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A Competitive Inhibition ELISA for the Differentiation of Serum Antibodies from Pigs Infected with Transmissible Gastroenteritis Virus (TGEV) or with the TGEV-related Porcine Respiratory Coronavirus

P. CALLEBAUT, M.B. PENSAERT and J HOOYBERGHS

Laboratory of Virology, Faculty of Veterinary Medicine, State University of Ghent, Casinoplein 24, B-9000 Ghent (Belgium)

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ABSTRACT

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A competitive inhibition ELISA was developed to detect non-neutralizing antibodies to the peplomer protein of transmissible gastroenteritis virus (TGEV) in porcine sera using a monoclonal antibody as an indicator. It was demonstrated that field strains of the TGEV-related porcine respiratory coronavirus (PRCV) did not induce this antibody, whereas the Miller strain and field strains of TGEV did. The sensitivity of the competitive inhibition ELISA appeared to be similar to that of the virus neutralization (VN) test. The test enables differentiation of pigs which were previously infected with TGEV or PRCV and which cannot be distinguished by the classical anti-TGEV neutralization test. The present test is useful for selective serodiagnosis.

INTRODUCTION

Transmissible gastroenteritis (TGE) of swine is a highly infectious disease causing high mortality in piglets <2 weeks of age (Doyle and Hutchings, 1946). The coronavirus which causes it, transmissible gastroenteritis virus (TGEV), replicates in the absorptive epithelial cells of the small intestine causing degeneration, villous shortening and diarrhea (Haelterman, 1972). Replication in the tonsils and in the respiratory tract has been reported, but appears to be secondary to the enteric infection (Kemeny et al., 1975; Furuuchi et al., 1978/1979). Virus spread routinely occurs by the oral–fecal route (Bohl, 1981).

In 1986, a TGEV-related porcine coronavirus was isolated, which causes a

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subclinical infection (Pensaert et al., 1986). It replicates to high titers in the respiratory tract and not in intestinal epithelial cells. This porcine respiratory coronavirus (PRCV) is transmitted aerogenically (Pensaert et al., 1987b). It has rapidly become very widespread among the swine population in several European countries (Brown and Cartwright, 1986; Jestin et al., 1987; Pensaert et al., 1987a).

In spite of these differences, the two viruses show complete cross-neutralization. Furthermore, serologic cross reactions have been found at the level of each of the viral structural proteins, i.e. the peplomer protein E2, the envelope protein E1 and the nucleoprotein N (Callebaut et al., 1988). Pigs infected either with TGEV or with PRCV, therefore, cannot be distinguished by the classical seroneutralization test. A problem has thus arisen in transporting swine on an international basis.

Recently, distinct antigenic differences have been demonstrated between the two viruses (Callebaut et al., 1988; Garwes et al., 1988; Laude et al., 1988). TGEV-specific monoclonal antibodies (MAbs), directed against E2 protein epitopes stimulating non-neutralizing antibodies, do not recognize PRCV. This indicates that the latter epitopes of TGEV are modified or absent in PRCV and that antibodies to these epitopes are not produced in PRCV-infected pigs.

A test for antibodies to these epitopes of TGEV would therefore permit selective detection of pigs infected with TGEV. In the present communication, we report on the development and the results of such an assay.

MATERIALS AND METHODS

TGEV production

The Purdue strain of TGEV was used as the antigen in the competitive inhibition ELISA. The virus, Passage 114 in primary porcine kidney cells, was adapted to grow in the swine testicle (ST) cell line by two more passages. The cells were grown in roller bottles in Dulbecco modified Eagle medium containing 10% fetal calf serum (FCS). After inoculation, the cells were maintained in the same medium without FCS. When approximately 90% of the cells showed cytopathic effect, the virus was harvested by freezing and thawing, followed by sonication (3×10 s). After clarification at $1000 \times g$ for 10 min, particulate material was sedimented at $100\,000 \times g$ for 60 min. This virus-enriched fraction was resuspended in phosphate-buffered saline (PBS), pH 7.2/glycerol (1/1) by sonication and was stored at $-20°C$.

