Title: Peptide ELISA for serodiagnosis of PRRSV. **NPB # 03-013**

Investigator: Peter G.W. Plagemann

Institution: University of Minnesota

Date Received: September 30, 2005

II. Abstract

I have developed indirect and competition ELISAs using synthetic peptides of the N-protein of porcine reproductive and respiratory syndrome virus (PRRSV) to measure anti-N-protein Ab responses in PRRSV in infected pigs and to characterize the epitopes by the pig Abs and by a battery of anti-N-protein mAbs. Four linear epitopes recognized by mAbs have been identified in the most hydrophilic segment of the N-protein (AA25-57). Similarly, at least four linear epitopes in this segment are immunogenic in PRRSV-infected pigs, but only one corresponds to one recognized by one of the mAbs (AA36-45). Most infected pigs generate Abs that bind to both peptides and HerdChek plates, which are commonly used in the sero-diagnosis of PRRSV infections, but the time courses of formation of peptide binding Abs and Abs that react with HerdChek plates differ greatly in individual pigs. This suggests that, although the peptide and HerdChek ELISAs may detect Abs to some of the same epitopes, they also seem to detect Abs to epitopes that are uniquely expressed by one and not the other. Some mAbs fail to bind to HerdChek ELISA plates and this is also the case for certain pig Abs. By peptide ELISA I have detected four herds in which most or all pigs possessed N-protein peptide binding Abs, even though they were HerdChek ELISA sero-negative and exhibited no other signs of PRRSV infection. Thus PRRSV infections may be more widespread than presently realized involving strains that cause asymptomatic infections. It will be important to identify such PRRSV strains since they may impede eradication of PRRS and may be the source of virulent strains. Thus the peptide ELISA should be used as an adjunct to the HerdChek ELISA or it could replace it since I have encountered only two serum samples among 450 tested that were HerdChek ELISA positive but peptide ELISA negative. The peptide ELISA is also considerably cheaper than the HerdChek ELISA.

III. Introduction

The HerdChek commercial ELISA using recombinant N-protein is presently widely used for the detection of N-protein Abs in the diagnosis of PRRSV infections. However this ELISA is expensive, complicated , not precise and suffers from other shortcomings : frequently some single pig sera are found to be positive without any other evidence of PRRSV infection of the herds from which they come and the epitope specificity of the Abs that bind to the plates is not known. Since the use of synthetic peptides as antigen in the indirect ELISA has greatly facilitated the identification of the primary neutralization epitope in the middle of the ectodomain of main envelope glycoprotein GP5, I have proposed to use the same approach to develop a simple, precise, universal, inexpensive indirect ELISA for the detection of anti-N-protein Abs. I proposed to measure the binding of sera from infected and uninfected pigs as well as of a battery of anti-N-protein mAbs to plates coated with synthetic peptides representing N-protein segments.

These research results were submitted in fulfillment of checkoff funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer reviewed

For more information contact:
National Pork Board, P.O. Box 9114, Des Moines, Iowa USA
800-456-7675, Fax: 515-223-2646, E-Mail: porkboard@porkboard.org, Web: http://www.porkboard.org/

IV. Objectives of original proposal.
To develop a less expensive, simpler and more reliable indirect ELISA for the serodiagnosis of PRRSV infected pigs than is presently available. It is based on measuring the binding of antibodies to the nucleocapsid (N) protein, the main Abs generated in infected pigs, to ELISA plates coated with synthetic peptides carrying known epitopes.

V. Materials and methods
2,1. Anti-N-protein mAbs
MAbs 1CA7, 1BD11 and 1CH5 were raised to the N protein of PRRSV strain Olot91(Rodriguez et al., 1997) and provided by M.Rodriguez. MAbs VO17, M30 and S30 were raised to whole virus of strain SD92-1509 and mAb SDOW17 to strain LV (Nelson et al., 1993; Wootton et al., 1998) and provided by E.Nelson. Mab 5H2.3B12 was raised to formalin-treated virus of strain LHVA93-3 and provided by R.Magar. MAbs 2D6 and 2G7 were raised to strain MN-1 and provided by D.Deregt (see Plagemann 2005). MAb ISU15A was raised to strain ISU-P (Yang et al.. 1999) and provided by K.B.Platt. MAbs ICA7, IBD11 and ICH5 were purified and dissolved to 1 mg/ml. The other mAbs represent crude preparations and their mAb concentrations are unknown. ISU15A was supplied in cell culture fluid.

