Development of a Recombinant Nucleoprotein-Based Enzyme-Linked Immunosorbent Assay for Quantification of Antibodies against Porcine Reproductive and Respiratory Syndrome Virus†

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A rapid, inexpensive enzyme-linked immunosorbent assay (ELISA) to quantitate antibodies to porcine respiratory and reproductive syndrome virus (PRRSV) in serum was developed using a recombinant PRRSV nucleoprotein (rN). The sensitivity (85.3%) and specificity (81.7%) of the Kansas State University ELISA were good, correlating well (82.4%) with the IDEXX HerdChek ELISA.

Since its emergence on three continents in the last decade (3, 4, 8, 12), porcine respiratory and reproductive syndrome virus (PRRSV), a member of the family Arteriviridae (15), has had a catastrophic economic impact on pork producers (19), causing respiratory disease and death in piglets and abortions, weight loss, and poor performance in adult animals. A chronic infection establishes itself in some animals, leading to repeated PRRSV infection “cycles” in swine herds (18). Because it is a high-throughput method (5), detection of serum antibodies to PRRSV by the IDEXX HerdChek enzyme-linked immunosorbent assay (ELISA) is currently used for herd screening prior to export shipping to prevent the spread of disease to uninoculated animals (2). This kit uses PRRSV-infected total cell lysates as a solid-phase antigen. However, improved testing strategies are needed (7). We have developed a rapid, inexpensive alternative method for detecting antibodies to PRRSV. For the Kansas State University (KSU) ELISA, the nucleocapsid (N) protein (15.1 kDa) derived from the PRRSV North American strain was chosen as the solid-phase antigen. Many N protein epitopes are conserved among North American and European isolates (11). It has been shown that the N protein is highly immunogenic (19), and antibodies to this protein are the earliest to develop after PRRSV infection.

An American PRRSV isolate, ATCC VR-2332 (1), was propagated in MARC-145 cells under standard conditions until a 50% cytopathic effect was evident (9). The QIAamp viral RNA kit (Qiagen, Valencia, Calif.) was used to extract viral RNA from the infected cell lysates for amplification of the open reading frame 7 (N protein) genome segment by one-tube reverse transcription-PCR. Nucleocapsid-specific primers were designed containing restriction enzyme sites (underlined) for BamHI (forward, 5'-GGATCCCCAAATAACACCGGCA AGCAGCAGA-3') and HindIII (reverse, 5'-AAGCTTATCA TGCTGGAGGGTGCTGTGA-3'). Reverse transcription was initiated by incubation at 60°C for 30 min. Conditions for amplification of cDNA were as follows: denaturation at 94°C for 2 min, 40 two-step cycles of denaturation at 94°C for 45 s and annealing and extension at 60°C for 45 s, and elongation at 72°C for 7 min. The final MgCl2 concentration was 2 mM, and the final Taq DNA polymerase concentration was 2.5 U/100 µl of reaction product. The PCR product was electrophoresed in an agarose gel, purified using a glass slurry (16), and cloned into plasmid pDK 101 T vector (10), which was transformed into NovaBlue competent cells (Novagen, Madison, Wis.). The insert was removed by double-enzyme digestion with BamHI and HindIII and then cloned into the QIAexpress pQE-30 vector for bacterial expression in Escherichia coli M15(pREP4) host cells by the procedure described in the QIA Expressionist manual (Qiagen). The cDNA insert was in the correct orientation and in frame and contained the entire coding sequence of PRRSV open reading frame 7 (13). Purification of the recombinant nucleocapsid (rN) protein was performed using the QIAexpress nickel-nitrotriacetic acid protein purification system (Qiagen) according to the manufacturer’s instructions. The rN protein was analyzed on 10% linear sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and either stained with GELOCODE Blue (Pierce, Rockford, Ill.) or transferred to nitrocellulose membranes by electroblotting. Membranes were blocked, for Western blot analysis, with 10% horse serum for 1 h and incubated either with a monoclonal antipolyhistidine antibody (1:3,000 dilution) (Sigma, St. Louis, Mo.) or with anti-PRRSV monoclonal antibodies (SWOW17 and SR30; 1:10,000 dilution) (National Veterinary Services Laboratory, Ames, Iowa). After being washed in Tris-buffered saline, they were incubated with horse anti-mouse horseradish peroxidase (HRPO)-labeled immunoglobulin G (IgG) (Vector Laboratories, Burlingame, Calif.) or goat anti-swine HRPO-labeled IgG (ICN Biomedicals, Aurora, Ohio). The immunoreactive rN protein bands were detected colorimetrically using metal-enhanced diaminobenzidine substrate (Pierce, Rockford, Ill.). Western immunoblotting was done to confirm the identity and the molecular mass of the PRRSV full-length N protein (~15 kDa), using hyperimmune anti-PRRSV serum and a monoclonal antibody against the histidine tag.