Monoclonal antibody (MAb)

MAb 1 DB12 ascites fluid, used as the indicator antibody in the blocking ELISA, was kindly provided by Dr. L. Enjuanes, Madrid, Spain. The prepa-

ration and characteristics of the MAb have been described elsewhere (Jiménez et al.., 1986). Briefly, it was a non-neutralizing MAb directed against the E2 protein of TGEV (Purdue strain) and belonging to the mouse IgG1 subclass. Using purified PRCV isolate TLM 83 (Pensaert et al., 1986) as the antigen, it has been shown by radio-immunoassay that MAb1 DB12 does not recognize PRCV (Callebaut et al., 1988).

Serum specimens

A total of 285 serum specimens, to be examined for antibody by competitive inhibition ELISA, were divided into three groups.

Group 1 consisted of antisera presumably directed against PRCV. This presumption was based on the demonstration that the sera contained TGEV-neutralizing antibody, but they were collected on farms in which no TGEV-induced diarrhea outbreak had occurred at least 2 years prior to the time of blood collection and in which no vaccination against TGE was performed. As no other selection criteria were available, however, and an enzootic TGEV infection on these farms could not be excluded, no absolute certainty could be obtained that these sera were PRCV-specific. A total of 130 antisera were collected on 18 Belgian farms. Between three and 23 serum samples were obtained on each farm from pigs of all ages.

Group 2 sera were from pigs infected with TGEV. Nine sera were obtained from three piglets, collected prior to experimental oral infection with the Miller strain of TGEV and at various intervals between 9 and 25 days thereafter. In addition, 49 TGEV-neutralizing sera were from fattening pigs and sows, on 11 Belgian farms with a natural outbreak of TGE, diagnosed by immunofluorescence staining on gut sections of euthanized animals. The latter sera were collected in 1977, i.e. before the appearance of PRCV. They were therefore considered to be free of PRCV antibody.

Group 3 antisera were from pigs which had antibodies to PRCV (actively formed or maternally derived) and were subsequently infected with TGEV. Four piglets were first inoculated oronasally with the TLM 83 isolate of PRCV (Pensaert et al., 1986); 3–6 weeks later, the piglets had built up TGEV-neutralizing serum antibodies and were orally inoculated with the Miller strain of TGEV. A total of 18 serum samples was collected at the time of inoculation with TGEV and at various intervals between 7 and 50 days thereafter. Additional serum samples were from eight piglets, nursed by sows which had been inoculated with the TLM 83 isolate of PRCV 1 month before farrowing and had formed TGEV-neutralizing antibodies in the absence of clinical illness; the piglets were orally inoculated with the Miller strain of TGEV at 3 days of age and paired sera were collected at that time and 39 days thereafter. On three farms, pigs had TGEV-neutralizing serum antibodies (although no TGE outbreak had occurred previously) at the time when a natural outbreak of TGE

was diagnosed by immunofluorescence staining as described above. On each farm, paired sera were collected from eight, seven and three sows, respectively, at the onset of the illness and 3–4 weeks thereafter. On two of these farms, paired sera were collected from six and eight piglets, respectively, which were born during the outbreak. Acute sera were obtained at the onset of diarrhea and convalescent sera 4 or 7 weeks later.