2.2.Pig sera
Serum samples from pigs infected with PRRSV strains JA142, VR2332, 17198-6, SDSU73, MN184,Abst-1 and Ingelvac ATP were provided by Dr. Michael Roof (Boehringer Ingelheim Vetmedica, Inc., Ames, Iowa). They were derived from an experiment conducted by Boehringer Ingelheim Vetmedica in which groups of ten 2-3 week old pigs were infected intranasally with 3000 TC $-ID_{50}$ of these PRRSV strains.
Various batches of pig serum were provided by E. Nelson and N. Ferrin (South Dakota State University). These sera had been submitted to the South Dakota Animal Disease Research and Diagnostic Laboratory for various types of analyses. These included: a. 25 individual serum samples submitted for the assay of virus neutralizing Abs. All were positive exhibiting focus forming neutralizing (FNN) titers varying between 4 to 256. b. 10 and 16 serum samples submitted by two farms (A-1-10 and B-1-16, respectively) for the assay of neutralizing Abs and anti-PRRSV Abs by HerdChek ELISA. All sera were negative in both assays. c. A batch of 7 serum samples submitted by a large swine producer (C-2, 4-9) for the assay of anti-PRRSV Abs by HerdChek ELISA and an immunofluorescent assay (IFA). All samples were negative in both tests. d. Serum samples from 60 pigs from the same swine producer but located at a different site (D-1-63). All were IFA and HerdChek ELISA negative. e. Serum samples from 11 and 18 pigs from two other farms (E-1-11; F-1-18). All were IFA positive (>1:40) and, except for 3 sera, HerdChek positive (S/P= 0.42-2.56).
Two pools of serum from LV-infected pigs, immune pig serum (IPS) 21 (van Nieuwstadt et al., 1996) and IPS8499 (from Boehringer-Ingelheim VetMedica) were kindly provided by Esther Wissink and Kay Faaberg, respectively. Serum samples from pigs born in the U.S. to two sows that had become infected with a European-like PRRSV (EuroPRRSV) SD02-11 (Ropp et al., 2004) were also provided by E.Nelson and N.Ferrin. The sera were obtained from 16 pigs bled when about 6 weeks of age. All sera lacked neutralizing Abs, but 8 were HerdChek ELISA positive (70-1-8); the remainder were negative (70-51-58). Another 7 pigs were bled when about 14 weeks of age. Their sera were FFN and HerdChek ELISA positive. Eight sets of serum samples containing HerdChek positive (considered "false positives") and negative samples were provided by Sagar Goyal (Department of Veterinary Diagnostic Medicine, University of Minnesota).

2.3. Indirect peptide ELISA for measuring anti-N-protein mAbs and pig sera for anti-N-protein Abs
The peptide ELISAwas conducted as previously described (Plagemann et al., 2002; Plagemann, 2005). In brief, the wells of Nunc Maxisorp ELISA plates were coated overnight with peptides dissolved in carbonate buffer, pH 9.6, at 4 μg/well. Control experiments showed that maximum Ab binding was achieved when coating wells with 4 μg of VR-P11 and VR-P12 per well or higher concentrations. When the concentration was reduced to 1 μg/well Ab binding was slightly reduced (data not shown). After peptide coating the wells were rinsed with PBS containing 0.1 (v/v) Tween 20 (PBS-Tween) and then incubated with blocking solution

composed of PBS, 0.5 % (v/v) Tween 20 and 1% (w/v) BSA at 37^0 C for 30 min and then with two-fold dilutions of anti-N-protein mAbs in blocking solution (as indicated in appropriate experiments) or pig serum in blocking solution (generally 1:50 to 1:200 or 1:400) at 37^0 C for 1 h. After rinsing with PBS-Tween the wells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma A-3562, diluted 1:500 in blocking solution) or rabbit anti-pig IgG (Sigma 1192; diluted 1:500 in blocking solution) at 37^0 C for 30 min, rinsed 4 times with PBS-Tween and once with 50 mM Tris-buffered saline (pH 7.4) and then incubated with alkaline phosphatase substrate (Sigma 104, 2 mg/ml of pH 9.6 carbonate buffer containing 5 mM $MgCl_2$; 200 µl/well) generally for 15 or 30 min. The alkaline phosphatase product was quantified by measuring absorbency at 405 nm (A_{405}) with an automatic plate reader. The sequences of the peptides used in the present study are shown in Table 1.Those not described previously (Plagemann, 2005) were synthesized by the Microchemical Facilities of the University of Minnesota or GLBiochem(Shanghai)LTD. MAb and anti-N-protein Ab titers in serum were expressed as the reciprocal of the highest dilution of mAb or serum that yielded an A_{405} value of 1.0 under standard assay conditions. The latter consisted of incubating ELISA plates coated with a single peptide (VR-P9-2, VR-P11 or VR-P12) with extended two-fold serum dilutions..

2.4 .Inhibition of Ab binding to immobilized large peptides by peptides in solution (competition ELISA)

ELISA plates were coated with the indicated peptides (4 µg/well). The plates were rinsed with PBS-Tween and then incubated with blocking solution for 30 min at 37°C followed by incubation for 60 min at 37°C with mixtures of 50 µl of an Ab solution in PBS and 100 µl of 2-fold dilutions of various peptides in PBS (generally from 20 to 0.6 µg/well). The wells were rinsed thrice with PBS-Tween and then incubated with the appropriate alkaline phosphatase-conjugated anti-pig IgG or anti-mouse IgG and substrate and finally read at 405 nm as described already. The dilutions of Ab or mAb used in the test were the highest dilutions that exhibited maximum binding to immobilized peptides.

2-5. Routine assay for anti-N-protein Abs by peptide ELISA

For the routine assay of anti-N-protein Abs by peptide ELISA I test two dilutions of a serum sample (1:50 and 1:100) for binding to VR-P12 (or VR-P9-2) and the irrelevant peptide VR-P22 (or uncoated wells). This allows the testing of 24 serum samples per ELISA plate. Examples are shown in Fig.12. Results for both VR-P12 and VR-P9-2 are presented indicating that similar Ab binding to both peptides is generally observed. However, as shown already, Ab binding to VR-P12 often exceeds binding to VR-P9-2 and the former is therefore preferred if only Ab binding to a single peptide is measured. Sera are considered Ab positive if A_{405} is well above 1.0 and several times higher than the value for VR-P22-coated or uncoated wells as is illustrated for two sera of the 60-serum set D (see earlier) that are HerdChek ELISA and IFA negative (Fig.13A; #11 and 19). If A_{405} is below 1.0 the sera are considered Ab negative (Fig.13A #39 and 27). Sometimes values only slightly higher than those for VR-P22-coated wells are observed. These might indicate the presence of very low levels of anti-N-protein Abs but are considered +/- and require further testing. The latter distinction is somewhat arbitrary, but so is the cut-off value of S/P=04 in the HerdChek ELISA. Fig.13B and C illustrate additional examples. Fig.13B shows the peptide ELISA results for two sera of set F that are HerdChek and IFA positive but lack Abs that bind to the N-protein peptides (#13 and 14). On the other hand, serum #5 is clearly peptide ELISA positive though it is HerdChek ELISA negative, similarly as serum #15 that is HerdChek ELISA positive. These samples are from a set of sera that yielded discrepant results in different diagnostic labs. Fig.13C illustrates the results for four sera of the set containing "false positives" (see earlier), which were all found to contain N-protein peptide binding Abs whether they were HerdChek ELISA positive or negative.

