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An ELISA for detection of antibodies against porcine epidemic diarrhoea virus (PEDV) based on the specific solubility of the viral surface glycoprotein

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ABSTRACT

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Viral proteins of porcine epidemic diarrhoea virus (PEDV) were extracted from the cytoplasm of infected Vero cells using hypotonic conditions and a non-ionic detergent. Both the pH and the NaCl concentration of the extraction buffer were varied in attempts to increase the solubility of the virion spike glycoproteins (S-protein) and of the nucleocapsid proteins (N-protein). Monoclonal antibodies, hyperimmune sera and convalescent pig sera were used to identify and monitor these proteins by immunoprecipitation and Western blots. The solubility of the S-protein was optimal at pH 4, whereas that of the N-protein was optimal at pH 9. Consequently, it was possible to enrich for either S-protein or N-protein; increases in the NaCl concentration of the buffer were of no advantage in this respect. Enriched preparations of the S-protein and N-protein were used as ELISA antigen for the S-ELISA and N-ELISA, respectively. The S-ELISA proved to be the more effective of the two immunoassays. Antibodies against S-protein remained detectable for longer periods of time than anti-N-protein antibodies in the sera of PEDV-infected pigs. Using this ELISA of increased sensitivity, it was observed that only a small number of farms in Switzerland had been infected with PEDV.

INTRODUCTION

Porcine epidemic diarrhoea virus (PEDV), a member of the coronaviridae, is the causative agent of severe diarrhoea in pigs. Although identified in 1978 by Pensaert and Debouck, it was not until 1988 that the virus could be propagated in cell cultures using Vero cells and medium containing trypsin

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(Hofmann and Wyler, 1988). The lack of *in vitro*-propagated PEDV made the diagnosis of infections extremely complicated and for this reason the available tests were of limited applicability. As reviewed by Möstl et al. (1990), viral antigens and anti-PEDV antisera had to be prepared from experimentally infected pigs. Using these materials, immunofluorescence and ELISA tests for the detection of PEDV antigens as well as antibodies could be developed.

Consequently, little is known about the antigenic properties and solubility of the structural PEDV proteins synthesized in infected cell cultures. Canavagh et al. (1990), proposed that there were at least three structural proteins for coronaviruses. Egberink et al. (1988), using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), had previously described three proteins in purified PEDV particles. The major glycoprotein had an apparent molecular weight of 85 kD, most likely representing the monomeric form of the spike protein (S-protein). A second, RNA-binding protein, which was probably the nucleocapsid protein (N-protein), had a molecular weight of 58 kD. The third protein, which was in fact a cluster of proteins with molecular weights ranging from 20 to 32 kD, was apparently a glycoprotein and may have represented the integral membrane glycoprotein (M-protein).

The application of structural PEDV proteins to ELISA was first reported by Hofmann and Wyler (1990). Their approach, however, had two disadvantages: (i) PEDV particles were purified by sucrose density-gradient centrifugation before being used as ELISA antigens. A less complicated method of antigen preparation would facilitate the application of ELISA for large-scale serological testing, e.g. for serological survey studies. (ii) The authors pointed out that their ELISA antigen consisted mostly of N-protein, with only a small number of spikes. N-proteins carry antigenic determinants which are conserved among the coronaviridae (Horzinek et al., 1982; Yaling et al., 1988; Jöhr, 1989), whereas the antigenic determinants of S-protein induce more specific antibodies (Talbot and Buchmeier, 1985; Jacobs et al., 1987; Correa et al., 1988). Less is known concerning the determinants of the M-protein but cross-reactions have also been described (Horzinek et al., 1982).

In order to develop a specific ELISA for the detection of antibodies to PEDV, our interest focused on the antigenic properties of the S-protein of the virus, in comparison with those of the N-protein. Furthermore, we took advantage of the replication of PEDV in the cytoplasm of infected cells. The use of cytoplasmic extracts in the place of sucrose gradient-purified virions simplified the production of antigens.

Consequently, we analysed the influence of various pH levels and electrolyte concentrations on the solubility of PEDV S- and N-protein using the soluble fractions of infected cell lysates. Viral proteins were identified by their migration patterns in polyacrylamide gels, and by their reaction with anti-PEDV specific monoclonal antibodies or hyperimmune sera.

It was possible to enrich the S-protein in a soluble form at pH 4, with the simultaneous removal of the N-protein. An ELISA which was established using this S-protein preparation as antigen, demonstrated that antibodies against the S-protein of PEDV remained detectable for longer periods of time than anti-N-protein antibodies. Furthermore, it was possible to confirm the report of Hofmann and Wyler (1987) that PEDV circulated in the Swiss pig population.

MATERIALS AND METHODS

Media

Cell growth medium consisted of minimal essential medium (MEM) (Hazelton, Denver, USA) buffered with 20 mM HEPES and 0.2% (w/v) bicarbonate, supplemented with 5% (v/v) foetal bovine serum (SEBAK, Aidenbach, Germany) and antibiotics (10 000 IU penicillin G, 10 mg dihydrostreptomycin, 5 mg neomycin, 10 000 IU polymyxin per ml).

