Detection of Antibodies to the Nucleocapsid Protein of PRRS Virus by a Competitive ELISA

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1. INTRODUCTION

The mature virions of the porcine reproductive and respiratory syndrome virus (PRRSV), a new porcine arterivirus, is made of three major structural proteins: a 25 kDa envelope glycoprotein (GP_5), an 18-19 kDa unglyco-sylated membrane protein (M), and a 15 kDa nucleocapsid (N) protein (Mardassi et al., 1995; Meulenberg et al., 1995). The N protein is the more abundant protein of the virion and is highly antigenic, which therefore makes it a suitable candidate for the detection of virus-specific antibodies and diagnosis of the disease (Loemba et al, 1996). It is also encoded by a relatively well conserved region of the viral genome, since a high degree of amino acid (aa) sequence identity has been observed among the N protein of North American (96-100%) and European (94-99%) strains (Meng et al., 1995; Suarez et al., 1994). Four to five domains of antigenic importance have been identified for the N protein, a common conformational antigenic site for European and North American strains being localized in the central region of the protein (Meulenberg et al., 1998; Wootton et al., 1998).

In this study, the genomic region encoding the N protein of a North American reference strain of PRRSV was cloned, expressed in *Escherichia coli*, purified and used as antigen in an indirect ELISA for detection of antibodies against PRRSV. Two MoAbs directed to highly conserved

The Nidoviruses (Coronaviruses and Arteriviruses).
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epitopes of North American PRRSV isolates were used in a competitive assay to improve the specificity of the test.

2. METHODOLOGY AND RESULTS

The entire ORF7 gene of the Quebec cytopathogenic IAF-Klop strain of PRRSV was amplified by RT-PCR using primer pairs ORF7-S (5'-CTAAA-TATGCCAAATAACAAC-3') and ORF7.AS (5'-CTCAAGAATGCCAGCTCA-3') (Gonin et al., 1999). The primers were designed according to the sequence of the Quebec reference strain (EMBL/ GeneBank accession number U64928) and contained two restriction sites for *Eco*RI (sense primers) and *Bam*HI (anti-sense primers) at their 5' end for directional cloning. The complementary DNA corresponding to the entire ORF7 gene of the IAF-Klop strain was inserted into procaryotic expression vector pGEX-4T-1 (Pharmacia) to yield plasmid pGEX-7 (Mardassi et al., 1996).

Following incubation in the presence of IPTG, competent *E. coli* strain BL21 (DE3) cells that were transformed with pGEX-7 expressed the GST-N recombinant fusion protein, essentially in the form of inclusion bodies. The molecular mass (M_r) of the GST-N recombinant fusion protein, determined following SDS-PAGE analysis of bacterial cell lysates, was estimated to 39.6 kDa, in accordance with the value determined previously from the aa sequence of the native N protein of the Quebec reference strain (Mardassi et al., 1995). The GST-N recombinant fusion protein could be recovered following solubilization of the pelleted inclusion bodies in the presence of lysozyme, triton X-100 and 8 M urea, then enriched and purified by affinity chromatography on glutathione-Sepharose 4B. Three to four additional protein bands were revealed following SDS-PAGE analysis of the second and third eluates with apparent M_r s of 35.9, 32.5, 31.5 and 29 kDa. Only the 39.6 and 31.5 species could be revealed following Western immunoblotting, using homologous porcine anti-PRRSV serum and rabbit anti-GST-N monospecific hyperimmune serum.

The optimal dilutions of the antigen and the test serum in indirect ELISA were determined by checkerboard titration. Using the homologous porcine hyperimmune serum, the optimal dilutions were found to be 1:50 for the serum and 1:1000 for the antigen, which corresponded to a final concentration of 0.1 to 0.5 μg of protein per well. The highest P/N ratios were obtained with PBS containing 0.05% Tween 80 and 5% goat serum as the blocking (saturation) and dilution buffer. Comparable results were obtained by incubating the plates at room T° or 37°C, with incubation periods of 45 min for the test serum and the anti-porcine IgG conjugate. Data obtained by Western immunoblotting suggested that part of the background

obtained with sow or adult sera was attributed to their reactivity with residual *E. coli* proteins (data not shown) that co-eluted with the GST-N recombinant usion protein following affinity chromatography. To avoid false-positive results due to bacterial proteins, a competitive ELISA was set-up where the capacity of clinical sera to interfere with the binding of anti-N MAbs (IAF-K8 and IAF-2B4), to the GST-N recombinant fusion protein was determined. Both MAbs were previously found to be directed against highly preserved conformational epitopes of North American isolates of PRRSV (Dea et al., 1996). Data for each test sera were expressed in percent competition, calculated by the following formula : % competition (sample) = 1 - (A_{450} (sample + MAb anti-N) / A_{450} (MAb alone)) X 100.

