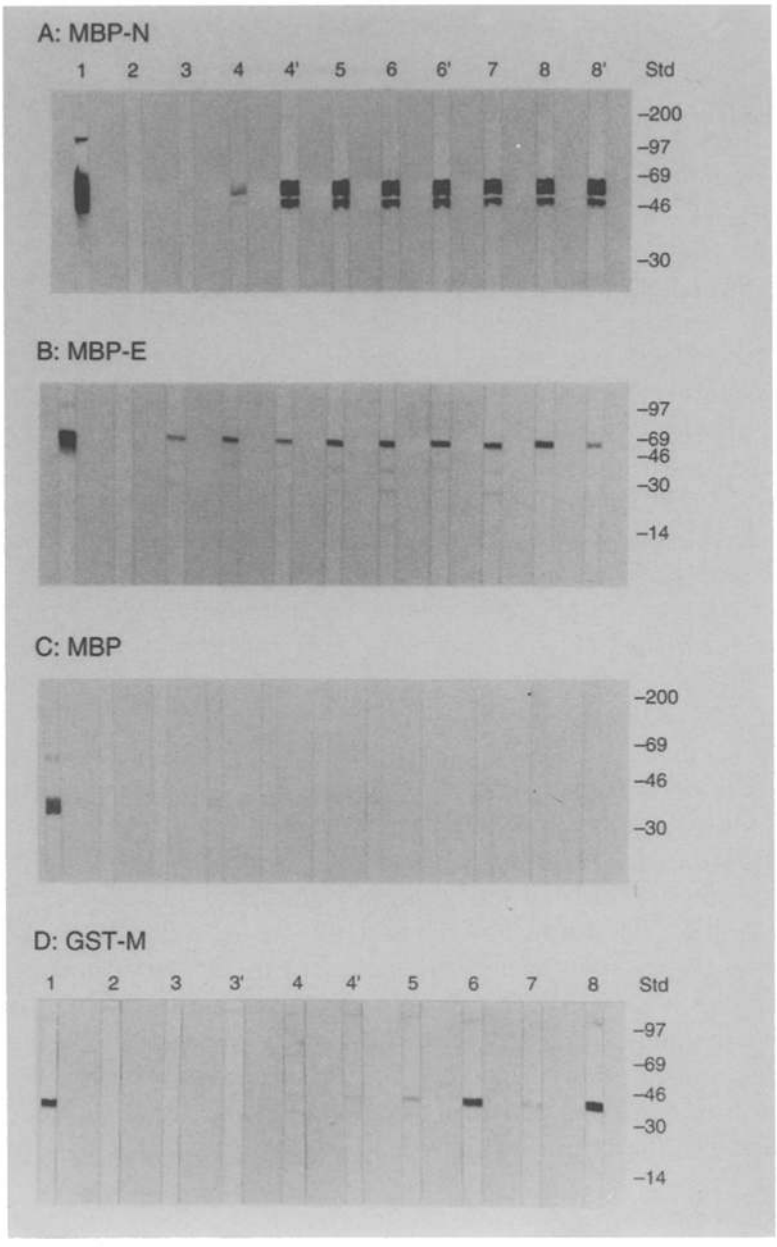

Fig. 2. Western immunoblotting analyses with PRRSV expressed ORFs 5 to 7 fusion proteins. Following IPTG induction, bacterial lysates were clarified and expressed fusion proteins were purified either by electroelution or by affinity chromatography, separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. **A**, **B** and **D** are immunoblots obtained with purified MBP-ORF7, MBP-ORF5, and GST-ORF6, respectively. Reactivity of the anti-PRRSV (IAF-Klop strain) porcine hyperimmune serum (1:100 dilution) toward each of the viral recombinant proteins is shown in *A1*, *B1* and *D1*. *C1* represents the reactivity of a rabbit hyperimmune serum raised against the MBP, alone. Sera from PRRSV-infected sentinel pigs (no. 22 and 33) were tested at a 1:25 dilution and were collected at day 0 (*2*), 7 (*3*, *3'* for no. 22 and no. 33, respectively), 14 (*4*, *4'*), 21 (*5*), 28 (*6*, *6'*), 35 (*7*), and 42 (*8*, *8'*) post-exposure to PRRSV-infected herd. No reactivity of the pig sera towards the MBP (**C**) and GST (data not shown) proteins has been observed. Protein markers (*Std*) are indicated on the right

As illustrated in Fig. 2, with clinical sera samples No. 22 and 33 (naturally-infected pigs), antibodies directed against the recombinant E protein could be detected by the end of the first week of infection, whereas antibodies directed against the recombinant N and M proteins could only be detected by the end of the second week of the PRRSV infection. No reaction has been noticed with MBP protein (Fig. 2c) and GST protein (data not shown) alone.