Virus infection medium consisted of MEM with 30 mM HEPES, 1% (v/v) 0.3 M NaOH, 0.3% (w/v) tryptose phosphate broth (Difco Laboratories, Detroit, MI, USA), 10 µg/ml trypsin (1:250, Difco) and antibiotics (Hofmann and Wyler, 1988).

Cell cultures and virus strains

Vero cells were used, between passage 2 to 23 after receipt from ATCC. Passage 12 of PEDV strain CV 777, adapted to propagation in Vero cell cultures, was kindly provided by M. Hofmann (Hofmann, 1987). PEDV strain V215/78, which is of low pathogenicity for pigs more than 12 weeks of age, was kindly provided by K.H. Witte (Witte et al., 1981).

Virus propagation

Three-day-old monolayers of Vero cells which had been seeded at 100 000 cells/ml (150-cm² flask) were washed twice with virus infection medium, then overlaid with 5 ml medium containing PEDV at a multiplicity of infection (m.o.i.) ranging from 0.01 to 0.1. The inoculum was replaced with fresh virus infection medium after an adsorption period of 2 h at 37°C.

After 12–24 h of incubation at 37°C, at which time the virus-specific CPE covered 95% of the cell monolayer, the virus was harvested. Cells and medium were frozen and thawed three times, and titers ranging from 10⁶–10⁸ TCID₅₀/ml were obtained.

Experimental animals

A fattening pig (No. 22) of 20 kg body weight was orally inoculated with 20 ml of a virus suspension [containing one part homogenized jejunal intestines (from a colostrum-deprived, PEDV-infected newborn piglet) and three

parts PBS]. A second fattening pig of similar size (No. 23) was kept in close contact with the inoculated pig.

After 37 h, both animals developed elevated rectal temperatures and diarrhoea which lasted for 3 days. Blood samples were taken from the two pigs both before and at weekly intervals after the infection.

Monoclonal antibodies and sera

The following antibody preparations and sera were used: (i) monoclonal antibody No. 24 (hybridoma supernatant), specific for S-protein of PEDV, kindly supplied by M. Roskopf (Institut für Virologie, Vet.-med. Fakultät, Zurich); (ii) monoclonal antibody No. 15 (hybridoma supernatant), specific for the N-protein of PEDV, kindly supplied by L. Jöhr (Jöhr, 1989; Institut für Virologie, Vet.-med. Fakultät, Zurich); (iii) serum samples from pig No. 23, collected before and at weekly intervals after contact infection; (iv) hyperimmune serum H1 (pig No. 922), collected from a pig infected with PEDV (strain V215/78) and subsequently reinfected several times (Hofmann, 1987), kindly provided by R. Wyler (Institut für Virologie, Vet.-med. Fakultät, Zurich); (v) hyperimmune serum H2, collected from a pig infected with PEDV strain CV 777 and subsequently reinfected several times (Möstl et al., 1990), kindly provided by F. Bürki (Institut für Virologie, Vet.-med. Universität, Vienna); (vi) 25 pig sera collected in four PEDV-free farms; (vii) sera from a group of 30 animals on a farm with a porcine epidemic diarrhoea (PED) history, bled at three different times during the fattening period. The first sampling was at the age of 4 months, the second at the age of 5 months, and the third on the day of slaughtering (age 6 months); (viii) 600 sera from the Swiss breeding pig serum bank (Berger et al., 1988; Palatini, 1988; Müller et al., 1990) were also tested. In 1985, 150 sera were obtained from the canton of Lucerne and another 150 from the canton of Berne. In 1989, another 150 sera were obtained from each of the regions mentioned.

Production of antigens

Monolayers of Vero cells were infected with 2–10 m.o.i. of PEDV. With each preparation, a separate control culture was mock-infected and treated as for the virus-infected culture. When the CPE reached 100%, the cells were washed twice with PBS, scraped into PBS with a rubber policeman, and centrifuged for 10 min at 120 g. The cells were then resuspended according to the experimental requirement in one of the various solutions as indicated below.

Soluble extracts of cell lysates. The cells were resuspended in double-distilled water to give 7.5×10^7 cells/ml. After 10 min, OBG (*n*-octyl-beta-*d*-glucopyranoside, Sigma, St. Louis, USA) was added to a final concentration of 1.5% (w/v) in order to disrupt the membranes of the cells (Ackermann et al., 1987). 1 M Tris-HCl with pH ranging from 1 to 10 was added at the rate of

25 $\mu\text{l}/\text{ml}$ to individual samples. When required, 0.125 or 0.25 M NaCl was added. The samples were then kept on ice for 10 min before being pelleted in Eppendorf tubes at 8800 g for 15 min in order to separate the soluble fractions from the insoluble components.

ELISA antigens derived from cell lysates. In order to prepare ELISA antigens, soluble fractions from the pH 4 lysates were supplemented with 3 μl phenylmethyl sulphonyl fluoride per ml of antigen (Sigma). Aliquots of 100 μl were stored at -70°C .

