ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF THE CORONAVIRUS-LIKE AGENT AND ITS ANTIBODIES IN PIGS WITH PORCINE EPIDEMIC DIARRHEA

P. CALLEBAUT, P. DEBOUCK and M. PENSAERT

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

(Accepted 15 February 1982)

ABSTRACT

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

An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of
the coronavirus-like agent in feces of pigs naturally affected with porcine epidemic diarrhea
(PED) or experimentally infected with the CV777 isolate. The assay was specific and more
sensitive than electron microscopy. An ELISA blocking assay is described for the detec-
tion and titration of antibodies. Specific antibody formation was demonstrated in pigs
experimentally infected with CV777 and in swine naturally affected with PED.

INTRODUCTION

A coronavirus-like agent (CVLA) is associated with diarrhea affecting swine
of all ages. This type of diarrhea has been called "porcine epidemic diarrhea"
(PED) (Pensaert, 1981). Based on its electron microscopic appearance, the
agent (isolate CV777) has been proposed as a member of the Coronaviridae
family (Pensaert and Debouck, 1978). To date, infections by this agent have
been diagnosed by the demonstration of the virus in intestinal epithelium
using immunofluorescence (IF) on gut sections and in feces using electron
microscopy (EM) (Debouck et al., 1981b). Antibodies to CV777 have been
demonstrated by indirect IF staining and by immunoelectron microscopy
(Pensaert et al., 1981). All these methods are rather cumbersome and time
consuming for large scale diagnostic and serological purposes. The epizootiol-
ogy of CVLA has, therefore, not been examined in detail.

The ELISA has found wide application in the detection of antibodies and
small amounts of antigen and seems to be particularly suitable for widespread
epizootiological studies. The assay has been used for the detection of bovine
coronavirus in feces (Ellens et al., 1978a) and for the serology of several
coronaviruses, including feline infectious peritonitis virus (Osterhaus et al.,
1979), murine hepatitis virus (Peters et al., 1979, Kraaijeveld et al., 1980a)

and human coronavirus strain 229E (Kraaijeveld et al., 1980a,b).

In the present report an ELISA is described for the detection of CVLA antigens in crude fecal suspensions and the results are compared with those of EM. It will also be shown that ELISA is useful for the detection of antibodies against CV777 in swine sera.

MATERIALS AND METHODS

Specimens of fecal material and of intestinal contents

Specimens, to be examined for CVLA antigens by ELISA, consisted either of fecal material, obtained directly from the rectum of live animals, or of contents collected from the caecum at the time of killing. The specimens originated not only from experimentally infected pigs but also from naturally infected animals and from controls. After collection all samples were stored at $-70°C$ until further processing.

Specimens from experimental pigs

Intestinal contents were obtained from two uninoculated, cesarean-derived colostrum-deprived (CDCD) piglets and from 17 CDCD piglets, orally inoculated at the age of 2 days to 2 weeks with approximately 10^4 pig-infective-doses (PID) of CVLA isolate CV777 (Debouck and Pensaert, 1980; Debouck et al., 1981a). Specimens were collected from three piglets that were killed during the incubation period (1 and 2 days after inoculation), from twelve piglets killed during the phase of severe diarrhea (2 to 5 days after inoculation) and from two pigs killed during the stage of recovery (6 to 8 days after inoculation).

Thirty-six samples of fecal material were collected from seven CDCD piglets (Nos. 1 to 7) during an 8 day observation period following oral administration of 10^4 PID of CV777 at the age of 2 weeks. From each piglet, a sample was collected prior to inoculation and during the incubation period 1 day after inoculation. Further samples were collected during the stage of severe diarrhea (3 to 5 days after inoculation) and towards the end of diarrhea (6 to 8 days) as indicated in Table II.

Fecal specimens were also obtained from an uninoculated conventional fattening pig and from two conventional fattening pigs during the acute phase of diarrhea, 2 days after oral inoculation with 10^4 PID of CV777.