Competitive inhibition ELISA

Polystyrene microtiter plates (Dynatech M 129 B) were coated by overnight incubation at 4°C with 100 μl per well of a previously determined optimal amount of viral antigen in PBS. The plates were washed three times with PBS containing 0.01% Tween 80 and 0.2% FCS. Then 50 μl of 2-fold serial dilutions of sera, starting at 1:2.5, were added to each antigen-coated well. Controls included two rows of wells, with 1:2.5 diluted TGEV antibody-positive and negative porcine serum, respectively. Following overnight incubation at room temperature and without removing the contents of the wells, 50 μl of MAb 1 DB 12 ascites fluid was added to each well and incubation was continued for 90 min at 37°C. The working dilution of the MAb was the highest dilution giving a maximum absorbence of 0.30 with TGEV antibody-negative serum when reading the test (see below). Test sera and MAb were diluted in 0.5 M NaCl, pH 7.2, containing 0.05% Tween 80 and 10% FCS. The plates were washed four times. Goat anti-mouse IgG1–horseradish peroxidase conjugate (Nordic) was diluted 1:4000 in 0.5 M NaCl, pH 7.2, containing 0.05% Tween 80 and 5% TGEV antibody-negative porcine serum; 100 μl of the dilution was added to each well for 1 h at 37°C. The plates were then washed six times and incubated overnight at 4°C following addition of 100 μl per well of substrate. The substrate solution contained 1 mg ml^{-1} 5-aminosalicylic acid–0.005% H_2O_2 in 0.01 M sodium phosphate, 1 mM Na_2 EDTA, pH 6.0. The absorbence at 450 nm was read with a Multiskan (Flow Laboratories). The reader was blanked on the row of wells with TGEV antibody-positive serum. The blocking titer of serum antibody was determined as the reciprocal of the highest dilution that gave an absorbence value of $< 45\%$ of the maximum absorbence reading. Maximum absorbence was taken as the mean of the row of wells with TGEV antibody-negative serum.

Virus neutralization (VN) test

VN tests with heat-inactivated (30 min at 56°C) sera were performed in SK6 cells using the microtiter method described previously (Voets et al., 1980). The virus used was the Purdue-114 strain of TGEV. Titers were expressed as the reciprocal of the final serum dilution which prevented cytopathic effect in 50% of the cultures.

RESULTS

Reactivity of sera from pigs presumably infected with PRCV in the competitive inhibition ELISA

The 130 antisera presumably directed against PRCV had VN titers between 12 and 384, geometric mean titer (GMT) 97. In the competitive inhibition ELISA, the lowest test dilution of these sera yielded absorbences with a mean value of 0.22 (range 0.29–0.16) corresponding to 73% (range 97–54%) of the maximum absorbence reading of 0.30 given by the TGEV antibody-negative control serum. These sera were scored as negative (titer <5). This was based on a preliminary criterion, considering a given test serum as negative if it produced an absorbence of $>50\%$ of the maximum absorbence. For all further tests, the reading criterion was derived from the above results with PRCV antisera taken as negative control sera. Their mean absorbence minus three standard deviations, i.e. 0.14 or 45% of the maximum absorbence, was considered the minimal absorbence which would be obtained with TGEV-antibody negative sera. Test sera producing an absorbence below that value were scored positive for TGEV antibodies, as mentioned in Materials and methods.

Reactivity of sera from pigs infected with TGEV in the competitive inhibition ELISA

The results obtained by competitive inhibition ELISA with the sera from piglets, experimentally infected with the Miller strain of TGEV, are shown in Table 1. The serum samples collected at the time of inoculation and 9 days later were negative (titer <5). One sample was collected 16 days after inoculation; it had a titer 96 by VN and 20 by ELISA. At 25 days, all piglets showed a positive reaction. They had VN titers of 256, 128 and 192; their corresponding blocking titers were 40, 20 and 160, respectively.

Of the 49 TGEV antisera from naturally infected pigs, 42 were positive by ELISA, i.e. between 50 and 100% of the number of serum samples collected on each of the 11 farms. The results of the comparative titrations of these sera by VN and ELISA are given in Fig. 1. The VN titers ranged from 2 to 1024 (GMT 16). Seven sera with VN titers between 2 and 8 were negative (titer <5) by ELISA. The remaining sera had blocking titers between 5 and 640 (GMT 26). The correlation coefficient was 0.792.