Immunoprecipitation. A total of 8×10^6 PEDV-infected cells were resuspended in 200 μl of immunoprecipitation (IP) buffer. IP buffer (pH 7.5) contained 50 mM Tris, 150 mM NaCl, and 1% (v/v) Triton X-100 (Maniatis et al., 1989). After 30 min of incubation at 4°C , the insoluble components were removed by centrifugation in an Eppendorf centrifuge (1 h, 10 000 rpm). 90 μl monoclonal antibody No. 24 was added to the supernatant and incubated for 5 h at 4°C . In the meantime, the equivalent of 10 mg protein-A sepharose (Pharmacia, Uppsala, Sweden) per sample was resuspended in IP buffer and washed six times. The concentration was adjusted to 100 mg/ml and rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) was then added to give a final dilution of 1:100. After 30 min at 37°C , the sepharose was washed extensively and resuspended in the initial volume of IP buffer. This suspension (100 $\mu\text{l}/\text{sample}$) was then added to the mixture of soluble lysate and monoclonal antibody, and incubated for 45 min at room temperature with occasional shaking. Following six washing steps with IP buffer, the precipitated immune complexes were solubilized with 200 $\mu\text{l}/\text{sample}$ of disruption buffer at 37°C for 30 min. Disruption buffer consisted of 2% (w/v) SDS, 0.5% (v/v), 1 M Tris-HCl, pH 7.0, and 2.8% (w/v) sucrose. The sepharose was removed by centrifugation before 20 μl of sample was loaded onto polyacrylamide gels.

SDS-PAGE

Whole cells, soluble fractions and precipitated immunocomplexes were solubilized for 30 min at 37°C (Sturman, 1977; Sturman and Holmes, 1983) in disruption buffer and homogenized by ultrasonication. A sample size equivalent to 7.5×10^5 cells was applied to 12% (w/v) polyacrylamide gels. Prestained SDS-PAGE Standards (Bio-Rad Laboratories, California, USA) were used as molecular weight markers. Polyacrylamide gel electrophoresis was performed for 1 h at 180 V using a mini-PROTEAN II system (Bio-Rad). The separated polypeptides were then transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) for 2 h at 100 V.

Immunoblotting

The nitrocellulose sheets with the transferred polypeptides were incubated in blocking solution [20 mM Tris-HCl, 500 mM NaCl, pH 7.5 (TBS) supplemented with 5% skimmed milk] for 1 h at 37°C with agitation. Anti-PEDV serum H1, diluted 1:40 in TBS supplemented with 1% skimmed milk, was then incubated with the nitrocellulose for 2 h at 37°C. Protein-A-peroxidase (Kirkegaard & Perry, MD, USA; diluted 1:500 in blocking solution) was then added for 1 h at 37°C, followed by the substrate solution of 0.18 mg/ml 4-chloro-1-naphthol in PBS containing 6% (v/v) methanol and 0.02% (v/v) H₂O₂ (Sigma) until the immunostained bands developed. Between each step, the nitrocellulose was washed three times with TTBS (TBS with 0.05% (v/v) Tween 20). The optical density of the stained bands was determined by scanning with a computing densitometer 300 A (Molecular Dynamics, Sunnyvale, California).

Glycoprotein detection

In order to detect and to quantify glycoproteins, a Glycan Detection Kit (Boehringer, Mannheim, Germany) was used according to the manufacturer's instructions. The digoxigenin-labelled glycoconjugates were subsequently visualized with an enzyme immunoassay using alkaline phosphatase-conjugated antibodies.

Protein assay

For protein quantification, the Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA) was used according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

Purified virus ELISA antigens

ELISA antigens were also derived from sucrose density gradient-concentrated virus particles. Virus and control antigens were prepared as described by Jöhr (1989).

ELISA

Indirect ELISA protocols using volumes of 200 µl per well, and with three washing cycles (PBS supplemented with 0.05% Tween 20) between each incubation step, were applied. Briefly, ELISA plates (Nunc, Immuno plate Maxisorp F96, Inter Med, Denmark) were sensitized with ELISA antigens diluted in coating buffer (0.05 M sodium bicarbonate, pH 9.6). Alternate rows of the plates were coated overnight at 37°C with virus or mock antigen, respectively. The plates were then blocked for 1 h at 37°C using 1% (w/v) bovine serum albumin in coating buffer. Sera, diluted 1:40 in PBS-Tween, were then added for 75 min at 37°C, followed by peroxidase-labelled conjugate [either rabbit anti-swine IgG, 1:200 (Dr. Bommeli AG, Bern, Switzer-

land) or rabbit anti-mouse IgG, 1:1000 (Dakopatts, Glostrup, Denmark)] for 1 h at 37°C. The reaction was visualized by the addition of the substrate (ABTS, Boehringer, Germany) after 30 min at 37°C. Positive and negative reference sera were included on each plate. Typical net absorptions (absorbance against virus antigen – absorbance against mock antigen) (referred to as 'Delta OD') of these sera were 0.5 Delta OD (± 0.1) at 405 nm for the positive reference and 0.0 Delta OD (± 0.05) for the negative reference, respectively. The values were determined with an Anthos reader 2001 (Anthos Labtec Instruments, Salzburg, Austria).

