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Enzyme-linked Immunosorbent Assay for the Detection of Porcine Epidemic Diarrhea Coronavirus Antibodies in Swine Sera

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(Accepted for publication 21 July 1989)

ABSTRACT

Hofmann, M. and Wyler, R., 1990. Enzyme-linked immunosorbent assay for the detection of porcine epidemic diarrhea coronavirus antibodies in swine sera. *Vet. Microbiol.*, 21: 263–273.

An enzyme-linked immunosorbent assay (ELISA) for detecting serum antibodies to the porcine epidemic diarrhea coronavirus (PEDV) was established by using cell culture-grown PEDV as antigen for coating. Ultracentrifugation through 20 and 45% (w/w) sucrose cushions proved to be the best antigen purification method. Examination of 1024 swine sera showed a high specificity and a greater sensitivity of the ELISA, when compared with indirect immunofluorescence. Reference sera with high antibody titers to PEDV originated from two pigs experimentally infected with PEDV. Three different antigen purification methods and the advantages of the ELISA compared with an immunofluorescence test are discussed.

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) (Pensaert and Debouck, 1978; Debouck and Pensaert, 1980; Debouck et al., 1981; Witte et al., 1981) is identified as the causative agent leading to diarrhea through destruction of the epithelial cells of the small intestine. Although it has been possible to demonstrate PEDV infections in many countries, extensive serological studies have been hindered by the lack of a simple, rapid and sensitive test to detect PEDV antibodies in porcine sera. For PEDV serology, three tests have been described in the literature, namely an indirect immunofluorescence test (IIFT) (Prager and Witte, 1981a, b; Hofmann and Wyler, 1987), an immunofluorescence blocking test (IFBT) (Witte and Prager, 1987) and an enzyme-linked immunosorbent assay (ELISA) blocking test (Callebaut et al., 1982). All these tests require an

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tigen from PEDV-infected piglets in the form of cryosections of the small intestine for IIFT and IFBT, or PEDV-containing crude fecal suspensions for the ELISA blocking test.

Recently, PEDV was adapted to Vero cell cultures (Hofmann and Wyler, 1988) thus rendering the production of large quantities of purified viral antigen possible. The aim of the present work was to establish a simple and sensitive ELISA for PEDV serology based upon cell culture-grown PEDV as immobilized antigen.

MATERIALS AND METHODS

Cells and virus propagation

Vero cells grown in 490-cm² roller bottles with Eagle's minimal essential medium (EMEM) were used to propagate PEDV. As described previously (Hofmann and Wyler, 1988), the PEDV adapted to Vero cells was at its 52nd passage. Briefly, confluent monolayers from which growth medium (containing 10% fetal calf serum) had been removed, were washed twice with phosphate-buffered saline (PBS, pH 7.2). Then 4×10^5 plaque-forming units (PFU) of PEDV diluted in 8 ml EMEM [without fetal calf serum, but containing 0.3% (w/v) tryptose phosphate broth and $10 \mu\text{g ml}^{-1}$ trypsin], were added to each roller bottle and virus was adsorbed for 1 h at 37°C. After adsorption, 45 ml of trypsin-containing EMEM were added to each roller bottle without removing the inoculum and cell cultures were further incubated at 37°C. Control vero cell cultures were mock inoculated in the same way using 1 ml of EMEM instead of 1 ml PEDV.

Purification of ELISA antigen

Three different methods for antigen production were compared.

