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Comparison of enzyme-linked immunosorbent assay and RT-PCR for the detection of porcine epidemic diarrhoea virus

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ABSTRACT

Porcine epidemic diarrhoea (PED) is a contagious enteric disease of pigs caused by a coronavirus. A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) based on the use of monoclonal antibodies was developed for the detection of porcine epidemic diarrhoea virus (PEDV). The DAS-ELISA was compared with RT-PCR in the examination of 506 specimens collected during 2006–2007 from pigs originating from different farms located in the Po valley. Both faecal samples obtained directly from the rectum of live animals showing clinical signs and intestinal samples collected from the caecum of deceased pigs were included in the study. The correlation between the two methods was higher when testing faecal samples ($K = 0.97$, 95% CI: 0.94–1.00) than testing intestinal samples ($K = 0.62$, 95% CI: 0.35–0.89). The use of ELISA technology provided an efficient and effective mean of evaluating the presence of coronavirus PED antigen in field samples and indicates that this procedure is a very useful tool in epidemiological studies.

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Porcine epidemic diarrhoea virus (PEDV) is a member of the order *Nidovirales,* family *Coronaviridae,* genus *Coronavirus* and is part of Group 1 species. PEDV was first reported in Belgium and United Kingdom in 1978 (Pensaert and DeBouck, 1978). Since then, several outbreaks of the disease have been reported in many swine-raising countries, most notably in Europe and East Asia (Pensaert and Sang-Geon, 2006).

PEDV induces an enteric disease similar to that caused by transmissible gastroenteritis virus (TGEV) in pigs of all ages with high morbidity and mortality related to the age. Mortality rate averages 50% but may be as high as 90% in pigs less than 7 days of age and very low or nil in pigs older than 2 weeks (Pensaert and DeBouck, 1978; Pensaert and Sang-Geon, 2006; Pritchard et al., 1999).

Since the 1990s PEDV has caused severe epidemics in some Asian countries such as Japan, Korea and China (Kweon et al., 1993; Takahashi et al., 1983). In these countries massive losses of piglets have been reported and mortality usually reached high percentages (30–80%) in pigs younger than 1 week (Shibata et al., 2001).

Between May 2005 and June 2006, an epidemic of watery diarrhoea in pigs of all ages, including piglets, occurred in Italy in the Po Valley, a densely pig populated area. In previous years, similar but sporadic outbreaks had been observed in growers and finishers but that was the first time that PED "re-emerged" in epidemic form in a European country in many years (Martelli et al., 2008).

The objective of the present study was to develop a double antibody sandwich (DAS) ELISA, based on the use of monoclonal antibodies for PEDV detection in swine intestinal and faecal samples useful for routine examinations of field samples. The diagnostic performances of the DAS-ELISA and the correlation with RT-PCR were evaluated by testing 506 samples collected from pig herds in the Po Valley during the 2006–2007 period. Two hundred and fifteen faecal samples were obtained directly from the rectum of live animals showing clinical signs and 291 intestinal samples were collected from the caecum of deceased pigs. These specimens were diluted with phosphate-buffered saline (PBS; 0.1 M, pH 7.2) to obtain a 10% suspension (v/v), clarified by centrifugation at 2000*g* for 10 min and then stored at −80 °C.

The PEDV reference strain (CV-777) was propagated in Vero cells grown in minimum essential medium Eagle (MEM) in the presence of trypsin (Hofmann and Wyler, 1988). As soon as the cytopathic effect was fully developed, the virus was released from the cells by repeated freezing/thawing. After centrifugation (2000*g* for 15 min), the supernatant of the infectious culture medium was kept at −80 °C before being used as positive control in the ELISA reaction. Negative control antigen was similarly prepared from non-infected cell lines.

Monoclonal antibodies (MAbs) were prepared using standard methods (Galfre and Milstein, 1981). Mice were immunised with partially purified CV-777 strain. Forty hybridomas were screened

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for secretion of desired antibodies by Indirect ImmunoFlorescence (IFI) and indirect ELISA, set up using PEDV infected and non-infected cells. Only six MAbs specific for the PEDV were selected and further characterised. Purified MAbs from hybridomas culture supernatants or ascitic fluids conjugated with horseradish peroxidase (HRPO) using a modification of the method described by Tjissen and Kurstak (1984).

