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Evaluation of the baculovirus-expressed S glycoprotein of transmissible gastroenteritis virus (TGEV) as antigen in a competition ELISA to differentiate porcine respiratory coronavirus from TGEV antibodies in pigs

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Abstract. The spike (S) glycoprotein of the Miller strain of transmissible gastroenteritis virus (TGEV) was recently cloned and expressed in baculovirus. The recombinant S protein was used as the coating antigen in a competition (blocking) enzyme-linked immunosorbent assay (ELISA) in combination with monoclonal antibodies to the S protein epitope A (conserved on TGEV and porcine respiratory coronavirus [PRCV]) or epitope D (present on TGEV only) to differentiate PRCV- from TGEV-induced antibodies. One set (set A) of 125 serum samples were collected at different times after inoculation of caesarean-derived, colostrum-deprived (n = 52) and conventional young pigs (n = 73) with 1 of the 2 porcine coronaviruses or uninoculated negative controls (TGEV/PRCV/negative = 75/30/20). A second set (set B) of 63 serum samples originated from adult sows inoculated with PRCV and the recombinant TGEV S protein or with mock-protein control and then exposed to virulent TGEV after challenge of their litters. Sera from set A were used to assess the accuracy indicators (sensitivity, specificity, accuracy) of the fixed-cell blocking ELISA, which uses swine testicular cells infected with the M6 strain of TGEV as the antigen source (ELISA 1) and the newly developed ELISA based on the recombinant S protein as antigen (ELISA 2). The sera from set B (adults) were tested for comparison. The plaque reduction virus neutralization test was used as a confirmatory test for the presence of antibodies to TGEV/PRCV in the test sera. The accuracy indicators for both ELISAs suggest that differential diagnosis can be of practical use at least 3 weeks after inoculation by testing the dual (acute/convalescent) samples from each individual in conjunction with another confirmatory (virus neutralization) antibody assay to provide valid and complete differentiation information. Moreover, whereas ELISA 1 had 10–20% false positive results to epitope D for PRCV-infected pigs (set A samples), no false-positive results to epitope D occurred using ELISA 2, indicating its greater specificity. The progression of seroresponses to the TGEV S protein epitopes A or D, as measured by the 2 ELISAs, was similar for both sets (A and B) of samples. Differentiation between TGEV and PRCV antibodies (based on seroresponses to epitope D) was consistently measured after the third week of inoculation.

Transmissible gastroenteritis virus (TGEV), a prominent cause of neonatal diarrhea, causes death in 90–100% of seronegative pigs under 2 weeks of age and costs the US swine industry nearly $200 million/year.[15,19] Surveys of TGEV antibodies in swine sera collected prior to the detection of porcine respiratory coronavirus (PRCV) indicated that 19–54% of swine herds in North America have antibodies,[17] but few differential serosurveys have been done since the detection of PRCV.[32] A variant of TGEV, referred to as PRCV, was isolated from pigs in Europe in 1986 and identified in the USA in 1989.[15,18,19] As reported from Iowa swine herds, a recent increase observed in TGEV/PRCV seroprevalence (91% of sera and 100% of herds tested) was most likely due to increased subclinical PRCV infections in these herds.[32] Experimental or natural infection of pigs with PRCV induces no diarrhea and mild or no respiratory disease in most cases,[15] but pneumonia has been reported.[30] The severity of PRCV respiratory disease may be enhanced in combination with porcine respiratory and reproductive syndrome virus infection.[9] Pigs previously exposed to PRCV show some reduction in the severity of disease caused by TGEV, which may explain why the severity of transmissible gastroentertis outbreaks in Europe has decreased following the appearance of PRCV.[15,18,19] PRCV is indistinguishable from TGEV by routine serologic diagnostic tests.[15]

The spike (S) glycoprotein is 1 of the 4 structural TGEV/PRCV proteins, but only the S protein contains epitopes that induce virus-neutralizing antibodies.[27] The S glycoprotein contains 4 major antigenic sites (A, B, C, D), antibodies to which can be found in the serum of TGEV-infected pigs. The loss of antigenic epitope D on PRCV strains enables the differential di-

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agnosis of TGEV and PRCV by the use of monoclonal antibodies (MAbs) to epitope D.

A panel of MAbs reactive with the TGEV and PRCV S protein (epitope A) and others nonreactive with the PRCV S protein but reactive with TGEV (epitope D) has been developed and used in a fixed-cell blocking enzyme-linked immunosorbent assay (ELISA) (ELISA 1) for the serologic differentiation of PRCV- and TGEV-infected pigs.[24] However, this assay is cumbersome for routine use by diagnostic laboratories because it requires a constant supply of standardized TGEV-infected fixed cells as an antigen source. To improve the assay and its potential application for routine use by diagnostic labs, a baculovirus recombinant TGEV S protein[20] was produced and adapted for use in a recombinant S protein ELISA (ELISA 2), thereby simplifying the immunoassay (Sestak K, et al.: 1996, Abstr Conf Res Workers Anim Dis 118).

The objectives were first to compare the 2 ELISA tests for accuracy using sera collected at various times from young pigs (set A) after inoculation with TGEV or PRCV or from uninoculated negative control pigs. Second, the development of antibodies in young pigs (set A) against S protein epitopes A and D was compared with results from adult sows (set B) either inoculated with PRCV and baculovirus-expressed TGEV S protein or mock inoculated and then exposed to virulent TGEV via challenge of selected litters.