Method i: virus purification by isopycnic density gradient ultracentrifugation through a linear sucrose gradient

Cell cultures were harvested 18–22 h after infection, when all the cells showed a characteristic cytopathic effect (Hofmann and Wyler, 1988). Intracellular virus was released by subjecting the cultures to three freezing and thawing cycles. This was followed by low-speed centrifugation at $4000 \times g$ for 30 min to remove coarse cell debris. The virus was then pelleted from supernatant fluid by high-speed centrifugation at $105\,000 \times g$ (maximum) at 4°C for 2 h, the resulting pellet resuspended overnight in 1% of the original volume of TNE (20 mM Tris-HCl, pH 7.2, 100 mM NaCl, 2 mM EDTA), layered on top of a 10–50% (w/w) continuous sucrose density gradient prepared in TNE and immediately centrifuged for 16 h at $85\,000 \times g$ (maximum) at 4°C. Gradients

were then fractionated with a Gilson 201 fraction collector (Gilson, Villiers-le-Bel, France). Each fraction was tested for its ELISA activity by diluting it 1:100 in coating buffer (50 mM carbonate buffer, pH 9.6) and coating overnight at 4°C onto wells of microtiter plates. ELISA was carried out as described below. All the fractions leading to a distinct reaction with a PEDV reference serum when tested in ELISA were pooled, diluted 1:3 in TNE and pelleted at $105\,000 \times g$ (maximum) for 2 h. The resulting pellets were then suspended in TNE and stored at -20°C. Fractions of the gradient showing a high reaction in ELISA were examined using electron microscopy.

Method ii: purification by ultracentrifugation through sucrose cushions

Cell cultures were harvested and virus was pelleted as described above. The pelleted virus was suspended in TNE to 5% of the original volume, used to fill a SW28 centrifuge tube, followed by underlayering two cushions of 12 ml 20% (w/w) and 8 ml 45% (w/w) sucrose solutions, prepared in TNE. The tubes were centrifuged at $146\,000 \times g$ (maximum) for 16 h in a Beckmann SW28 rotor. The resulting band between the two sucrose concentrations was collected with a syringe and used as ELISA antigen without further processing.

Method iii: solubilization of protein by detergent treatment followed by density gradient centrifugation

Harvesting of the cell cultures and virus pelleting were carried out in the same way as in Methods i and ii. The virus pellet was suspended in TNE containing 1% Nonidet P-40 (NP40, Fluka AG, Buchs, Switzerland) in 2% of the original volume. After standing at room temperature for 1 h, the detergent-solubilized virus was centrifuged at $10\,000 \times g$ for 30 min to remove insoluble material. The supernatant was then layered on top of a 20–40% (w/w) continuous sucrose gradient prepared in TNE containing 0.1% NP40 and the gradients centrifuged for 16 h at $280\,000 \times g$ (maximum) in a Beckmann SW40 rotor. After sedimentation, the gradients were fractionated and each fraction was tested for its ELISA activity as described in Method i. PEDV antigen-containing fractions were pooled and subsequently dialyzed against TNE overnight at 4°C. The dialyzed preparations were stored at -20°C until further use.

Mock-infected cell cultures serving as controls in the ELISA were always processed in the same way as virus-containing fluids.

Two main criteria were used to compare the three different antigen preparations. First, the maximum extinction (ΔOD) reached when assayed with anti-PEDV reference serum was detected. Second, the maximum working dilution leading to a ΔOD of 1.5–2.0 when assayed with anti-PEDV reference serum diluted 1:40 was determined. The maximum amount of antigen which may be bound to ELISA plates, and consequently the sensitivity of the ELISA, depend on the absolute and relative content of the antigen in the coating buffer. High

amounts of cellular proteins compete with the viral antigen for binding sites on the well wall even if the viral antigen is present in high concentrations.

Production of antisera against PEDV

Two pigs (8 weeks of age) were used for antiserum production. Animal 931 was infected with PEDV of gut origin; Pig 922 served as a contagion control and was infected by the virus-containing feces of the experimentally infected animal. Both pigs were subsequently boosted four times at 3-week intervals by intramuscular injection of gut origin PEDV emulsified in the same volume of complete Freund's adjuvant. After 12 weeks, both animals were re-infected orally with PEDV-containing diarrheal feces from their first diarrhea episode and slaughtered 1 week later. The animals were bled on the day of primary infection and 2, 3, 6, 8, 10 and 11 weeks later. The serum of Pig 931 was used as PEDV-positive reference serum. All the serum samples were screened for the presence of transmissible gastroenteritis virus (TGEV) antibodies by IIFT using TGEV-infected cell cultures as antigen and by ELISA using TGEV antigen-coated plates (kindly provided by Eidg. Vakzine-Institut, Basel, Switzerland).