To directly compare the two ELISAs, reagents and a protocol similar to those for the IDEXX ELISA were used with the KSU ELISA, except for sample and substrate incubation times. Standard checkerboard titration was performed to determine the optimal concentrations for the solid-phase antigen (50 ng of rN protein/well) and serum sample (1:40 dilution) to be employed in our test. Immulon I microtiter plates (Dynal.
tech Labs Inc., Alexandria, Va.) (14) that were coated with purified rN protein were incubated overnight at 4°C and then washed with 0.01 M phosphate-buffered saline–0.5% Tween 20 buffer (PBS-T). After blocking with 100 μl of 0.05% glycine–PBS per well at 37°C for 40 min, the plates were washed with PBS-T. Positive and negative controls and swine serum samples (from 30 swine herds) (n = 505) diluted in PBS-T were run in triplicate (100 μl/well). The plates were incubated at 37°C for 1 h and washed five times with PBS-T, and then 100 μl of goat anti-swine HRPO-labeled IgG (ICN Biomedicals)/well (1:10,000 dilution) was added. After incubation at 37°C for 30 min, the plates were washed five times with PBS-T, 100 μl of fresh tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.)/well was added, and the plates were incubated at 37°C for 30 min before color development was terminated with 100 μl of 1 N H₂SO₄/well. Absorbances were measured at 450 nm. The greatest outlier of the three optical density (OD) readings was discarded, and the remaining two values were averaged to obtain an adjusted OD. Signal-to-positive (S/P) ratios for each sample were determined according to the following formula: (adjusted ODSample − adjusted ODNeg)/(adjusted ODPos − adjusted ODNeg), where adjusted ODSample is the average OD for the swine serum sample, adjusted ODPos is the average OD for the positive control, and adjusted ODNeg is the average OD for the negative control. Samples were judged to be positive at S/P values above a cutoff point calculated with the following formula: [average adjusted ODNeg/(avg. adjusted ODPos − average adjusted ODNeg)] × 2.

The data were analyzed with SAS software (SAS Institute, Cary, N.C.). Analysis of variance was used to compare results between different sera, and the least-squares means method was used to compare results within the same case. A regression analysis was performed for the graph of IDEXX S/P ratios versus KSU S/P ratios, and a regression coefficient was calculated. No significant difference was found between the two tests for the average S/P ratios. When average S/P ratios were compared on a case-by-case basis, 12 of 30 were determined to have a significant difference at a P value of 0.05. When results from the two ELISA methods were compared, it was found that 93 samples were positive by both (true positive) and 323 were negative by both (true negative); also, 73 samples were negative by the KSU ELISA but positive by the IDEXX assay (false negative), and 16 were positive by the KSU assay but negative by the IDEXX assay (false positive). The IDEXX ELISA identified more samples as positive (n = 166) and fewer as negative (n = 339) than did the KSU ELISA (n = 106 and n = 396, respectively), but correlation between the two tests is 82.4%. When McNemar’s test was applied to false-positive and false-negative results, a significant difference was found at a P value of 0.05. Because no universal “gold standard” has been established for PRRSV antibody testing, sensitivity and specificity were calculated using either test as the reference method of detection for the presence of anti-PRRSV antibodies in swine sera (n = 505). The IDEXX ELISA sensitivity and specificity were determined using the KSU ELISA as the reference assay.