Materials and Methods

Serum samples

Set A (n = 125). Seventy-three 11-day-old conventional pigs from cross-bred sows seronegative for TGEV/PRCV neutralizing antibodies were inoculated oronasally as previously reported[28] with 1 of the following viruses: virulent TGEV M5C (5.2×10^5 plaque-forming units [PFU]/pig, 32 pigs), attenuated TGEV P115 (5.2×10^5 PFU/pig, 4 pigs), PRCV ISU-1 (2×10^8 PFU/pig, 26 pigs), or mock cell culture fluid (11 control pigs). Fifty-two caesarean-derived, colostrum-deprived pigs that were TGEV/PRCV seronegative were inoculated orally at 4 wk of age with viral suspensions of unspecified doses of TGEV Miller (39 pigs) or PRCV Indiana (4 pigs), causing clinical disease or virus shedding, respectively. Nine pigs were uninoculated negative controls. Set A serum samples were collected weekly up to 42 postinoculation days (PID). All samples from set A were tested by both ELISAs to determine seroconversion against TGEV S protein epitopes A and D. The 125 set A sera were divided into 5 major subsets based on PID: 0, 7–9, 11–17, 21–28, and 31–42 PID.

Set B (n = 63). Sera were collected prior to each inoculation and challenge from 7 large white breed TGEV/PRCV-neutralizing antibody seronegative sows. Sows were oronasally inoculated at 42 and 28 prepartum days (PPD) with 2×10^8 PFU of PRCV (ISU-1) and at 14 PPD intramuscularly (IM) with 1 mg of the baculovirus-expressed recombinant TGEV S protein (containing antigenic sites A, B, C, and D; sows 36-4, 49-8, 87-4, 40-5, and 87-3) or wild type baculovirus protein in incomplete Freund's adjuvant, (sows 50-8 and 40-5B).[20] Nursing pigs of 4 sows (except litters from sows 36-4, 49-8, and 87-4) were inoculated oronasally 5 days post partum with 100 PDD_{50} (pig diarrhea dose-50%) (5.2×10^5 PFU) of virulent M5C TGEV.[20]

Sera analysis

Seventy-two sera from set A (conventional pigs only) and 63 sera from set B (135 total) were tested by a plaque-reduction virus neutralization (VN) test for the presence of TGEV/PRCV-neutralizing antibodies to compare the results obtained by the 2 ELISA systems with those from a conventional diagnostic (VN) test. All sera from both sets (188 total) were tested by both ELISAs to determine the presence of antibodies against TGEV S protein epitopes A and D.

Monoclonal antibodies to TGEV/PRCV

The 2 MAbs used in this study were 25C9 and 44C11.[23] The 25C9 MAb recognizes epitope A of TGEV/PRCV S protein, whereas MAb 44C11 recognizes epitope D of the TGEV S protein (no reaction with PRCV).[23] The MAbs were purified from mouse ascitic fluids by Avidchrom-gel[a] chromatography and biotinylated.[23]

Competition ELISA using the TGEV M6 infected ST cells (ELISA 1)

Swine testis cells (ST) were grown to confluency in 96-well cell culture plates, and the cells were infected with the titer of the M6 strain of TGEV that produced infectivity in at least 80% of the cells by 20 postinoculation hours.[24] Expression of the viral S protein within the cell cytoplasm was confirmed by an enzyme-linked immunospot assay (Sestak K, et al.: 1996, Abstr Conf Res Workers Anim Dis 118). Mouse hybridoma cells producing IgG antibodies against the TGEV S protein (A or D epitope specific) were incubated in RPMI medium for 6 hr at 37 C on monolayers of the acetone-fixed, M6-infected ST cells. Biotin-labeled anti-mouse IgG,[b] horseradish peroxidase-conjugated streptavidin,[c] and TMB-chromogenic substrate[b] were used to develop the reaction, which resulted in dark blue spots for individual hybridoma cells. After fixation with acetone, if not used immediately for ELISA 1, the 96-well plates were stored at −20 C for up to 10 days.

The ELISA 1 was conducted as described previously.[24] The M6-infected fixed ST cells were thawed from −20 C, rehydrated, and blocked with TBS-T (20 mM Tris-HCl, pH 8.0, 0.1% v/v Tween 20) for 2 hr at 25 C. Serum samples were serially diluted 3-fold in TBS-T starting at 1:5 and incubated on M6-ST plates at 4 C overnight. After incubation with sera, the biotinylated MAbs (bMAbs) against epitopes A or D of TGEV S protein were added (without washing the plates) at a concentration of 0.1 or 2.5 μg/ml, respectively, and incubated at 25 C for 2 hr. Horseradish peroxidase-conjugated streptavidin[c] was applied prior to washing and adding the chromogenic substrate.[24] The 50% blocking dilution was assessed as a cut-off value for determining the serum titer.

Competition ELISA using the baculovirus–expressed TGEV S protein (ELISA 2)

The recombinant baculovirus expressing a TGEV M6 S protein of 789 amino acids (R2-2), containing the 4 major antigenic sites (A, B, C, D),[20] was used to infect an insect (Sf9) cell line at a multiplicity of infection of 10 (Sestak K, et al.: 1996, Abstr Conf Res Workers Anim Dis 118). The recombinant S (rec-S) protein was harvested in soluble form by low speed centrifugation (1,000 × *g*, 10 min) of cell culture supernatants and used to coat the 96-well plates[d] in phosphate-buffered saline (PBS) (pH 7.4). The conformational stability of rec-S protein, its reactivity with serum from TGEV/PRCV hyperimmunized animals, and its presence in infected supernatant fluids was confirmed by Western blot.[20,26] Approximately 0.05 μg/ml (epitope A competition) and 1 μg/ml (epitope D competition) of rec-S protein was used for coating the ELISA plates.

The 96-well plates were coated with rec-S protein as described above at 4 C overnight. After antigen coating, plates were blocked with 1% nonfat dry milk in PBS (pH 7.3) for 1 hr at 25 C. Plates were then washed with TBS-T, and test sera were applied in serial dilutions as described for ELISA 1 and incubated for 2 hr at 25 C or 4 hr at 4 C. After incubation with sera, bMAbs against epitopes A or D of TGEV S protein were added (without washing the plates) at a concentration of 0.1 or 8.0 μg/ml, respectively, and incubated at 25 C for 2 hr. The rest of the procedure for ELISA 2 was identical to that for ELISA 1.