Serum samples

A total of 1024 sera of flattening pigs and breeding sows, which had been tested previously for the presence of PEDV antibodies by the IIFT (Hofmann and Wyler, 1987) and for the presence of TGEV antibodies by means of the neutralization test (Bereiter et al., 1988), were examined to demonstrate the sensitivity and specificity of the ELISA.

ELISA procedure

The ELISA was carried out in 96-well microtiter plates (Microelisa M129A, Dynatech Produkte AG, Kloten, Switzerland). For coating, the antigen, of which the optimum working dilution had been previously titrated, was diluted in coating buffer (50 mM carbonate buffer, pH 9.6). The wells were coated by adding 200 μ l per well of diluted positive or negative antigen in alternating vertical rows of eight wells and by incubating the plates overnight at 4°C in a humid chamber. The antigen was then poured off and the remaining free binding sites on the plastic surface were blocked without previous washing by filling each well with 300 μ l of 0.2% bovine serum albumin (BSA, fraction V, Fluka AG, Buchs, Switzerland) diluted in coating buffer, and incubating the plates for 1 h at 37°C in a water bath. After the blocking step, the BSA solution was poured off and the plates were air dried, packed in evacuated plastic bags and stored at 4°C.

To reduce non-specific serum binding reactions, sera to be tested for the presence of PEDV antibodies were diluted in PBS containing 1% Tween 20 (Bender + Hobein AG, Chemicals, Zurich, Switzerland) in place of the 0.5% usually recommended (Kenny and Dunsmoor, 1983). Samples were added to a horizontal row of four wells in 200- μ l quantities (alternating negative and positive antigen-coated wells) and the plates warmed to 37°C for 1 h in a water bath. Subsequently, the plates were washed three times with PBS containing 0.1% Tween 20 (PBST). Then 200 μ l of horseradish peroxidase-conjugated rabbit anti-porcine immunoglobulin G antibody, diluted 1:6000 in PBST, were added to each well. After incubating further for 1 h in a water bath at 37°C, plates were washed three times with PBST, shaken dry and 200 μ l of the substrate indicator solution, consisting of 2 mM 2,2'-azino-di[3-ethyl-benzthiazoline-sulfonate (6)] (ABTS) in 20 mM acetate, pH 4.2, plus 2.5 mM H₂O₂ were added to each well. Finally, the plates were incubated at room temperature in the dark and extinctions measured at 405 nm with a Titertek Multiskan photometer after 1 h.

The detection limit in ELISA was defined as $\geq 0.200 \Delta OD$. Sera with a ΔOD of < 0.163 [mean value of negative reference sera (0.058) plus three times their standard deviation (0.035)] were considered to contain no PEDV antibodies. Values < 0.200 and ≥ 0.163 were classified as questionable reactions. Positive reactions of 0.200–0.400 ΔOD were scored +positive, those of 0.400–0.800 ++positive and those > 0.800 +++positive.

RESULTS

Comparison of ELISA activities of different antigen preparation methods

The three methods tested to purify ELISA antigen yielded preparations with different activities.