1. Benfield, D. A., E. Nelson, J. E. Collins, L. Harris, S. M. Goyal, D. Robinson, W. T. Christianson, R. B. Morrison, D. Gorcyca, and D. Chladek. 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Invest. 4:127–133.
2. Botner, A. 1997. Diagnosis of PRRS. Vet. Microbiol. 55:295–301.
3. Carman, S., S. E. Sanford, and S. Dea. 1995. Assessment of reproductive and respiratory syndrome (PRRS) virus in swine herds in Ontario—1979 to 1982. Can. Vet. J. 36:779–777.
4. Cheon, D. S., C. Chae, and Y. S. Lee. 1997. Seroprevalence of antibody to porcine reproductive and respiratory syndrome virus using enzyme-linked immunosorbent assay in selected herds in Korea. J. Vet. Diagn. Invest. 9:434–436.
5. Cho, H. J., D. Deregt, and H. S. Joo. 1996. An ELISA for reproductive and present in sera result in false-positive reports. Preparation of the solid-phase antigen used in either assay may account for discrepancies between the two tests. PRRSV-infected cell culture lysates are used in the IDEXX ELISA. Cell cultures often contain endogenous viruses and are also susceptible to contamination with Mycoplasma. Mycoplasma infections are common to pigs; thus, significant titers of antibody to this organism are often present in swine, and false positives can occur by reaction with Mycoplasma contaminants. The IDEXX ELISA uses noninfected cell culture lysate reference wells to control for this effect. However, it is possible that a specific antibody response to the PRRSV antigens and a nonspecific immune response to the cell culture antigens could occur in the same sample, causing false-negative readings.

Bacterial expression systems for antigen production reduce the potential for contamination with microbial agents that is inherent to cell culture-prepared extracts of PRRSV antigen (17). Arguably, the use of a purified PRRSV rN protein strain as a solid-phase antigen in the KSU ELISA explains why fewer PRRSV antibody-positive sera are identified by this assay than by the IDEXX ELISA. We contend that the IDEXX protocol may lead to false-positive test results. An additional advantage to using cloned, bacterially expressed antigen for the ELISA is that batch-to-batch antigen variation will be lower than that for cell culture-derived PRRSV preparations. RNA viruses naturally show greater variability due to lower proofreading fidelity during replication. With bacterially expressed antigens, greater quantities may be economically produced than with cell culture, contributing to the purity of the final antigen preparation, with a consequent improvement in the specificity of the immunoassay.

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REFERENCES
1. Benfield, D. A., E. Nelson, J. E. Collins, L. Harris, S. M. Goyal, D. Robinson, W. T. Christianson, R. B. Morrison, D. Gorcyca, and D. Chladek. 1992. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J. Vet. Diagn. Invest. 4:127–133.
2. Botner, A. 1997. Diagnosis of PRRS. Vet. Microbiol. 55:295–301.
3. Carman, S., S. E. Sanford, and S. Dea. 1995. Assessment of reproductive and respiratory syndrome (PRRS) virus in swine herds in Ontario—1979 to 1982. Can. Vet. J. 36:779–777.
4. Cheon, D. S., C. Chae, and Y. S. Lee. 1997. Seroprevalence of antibody to porcine reproductive and respiratory syndrome virus using enzyme-linked immunosorbent assay in selected herds in Korea. J. Vet. Diagn. Invest. 9:434–436.
5. Cho, H. J., D. Deregt, and H. S. Joo. 1996. An ELISA for reproductive and