RESULTS

Identification of PEDV S- and N-protein

In order to identify the major viral antigens in PEDV-infected cells, lysates of both PEDV-infected and mock-infected cells were separated on polyacrylamide gels and transferred to nitrocellulose sheets for visualization. The results (Fig. 1) showed the following:

(i) in lysates of mock-infected cells, five bands with molecular weights of 96, 89, 63, 56 and 53 kD were stained with sera obtained both from PEDV-infected and PEDV-free pigs (lane 1).

(ii) In lysates from PEDV-infected cells, three additional prominent bands with apparent molecular weights of 200, 180 and 58 kD were stained by the anti-PEDV hyperimmune serum (lane 2). These bands appeared to represent the major viral antigens. Of the cellular proteins, the 96- and 89-kD bands were stained at a comparable intensity, whereas the others appeared fainter.

(iii) In order to detect viral glycoproteins, the transferred lysates were stained with the Glycan Detection Kit. Many glycoproteins were stained in both mock- and virus-infected cell lysates, but the presence of a 200/180-kD double band in the PEDV-infected lysate suggested that these two bands represented viral glycoproteins, most probably the spike glycoprotein (lane 3 and 4).

(iv) S-protein could not be stained on the Western blots by the use of anti-S specific monoclonal antibodies (data not shown). However, following immunoprecipitation of soluble lysates of PEDV-infected cells with monoclonal antibody, No. 24, only the 200- and 180-kD protein bands appeared on the Western blots. These bands could be stained immunologically with anti-PEDV hyperimmune serum (lane 5). This result, in combination with the above observations (ii and iii), indicated that monoclonal antibody No. 24 was specific for S-protein. As determined previously by radioimmunoprecipitation, this monoclonal antibody did not react with proteins specified by TGE (transmissible gastroenteritis) virus or PRCV (porcine respiratory coronavirus) (data not shown).

(v) According to the apparent molecular weight, the 58-kD viral antigen

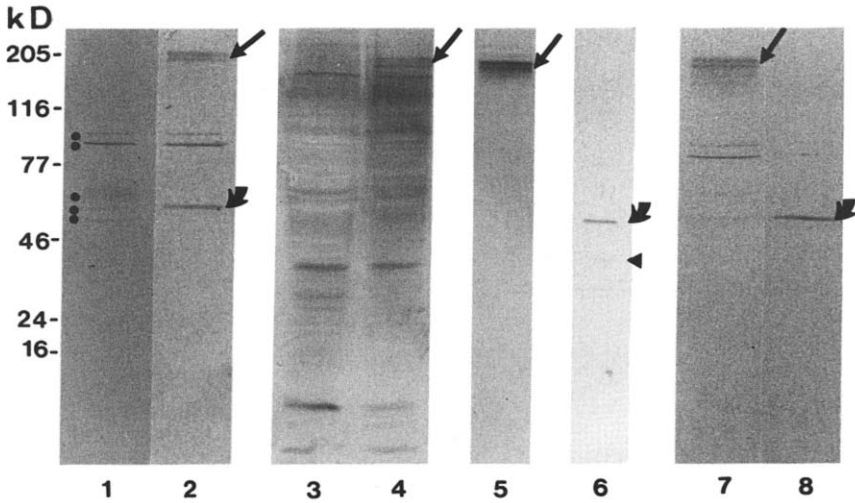


Fig. 1. Immunoblotting (lanes 1–2 and 6–8) or glycan staining (lanes 3–4) of electrophoretically separated PEDV- or mock-infected proteins, and the electrophoresis of immunoprecipitated S-protein (lane 5). Lane 1, mock-infected cell lysates stained with H1 antiserum; lane 2, PEDV-infected cell lysates stained with H1 antiserum; lane 3, mock-infected cell lysates stained for glycan; lane 4, PEDV-infected cell lysates stained for glycan; lane 5, S-protein precipitated with monoclonal antibody No. 24, electrophoresed, transblotted and then stained with H1 antiserum; lane 6, PEDV-infected cell lysates, stained with anti-N-protein monoclonal antibody No. 15; lane 7, soluble (pH 4) extract of PEDV-infected cells stained with H1 antiserum; lane 8, insoluble (pH 4) extract of PEDV infected cells stained with H1 antiserum. S-protein, upper (straight) arrow (lanes 2, 4, 5, 7); N-protein, lower (curved) arrow (lanes 2, 6, 8); degraded N-protein, arrow-head (lane 6); host proteins, circles (lane 1).

most likely represented the nucleoprotein (N) of PEDV. This was confirmed by staining the Western blots using monoclonal antibody No. 15 (lane 6, curved arrow).

(vi) The integral membrane glycoprotein could not be detected on Western blots.

Solubility of S-protein and N-protein

The solubility of the S- and N-proteins was studied under pH conditions ranging from pH 1 to 10. Soluble supernatants and resuspended pellets which resulted from these treatments were electrophoresed and stained on Western blots with anti-PEDV hyperimmune serum.