Method i

When individual fractions prepared from a linear sucrose gradient were used to coat the plates, PEDV antigens could be detected over a wide range of the gradient resulting in ELISA reactions comparable with those of the virus band which could be clearly identified in the gradient [Fractions 5–8, at $\sim 40\%$ (w/w) sucrose concentration and corresponding to a density of 1.18] (Fig. 1). When examined by electron microscopy, PEDV particles in the virus band had lost most or all of their spikes (Fig. 2). Coating virus band Fractions 5–8 alone resulted in an ELISA activity of ΔOD 2.4, but the antigen could not be diluted higher than 1:20. In contrast, Fractions 10–16 contained numerous morphologically irregular vesicles and membrane fragments covered by 15-nm long club-shaped projections typical of coronavirus spikes (Fig. 3). No such projections were ever detected in the corresponding fractions of control preparations.

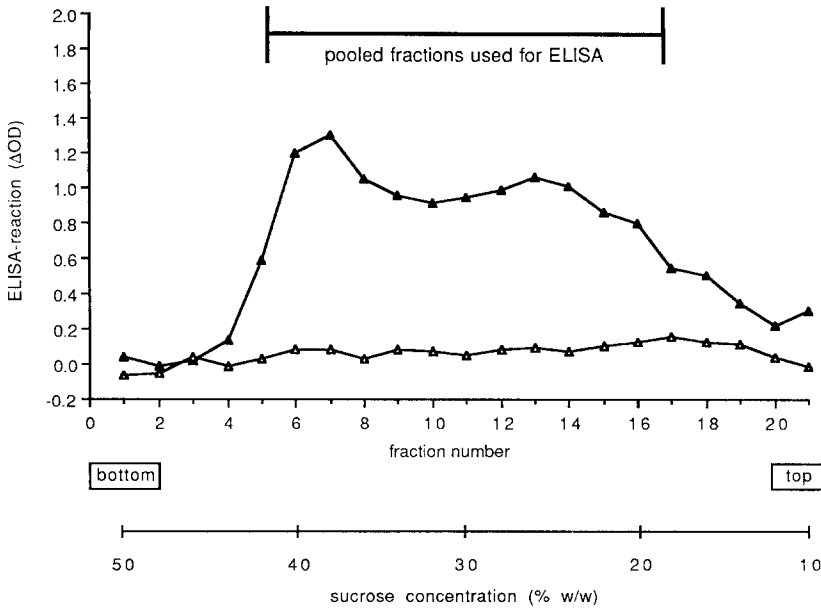


Fig. 1. ELISA reactions of fractions from a 10–50% linear sucrose gradient. ▲, anti-PEDV reference serum; △, PEDV-negative serum.

The maximum Δ OD of Fractions 10–16 was 1.5. Pooling Fractions 5–16 of the linear sucrose gradient resulted in an ELISA activity of Δ OD of 1.9 and the maximum working dilution of the antigen was 1:100.

Method ii

Antigen purified by centrifugation through 20 and 45% (w/w) sucrose cushions contained approximately the same amount of PEDV proteins (Δ OD 2.2) as the pooled Fractions 5–16 of the linear sucrose gradient (Δ OD 1.9), but was suspended in a small volume of sucrose solution and could therefore be used further without an additional pelleting step to remove the sucrose. The maximum working dilution of the antigen was 1:150.

Method iii

When NP40-solubilized antigen was used, no ELISA reaction was detected at low dilutions. Only with dilutions > 1:150 was an extinction of Δ OD 0.9 reached. Electron microscopy revealed that spike proteins (density 1.12, Sturman et al., 1980) were the only structural components of the virus which could be found in the gradient. The nucleoprotein was pelleted through the gradient (density 1.28, Sturman et al., 1980) and the matrix protein (density 1.06, Sturman et al., 1980) floated on top of the gradient, where the concentration of NP40 was so high that no protein could be coated onto the ELISA plates.