Reactivity of sera from pigs with antibodies to PRCV and subsequently infected with TGEV in the competitive inhibition ELISA

As shown in Table 1, all the sera of the experimental pigs with actively formed antibodies to PRCV were negative (titer <5) by ELISA at the time of inocu-

TABLE 1

Seroconversion to TGEV in experimentally infected piglets demonstrated by competitive inhibition ELISA

Pig description	Sera		Geometric mean VN titer (range)[b]	ELISA positive sera	
	No.	Time of collection (d.p.i.)[a]		No	Geometric mean titer (range)[b]
TGEV-infected	3	0	<2	0	<5
	2	9	6(4-8)	0	<5
	3	25	181(128-256)	3	50(20-160)
PRCV/TGEV-infected[c]	4	0	117(12-512)	0	<5
	4	7	5312(2048-12288)	0	<5
	4	14	10624(3072-65536)	1	5
	4	25	3251(2048-6144)	4	8(5-20)
	2	50	1722(1536-2048)	2	20(20-20)
PRCV mothers/TGEV-infected[d]	8	0	95(12-384)	0	<5
	8	39	33(16-64)	8	31(20-40)

[a]d.p.i. = days post inoculation with TGEV.
[b]Titers expressed as the reciprocal of the final positive serum dilution
[c]Infected with PRCV and subsequently infected with TGEV.
[d]With antibodies passively obtained from PRCV-infected mothers and subsequently infected with TGEV

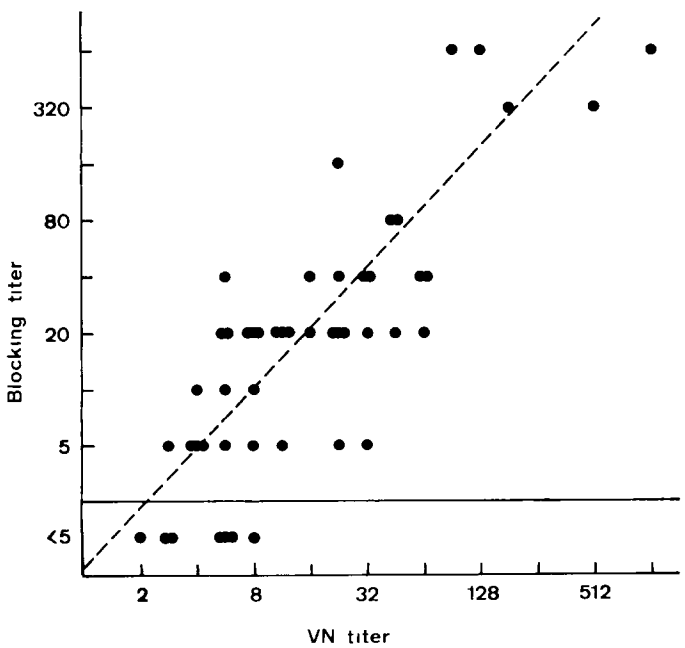

Fig. 1 Comparison of VN and blocking antibody titers in sera from naturally TGEV-infected pigs (titers expressed as reciprocal of final positive dilution; dashed line represents regression line)

TABLE 2

Comparison of VN and blocking titers of paired sera from experimentally infected piglets

Pig description	Serum Pair No.[a]	Titer[b]			
		VN		ELISA	
		Acute	Convalescent	Acute	Convalescent
PRCV/TGEV-infected[c]	1	192	6144	<5	10
	2	192	2048	<5	20
	3	12	3072	<5	5
	4	512	3072	<5	5
PRCV mothers/TGEV-infected[d]	5	192	64	<5	40
	6	96	32	<5	40
	7	384	24	<5	20
	8	64	16	<5	40
	9	384	32	<5	20
	10	192	32	<5	40
	11	12	48	<5	40
	12	24	48	<5	20