### TABLE 1. Sensitivities and specificities of the KSU ELISA and the IDEXX HerdChek ELISA

| Reference method | Sensitivity (%) | Specificity (%) | Correlation (%) |
|------------------|----------------|----------------|----------------|
| KSU ELISA       | 81.7           | 85.3           | 82.4           |
| IDEXX ELISA     | 95.3           | 56.0           | 82.4           |

* Sensitivity and specificity of the KSU ELISA were calculated using the IDEXX HerdChek ELISA as the reference method of detection for the presence of anti-PRRSV antibodies in swine sera (n = 505). The IDEXX ELISA sensitivity and specificity were determined using the KSU ELISA as the reference assay.

* Sensitivity: [TN/(TN + FP)] × 100, where TN is the number of true-negative results and FP is the number of false-positive results.

* Specificity: [TP/(TP + FN)] × 100.

* Correlation between the two tests, (TP + TN)/(TP + FP + FN).
respiratory syndrome: production of antigen of high quality. Can. J. Vet. Res. 60:89–93.

6. Cho, H. J., S. C. Entz, R. Magar, and H. S. Joo. 1997. Performance of ELISA antigens prepared from 8 isolates of porcine reproductive and respiratory syndrome virus with homologous and heterologous antisera. Can. J. Vet. Res. 61:299–304.

7. Dee, S. A., and T. W. Molitor. 1998. Elimination of porcine reproductive and respiratory syndrome virus using a test and removal process. Vet. Rec. 143:474–476.

8. Garner, M. G., L. J. Gleeson, P. K. Holyoake, R. M. Cannon, and W. J. Doughty. 1997. A national serological survey to verify Australia’s freedom from porcine reproductive and respiratory syndrome. Aust. Vet. J. 75:596–600.

9. Kim, H. S., J. Kwang, I. J. Yoon, H. S. Joo, and M. L. Frey. 1993. Enhanced replication of porcine reproductive and respiratory syndrome (PRRS) virus in a homogenous subpopulation of MA-104 cell line. Arch. Virol. 140:1405–1418.

10. Kovalic, D., J. H. Kwak, and B. Weisblum. 1991. General method for direct cloning of DNA fragments generated by the polymerase chain reaction. Nucleic Acids Res. 19:4560.

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

12. Nodelijk, G., L. A. Van Leengoed, E. J. Schoevers, A. H. Kroeghe, M. C. De Jong, G. Wensvoort, and J. M. Verherijden. 1997. Seroprevalence of reproductive and respiratory syndrome virus in Dutch weaning pigs. Vet. Microbiol. 56:21–32.

13. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.

14. Schoenthaler, S. L., and S. Kapil. 1999. Development and applications of a bovine coronavirus antigen detection enzyme-linked immunosorbent assay. Clin. Diagn. Lab. Immunol. 6:130–132.

15. Snijder, E. J., and J. J. M. Meulenberg. 1998. The molecular biology of arteriviruses. J. Gen. Virol. 79:961–979.

16. Vogelstein, B., and D. Gillespie. 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA 76:615–619.

17. Woods, R. D., R. A. Kunkle, J. A. Ridpath, and S. R. Bolin. 1999. Bovine viral diarrhea virus isolated from fetal calf serum enhances pathogenicity of attenuated transmissible gastroenteritis virus in neonatal pigs. J. Vet. Diagn. Invest. 11:400–407.

18. Zimmerman, J., T. Sanderson, K. A. Eernisse, et al. 1992. Transmission of SIRS virus from convalescent animals to commingled penmates under experimental conditions. Am. Assoc. Swine Pract. Newsl. 4:25.

19. Zimmerman, J., K. J. Yoon, G. Stevenson, and S. A. Dee. 1998. The 1998 PRRS compendium: a comprehensive reference on porcine reproductive and respiratory syndrome for pork producers, veterinary practitioners, and researchers. National Pork Producers Council, Des Moines, Iowa.