The evaluation of the competitive ELISA was carried out by first testing 95 pig sera obtained from previous experimental inoculation studies. A total of 20 of these pig sera were considered as negative by IIF (antibody titers < 16) and HerdCheck® ELISA (P/N values < 0.4). For both, the Herd-Check®ELISA and K8-ELISA, a linear correlation was obtained between P/N ratio or % competition values, with antibody titers determined by IIF. Comparison with HerdCheck® ELISA and IIF allowed the definition of a threshold range between 20 and 30% competition. In general, clinical sera given P/N values of > 2.0 in HerdCheck® ELISA showed >85% competition in the K8-ELISA corresponding to IIF titers > 1:512. Those sera for which P/N values varied between 1.0 and 2.0 in HerdCheck® ELISA yielded >50% competition in the K8-ELISA corresponding to IIF titers ranging from 1:64 and 1:256. Finally, sera with IIF titers ranging from 1:16 to ≤ 1:64 displayed P/N values of >0.4 < 1.0 in HerdCheck® ELISA, with percent competition values in K8-ELISA that varied from ≥ 20 to ≤ 50 %.

Comparison of data obtained in K8-ELISA with those obtained in IIF and HerdCheck® ELISA also allowed a comparison of the sensitivity and the specificity of these tests (Table 1). The data obtained with sera from experimentally-infected pigs showed that the K8-ELISA was capable of detecting anti-PRRSV antibodies in 86.7% (65/75) and 92.6 % (63/68) of pig sera that were considered as seropositive in IIF (titers > 16) and in HerdCheck® ELISA (P/N ratio > 0.4), with specificity values of 100% and 96.2 %, respectively. If a cut-off value of 1:32 rather than 1:16 was considered for IIF, the sensitivity and specificity of the K8-ELISA increased to 95.5 % and 96.5 %, respectively, but the performance of the test remained unchanged when compared to the HerdCheck® ELISA. Compa-rable results were obtained when using MAb IAF-2B4 in the competitive ELISA (data not shown). When tested on clinical samples (542 sera) from 28 positive and 28 negative pig herds, the K8-ELISA performed in a similar way to HerdCheck® and IIF tests as shown by Kappa values of 0.762 and 0.803. The sensitivity and specificity of K8-ELISA were 100% on a herd basis,

whereas sensitivity a specificity of 98.7% were determined on an individual basis in comparison to HerdCheck®and IIF tests.

Table 1. Comparison of sensitivity and specificity of the 3 serological tests used for the validation of the K8-ELISA, n= 95

Test X	Cut-off values	Reference test					
		IIF		IDEXX® ELISA		K8-ELISA	
		Se[a]	Sp[b]	Se	Sp	Se	Spe
IIF	1/16			100	74.1	100	66.7
IDEXX ELISA	P/N = 0.4	90.7	100			96.2	86.7
K8-ELISA	20%	86.7	100	92.6	96.2		
IIF	1/32			97.0	100	96.9	93.3
IDEXX ELISA	P/N = 0.4	100	93.1			96.3	86.7
K8-ELISA	20%	95.5	96.5	92.6	96.2		

[a] Sensitivity (test X) = 100 x number of positives in both X and reference test / total number of positives in the reference test.
[b] Specificity (test X) = 100 x number of negatives in both X and reference test / total number of negatives in the reference test.

3. DISCUSSION

Using sera from experimentally infected pigs, linear correlations were obtained by comparing the % of competition values to P/N ratios determined by Herdcheck® ELISA, as well as with IIF antibody titers, with relatively small standard deviation values. None of the sera that scored negative by Herdcheck ® ELISA and by IIF exhibited reactivities > 30% in the K8-ELISA which therefore confirms the high specificity of this competitive ELISA. Furthermore, practically 100% correlation (sensitivity and specificity) was obtained with K8-ELISA if P/N ratios of > 0.5 were considered as the threshold of the Herdcheck® ELISA rather than > 0.4.

Although a study of the kinetics of antibody production was not per se using K8-ELISA, this new competitive ELISA should permit identification of pigs as early as 7 to 10 day after infection, since it has been previously demonstrated that IIF antibody titers of > 1:16 were usually obtained at that time post-infection using the same experimental pig sera (Loemba et al., 1996). Since MAb IAF-K8 was found to be directed to a well preserved epitope of the North American and European strains of PRRSV (Dea et al. 1996), we expect that a similar efficacy of the K8-ELISA should be obtained with clinical sera from European pig herds.

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