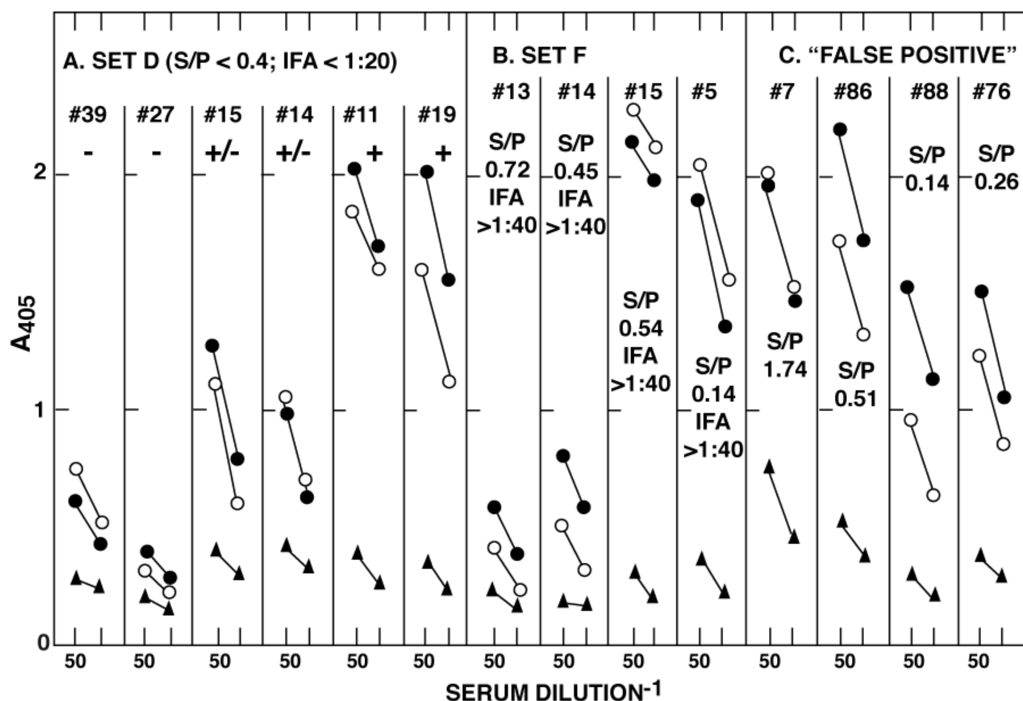

Fig.13. Routine peptide ELISA of serum samples from a number of pigs in set D (A), set F (B) and a set of sera containing "false positive" HerdChek ELISA results. The ELISA plates were coated with peptides VR-P9-2 (❍-❍), VR-P12 (●-●) and VR-P22 (▲-▲).

VI and VII.Results and Discussions

I have established both indirect and competition peptide ELISAs using synthetic peptides representing parts of AA segment 25-92 of the N-protein of VR2332 and equivalent peptides repressing the N-protein of PRRSV LV to study the formation of anti-N-protein Abs in PRRSV-infected pigs and to characterize the epitopes recognized by these pig Abs as well as by a battery of anti-N-protein mAbs obtained from various investigators (Plagemann, 2005). Details of the results I have obtained are presented in the attached manuscript. The following is a summary of the results and the discussion of the results.

Using these ELISAs I have further delineated the location of four linear epitopes of the PRRSV N-protein some of which had been studied earlier (Rodriguez et al., 1997; Meulenberg et al., 1998; Wootton et al.. 1998). One epitope is located in AA segment 48-56 and is highly conserved between European and North American PRRSV isolates, which explains that mAbs generated to a European PRRSV and a North American PRRSV (1CA7 and ISUA15, respectively) recognize the same epitope. AA54-56 represent an essential component of the epitope. The second epitope is located in VR2332 AA segment 36-45 being specific for mAb 5H2. Substitutions of P at AA39 and I or S at AA45 had no effect on mAb binding (see Table 1) since the mAb bound as well to MN-P12 and JA-P12 as to VR-P12 (Table 3) and VR-P16M was as effective in inhibiting mAb binding to immobilized larger peptides as VR-P16. On the other hand, failure of mAb 5H2 to bind to MD-P12 indicates that ^{38}V or ^{43}K or both play a major role in epitope specificity, which may also explain the failure of this mAb to bind to LV-specific peptides. The third epitope, recognized by mAb NS99, is located in LV-specific AA segment 25-34 in agreement with a previous report (Meulenberg et al., 1998). The location of the fourth epitope recognized by mAb IBD11 could not be definitively identified, except that it is present in LV AA segment 31-50. The epitope(s) recognized by mAbs VO17, 2D6 and 2G7 could also not be identified. Contrary to a previous report (Wootton et al.,1998), the mAbs are broadly specific and seem to recognize an epitope(s) in AA segment 30-48 rather than 52-69 plus 112-123. The epitopes recognized by mAbs SDOW17, ICH5 and SR30 remain ill or undefined (Table 3). ICH5 binds with low titer to many peptides, similarly as mAbs VO17, 2D6 and 2G7, whereas mAb SR30 binds only with low titer to the C-end peptide VR-P13, which is consistent with the view that the latter recognizes a discontinuous epitope in AA segment 69-123 (Wootton et al., 1998). MAb SDOW17 has been reported to recognize a broadly specific discontinuous epitope but different AA

segments have been implicated in harboring the epitope (Meulenberg et al., 1998; Wootton et al., 1998). The mAb fails to bind significantly to any of the peptides even those containing the implicated epitope segments.