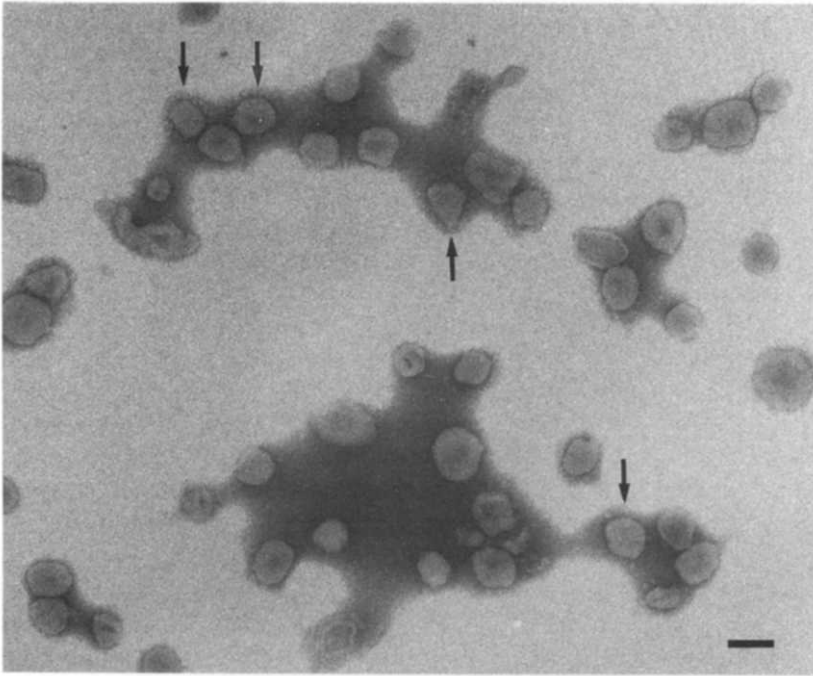


Fig. 2. Electron micrograph of virus-band-containing Fraction 7 of a 10–50% (w/w) linear sucrose gradient. Note the peplomer loss in most of the purified PEDV; only a few virus particles have partially retained spikes (arrows). The bar represents 100 nm. $\times 59\,200$.

Although a weak background reaction was observed, the concentration of PEDV-specific proteins was too low to induce a satisfactory ELISA reaction.

Hence Method ii, purifying the virus through sucrose cushions of 20 and 45% (w/w), proved to be the most suitable procedure to provide PEDV antigen for the ELISA. The optimum working dilution of the antigen prepared by Method ii was defined as the highest possible dilution leading to a ΔOD of 1.5–2.0. Under these conditions, antigen preparations could be diluted 1:100–1:200.

ELISA sensitivity and specificity testing

For testing the sensitivity and specificity of the ELISA, sera taken from experimentally infected pigs at different times after primary infection and checked previously for the presence of PEDV antibodies by an IIFT were used. The IIFT, as well as the ELISA, revealed a typical PEDV antibody titer rise after infection (Fig. 4), yet both pigs were seronegative before primary infection. None of the serum samples contained antibodies to TGEV as determined by IIFT and ELISA (data not shown).