The different combinations of the six selected MAbs were tested in DAS-ELISA using as antigen both VERO cells infected or not with PEDV and cells infected with other coronaviruses or other pig viruses. The intensity and specificity of the reaction using each MAb as catcher or tracer were considered as criteria for selection of the best combination, i.e. MAb 4C3 as antigen catching antibody and 1F12 as conjugated MAb.

In the standard procedure, Nunc Maxisorb Immunoplates were coated overnight at 4 °C with purified MAb 4C3 optimally diluted in 0.05 M carbonate–bicarbonate buffer, pH 9.6 (2 μg/well). Samples were dispensed to duplicate wells and, after incubation a 37 °C for 1 h, the HRPO-conjugated MAb 1F12 was added at a pre-determined optimal dilution. Positive and negative controls for PEDV were included in each plate. The diluting buffer consisted of PBS (pH 7.4) with 0.05% Tween 20 and 1% yeast extract. Following incubation at 37 °C for 1 h, the substrate solution (orthophenylenediamine dihydrochloride 0.5 mg/ml and 0.02% H_2O_2 in 50 mM phosphate citrate buffer, pH 5.0) was then added. The colorimetric reaction was stopped after 10 min by addition of 2 N sulphuric acid and the absorbance values were read at 492 nm using a spectrophotometer. Results were expressed as an optical density (OD). Fifty microliters per well of reagent were dispensed; three washings with PBS-Tween 20 were performed after each incubation.

A panel of four faecal samples, that had a different degree of viral load (from high positive to negative) when examined in dilution in RT-PCR (data not shown), were analyzed by DAS-ELISA in serial two fold dilutions (from 1/1 to 1/512) in order to select the optimal dilution for test samples. The titration curves based on the OD values provided evidence of a clear separation between the positive and negative samples (Fig. 1). The dilutions 1/1 and 1/2 provided the widest window between them and were selected as screening dilutions combined with an OD cut-off value 0.2.

All the 506 field samples were examined in parallel using DAS-ELISA and a RT-PCR performed using primers specific for the spike protein (S-protein) as previously described (Kim et al., 2001). The agreement between PCR and ELISA techniques was measured with the kappa statistic value (Landis and Koch, 1977).

Table 1
Comparison of RT-PCR and DAS-ELISA for the detection of PEDV in (A) faecal and (B) intestinal samples.

DAS-ELISA	RT-PCR		
	Positive	Negative	Total
(A)	$K = 0.97$		
Positive	42	0	42
Negative	2	171	173
Total	44	171	215
(B)	$K = 0.62$		
Positive	6	7	13
Negative	0	278	278
Total	6	285	291

The results obtained by examining, respectively, faecal and intestinal samples with both methods are shown in Table 1. When examining faeces, only two samples gave discordant results, i.e. they were negative by ELISA, but were identified as PEDV-positive by RT-PCR. The kappa value was high (0.97), suggesting an almost perfect agreement between the two methods. The ELISA test may fail to detect antigens in faecal samples with very low viral titres specially when clinical specimens are collected in the recovery phase of the disease. Other reasons could be the presence in faeces of specific antibodies that form immunocomplexes, or an excessive delay between collection and examination resulting in sample degradation and loss of antigenic sites.