An example of these experiments is shown in Figure 1 (lanes 7 and 8): at pH 4 much of the S-protein, as well as significant amounts of the 96- and 89-kD cellular proteins, were found in the supernatant (lane 7). A large proportion of N-protein and minor amounts of the cellular proteins remained in the pellet (lane 8). The relative solubilities of the S- and N-proteins at the various pH levels were determined by measuring the optical densities of the protein

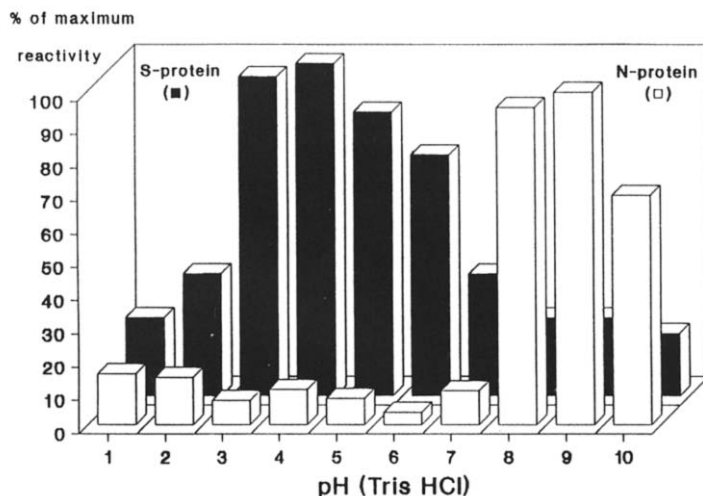


Fig. 2. Relative absorbance at 405 nm (% of maximum reactivity; maximum reactivity = maximum absorbance obtained with the respective protein) (Y-axis) of Western blots of viral protein bands obtained after solubilization at different pH levels (X-axis) of PEDV-infected cell proteins. The soluble material was separated by centrifugation from the insoluble mass, electrophoresed (PAGE), and immunoblotted on nitrocellulose filters using the H1 antiserum. The absorbances were determined by computing densitometry. Filled columns, S-protein; empty columns, N-protein.

bands obtained from such treatments on the Western blots. Figure 2 shows that the maximum solubility of S-protein occurred at pH 3–4, whilst the N-protein was most soluble under basic conditions (pH 8–9).

Slightly more S-protein could be accumulated in the soluble fractions when 0.125 or 0.25 M NaCl was added to the lysates (data not shown). However, the solubility of N-protein also improved with the addition of NaCl, and the gain, in terms of accumulating S-protein whilst removing N-protein, was marginal. Furthermore, with the addition of NaCl, a second, faster migrating protein (45 kD) was detected with the N-specific monoclonal antibody No. 15 (Fig. 1, lane 6). The original 58-kD band, on the other hand, appeared fainter (data not shown). For these reasons, the addition of NaCl was omitted in the remaining experiments.

Analysis of the influence of the non-ionic detergent in the extraction buffer was made by varying its concentration. No effect upon the solubility of the virion proteins was observed (data not presented).

Coating of enriched S-protein onto ELISA plates

Serial dilutions of soluble cell extracts which had been prepared at pH 4 were used to sensitize ELISA plates, and were compared with respect to their PEDV antigen content (by reaction of anti-PEDV hyperimmune serum H2)

and their glycoprotein content (by the Glycan Detection Kit). The results are shown in Figure 3a (virus antigen) and in Figure 3b (mock antigen). At dilutions between 1 : 100 and 1 : 800 for the viral antigens, a strong reaction was seen with the anti-PEDV hyperimmune serum. At higher dilutions, this reaction decreased rapidly. A close relationship between the detection of PEDV antigen and that of total glycoprotein was evident. Thus, S-protein seemed to be a major component of the antigen. Using antigens obtained from mock-infected cells, no specific reaction of the hyperimmune serum was observed (Fig. 3b). In contrast, cellular glycoproteins were detected, in particular between dilutions of 1 : 1600 and 1 : 6400. This indicated that the coating of cellular glycoproteins was competed by other cellular components in the mock antigen preparation at dilutions of less than 1 : 1600.