Of 1024 pig sera from the slaughterhouse, 888 samples proved to be negative

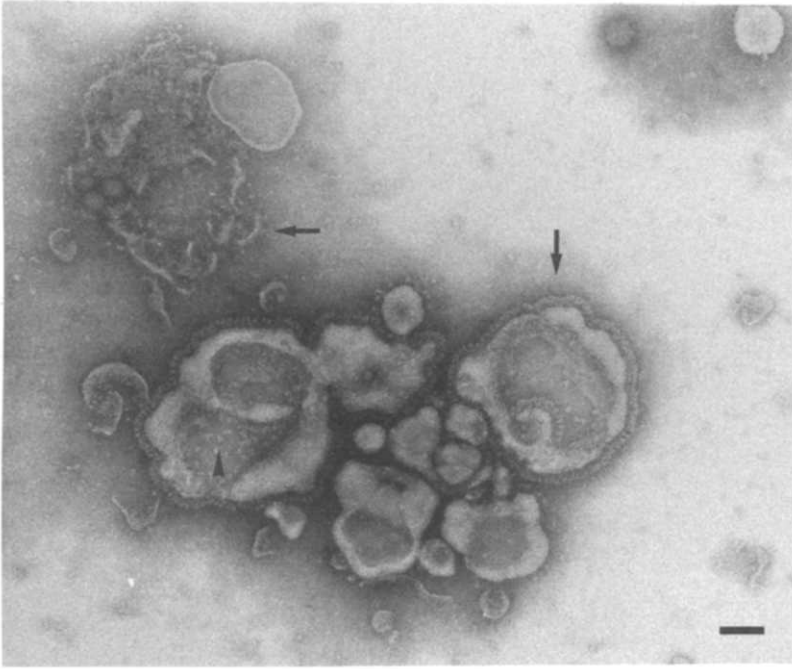


Fig. 3. Electron micrograph of Fraction 13 of a 10-50% (w/w) linear sucrose density gradient displaying numerous spike-covered structures (arrows) which, in turn, are surrounding spike-bearing structures (arrowhead). The bar represents 100 nm. $\times 56\ 100$.

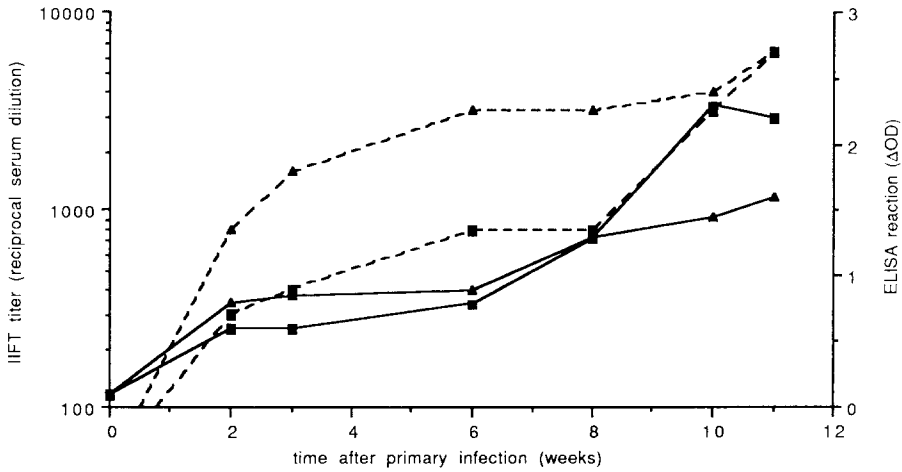


Fig. 4. Rise in PEDV antibody titers of the two experimentally infected pigs, as determined by IIFT and ELISA. ■, Pig 922; ▲, Pig 931; —, ELISA; ----, IIFT.

TABLE 1

Comparison of PEDV antibody detection by ELISA and IIFT in 1024 pig sera

ELISA reaction	IIFT reaction		ELISA total
	Negative	Positive	
Negative	670	15	685
Questionable	82	6	88
Positive	136	115	251
IIFT Total	888	136	1024