A lower number of intestinal samples resulted PEDV positive in both arrays (6 vs 42 faecal samples) and, in addition, the two methods gave more discordant results, indicating just a substantial agreement ($K = 0.62$). In particular, 6 of the 291 intestinal samples were positive by both methods, whereas 7 samples resulted ELISA positive, but RT-PCR negative. Such disagreement could be due to the presence of PCR inhibitors and DNA damaging substances in stool samples, but can also result from poor-quality specimens, e.g. extremely autolysed tissues or those stored at room temperature for prolonged periods. In addition, false-positive reactions in the ELISA assay may be due to the non-specific binding of antibodies used as reagents to intestinal bacteria or their products (Brandt et al., 1981). However, this was probably

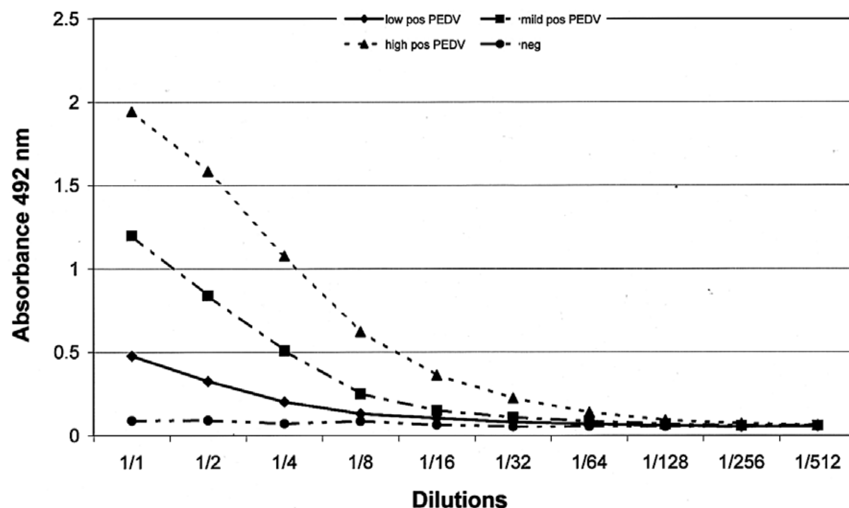

Fig. 1. Dose relationship between absorbance and sample dilutions. Absorbances for a weakly, mildly and strongly PEDV positive faecal samples and a negative faecal sample are shown.

not the case since the results did not change, even after incubation of ELISA pos/RT-PCR neg samples with a negative mouse serum in order to block a potential binding between murine antibody (MAbs) and pig specimens.

It is already known that ELISA results depend on the clinical and pathological data: in intestinal contents and faecal specimens obtained from experimentally infected PEDV piglets, the virus shedding is detected with high consistency during the acute phase of disease, but much less frequently during the incubation period and the recovery phase (Callebaut et al., 1982). Our data confirm that for a successful and correct diagnosis of PEDV it is advisable to collect faecal materials at the onset of illness rather than taking intestinal contents from dead or suppressed animals with prolonged clinical signs.

Using DAS-ELISA the presence of PEDV was found in 55 of 506 (10.8%) field samples of pigs with diarrhoea originating from different farms located in the Po Valley.

A high proportion of positive results was detected in samples taken between January and June 2006 (20 of 30 examined, 66.6%), during an epidemic of diarrhoea in the same area of Italy (Martelli et al., 2008), from pigs of all ages. It is therefore possible that virus persisted after the acute phase in some groups of animals, for example after weaning, but this aspect was not specifically investigated by serology.

During the latter part of 2006 and 2007, the PEDV was detected in field samples at a lower rate, i.e. 35 of 476 (7.3%). The rapid decrease of the frequency of detection is probably also related to the quick development of a sufficient level of immunity in the population that caused a reduction of virus circulation. These findings are similar to those found in other countries with developed swine production (Pensaert and Sang-Geon, 2006).

On the whole the results obtained by testing samples of naturally infected swine indicate that the DAS-ELISA here described could be considered as a reliable and accurate assay for the diagnosis of PEDV in clinical specimens. Such data agree with previous studies in which the PEDV-ELISAs were employed for the screening for pig herds during epizootic outbreaks (Carvajal et al., 1995; Rodak et al., 2005; Van Nieuwstadt and Zetstra, 1991).

The advantages of the DAS-ELISA compared with RT-PCR assay derive from the simple and rapid procedure, suitable for the screening of a large number of specimens, and from the use of MAbs, that ensure standardisation and reproducibility. When taking into consideration the epidemiological characteristics of PEDV, the rapid identification of the etiological agent would facilitate the implementation of effective control measures.