Plaque-reduction VN assay

The TGEV/PRCV neutralizing antibody titers were determined against M6 TGEV by a plaque reduction test, and titers were expressed as the reciprocal of the highest serum dilution that produced an 80% reduction in plaques as compared with untreated controls.[1]

Statistical analysis

A 2-sample *t*-test was used to evaluate significant differences ($P < 0.05$) between ELISA blocking titers at different times, comparing seroconversion against epitope A of TGEV/PRCV serum samples versus negative control serum samples and seroconversion against epitope D of TGEV serum samples versus PRCV and negative control serum samples.

Correlations. The confirmatory (epitope A) and differentiation (epitope D) competition results obtained by both ELISAs were compared with VN test results for 30 endpoint-titered TGEV serum samples from set A by calculation of correlation coefficients. The presence of antibodies to both epitopes A and D of the TGEV S protein in the test samples was a prerequisite for selecting sera for correlation of site A or D blocking ELISA titers with VN titers; therefore, the PRCV sera were not included. The correlation coefficients were calculated, and regression lines were plotted with 95% confidence intervals using the Minitab[e] software.

Sensitivity, specificity, and accuracy. All serum samples from TGEV/PRCV-inoculated animals were considered positive to maintain constant evaluation criteria for accuracy indicators of both blocking ELISA tests. Although the VN test was used for calculations of correlation coefficients and drawing the regression lines against ELISA results, it was not used as a ''gold standard'' test. Only sera from set A were used for calculations of sensitivity, where sensitivity = true positives/(true positives + false negatives), specificity, where specificity = true negatives/(false positives + true negatives), and accuracy, where accuracy = (true positives + true negatives)/total for both ELISA tests. To demonstrate overall increases in these indicators with time after virus exposure and to simplify the number of calculations, ELISA results were pooled into 2 major time intervals, 7–17 PID and 21–42 PID. Each accuracy indicator was calculated with the same 32 samples (16 negative with expected S protein seroconversion characteristic A−D−, 8 TGEV positive with expected S protein characteristic A+D+, and 8 PRCV positive with expected S protein characteristic A+D−) for the 2 antigenic epitopes (A, D) for the 2 major time intervals.

Results

Optimal concentrations of epitope A and D bMAbs used for competition ELISA with serum antibodies were 2–10-fold lower than the concentrations that resulted in maximal ligand binding (Fig. 1) because the average polyclonal serum antibody avidity was generally lower than the avidity of the MAbs.[24] The optimal dilutions were determined empirically as reported previously[24] by serial dilutions of bMAbs and reactions with reference TGEV/PRCV-negative control sera (Fig. 1). For ELISA 1 (M6-infected fixed ST cells), the optimal concentration of bMAbs that competed with serum antibody for site A was 0.05 μg/ml and that for site D was 3 μg/ml (Fig. 1A, 1B). For ELISA 2, which used S protein as antigen substrate, the optimal concentration of the site A bMAb was the same as above, but for site D the bMAb was 10–15 μg/ml (Fig. 1C, 1D). Each serum sample with more than 50% blocking of bMAb-associated ELISA signal was considered positive, and the blocking titer was defined as the reciprocal of the highest serum dilution that resulted in ≥50% blocking.[24]

Analysis of TGEV/PRCV-negative blocking titers of the set A sera showed that epitope A seroconversion among TGEV- or PRCV-inoculated pigs was first consistently measured at PID 11–17 ($P < 0.05$, 88–100% of TGEV/PRCV-inoculated pigs positive), although some blocking titers were detected earlier (PID 7–9), especially for TGEV-inoculated pigs (72–78% of pigs; Table 1, Fig. 2). Using the VN test, TGEV/PRCV-specific seroconversion was detected from PID 7–9 on (Table 2) for most TGEV-exposed pigs (83%) but not until PID 11–17 for PRCV-exposed pigs (100%). For seroconversion against epitope A for either TGEV- or PRCV-inoculated pigs (Fig. 2, A curves and C; Table 1), higher blocking titers were generally measured by ELISA 2 than by ELISA 1. For serum samples from TGEV-inoculated pigs, epitope D seroconversion (Fig.