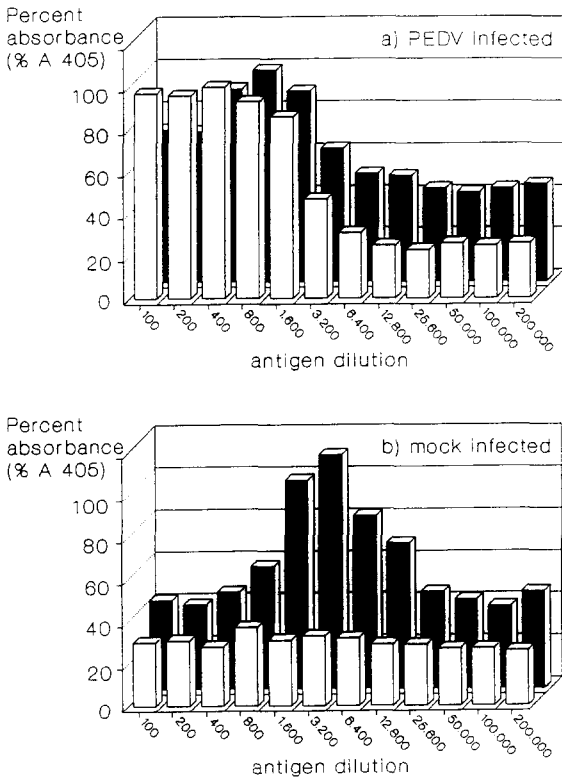


Fig. 3. Relative absorbance values at 405 nm (% A₄₀₅ with respect to the maximum value obtained in Figure 3a (y-axis) of serial dilutions of soluble (pH 4) lysates from PEDV-infected (a) and mock-infected (b) cells coated on ELISA plates. Proteins which had bound were detected using either hyperimmune serum H2 (empty columns) or the Glycan Detection Kit (filled columns). The antigen dilutions (reciprocal values) are indicated on the x-axis.

ELISA

The pH 4 extracts were used to coat ELISA plates at dilutions of up to 1:800, which corresponds to an equivalent of 18 750 cells or 800 ng of protein per well. The monoclonal antibodies No. 24 (specific for S-protein) and No. 15 (specific for N-protein) were used to test the coated ELISA plates. The results indicated that S-protein, contaminated by traces of N-protein, coated most efficiently. Therefore, this ELISA was referred to as S-ELISA.

The same monoclonal antibodies were used to test ELISA plates coated with sucrose gradient-purified virus particles. This antigen consisted mostly of N-protein with minor amounts of S-protein. Therefore, this ELISA was referred to as N-ELISA.

The two ELISAs were applied to the determination of anti-S protein and anti-N protein-specific antibody titres in two hyperimmune pig sera, and in sera obtained from a pig both before and after PEDV infection. As shown in Table 1, similar titres were found in the two tests, indicating that antibodies against S-protein and N-protein had been induced. The specificity of the antibodies in the different preparations for S-protein and N-protein was confirmed by immunoblotting (Table 1).

Positive cut-off values of the two ELISAs

Pig sera obtained at weekly intervals following PEDV infection were tested by measuring the net absorbance (Delta OD) at a constant serum dilution of 1:40 in the two ELISA systems. Figure 4 shows the results obtained with the sera from pig No. 23 (infected by contact). The sera from pig No. 22 gave a similar picture. The following observations were made: (i) both before infection and 1 week post-infection (p.i.) no specific antibodies could be detected; (ii) 2 weeks p.i., a definite reaction of the sera with the viral antigens was observed; (iii) between 3 and 8 weeks p.i., a steady increase in the net absorption of the sera in both ELISAs was observed; (iv) the sera collected be-

TABLE 1

Antibody titers in S-ELISA and N-ELISA

Serum	Titer ¹		Immunoblot ²	
	S-ELISA	N-ELISA	S-protein	N-protein
Pig 23 (preimmune)	neg	neg	neg	neg
Pig 23 (3 weeks p.i.)	160	320	++	+
H1 (hyperimmune)	3200	6400	+	+
H2 (hyperimmune)	12800	5120	+++	(+)

¹Reciprocal antibody dilution which gave Delta OD 0.1 (absorbance against virus antigen-absorbance against mock antigen).

²Relative strength of reaction is denoted by the number of + signs.

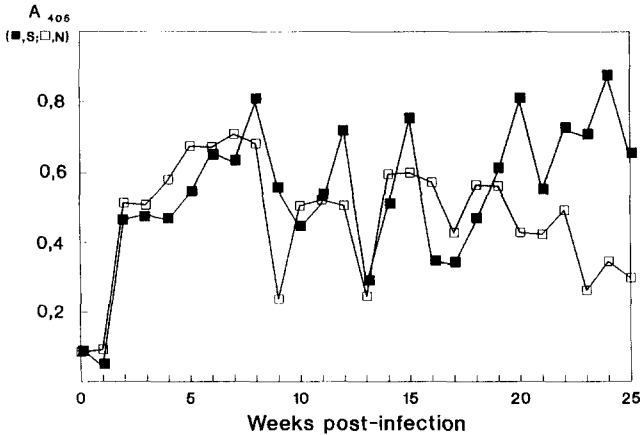


Fig. 4. Reactions in the S-ELISA (black squares) and N-ELISA (open squares) of consecutively sampled sera from pig No. 23 after PEDV infection. The times of sampling (weeks post-infection) are indicated on the x-axis; the net absorption values (Delta OD 405 = absorption against virus antigen – absorbance against mock antigen) are indicated on the y-axis.

tween 9 and 19 weeks p.i. remained ELISA-positive, but were variable in terms of the net absorption obtained; (v) from 20 weeks p.i., the reactions in the N-ELISA had a tendency to decrease whilst those in the S-ELISA remained relatively elevated.

The serum obtained 2 weeks p.i. was used as a weak positive reference serum. The reaction of this serum was set as 100%, and the reactions of test sera were expressed in relation to this standard as %OD. Sera giving a %OD of at least 100% were considered positive.