The finding that mAb ICA7 and ISU15A, IBD11 and NS99 bind with similar titers to peptides and HerdChek ELISA plates (Table 3) indicates that the epitopes recognized by these mAbs are efficiently expressed by both. In contrast, the finding that mAbs 5H2, VO17, 2D6 and 2G7 fail to bind to HerdChek plates suggest that the epitopes recognized by these mAbs are not expressed on these plates, perhaps because the epitopes are obscured or altered during immobilization/denaturation of the antigens in the wells. This has important implications in the case of mAb 5H2 since its epitope may be one of the primary N-protein epitopes immunogenic in pigs (see below). Thus, in summary, some epitopes specific for this group of mAbs are expressed by both the peptides and HerdChek antigens, but others are expressed by one and not the other. This is important in understanding the lack of correlation between results from the peptide and HerdChek ELISAs.

Little was known about the immunogenicty of the epitopes specific for the mouse mAbs in pigs. The results from the indirect and competition peptide ELISAs indicate pigs infected by North American PRRSVs generate Abs to at least three epitopes contained in the peptides used in the present study but only one might correspond to an epitope, **^{36}SRGKGPGKKNK,** recognized by a mAb, namely mAb 5H2. An epitope in this segment is immunogenic in many but not all pigs (see Table 1). Other pigs generate Abs to a slightly more downstream but overlapping epitope **^{42}GKKNKKKNP**. In addition many pigs generate low levels of Abs to epitopes located in overlapping downstream VR2332-specific AA segments, **^{24}LGKIIAQQN** and **^{29}IAQQNQSRG**. I have not been able to prove by Ab competition experiments that mAb 5H2 and Abs from PRRSV-infected pigs bind to the same epitope, but the failure of the mAb and the pig Abs to compete in binding to various peptides might be due to differences in affinity or recognition of slightly different forms of the epitope when immobilized on the plate. That various forms of this epitope may exist is indicated by the finding that mAb 5H2 does not bind to MD-P12, whereas Abs from pigs infected by the various PRRSV strains or from field sera bind to varying degrees to this peptide.

The epitope in AA segment 42-50 falls in the same segment as the epitopes recognized by mAbs VO17, 2D6 and 2G7 but whether they are related is unknown. The two epitopes recognized by pig Abs in AA segment 24-37 (see above) have no mAb counterparts. On the other hand, I found no evidence that the epitope specific for mAbs ICA7 and ISU15A is immunogenic in pigs; VR-P18 had no effect on the binding of pig Abs to larger immobilized peptides, whereas it strongly competed with the binding the mAbs. In general, pigs infected with European type PRRSV generate Abs to the same epitopes as their North American counterparts. In addition, some of the pigs seem to generate low levels of Abs to epitopes located in LV AA segments 20-31 and 54-92. The first epitope could correspond to that recognized by mAb NS99, but further work is required to explore this potential relationship. The nature of the second epitope is unclear. There is also no evidence that the LV-specific epitope recognized by mAb IBD11 is immunogenic in pigs infected by European PRRSVs. These results indicate that though certain N-protein segments might be immunogenic in mice they may not be so in pigs,

Pigs generate Abs to many N-protein epitopes, some of which are not represented by the panel of mAbs studied here. Some of the epitopes are expressed both on the peptides and HerdChek plates. This is indicated by a good correspondence between positive results in both tests and the finding that adsorption of the sero-positive pig sera on HerdChek PRRS wells reduced peptide-binding activity of the sera, though less than adsorption on peptide-coated wells. Unexplained is the finding that adsorption of sero-positive pig sera on HerdChek PRRS wells did not reduce Ab binding to peptides and only minimally their binding to the HerdChek PRRS wells. The latter results were not due to lack of linearity between A_{650} values obtained in the ELISA and serum concentration. One problem in interpreting HerdChek ELISA results is the lack of knowledge of the specificity of the Abs that bind to the plates. It has been concluded that PRRSV-infected pigs generate Abs to the epitope recognized by mAb SDOW17 because they block the binding of the mAb to a recombinant N-protein (Ferrin et al., 2004). However, I have not been able to duplicate this result using HerdChek plates. Incubation of HerdChek wells with sera from PRRSV-infected pigs failed to reduce the binding of mAb SDOW17 to the wells and vice versa incubation of the wells with mAb SDOW17 did not reduce the binding of the pig Abs to the wells. This difference could be due to differences in the nature of the recombinant N-proteins used in these studies.

Regardless, although sera that are positive in the HerdChek ELISA are generally also positive in the peptide ELISA, clear differences exist. This is clearly indicated by a lack of correspondence between the time courses of Ab formation measured by the two ELISAs and the Ab titers obtained (Fig.3). These differences are unrelated to the great heterogeneity in the Ab responses of pigs to PRRSV infection. This heterogeneity of the Ab responses of individual pigs is apparent in differences in the time courses of formation of both peptide binding and HerdChek plate binding Abs and the Ab levels attained (Fig.3) as was also observed for the formation of Abs to the GP5 neutralization epitope (Plagemann et al., submitted for publication) This heterogeneity in Ab response of individual infected pigs is not PRRSV strain related. It had been concluded that the anti-N-protein responses of pigs infected with PRRSV strains Abst.-1 and Ingelvac ATP were lower than those of pigs infected with the five field strains ((Johnson et al., 2004) but this conclusion was based on means of ten pigs and did not consider that some of Abst,-1 and Ingelvac ATP exposed pigs had not become infected. The Ab responses of the pigs that had become infected with the latter strains were comparable to those of the pigs infected with the five field strains (Fig.3 and Plagemann et al., submitted for publication).