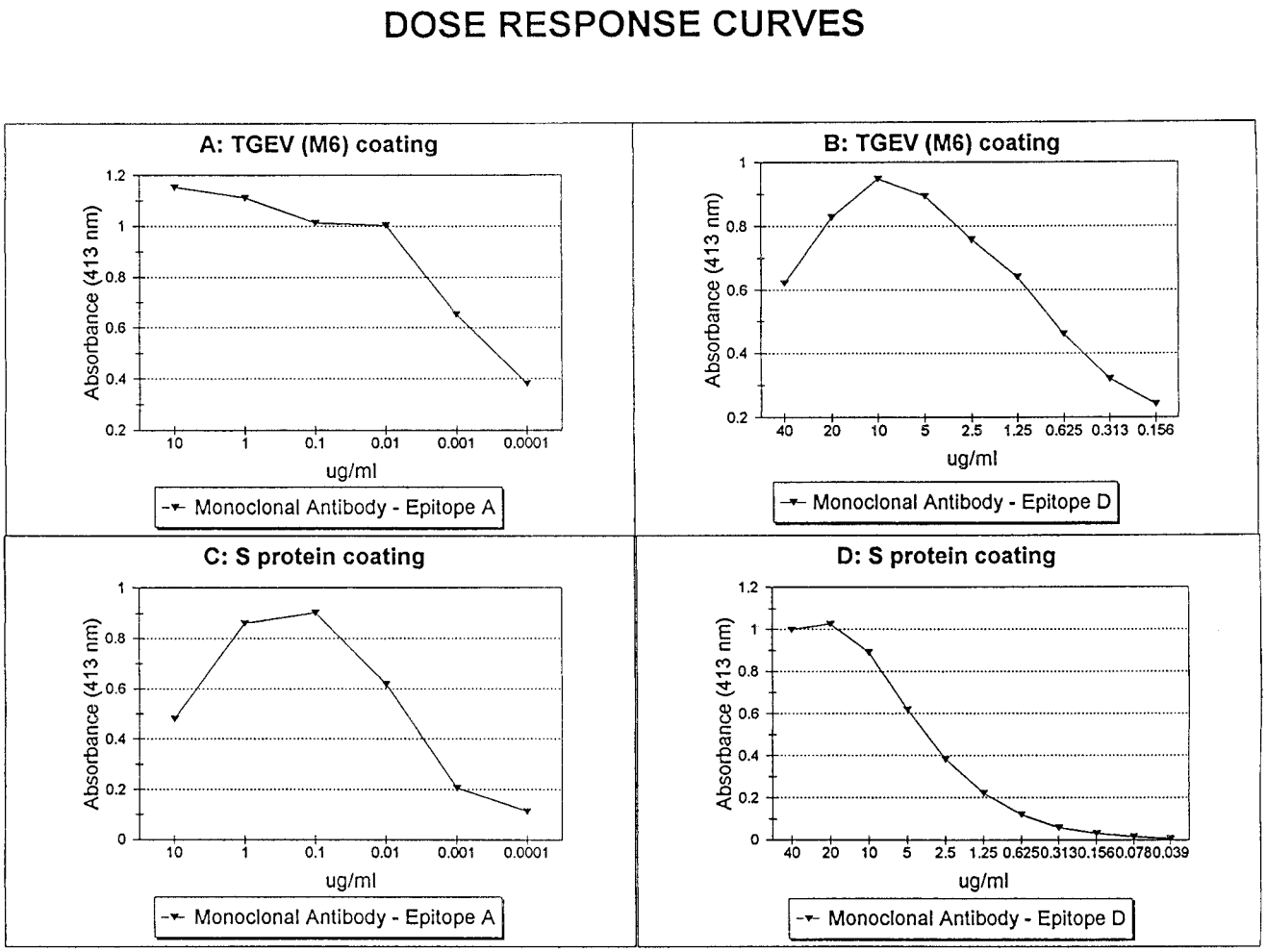

Figure 1. Monoclonal antibodies against TGEV S glycoprotein epitopes A and D were affinity purified and biotinylated, and the dose–responses titrated by using the 2 different antigens (recombinant S protein and M6-infected ST cells) as the coating for ELISA. **A, B.** Dose–response curves reflect binding between fixed M6-infected ST cells and bMAbs against epitopes A and D of TGEV S protein. The bMAbs were serially diluted to determine the concentrations that resulted in optimal antigen–antibody binding reactions. **C, D.** Binding reactions between recombinant TGEV S protein and epitope A and D bMAbs.

2B, Table 1) was detected in the majority (≥88%) of pigs only after 21–28 PID. Testing for epitope D seroconversion among PRCV-inoculated pigs showed low blocking titers in 10–20% of pigs after PID 11–17 with ELISA 1 (indicative of false positives) but no seroconversions detected with ELISA 2 (Table 1; Fig. 2D). However, only 5 PRCV-inoculated animals were tested at PID 21–28.

Analysis of 63 set B sera from sows inoculated during pregnancy with PRCV, baculovirus-expressed rec-S TGEV protein, or baculovirus wild type protein and then exposed to the virulent M5C strain of TGEV (4 sows upon challenge exposure of their litters) showed similar seroconversion patterns as detected for set A (Table 3). Epitope A blocking titers were detected by ELISA 2 and TGEV antibodies were detected by the VN test by PID 14 after the first PRCV inoculation for all sows (Table 3). Only by PID 28 were all sows seropositive to epitope A by ELISA 1 (Table 3). The IM administration of the rec-S TGEV protein (5 sows) at 14 PPD (28 PID with PRCV) resulted in epitope D seroconversion that was detected by both ELISAs 14 days later (3 of 5 animals, Table 3); 1 more sow seroconverted (ELISA 2) 5 days later at the day of litter challenge. At 26 days after rec-S TGEV protein administration or 7 postchallenge days. (PCD), all 7 inoculated sows seroconverted to epitope D as demonstrated by both ELISAs (Table 3). Moreover, serum titers against epitope D were first detectable (no S protein inoculation, sows 50-8 and 40-5B) or increased (sow 87-3) by 7 days after exposure of sows to virulent TGEV shedding by their challenged litters. Peak VN antibody titers in sera of most set B sows (but not sow 36-4) were observed 7 days (PID 54) after virulent TGEV challenge inoculation of their suckling pigs (Table 3).

Table 1. Comparison of ELISA 1 and 2 epitope A and D 50% blocking titers using the sera from 125 1–4-week old experimentally infected pigs (TGEV/PRCV/negative = 75/30/20). Blocking titers were expressed as geometric mean and 95% confidence interval for each postinoculation day (PID) interval.

	TGEV				PRCV				Negative			
	ELISA 1		ELISA 2		ELISA 1		ELISA 2		ELISA 1		ELISA 2	
PID	A	D	A	D	A	D	A	D	A	D	A	D
0	…	…	…	…	…	…	…	…	<5	<5	<5	<5
	…	…	…	…	…	…	…	…	<5	<5	<5	<5
	…	…	…	…	…	…	…	…	2/20	0/20	2/20	0/20
	…	…	…	…	…	…	…	…	10	0	10	0
7–9	10*	<5	6	<5	<5	<5	<5	<5	…	…	…	…
	7–20†	<5–9	11–30	<5	<5–10	<5	<5–10	<5	…	…	…	…
	14/18‡	4/18	13/18	6/18	1/3	0/3	1/3	0/3	…	…	…	…
	78§	22	72	33	33	0	33	0	…	…	…	…
11–17	38	5	99	9	21	<5	109	<5	…	…	…	…
	48–88	5–29	95–129	15–49	16–56	<5–5	94–139	<5	…	…	…	…
	23/26	14/26	26/26	17/26	11/12	2/12	12/12	0/12	…	…	…	…
	88	54	100	65	92	17	100	0	…	…	…	…
21–28	65	30	135	84	72	<5	120	<5	…	…	…	…
	54–99	33–90	135	85–132	<5–149	<5–34	78–162	<5	…	…	…	…
	17/17	15/17	17/17	16–17	4/5	1/5	5/5	0/5	…	…	…	…
	100	88	100	94	80	20	100	0	…	…	…	…
31–42	95	43	135	58	86	<5	113	<5	…	…	…	…
	94–136	35–91	135	51–109	68–119	<5–9	96–144	<5–4	…	…	…	…
	14/14	14/14	14/14	14/14	10/10	1/10	10/10	0/10	…	…	…	…
	100	100	100	100	100	10	100	0	…	…	…	…