[a]Collected at time of inoculation with TGEV (acute) and 25 days (Serum Pair Nos. 1-4) or 39 days (Serum Pair Nos. 5-12) after inoculation with TGEV (convalescent)
[b]Titers expressed as the reciprocal of the final positive serum dilution.
[c]Infected with PRCV and subsequently infected with TGEV.
[d]With antibodies passively obtained from PRCV infected mothers and subsequently infected with TGEV

lation with TGEV and 7 days later. Their VN titers against TGEV varied between 12 and 512 (GMT 117). By the fourteenth day after inoculation, only one of the piglets showed a positive reaction in the ELISA; its VN titer was 3072. All sera collected 25 and 50 days after inoculation were positive. The antibody titers of each animal at the time of inoculation and 25 days thereafter are given in Table 2. The eight piglets with maternally derived serum antibodies to PRCV (VN titers between 12 and 384, GMT 95) were negative (titer <5) by blocking ELISA; following inoculation with TGEV, the convalescent sera from all piglets contained blocking antibodies (Tables 1 and 2).

The results obtained with pigs from three field outbreaks of TGE are shown in Table 3. At the start of the outbreaks, the sera of the sows had VN titers between 32 and 256 (GMT 98) and were negative in the ELISA. Three to four weeks later, blocking antibodies were detected in the sera from 13 of the 18 sows. The sera of the five remaining sows, representing 14–33% of the number tested on each farm, were negative (titer <5). The acute sera of the 14 piglets had VN titers between 64 and 256 (GMT 155) and were negative (titer <5)

TABLE 3

Comparison of VN and blocking titers of paired sera from pigs with antibodies presumably to PRCV on farms with a natural TGE outbreak

Pig description	Serum Pair No.[a]	Titer[b]			
		VN		ELISA	
		At outbreak	Convalescent	At outbreak	Convalescent
Presumably PRCV/TGEV-infected[c]	1	96	1024	<5	5
	2	192	256	<5	20
	3	192	3072	<5	40
	4	64	2048	<5	5
	5	256	512	<5	20
	6	192	256	<5	<5
	7	128	1024	<5	5
	8	192	128	<5	<5
	9	98	512	<5	10
	10	64	256	<5	10
	11	32	48	<5	5
	12	128	64	<5	<5
	13	128	8192	<5	5
	14	96	512	<5	<5
	15	64	192	<5	20
	16	192	192	<5	<5
	17	192	512	<5	5
	18	128	512	<5	5
Presumably PRCV mothers/TGEV-infected[d]	19	96	32	<5	5
	20	128	3	<5	<5
	21	256	32	<5	10
	22	256	6	<5	10
	23	192	12	<5	20
	24	96	4	<5	10
	25	256	24	<5	20
	26	256	8	<5	20
	27	64	12	<5	10
	28	256	96	<5	20
	29	96	24	<5	10
	30	96	32	<5	10
	31	192	16	<5	10
	32	256	32	<5	40

[a]Serum Pair Nos 1-7 collected on Farm 1; Serum Pair Nos 8-15 and Nos 19-26 collected on Farm 2; Serum Pair Nos 16-18 and 27-32 collected on Farm 3

[b]Titers expressed as the reciprocal of the final positive serum dilution

[c]Previously infected presumably with PRCV at the time of the TGE outbreak.

[d]With antibodies passively obtained from presumably PRCV infected mothers at the time of the TGE outbreak

in ELISA. In their convalescent sera, however, all except one of the piglets had blocking antibodies.

DISCUSSION

The antisera, presumed to be directed against PRCV and representing a survey of 18 different field cases, contained no antibodies which blocked the binding of MAb 1DB12 to a significant extent. This finding confirms that the criteria for selection of the sera were appropriate and that the antisera were PRCV-specific. It also implies that in all field strains of PRCV involved in these cases, the E2 protein epitope of TGEV defined by MAb 1 DB12, is absent or modified. On the contrary, the results obtained with antisera to the Miller strain and to field strains of TGEV on 14 farms demonstrate that MAb 1 DB12 defines an epitope, which is conserved by these TGEV strains. The absence or presence of blocking antibodies therefore appears to be a reliable criterion to identify antisera to PRCV or to TGEV, respectively.