The differences in Ab measurements in the peptide and HerdChek ELISAs could in part reflect differences of the affinity of Abs to the same epitope when presented in immobilized peptides and the HerdChek antigens. But is seems more likely that besides detecting Abs to the same epitope they detect Abs that bind epitopes presented only by the peptides or the HerdChek antigens. For example, if pigs generate Abs to the epitope recognized by mAb SDOW17, these would be missed by the peptide ELISA. However, I have detected only two serum samples that were reported to be HerdChek ELISA and IAF positive but lacked peptide binding Abs (Fig.13B). On the other hand, pig Abs to the epitope recognized by mAb 5H2 would not be detected by the HerdChek ELISA. Since this or a closely related epitope is a major N-protein epitope in pigs, the failure to detect Abs to it is a considerable shortcoming of the HerdChek ELISA and may account for the detection of peptide binding Abs in sera of pigs of several herds that were HerdChek ELISA negative (Fig.13B and C). In fact, competition ELISA showed that the binding of sera A-2/8 and B-3/6, which are HerdChek ELISA negative, to immobilized VR-P12 and MN-P12 was inhibited by VR-P16, just as the binding of mAb 5H2 and Abs from some pigs infected with the five field strains. Abs to this epitope might be the primary Abs generated in the pigs of these herds and thus not be detected by the HerdChek ELISA. However, these pigs may have also generated Abs to additional epitopes presented by the peptides but not the HerdChek antigen perhaps in response to infection but PRRSV strains that differ from those commonly recognized.

The finding of pigs of herds that are HerdChek ELISA negative but are peptide binding Ab positive suggests that PRRSV infections may be more widespread than presently realized. Since these herds were reported to be free of any clinical signs of PRRSV infection they may have been infected by strains that cause asymptomatic infections. The presence of such strains would not be unexpected since all other arteriviruses cause asymptomatic infections (Plagemann, 1996). The closely related LDV as well as simian hemorrhagic fever virus (SHFV) only cause asymptomatic infections in their respective hosts. These viruses would never have been discovered if an LDV infection would not be associated with an elevation of lactate dehydrogenase in the circulation and SHVF would not cause a lethal disease when artificially transmitted from their natural host, African monkeys, to Asian rhesus monkeys (Plagemann, 1996).

It will be important to identify the PRRSVs that cause asymptomatic infections of pigs since they may impede eradication of PRRS and may have been and may be so in the future the source of virulent strains. They may greatly differ in genomic sequence and their proteins in antigenic properties and specificity from those of presently studied strains and thus their detection may require special approaches. The peptide ELISA should be used as an adjunct to the HerdChek ELISA since it detects sero-positive pigs that are missed by the latter and the epitope specificity of the Abs detected is largely known, which is not the case for the HerdChek ELISA. In fact, it could replace the HerdChek ELISA since I have encountered only two serum samples among 450 tested that were HerdChek ELISA positive but peptide ELISA negative (Fig.13B) though these sera came from a set of sera that yielded discrepant results in diagnostic tests. The peptide ELISA is also considerable cheaper than the HerdChek ELISA. Including positive and negative control sera one ELISA plate will allow testing 22 serum samples/plate with a cost of less than $ 2 for materials and reagents. The peptide ELISA also provides flexibility since by design of specific peptides the epitope specificity of anti-N-protein Abs can be explored. One problem still to be resolved is the finding that IgG of some serum samples binds to a greater extent to N-

protein peptide coated wells than to uncoated wells or wells coated with an irrelevant peptide (considered +/-). This could indicate the presence of low levels of anti-PRRSV Abs but other reasons have not been ruled. The same problem pertains to S/P values around 0.4 obtained in the HerdChek ELISA.

VIII. Lay Interpretation. I have developed a new type ELISA using synthetic peptides as antigen for the detection of antibodies to the nucleocapsid protein of porcine reproductive and respiratory syndrome virus (PRRSV). This ELISA has several advantage over the presently available commercial ELISA (HerdChek) used commonly in the diagnosis of PRRSV infections of pigs, This new ELISA also has detected Abs in pigs of herds which are thought to be free of PRRSV since the pig sera were negative in the commercial ELISA and the herds were free of clinical symptoms. These results suggest that PRRSV infections may be more widespread than presently recognized probably involving PRRSV strains that cause only asymptomatic infections. The prevalence of such strains would impede eradication of PRRS and could be the source of new virulent viral strains.