* Geometric mean of 50% blocking ELISA titer (for calculations: <5 = 2.5).
† 95% confidence interval.
‡ Ratio of positive/total serum samples tested.
§ Percentage of pigs that tested positive.

Calculations of sensitivity, specificity, and overall accuracy of both blocking ELISAs at an early stage of TGEV/PRCV infection (7–17 PID, set A sera) and at a later stage of TGEV/PRCV infection (21–42 PID, set A sera) revealed marked differences over time. At an early stage of infection, the sensitivity of blocking ELISA 1 was <40% and for ELISA 2 it was approximately 50%. The specificity of blocking ELISAs at this time was 94% (ELISA 1) and 100% (ELISA 2). At 7–17 PID, the accuracy for ELISA 1 was 66% and that for ELISA 2 was 88%. These sensitivities and accuracies indicate a reduced ability to differentiate between TGEV and PRCV seroconversion less than 3 weeks after infection. As indicated (Fig. 2A, 2C) both ELISAs detected epitope A seroconversion to TGEV or PRCV after 11–17 PID (88–100% positive). No significant blocking titers for epitope D were detected at 11–17 PID by either ELISA 1 or ELISA 2 (Fig. 2B) for either TGEV or PRCV serum samples. However, as time after infection increased (21–42 PID), more pronounced seroconversion against epitope D of the TGEV S protein in TGEV-infected pigs (not found on the PRCV S protein) resulted in an improvement in the discriminatory power of both ELISAs. After PID 21, differences in epitope D blocking titers between TGEV and PRCV sera became significant, (both ELISAs; Fig. 2, curve B). The sensitivity increased to 94% for ELISA 1 and to 99% for ELISA 2. There was a higher tendency for epitope D false-positive results (ELISA 1) among PRCV sera with high epitope A blocking titers than among PRCV samples with lower epitope A blocking titers. Accuracy values improved to 88% for ELISA 1 and to 94% for ELISA 2.

Regression analysis and calculation of correlation coefficients (R) revealed moderate to high correlation between the results obtained by VN tests and those of both ELISAs (Fig. 3). Seroconversion against epitope A measured by both ELISAs correlated highly ($R > 0.8$) with VN titers. Only moderate correlation ($R > 0.5$), was found between VN titers and blocking titers against epitope D, as measured by both ELISAs.

Discussion

Various methods have been described to differentiate TGEV from PRCV infections.[2,3,5,10–12,16,24,25,29,31] In general, these methods are based on antigenic or genomic differences between TGEV and PRCV S proteins/genes. Blocking or competition ELISAs have been used by several authors, with TGEV as the coating antigen.[2,3,5,10,16,24,29] Although several tests have