Negative cut-off values of the two ELISAs

25 pig sera were collected randomly from five PEDV-free premises. The ELISA reactions of these sera were low in both tests; between 0% and 36% (relative to the weak positive reference serum) in the S-ELISA, and between 0% and 61% in the N-ELISA. Consequently, ELISA reactions within the range of the mean value plus 2 standard deviations were regarded as negative. Thus, in the S-ELISA the negative cut-off value was 33% and in the N-ELISA, 56%. Reactions of test sera which fell in the range between the negative and positive cut-off values (33% or 56%, and 100% respectively) were uninterpretable.

Comparison of S-ELISA and N-ELISA

In order to compare the S-ELISA and the N-ELISA, serum samples were taken at monthly intervals from 30 pigs which originated from a PEDV-positive farm. The pigs were bled at the age of 4, 5 and 6 months.

The results are shown and compared in Figure 5. At the age of 4 months (Fig. 5a), most of the pigs apparently had antibodies against PEDV. In the S-

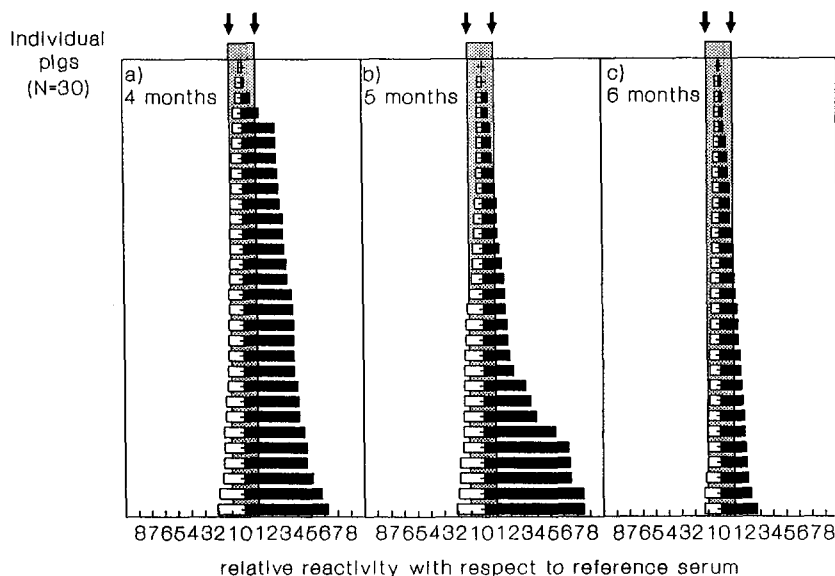


Fig. 5. Comparison of S-ELISA (filled columns) and N-ELISA (empty columns) for their ability to detect antibodies in sera from 30 pigs on a PEDV-positive farm. The sera were collected when the pigs were 4 months old (a), 5 months old (b) or 6 months old (c). The positive cut-off values are indicated by arrows; between these arrows is the uninterpretable zone plus the negative value range. Sera were aligned by their reactivity in N- and S-ELISA, and were not identified individually. With respect to the x-axis (relative reactivity), 1 = 100%; i.e. the same reactivity as the positive reference serum; 2 = 200%, etc.

ELISA, 27 of the 30 sera (90%) gave positive reactions, two were negative and one was uninterpretable. With the N-ELISA, only 18 sera (60%) could be regarded as positive, two were negative and ten sera were uninterpretable.

One month later (Fig. 5b), 22 sera and 14 sera were positive in the S-ELISA and the N-ELISA, respectively. After a further month (Fig. 5c), 17 pigs (56%) were seropositive by the S-ELISA, but only three (10%) were seropositive in the N-ELISA.

Serological survey for PEDV in Swiss pig breeding herds

In order to determine if PEDV circulated in Swiss breeding pig herds, the S-ELISA was used to analyse 600 serum samples of sows and boars collected in the slaughterhouses of two cantons (Lucerne and Berne). Half of the sera were collected in 1985 and half in 1989. Each sample tested originated from a different herd. Seven samples, collected in 1985 in the canton of Lucerne, were positive for anti-PEDV antibodies, whereas all other sera apparently were negative (data not presented). This result indicated that Swiss pig breeding herds have a low prevalence of antibodies against PEDV.

DISCUSSION

Since it was not possible until recently to propagate porcine epidemic diarrhoea virus in cell culture (Hofmann and Wyler, 1988), the diagnostic tests for the detection of anti-PEDV antibodies were complicated to establish and expensive to perform (Möstl et al., 1990). Therefore, their application was clearly limited and large-scale serological surveys could not be performed. In addition, little was known about the physicochemical and antigenic properties of the viral structural proteins.

The studies described in this report provide new information with regard to:

(i) identification and partial characterization with monoclonal antibodies and immune sera of PEDV surface protein (S-protein) and nucleoprotein (N-protein);

(ii) conditions required for the solubilization and/or precipitation of PEDV S-protein and N-protein;

(iii) establishment of a specific and easy-to-use S-ELISA which is superior to the N-ELISA described earlier (Hofmann and Wyler, 1990) in terms of sensitivity and specificity;

(iv) duration of detectable concentrations of anti-N-protein antibodies and anti-S-protein antibodies in the sera of pigs experimentally or naturally infected with PEDV;

(v) application of the ELISA to a serological survey.