Peter G.W.Plagemann,Ph.D.,Professor
Department of Microbiology
University of Minnesota
1460 Mayo Memorial Bldg.,Box 196
420 Delaware Str.S.E.
Minneapolis,MN 55455-0312
e-mail:plage001@umn.edu
fax: 612-626-0623
phone:651-: 633-0466 or 612-624-3187

Acknowledgements

The author thanks M. Rodriguez, E. Nelson, D. Deregt, R. Magar and K.Platt for providing mAbs, M. Roof for providing the serum samples from the Boehringer Ingelheim VetMedica experiment (Johnson et al., 2004) and HerdChek ELISA data for the sera (Fig.3), R.R. Rowland for providing serum samples and HerdChek data from the in utero infected pig (Fig.8), E. Nelson, N.Ferrin and S. Goyal for providing serum samples and results from diagnostic tests, E.Wissink and K.Faaberg for provided serum pools from LV-infected pigs and K.Faaberg for providing N-protein sequence data for North American PRRSV isolates. This work was in part supported by a grant from the National Pork Board and by some funds from the Symgen

References

Albina, E., Leforban,Y., Baron, T., Plana-Duran,,J., Vannier, P., 1992. An enzyme-linked immunosorbant assay (ELISA) for the detection of antibodies to the porcine reproductive and respiratory syndrome (PRRS) virus. Ann.Rech.Vet. 23,167-176.

Benfield, D.A., Collins, J.E., Dee, S.A., Halbur, P.G., Joo, H.S., Lager, K.M., Mengeling, W.L., Murtaugh, M.P., Rossow, K.D., Stevenson, G.W., Zimmerman, J.J. 1999. Porcine Reproductive and Respiratory Syndrome, p. 201-232. In B. E. Straw, S. D'Allaire, W. L. Mengeling, and D. J. Taylor (ed.), Diseases of the Swine, 8th ed. Iowa State University Press, Ames, Iowa.J.,

Cho,H.J., Deregt,D., Joo,H.J. (1996) An ELISA for porcine reproductive and respiratory syndrome:production of antiogen of high quality. Can.J.Vet.res. 60,89-93.

Dea, S., Ganon, C.A., Mardassi, H., Pirzadeh, B., Rogan, D. 2000a. Current knowledge on the structural proteins for porcine reproductive and respiratory syndrome (PRRS) virus: comparison of the North American and European isolates. Arch. Virol. 145:659-688.

Dea, S., Wilson, L., Therrien, D., Cornaglia, E. (2000b). Competitive ELISA for detection of antibodies to porcine reproductive and respiratory stndrome virus using recombinant *E.coli*-expressed nucleocapsid protein as antigen. J.Virol.Meth. 87, 109-122.

Denac, H., Moser, C., Tratschin,, J.D., Hofmann, M.A. (1997) An indirect ELISA for the detection of antibodies against porcine reproductive and respiratory syndrome virus using recombinant nucleocapsid protein as antigen. J.Virol.Meth. 65,169-181.

Eisenberg, D., Schwarz, E., Komaromy, M, Wall, R. (1984) Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J.Molec. Biol. 179, 125-142.

Ferrin,N.H., Fang, Y., Johnson, C.R., Murtaugh, M.P., Polson, D.D., Torremorell, M., Gramer, M.L., Nelson, E.A. (2004) Validation of a blocking enzyme-linked immunosorbent assay for detection of antibodies against porcine reproductive and respiratory syndrome virus. Clin.Diagn.Lab.Immunol. 11, 503-514,

Gonin, P., Pirzadeh, B., Gagnon, C.A., Dea, S. 1999. Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. J. Vet. Diagn. Invest. 11:20-26.

Johnson W, Roof M, Vaughn E, Christopher-Hennings J, Johnson CR, Murtaugh MP (2004) Pathogenic and humoral immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) are related to viral load in acute infection. Vet Immunol Immunopath 102:233-247.

Mengeling, W.L., Lager, K.M. (2000) A brief review of procedures and potential problems associated with the diagnosis of porcine reproductive and respiratory syndrome. Vet. Res. 31,61-69

Meulenberg, J.J.M. 2000. PRRSV, the virus. Vet. Res. 31:11-21.

Meulenberg, J.J.M., Peterson-den Besten, A., de Kluyver. E.P., Moormann, R.J.M., Schaaper, W.M.M., Wenvoort, G. (1995). Characterization of proteins encoded by Orfs 2-7 of Lelystad virus. Virology 206, 155-163.

Meulenberg, J.J.M., van Nieuwstadt, A.P., van Essen-Zandbergen, A., Bos-de Ruijter, J.N.A., Langeveid, J.P.M., Meloen, R.H. 1998. Localization and fine mapping of antigenic sites on the nucleocapsid protein N of porcine reproductive and respiratory syndrome virus with monoclonal antibodies. Virology 252:106-114.

Nelsen, C.J., Murtaugh, M.P., Faaberg, K.S. 1999. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J.Virol. 71:270-280.

Nelson, E.A., Christopher-Hennings, J., Benfield, D.A. 1994. Serum immune responses to the proteins of porcine reproductive and respiratory syndrome (PRRS) virus. J. Vet. Diagn. Invest. 6:410-415.

Nelson, E.A., Christopher-Hennings, J., Drew, T., Wensvoort, G., Collins, J.E., Benfield, D.A. 1993. Differentiation of U.S. and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J. Clin. Microbiol. 31:3184-3189.

Plagemann, P. G. W. (1996). Lactate dehydrogenase-elevating virus and related viruses, p. 1105-1120. In B. N. Fields, D. M. Knipe, P. M. Howley(eds.), Virology, 3rd edition. Raven Press, New York

Plagemann, P.G.W. 2001. Complexity of the single linear neutralization epitope of the mouse arterivirus lactate dehydrogenase-elevating virus. Virology 290:11-20.

Plagemann, P.G.W. 2003. Porcine reproductive and respiratory syndrome virus: origin hypothesis. Emerg. Inf. Dis. 9:903-908.

Plagemann, P.G.W. 2004a. GP5 ectodomain epitope of porcine reproductive and respiratory syndrome virus, strain Lelystad virus. Virus Res. 102: 225-230.

Plagemann, P.G.W. 2004b. The primary GP5 neutralization epitope of North American isolates of porcine reproductive and respiratory syndrome virus. Vet.Immunol.Immunopath. 102:265-278.