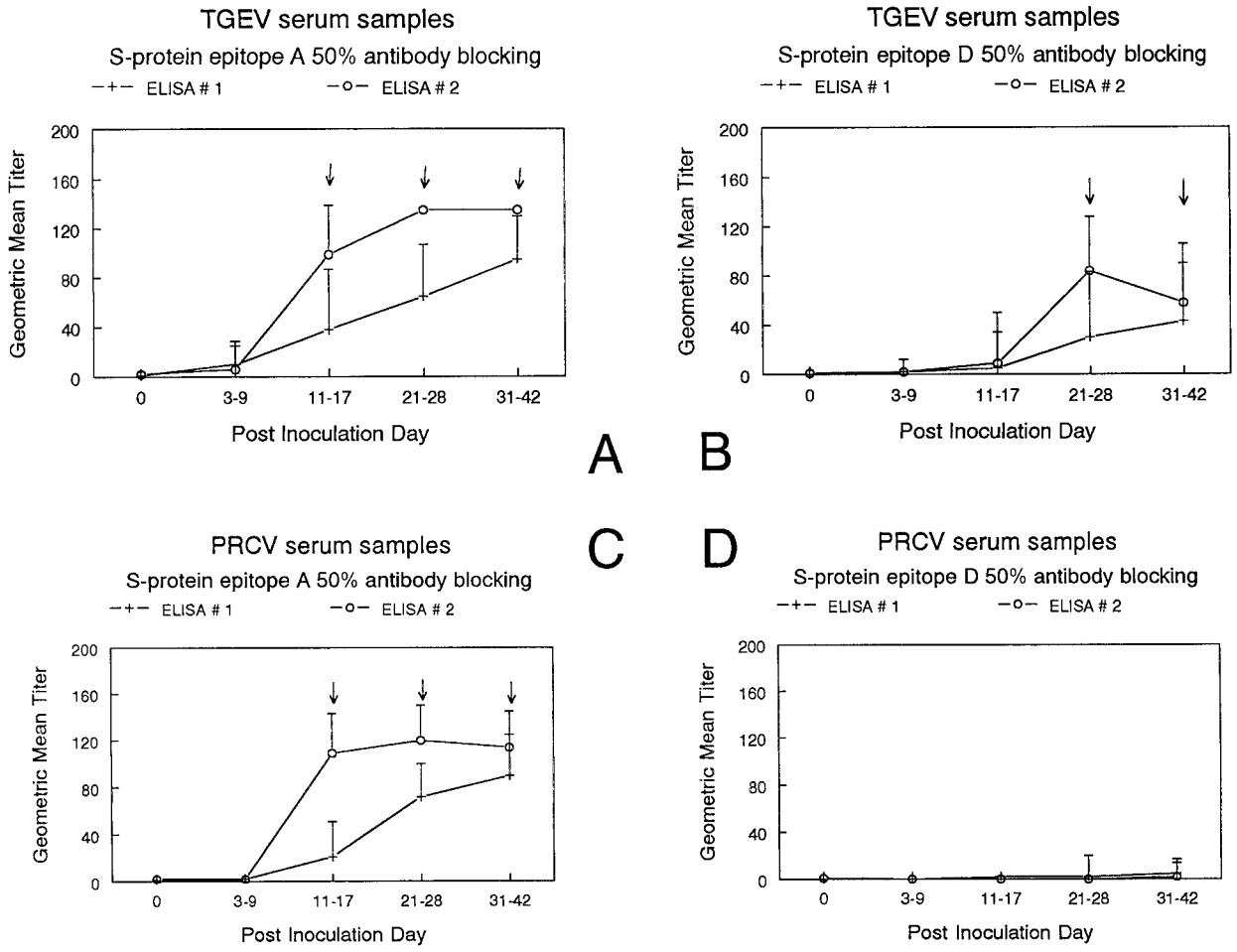

Figure 2. Serum samples (n = 125) from 1–4-week-old experimentally inoculated (TGEV/PRCV/negative = 76/30/20) pigs were tested at 5 subsequent postinoculation times by the 2 blocking ELISAs (1 and 2) to determine seroconversion against epitopes A and D of TGEV/PRCV S protein. Blocking titers were expressed as the geometric mean titer + SD of 50% antibody blocking titers. Arrows designate significantly elevated ($P < 0.05$) 50% blocking titers as measured by ELISAs 1 and 2. **A**, **C**. TGEV/PRCV samples above negative serum samples. **B**, **D**. TGEV samples above PRCV samples and negative control.

been developed to differentiate TGEV from PRCV seroconversion, to date only a few serologic surveys have been conducted nationally.[2,5]

The existence of at least four antigenic sites (A, B, C, D) located at the N-terminus of the mature S protein has been described for TGEV.[4,7] The S protein epitopes can be subdivided into 2 categories based on their ability to induce VN antibodies. Generally, TGEV/PRCV S protein VN antibody-inducing epitopes are highly conserved, and antibody formed against them possesses high affinity.[12] In contrast, some low VN titer or no VN antibody-inducing S protein epitopes induce seroconversion to TGEV only and can be used for differentiation between TGEV and PRCV. However, these antibodies usually possess lower affinity than do S protein VN-inducing antibodies.[14,22] The deletion of 224 amino acids from the S protein of a European PRCV strain and 227 amino acids from the S protein of a US PRCV strain resulted in a truncated PRCV S protein (170 kD) compared with the TGEV S protein (220 kD) and the loss of antigenic epitope D.[8,15]

This is the first report using a baculovirus-expressed TGEV S protein for the differential serologic diagnosis of TGEV/PRCV infection. Because the baculovirus expression system permits the production of high quantities of foreign proteins in secreted form and the preservation of the native conformation of glycoproteins, including posttranslational modifications such as N-glycosylation, this system represents an attractive method for production of diagnostic reagents.[20,27]

The objectives of this study were to simplify blocking ELISA procedures by using recombinant baculovirus S protein antigen for coating and to evaluate its potential for large-scale testing. Comparison of the sera of TGEV-infected, PRCV-infected, and negative control animals revealed that at 21–28 PID blocking

Table 2. VN titers of sera from 72 1–4-week old inoculated pigs (TGEV/PRCV/negative = 35/26/11). Titers were expressed as geometric mean titers and 95% confidence interval for each post-inoculation day (PID) interval.

PID	TGEV	PRCV	Negative
0	…	…	<1
	…	…	<1
	…	…	0/11
	…	…	0
7–9	4.5*	<2	…
	2.7–15†	<2	…
	10/12‡	0/3	…
	83§	0	…
11–17	35.4	24.5	…
	20–146	17–52	…
	16/16	12/12	…
	100	100	…
21–42	110	29	…
	77–152	16–75	…
	7/7	11/11	…
	100	100	…

* Geometric mean titer.

† 95% confidence interval.

‡ Ratio of positive/total serum samples tested (for calculations: <1 = 0.5).

§ Percentage of pigs that tested positive.

ELISA antibodies were present, which enabled differentiation between TGEV and PRCV seroconversion with an accuracy of approximately 90% for both ELISA tests. For both sets of serum samples (A and B), seroconversion against epitope A and D was reliably detected after PID 11–17 and 21–28, respectively.

The present results are consistent with those of previous studies[13] that demonstrated seroconversion by blocking ELISA 1 in sows experimentally exposed during pregnancy to TGEV or PRCV and subsequently exposed to TGEV as a result of challenge of their suckling pigs 5 days after parturition. Only the sows exposed to TGEV during pregnancy seroconverted to epitope D with competition ELISA geometric mean titer (GMT) > 10 at 14–21 PCD of their suckling pigs, whereas sows inoculated with PRCV during pregnancy showed low or no epitope D seroconversion (GMT < 10) at 14–21 PCD. Similar to our results, antibodies to epitope A were detected at 7–14 PCD for the same group of sows.[13] In another study of pigs experimentally inoculated with TGEV, blocking ELISA antibodies to the epitope absent on PRCV were not consistently detected until PID 25 (in 100% of inoculated pigs).[3] The same authors found 7 TGEV false negatives of 49 samples from animals naturally infected with TGEV, all of which were positive by the VN test (R = 0.79 for VN vs. ELISA antibodies).[3] Only the differentiating MAb reactive with TGEV and not with the PRCV S protein was used.[3] The immunodominance of the S protein epitope A in serum antibody responses of TGEV- or PRCV-inoculated sows was previously demonstrated.[6] These authors proposed that epitope A, subsite Ab, plays a dominant role in the induction of TGEV-neutralizing (protective) antibodies. In contrast, others[16] found no significant correlation between VN antibody titers and blocking ELISA titers to common S protein epitopes of porcine, feline, and canine coronaviruses. The present study shows a strong positive correlation between epitope A seroconversion and VN titers and a low to moderate correlation between epitope D seroconversion and VN titers (Fig. 3), reflecting the fact that epitope A is immunodominant and elicits VN antibodies whereas epitope D induces no or low titers of VN antibodies.[6]