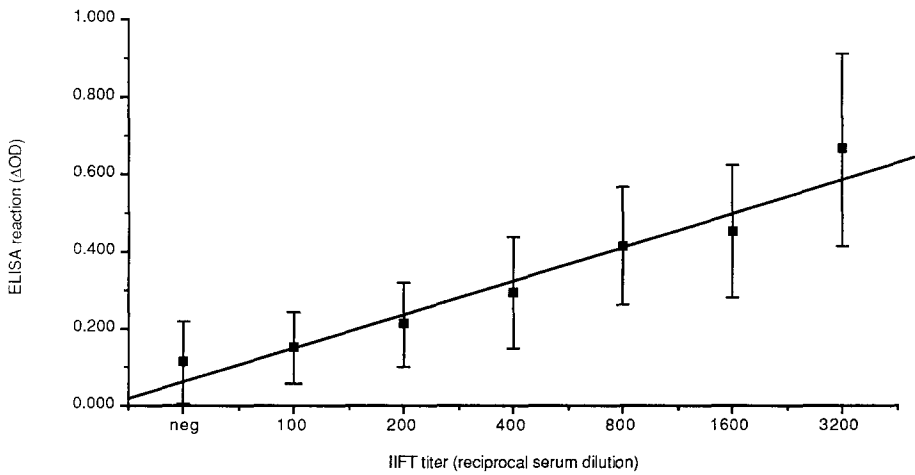


Fig. 5. Results of PEDV antibody detection in 1024 slaughterhouse sera using IIFT and ELISA. Mean values and standard deviation of ΔOD obtained in ELISA are compared with IIFT titers.

in the IIFT, but only 685 were negative when tested in the ELISA. Whereas 15 sera were found to be negative in the ELISA but positive in the IIFT, 136 IIFT-negative sera proved to be positive in the ELISA (Table 1, Fig. 5). None of the 23 sera known to contain TGEV antibodies showed a positive reaction in the PEDV ELISA. As demonstrated by these results, the sensitivity of the ELISA was higher than that of the IIFT.

DISCUSSION

Of the three methods tested to prepare an ELISA, antigen purification by ultracentrifugation through sucrose cushions (Method ii) proved to be optimal. Electron microscopy revealed that the preparation was comprised of a great number of vesicles and envelope fragments carrying spikes comparable

with those displayed in Fig. 3. The structures seen in Fig. 3 cannot be defined unequivocally. It is probable that the structures represent steps of viral morphopoiesis (Siddell et al., 1983) whereby the membranes stem from the endoplasmic reticulum and the spikes represent viral peplomers embedded in the membranes. Peplomer glycoproteins are known to be the most effective immunogenic proteins of coronaviruses (Sturman and Holmes, 1983; Jimenez et al., 1986); therefore, the high number of spikes is reflected in the high ELISA reaction of this fraction pool.

After centrifugation through a linear sucrose gradient (Method i), satisfactory antigen preparations were obtained only by pooling Fractions 5–16. On the whole, Method i proved to be inferior to Method ii.

With NP40-solubilized antigen (Method iii), no specific ELISA reaction was detectable in low antigen dilutions due to the inhibiting effect of non-ionic detergents on the protein coating of polystyrene plates (Gardas and Lewartowska, 1988). Only with dilutions $> 1:150$, probably below the critical micelle concentration (Gardas and Lewartowska, 1988), was an extinction of ΔOD 0.9 reached.

Inconsistent results using IIFT and ELISA to detect antibodies to PED in pig sera could be explained by the high background reaction exhibited by single sera in the IIFT. Due to these unspecific reactions, serum samples had to be diluted 1:100 (Hofmann and Wyler, 1987) to reduce the background reaction to a negligible level. For the ELISA, the standard dilution was only 1:40 and even if there was a high unspecific reaction, ΔOD values could be determined without difficulty.

Three criteria may be put forward for the specificity of the ELISA: (i) the parallel antibody titer rise in the two experimentally infected pigs; (ii) high PEDV antibody titers of field sera detected by the IIFT were parallel with high ΔOD values in the ELISA; (iii) the lack of cross-reactions between TGEV and PEDV.

The ELISA described in the present study has several advantages compared with the IIFT. Antigen can be prepared by infecting cell cultures and one does not have to rely on cryosections of PEDV-infected piglets. Cryosections are usually contaminated by intestinal enteric viruses or bacteria which are responsible for a high degree of unspecific serum binding reactions in IIFT. Moreover, sera only have to be diluted 1:40 for ELISA instead of 1:100 as in IIFT.

Finally, an ELISA is a more rapid test than IIFT and reactions can be read automatically and without training, whereas experience in judging immunofluorescence reactions to distinguish specific from unspecific reactions is needed for IIFT.

ACKNOWLEDGEMENTS

We thank Dr. Georgiana Hanselmann for help in preparing the manuscript.

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