Plagemann PGW (2005) Epitope specificity of monoclonal antibodies to the N-protein of porcine reproductive and respiratory syndrome virus by ELISA with synthetic peptides. Vet Immunol Immunopath 104:50-68

Plagemann, P.G.W., Rowland, R.R.R., Faaberg, K.S. 2002. The primary neutralization epitope

of porcine reproductive and respiratory syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch. Virol. 147:2327-2347.

Rodriguez, M.J., Sarraseca, J., Garcia, J., Sanz, A., Duran, P., Casal, J.I. 1997. Epitope mapping of the nucleocapsid protein of European and North American isolates of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 78:2269-2278.

Ropp,.S.L., Mahlum Wees, C.E., Fang, Y., Nelson, E.A., Rossow, K.D., Bien, M., Arndt, B., Preszler, S., Steen, P., Christopher-Hennings, J., Collins, J.E., Benfield, D.A., Faaberg. K.S. (2004) Characterization of emerging European-like porcine reproductive and respiratory syndome virus isolates in the United States. J.Virol. 78,3684-3703.

Seuberlich,T., Tratschin, J.D., Thur, B., Hofmann, M.A. (2002) Nucleocapsid protein-based enzyme-linked immunosorbent assay for detection and differentiation of antibodies against European and North American porcine reproductive and respiratory syndrome virus. Clin.Diagn.Lab.Immunol. 9,1183-1191.

Snijder, E.J., Meulenberg, J.J.M. 2001. Arteriviruses. In: *Fields Virology*, 4th Edition, (D. M. Knipe, P. M. Howley, D. E. Griffin, M. A. Martin, R. A. Lamb, B. Roizman, and S. E. Strauss, eds.), pp. 1205-1220. Lippincott, Williams, and Wilkins, Philadelphia, PA.

Van Nieustadt, A.P., Meulenberg, J.J.M., van Essen-Zandbergen, A., Petersen-den-Besten, A., Bende, R.J., Moorman, R.J.M., Wensvoort, G. (1996) Proteins encoded by open reading frames 3 and 4 of the genome of Lelystad virus (*Arteriviridae*) are structural proteins of the virion. J.Virol. 70,4767-4772.

Wootton, S.K., Nelson, E.A., Yoo, D. 1998. Antigenic structure of the nucleocapsid protein of porcine reproductive and respiratory syndrome virus. Clin. Diagn. Lab. Sci. 5:773-779.

Yang, L., Yoon, K.-J., Lee, J.-H., Zimmerman, J.J., Frey, M.L., Harmon, K.M., Platt, K.B. (1999) Antigenic and genetic variations of the 15 kD nucleocapsid protein of porcine reproductive and respiratory syndrome virus isol,ates. Arch.Virol. 144,525-546.

Fig.3. Time courses of formation of anti-N-protein Abs of representative pigs infected with PRRSV strains VR2332 (A-C), 17198-6 (D-F), JA142 (G-J), SDSU73 (K and L), MN185 (M and N), Abst.-1 (O and P) and Ingelvac ATP (R and Q) as measured by peptide ELISA using the indicated peptides (open symbols, serum dilution 1:50) and HerdChek ELISA (solid circles; serum dilution 1:40; S/P values are corrected for background =0.4; the values for individual pigs were provided by Mike Roof; means for the 10 pigs of each group were presented in Johnson et al., 2004).

.