The 2 MAbs used in this study, 25C9 (epitope A) and 44C11 (epitope D), have been previously described.[23] The MAb 25C9 competes with sera from TGEV- or PRCV-infected animals because epitope A is conserved on both TGEV and PRCV S proteins.[8,24] Therefore, competition of serum samples with bMAb 25C9 resulted only in a confirmatory and not a differentiating ELISA reaction, similar to the VN test used to confirm the presence of TGEV/PRCV antibodies. Comparison of bMAb 25C9 with bMAb 44C11 showed that maximum binding occurs for each MAb under different conditions. For bMAb 25C9 (epitope A), concentrations of 0.01–1 μg/ml produced maximal binding, but for bMAb 44C11 (epitope D) at least 10 times higher concentrations were necessary to produce maximal binding.[24] Thus, standardization of dose–response curves for each new batch of antigen coatings (ST cells expressing TGEV antigens or rec-S S protein) was crucial to optimize the blocking ELISA and the ELISA cut off absorbance, factors that influenced the specificity and sensitivity of the assay. To establish sensitivity values >90%, inevitably some false-positive results may emerge (Table 1), decreasing the accuracy. The concentration of bMAb used for the competition reaction with serum antibody was less than the 100% saturating dose, but it still produced a strong ELISA signal (absorbance ≥ 0.8). A limitation of the blocking ELISAs to distinguish TGEV from PRCV seroconversion is the relatively low affinity of the differentiating (epitope D) MAb and the generally lower and later antibody titers induced to epitope D in TGEV-inoculated pigs. The probability of a false-negative result is therefore higher for epitope D than for epitope A, necessitating the use of higher concentrations of bMAb 44C11 (epitope D) for the blocking reaction than of bMAb 25C9 (epitope A).

Testing the samples in parallel with other confirmatory tests (VN epitope A ELISA) is important to provide additional information on the serostatus of the animals. Furthermore, a history of clinical symptoms

Table 3. Comparison of ELISA 1, ELISA 2, and virus neutralization tests (VNT) to measure the antibody titers in 7 sows inoculated during pregnancy with PRCV baculovirus-expressed TGEV S protein, or wild-type baculovirus protein and then exposed to virulent TGEV after challenge of their litters at 5 days of age.

Sow number	Test	Postinoculation days (±days pre- or postfarrowing)								
		0 (−42)	14 (−28)	28 (−14)	42 (0)	47 (+5)	54 (+12)	61 (+19)	68 (+26)	75 (+33)
36-4	VNT	<1*	15*	105†	600	440	370	360	26	92
	ELISA 1‡	<5/<5	<5/<5	>45/<5	>45/11	>45/30	>45/32	>45/24	>135/22	46/11
	ELISA 2‡	<5/<5	8/<5	100/<5	>135/12	>135/38	>135/18	>135/26	>135/30	>135/30
49-8	VNT	<1*	36*	130†	460	5,200	7,000	6,000	2,700	2,500
	ELISA 1	<5/<5	>45/<5	80/<5	>45/<5	>45/<5	>45/21	>135/20	>45/17	>45/>45
	ELISA 2	<5/<5	120/<5	>135/<5	>135/<5	>135/>135	>135/>135	>135/>135	>135/130	>135/>135
87-4	VNT	<1*	42*	25†	320†	400	2,500	2,000	1,100	460
	ELISA 1	<5/<5	<5/<5	6/<5	>45/>45	>45/>45	>45/>45	···§	···§	···§
	ELISA 2	<5/<5	30/<5	45/<5	>135/>135	>135/100	>135/>135	···§	···§	···§
40-5	VNT	<1*	10*	120†	400	375‖	540	540	110	385
	ELISA 1	<5/<5	32/<5	>45/<5	>45/18	>45/16	>45/20	>45/6	>45/42	>45/<5
	ELISA 2	<5/<5	10/<5	>135/<5	>135/42	>135/30	>135/18	>135/40	>135/90	>135/50
87-3	VNT	<1*	7*	20†	130	300‖	2,900	2,100	1,450	1,250
	ELISA 1	<5/<5	45/<5	>45/<5	>45/<5	>45/<5	>45/>45	>45/>45	>45/>45	>45/>45
	ELISA 2	<5/<5	45/<5	17/<5	>135/<5	>135/<5	>135/>135	>135/>135	>135/>135	>135/90
40-5B	VNT	<1*	64*	96¶	24	27‖	4,300	1,400	1,150	700
	ELISA 1	<5/<5	10/<5	>45/<5	16/<5	20/<5	>45/>45	···§	>45/40	>135/32
	ELISA 2	<5/<5	30/<5	38/<5	70/<5	110/<5	>135/45	···§	>135/38	>135/>135
50-8	VNT	<1*	21*	64¶	25	14‖	5,300	1,950	2,150	1,250
	ELISA 1	<5/<5	9/<5	10/<5	12/<5	6/<5	>45/>45	>45/>45	···§	>45/>45
	ELISA 2	<5/<5	35/<5	32/<5	32/<5	38/<5	>135/32	>135/60	···§	>135/>135

* Inoculation with PRCV (2×10^8 PFU/sow).
† Vaccination (1 mg/sow) with recombinant TGEV S glycoprotein.
‡ Epitope A/D 50% blocking ELISA titer.
§ Low volume sample, not tested.
‖ Challenge exposure of litter with 100 PDD_{50}/suckling pig of virulent TGEV M5C strain.
¶ Vaccination with wild-type baculovirus protein.