In the present study, PRRSV-infected piglets generally developed mild clinical signs of a respiratory disease within the first week post-exposure to the virus that lasted not more than 3 to 7 days. However, all the animals tested remained viremic till the end of the 42 or 63-day observation period. Our data are in agreement with previous observations by others who demonstrated that PRRSV may persist for many weeks, even months in the infected pigs, despite their relatively high IIF antibody titers [14, 17, 23]. Noteworthy, our results on the detection of IgG antibodies to PRRSV in infected pigs sera are also compatible with previous findings that specific IgG antibodies can be detected by IIF from 11–14 days p.i. up to several weeks (more than 4–5 months) after exposure to the virus [14, 17]. In general, IgG antibody titers detected by IIF peaked by the end of the third or fourth week post-exposure. More recently, Nelson et al. [17] demonstrated that peaked IgG antibody titers to PRRSV (IIF titers > 1024) may persist in experimentally-infected pigs for more than 3 months, then decrease progressively to reach very low levels (IIF titers < 20) after more than 300 days p.i. Consequently, detection of IgG antibodies in pig sera may indicate that the animals have been infected by PRRSV in a recent or distant past. Since no correlation could be demonstrated between the levels of IgG antibody titers determined by IIF and the viremic status of the animals, one cannot precisely pin point when the exposure to the virus had occurred or whether the pigs had been carrying the virus for a long time. Interestingly, our results indicate that detection by IIF of IgM antibodies to PRRSV may provide more precise information in the serological diagnosis of PRRSV infection. Indeed, the short-term persistence of circulating specific IgM antibodies may be considered in the differentiation between acute and chronic PRRSV infections in pigs.

Also in agreement with previous findings by other authors, antibodies with in vitro VN activity were relatively slow to appear and were not detected until 4–5 weeks p.i. [14, 17, 22]. Although the VN test has been reported to be less sensitive than the IIF tests and immunoblotting [17], the long term persistence of PRRSV in the experimentally- or naturally-infected animals despite high levels of antibodies to PRRSV, raises the question as to what role the humoral immune response has in the protection to PRRSV infection. Also, it is possible for antibodies to enhance viral infection of Fc-receptor-positive cells such as macrophages, by forming immune complexes that use the Fc receptor to bind to the macrophages [4]. Thus, the putative protective role of neutralizing antibodies in PRRSV infection needs to be further documented.

Western blotting analyses demonstrated that the immune response of experimentally- and naturally-infected pigs was directed to the three major viral

structural proteins N, M and E, as previously reported in cases of pigs that have been experimentally-infected with the reference ATCC-VR2332 US strain [17]. These preliminary observations were further confirmed by testing the reactivity of the porcine anti-PRRSV sera against *E. coli*-expressed ORFs 5 to 7 gene products.

In light of our observations, it appears that antibody development to the various viral proteins in PRRSV-infected pigs is chronological, initial detection of antibodies to the N, M and E proteins varied among pigs, ranging from 6 to 28 days p.i. These results are in agreement with previous findings by others [17, 24]. However, it has been postulated that the stronger signal observed to the N and M proteins may reflect a higher molar ratio of these proteins in the virion relative to the E protein, or it may suggest a greater immunogenicity of these proteins in the pigs [17, 24]. The results obtained using *E. coli*-expressed ORFs 5 to 7 are in favor of the first hypothesis. Indeed, antibodies to the recombinant MBP-E protein could be demonstrated as early as 6 days pi, whereas antibodies to the N and M proteins could only be detected by the end of the second week p.i. This finding was not expected in case of the recombinant MBP-E protein since reactivity towards the viral envelope glycoprotein in immunoblotting experiments, using purified virus, could not be detected until the end of the third or fourth week p.i. This discrepancy may be due to the fact that when dealing with recombinant proteins, comparable amounts of the various proteins could be transferred onto the nitrocellulose membranes, whereas with purified viral preparations, the N and M proteins are expected to be present at a higher molar ratio comparative to the E glycoprotein [13, 16]. Indeed, in the case of equine arteritis virus it has been demonstrated that with the exception of Gs (small envelope glycoprotein), the proteins are approximately equal in abundance, being present at a molecular ratio of 3 (N): 2 (M): 3 (Gl: large envelope glycoprotein) [7]. The Gs protein, which is expressed at a level similar to that of M in infected cells, is strikingly underrepresented in virus particles (1 to 2%) [7]. Thus, it can be expected that for PRRSV, the immunogenicity of the E glycoprotein in the pig is likely comparable to that of the N and M proteins.

From results obtained with recombinant proteins, as well as with purified virus, there was no clear correlation between the appearance of circulating antibodies as detected by VN and IIF tests, viremia and protein specificities of the circulating antibodies after various time p.i. The neutralization process may be the result of virus interaction with antibodies directed against epitopes located on different viral proteins. Recently, we have been able to demonstrate that individually recombinant N, M and E proteins expressed by *E. coli* cells can induce the production of specific antibodies in rabbits and pigs; although IIF antibody titers to IAF-Klop strain ranged between 256 to 2048, the antisera are devoid of neutralization activity. Expression of recombinant viral proteins in eucaryotic vectors should permit us to demonstrate if the conformation and glycosylation of viral envelope proteins are essential requirements for the expression of epitopes involved in virus neutralization.

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