In an initial series of experiments, a 200/180-kD glycoprotein band which represented the S-protein of PEDV was identified, in agreement with Jöhr (1989). Egberink et al. (1988) had proposed that the S-protein of purified virus particles had apparent molecular weights of 85, 110 and 135 kD, although monomeric forms and other degradation products may have been present due to the use of β -mercaptoethanol in the disruption buffer. King and Brian (1982) reported that the peplomer protein (S-protein) of bovine coronavirus segregated into monomers following β -mercaptoethanol treatment. Depending on the antigen batch, an 85-kD protein could also be seen in our experiments (data not presented).

A 58-kD virus-specific protein detected in infected cell lysates was taken to be the N-protein; its molecular weight corresponded to that of the RNA-binding protein of PEDV (Egberink et al., 1988), and it specifically reacted with an anti-N-protein monoclonal antibody. It was possible to separate the S- and N-proteins due to differences in relative solubility. The S-protein was most soluble at pH 4, whereas the N-protein precipitated under these conditions, being most soluble at pH 9. These observations were not unexpected since variable solubility of constituent virion proteins under different ionic conditions has also been reported for other viruses (Ackermann et al., 1984, 1986).

Protein solubility may also be influenced by the concentration of electro-

lytes in the medium. Increasing the NaCl concentration in the protein extraction buffer had a slight influence on the solubility of both S-protein and N-protein, which rendered the procedure unusable for our purposes. In addition, a 45-kD protein, detected with the N-specific monoclonal antibody in immunoblots, appeared at the expense of the original 58-kD band when high NaCl concentrations were used. This argues against the 45-kD band being the integral membrane glycoprotein of PEDV, as proposed by Jöhr (1989), and in favour of the conclusion by Robbins et al. (1986) that such faster migrating bands represented cleavage products of N.

Using the S-protein solubilized at pH 4, an indirect ELISA was established. The ELISA system was chosen due to the non-adaptability of the serum neutralization test for PEDV (serum in the culture medium of this assay has the potential to inhibit virus replication either directly through virus neutralization, or indirectly by the inhibition of the trypsin required to permit virus entry into the cells).

An ELISA in which the antigen was mainly N-protein (Hofmann and Wyler, 1990) was used for comparison. The positive cut-off values of the two ELISAs were established by using sera of pigs which had been experimentally infected with a low-virulence strain of PEDV. Accordingly, weakly positive sera could be used as references, thus helping to increase the sensitivity of the test. On the other hand, at first sight, the background levels obtained with negative sera were relatively high, with negative cut-off values of 33% for the S-ELISA and 56% for the N-ELISA. It should be mentioned that pigs which had been experimentally infected with other coronaviruses, i.e. TGE virus and PRCV, did not seroconvert for PEDV. Sera of these pigs had been collected at regular intervals before and for several weeks after experimental TGE and PRCV infection. Whereas a marked seroconversion against TGE and PRCV was observed, the reactions of these sera remained clearly in the PEDV-negative range of between 0 and 14% relative to the control serum (data not shown).

The two anti-PEDV ELISAs gave similar results with positive sera taken shortly after PEDV infection, but with increasing time after infection, the N-ELISA became more variable. Anti-N-protein antibodies seemed to be cleared from sera more rapidly than anti-S-protein antibodies. This observation was true for both experimentally and naturally PEDV-infected pigs. A relatively short persistence of anti-PEDV antibodies was also observed by Möstl (personal communication), while Pensaert (1985) reported that the immunity against PEDV did not last longer than 5 months.

The S-ELISA was more efficient than the N-ELISA at identifying seropositive individuals. Comparison with previously published ELISA work is not possible, since the earlier analyses did not differentiate between reactions against individual viral proteins. In addition, Hofmann and Wyler (1987) did not specify the time post-infection of serum sampling. Callebaut et al.

(1982) reported seropositive reactions for 180 days after clinical PED, but it was not clear if reinfection had occurred. Our experiments showed that the persistence of the antibody response in one piglet could be explained by a chronic infection, manifested by transient periods of diarrhoea (data not shown). Garreis and Stahl (1981) reported that PEDV could circulate in pig fattening farms for more than 1 year, provided that new piglets were introduced at weekly intervals.

With the detection of seven seropositive herds in the canton of Lucerne in 1985, it could be concluded that PEDV was circulating at that time among breeding pigs in that part of Switzerland. Hofmann and Wyler (1987) had previously reported an anti-PEDV seroprevalence of 1.6% in Switzerland. However, in 1989 we were unable to detect seropositive animals in the cantons of Lucerne or Berne. Nevertheless, in order to perform a sero-epidemiological survey, the sample size would have to be increased, samples from all relevant areas of Switzerland would be required, and, above all, fattening pigs would have to be included. It is clear, however, that the S-ELISA would be the method of choice for such an analysis, since it provides a rapid, sensitive and defined assay for the detection and analysis of the antibody response against PED virus.

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