References

Brandt, C.D., Kim, H.W., Rodriguez, W.J., Thomas, L., Yolken, R.H., Arrobio, J.O., Kapikian, A.Z., Parrott, R.H., Chanock, R.M., 1981. Comparison of direct electron microscopy and rotavirus enzyme-linked immunosorbent assay for detection of gastroenteritis viruses in children. Journal of Clinical Microbiology 13, 976–981.

Callebaut, P., Debouck, P., Pensaert, M., 1982. Enzyme-linked immunosorbent assay for the detection of the coronavirus-like agent and its antibodies in pigs with porcine epidemic diarrhea. Veterinary Microbiology 7, 295–306.

Carvajal, A., Lanza, I., Diego, R., Rubio, P., Carmenes, P., 1995. Evaluation of a blocking ELISA using monoclonal antibodies for the detection of porcine epidemic diarrhoea virus and its antibodies. Journal of Veterinary Diagnostic Investigation 7, 60–64.

Galfre, G., Milstein, C., 1981. Preparation of monoclonal antibodies, strategies and procedure. Methods in Enzymology 75, 3–53.

Hofmann, M., Wyler, R., 1988. Propagation of the virus of porcine epidemic diarrhoea in cell culture. Journal of Clinical Microbiology 26, 2235–2239.

Kim, S.Y., Song, D.S., Park, B.K., 2001. Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhea virus by RT-PCR. Journal of Veterinary Diagnostic Investigation 13 (6), 516–520.

Kweon, C.H., Kwon, B.J., Jung, T.S., Kee, Y.J., Hur, D.H., Hwang, E.K., Rhee, J.C., An, S.H., 1993. Isolation of porcine epidemic diarrhea virus (PEDV) infection in Korea. Korean Journal of Veterinary Research 33, 249–254.

Landis, J.R., Koch, G.G., 1977. The measurement of observer agreement for categorical data. Biometrics 33 (1), 159–174.

Martelli, P., Lavazza, A., Nigrelli, A.D., Merialdi, G., Alborali, L.G., Pensaert, M.B., 2008. An epidemic of diarrhoea caused by Porcine Epidemic Diarrhoea virus in Italy. Veterinary Record 162 (10), 307–310.

Pensaert, M.B., DeBouck, P., 1978. A new coronavirus-like particle associated with diarrhea in swine. Archives of Virology 58 (3), 243–247.

Pensaert, M.B., Sang-Geon, Y.E.O., 2006. Porcine epidemic diarrhea. In: Straw, B.E., Zimmerman, J.J., D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine, 9th ed. Blackwell Publishing, Oxford, pp. 367–372.

Pritchard, G.C., Paton, D.J., Wibberley, G., Ibata, G., 1999. Transmissible gastroenteritis and porcine epidemic diarrhoea in Britain. Veterinary Record 144 (22), 616–618.

Rodak, L., Valicek, L., Smid, B., Nevorankova, Z., 2005. An ELISA optimized for porcine epidemic diarrhoea virus detection in faeces. Veterinary Microbiology 105 (1), , 9–17.

Shibata, I., Ono, M., Mori, M., 2001. Passive protection against porcine epidemic diarrhoea (PED) virus in piglets by colostrum from immunized cows. The Journal of Veterinary Medical Science 63, 655–658.

Takahashi, K., Okada, K., Ohshima, K., 1983. An outbreak of swine diarrhoea of a new type associated with coronavirus-like particles in Japan. The Japanese Journal of Veterinary Science 45, 829–832.

Tjissen, P., Kurstak, E., 1984. Highly efficient and simple methods for the preparation of peroxidase and active peroxidase–antibodies conjugates for enzyme immunoassays. Analytical Biochemistry 136, 451–457.

Van Nieuwstadt, A.P., Zetstra, T., 1991. Use of two enzyme-linked immunosorbent assays to monitor antibody responses in swine with experimentally induced infection with porcine epidemic diarrhoea virus. American Journal of Veterinary Research 52 (7), 1044–1050.