Since antigenic variation between TGEV strains may occur, however (Jiménez et al., 1986; Laude et al., 1986; Hohdatsu et al., 1987), the possibility that some field strains of TGEV exist which lack or vary in the epitope recognized by MAb 1DB12 cannot be excluded. Such strains would give rise to false-negative results in the present competitive inhibition ELISA using the single MAb 1DB12 as the indicator antibody. Therefore, we are currently identifying other MAbs which recognize epitopes which are absent in PRCV strains and present in TGEV strains. The use of a pool of these MAbs in the ELISA will reduce the possibility of scoring false-negative results, as the chance that a particular TGEV field strain would have lost more than one epitope is likely to be very small.

The ELISA is an efficient assay for the detection of an antibody response to the E2 epitope of TGEV as shown by the clear-cut seroconversion found in the sera of all experimentally infected piglets, with or without actively formed or passively obtained antibodies to PRCV at the time of inoculation with TGEV. These results correlate with the results of the VN test only in the group of TGEV-infected pigs without antibodies to PRCV. There is no correlation in the other groups. At the time of inoculation with TGEV, pigs with antibodies to PRCV were positive by VN and negative by ELISA. Following inoculation with TGEV, pigs with actively formed PRCV antibodies showed a secondary VN antibody response as indicated by the rapid formation of high VN antibody titers. Pigs with passively obtained PRCV antibodies and infected with TGEV generally showed a VN antibody titer drop in paired sera. The results obtained with pigs from farms with a natural TGE outbreak are similar. The convalescent sera from 42 of 49 fattening pigs and sows with a diagnosed natural TGEV infection and collected in 1977 contained blocking antibodies. The titers of these antibodies were similar to the VN antibody titers, confirming that these

pigs had been infected with TGEV only. Furthermore, the acute sera from 18 sows and 14 piglets on farms with a natural TGE outbreak were positive by VN, but negative by ELISA. This confirms that they had antibodies against PRCV at the onset of the TGE outbreak. The convalescent sera of 15 sows had an increased VN titer indicating that their PRCV antibodies had been actively formed; titer rises varied largely between individual animals, the reason for which is unknown at present. In 13 of the latter 15 sows seroconversion was demonstrated by ELISA. The VN titers of the convalescent sera from the 14 piglets were decreased, indicating that the PRCV antibodies in the acute sera from these animals had been passively obtained. By ELISA, 13 piglets showed seroconversion to TGEV. These results show that following a PRCV infection pigs develop antibodies which cross-neutralize TGEV, as previously reported (Hooyberghs et al., 1988; Callebaut et al., 1988). Current work is in progress to determine the significance of these antibodies for protection against diarrhea upon challenge with TGEV.

The finding that blocking antibodies were detectable in experimental piglets at 2–3 weeks after inoculation indicates high sensitivity of the test. A further indication is that antibody titers in 42 of 49 TGEV antisera were of the same order of magnitude in VN and in ELISA. The ratio between VN and blocking titers, however, varied largely between individual sera. This may explain the false-negative results in ELISA with seven of the 49 TGEV sera which reacted weakly positive in VN. In five sows and one piglet with PRCV antibodies on farms where a natural TGE outbreak occurred, no blocking antibody response could be detected, yet two sows showed a VN titer rise in their paired sera, whereas the convalescent serum of the piglet had a low VN titer. Further work will be needed to find out if this is due to a lack of sensitivity of the blocking ELISA.

In conclusion, the present competitive inhibition ELISA permits the detection of TGEV-specific serum antibodies without interference of antibodies to PRCV. Since PRCV is at present very widespread in the swine population in different European countries, the availability of a differential test will be of great value for the serodiagnosis of TGEV infections; it will provide the serological evidence that pigs are free from TGEV infection that is often required for the export of pigs. It will also enable estimation of the efficacy of vaccination experiments for eliciting a TGEV-specific antibody response in PRCV antibody-positive pigs. False-positive results were not obtained in the present study and false-negative results were rare. Positive results can be accepted with confidence; a negative result, however, must be confirmed using several serum samples from the same farm.

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