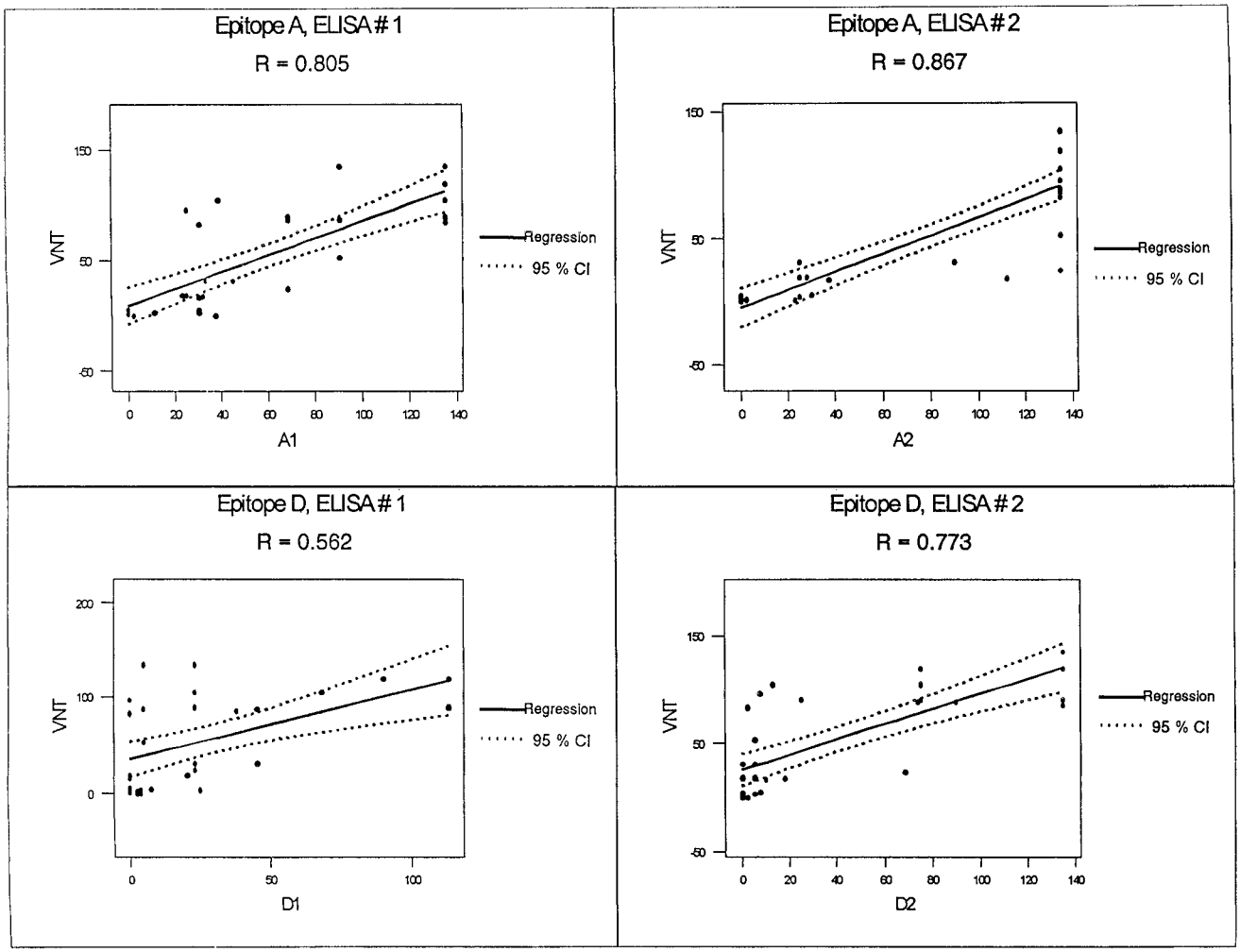

Figure 3. Fitted line plots including the 95% confidence intervals and correlation coefficients (R) from regression analysis of serum titers obtained by VN test and blocking titers of both ELISA tests. Upper graphs reflect high correlation ($R > 0.8$) between seroconversion against epitope A of TGEV S protein as measured by ELISAs 1 and 2 and VN titers. Lower graphs depict moderate correlation ($R > 0.5$) between seroconversion against epitope D of TGEV S protein and VN titers.

of disease (diarrhea, mortality in suckling pigs, respiratory symptoms) and histopathologic findings (villous atrophy, lung lesions) are also useful for differential diagnosis of TGEV/PRCV in conjunction with the serologic tests. To avoid false-negative results, especially before 3 weeks of infection when epitope D seroconversion is not yet fully developed and before 2 weeks of infection when neither epitope A or D seroconversion is fully induced, paired sera from individual pigs or multiple serum samples from each herd should be collected and tested. In these situations, a single unpaired serum sample is not sufficient for reliable differentiation.

In summary, findings suggest that blocking ELISA 2 based on the S glycoprotein solid-phase antigen (which could be developed commercially as part of diagnostic kits or provided by laboratories possessing recombinant baculovirus expressing the TGEV S glycoprotein) may be a practical, cheaper, and less labor-intensive diagnostic tool than ELISA 1 for differentiating TGEV from PRCV seroconversion. However, the limitations need to be considered for both ELISA tests.

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Sources and manufacturers

a. Unisyn Technologies, Hopkinton, MA.
b. Kirkegaard & Perry Laboratories, Gaithersburg, MD.
c. Boehringer-Mannheim, Indianapolis, IN.
d. Maxisorb-Nunc, Roskilde, Denmark.
e. Minitab, State College, PA.

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