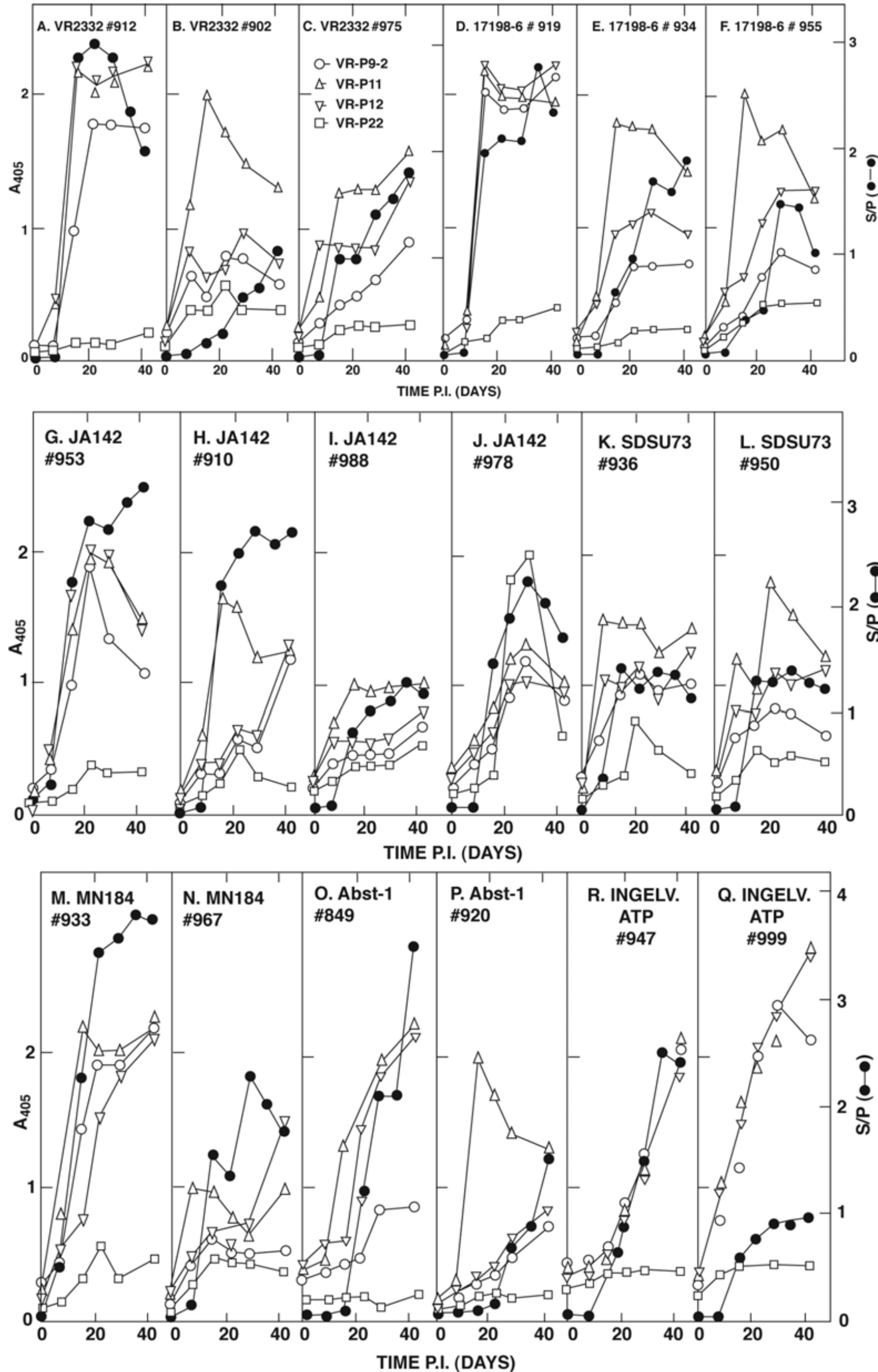

Table 3. Peptide specificity of anti- anti-N-protein mAbs

Peptide	Titer of mAb										
	1CA7 (43-56)[a]	1BD11 (31-50)	1CH5 (30-50)	SDOW17 (31-58)	VO17 (30-48)	SR30 (?)	NS99 (23-33)	5H2 (30-48)	2D6 (30-48)	2G7 (30-48)	ISU15A (43-56)
VR-P9-2	300	2000	200	< 50	400	<50	2,000	**200,000**	1,600	400	<50
LV-P9	300	**100,000**	ND	<50	<50	<50	**12,000**	400	1,200	400	<50
VR-P10	**100,000**	200	100	<50	<50	<50	<50	400	100	<50	**4000**
LV-P10	**100,000**	**8,000**	50	<50	<50	<50	<50	<50	100	50	**4000**
VR-P11	**100,000**	800	200	<50	100	<50	<50	**100,000**	800	400	**4000**
LV-P11	**100,000**	**100,000**	200	200	200	<50	<50	400	1,600	400	**4000**
VR-P12	100	< 50	100	<50	150	<50	<50	**100,000**	400	400	<50
MN-P12	<50	<50	100	<50	200	<50	<50	**200,000**	800	400	<50
JA-P12	<50	<50	100	<50	150	<50	<50	**200,000**	400	400	<50
MD-P12	<50	<50	100	<50	150	<50	<50	<50	400	ND	ND
VR-P13	<50	<50	<50	<50	<50	800	<50	<50	50	ND	ND
VR-P20	ND	2,000	<50	<50	<50	<50	ND	ND	200	200	<50
LV-P20	ND	50	<50	<50	100	100	ND	ND	400	400	ND
HerdChek	**120,000**	**80,000**	**8,000**	**300,000**	<50	**26,000**	2,000	800	<50	<50	**4000**

[a] Segment containing epitope recognized by mAb.
ND=not determine

Table 1.Amino acids 19-92 of N-protein of PRRSV strain VR2332 and corresponding sequence of N-protein segment of PRRSV strain LV and sequences of PRRSV N-protein specific peptides used in peptide ELISA

```
VR2332    [19]VN QLCQMLGKII AQQNQSRGKG PGKKNKKKNP EKPHFPLATE DDVRHHFTPS ERQLCLSSIQ TAFNQGAGTC TL[92]
LV        [23]-- ----L--AM- KS-***-QQ* --GQA---K- --------A- --I---L-QT --S---Q--- ---------A S-
VR-P9-1          ---------- --------** --------
VR-P9-2          ---------- ---------- --------
LV-P9         -- ----L--AM- KS-***-QQ* --GQA---K
VR-P10                                   -------- ---------- ----------
LV-P10                                   GQA---K- --------A- --I---L-QT
VR-P11                    - ---------- ---------- -------
LV-P11                 -AM- KS-***-QQ* --GQA---K- -------
VR-P12                ----- ---------- ---------- ---
MN-P12                ----- ---------- ----S---S- ---
JA-P12                ----- ---------- ----I-N--- ---
MD-P12                ----- ---S---V-- --R------- ---
VR-P20                                                         -------- ---------- ---------- --
LV-P20                                         K- --------A- --I---L-QT --S---Q--- ---------A S-
VR-P14                ----- ----
VR-P15                    - --------
VR-P16                           ----- -----
VR-P16M                          ---P- -----
VR-P17                                  ---------
VR-P18                                        --- -------
VR-P19                                               ------- ---
LV-P13 QPVNV     ----L-
LV-P14           ----L--AM-
LV-P15                 -AM- KS-***-QQ
LV-P16                            -QQ* --GQA--
LV-P17                                     A---K- ----
LV-P14/15           -L--AM- KS-
LV-P15/16                   KS-***-QQ* --GQ
LV-P16/17                           Q* --GQA---K-
LV-P13                                                      [102]EFSLPTHH TVRLIRVTAS PSA
```