Specimens from affected swine in the field

A fecal specimen was collected from each of 23 conventional pigs of varying ages originating from four different farms on which a natural outbreak of PED had been diagnosed by IF staining on gut sections of sacrificed animals. At the time of collection, 18 of these pigs had diarrhea while the remaining five pigs had been convalescent for more than 1 week.

Control specimens

Control fecal samples, used to determine the limit between positive and negative absorbance values in ELISA, consisted of 25 non-diarrheal specimens obtained from conventional pigs of all ages. Control samples, to test the specificity of ELISA, consisted of 23 fecal specimens from piglets with diarrhea, caused by transmissible gastroenteritis virus (TGEV) or porcine rotavirus.

Serum specimens

A total of 62 serum samples, to be examined for antibody by ELISA blocking, were collected from four CDCD piglets and seven conventional experimental fattening pigs prior to inoculation with CV777 and at various intervals between 7 and 81 days thereafter. Additionally, 76 serum specimens were obtained from 42 conventional pigs of all ages on five different farms. The latter samples were collected from 5 to 180 days after the onset of an outbreak of PED.

Control serum specimens consisted of a convalescent serum from two conventional pigs recovered from TGE- and rotavirus infection and of three hyperimmune pig sera prepared respectively against a virulent Belgian strain of TGEV, hemagglutinating encephalomyelitis virus (HEV) strain VW572 (Pensaert and Callebaut, 1974) and porcine rotavirus strain RV277 (Debouck and Pensaert, 1979). These hyperimmune sera were prepared as described elsewhere (Pensaert et al., 1981). All sera were stored at −20°C until used.

ELISA procedure for the detection of CVLA antigen

For the detection of CVLA antigen by ELISA, the "double antibody sandwich" form of the assay was used. It was conducted essentially as described by Ellens et al. (1978,b) in flat-bottomed microplates (Cooke Microtitre M 129 B), using 100 μl volumes of reagents per well. Plates were coated with a 1/2000 dilution of anti-CV777 globulin, prepared according to the method of Purcell et al. (1973) from a hyperimmune porcine antiserum, obtained as described elsewhere (Debouck and Pensaert, 1980). Samples to be examined were first homogenized by shaking for 1 h at 4°C in four volumes of phosphate buffered saline, pH 7.2 (PBS), containing 0.01% Tween 80, and each was then added to two coated wells.

Controls included PBS and a series of twofold dilutions of a standard CV777 antigen preparation. The latter preparation was intestinal perfusate (Debouck and Pensaert, 1980). Briefly, a CDCD piglet was inoculated orally with a bacteria-free filtrate of a 20% intestinal homogenate containing CV777. After 27 h, when the diarrhea started, the small intestinal lumen of the anesthetized piglet was perfused with 1500 ml Eagle's minimal essential medium during 12 h at 37°C. The perfusate was subsequently centrifuged at 3000 × *g* at 4°C for 1 h. The supernatant, containing CV777 antigen, was known to contain 320 ELISA units (E.U.) in previous titrations.

Specificity testing was performed by a blocking assay. The first sample well was treated with a porcine negative control serum, diluted 1/10; to the second sample well a 1/10 dilution of porcine anti-CV777 serum was added. Both sera were obtained from pigs, different from that which provided the serum used for preparation of the anti-CV777 globulin-fraction.

The conjugate used was the anti-CV777 globulin preparation, labelled with horseradish peroxidase (Grade I, Boehringer) according to the method described by Wilson and Nakane (1978). The optimal working dilution was 1/600. The amount of conjugate bound was determined by adding the enzyme substrate solution, containing 1 mg/ml of recrystallized 5-aminosalicylic acid, 0.005% hydrogen peroxide, 1 mM Na_2 EDTA and 0.01 M sodium phosphate, final pH 6.0. After overnight reaction at 4°C, the absorbance of each well was measured at 450 nm against the PBS blank using a Multiskan colorimeter (Flow Laboratories). A sample was scored positive if the absorbance in the well treated with negative control serum was equal to or higher than that of the 1/320 dilution of the standard CV777 preparation and if the absorbance value was reduced by > 50% in the well treated with anti-CV777 serum.

ELISA procedure for the detection of antibody to CV777

Antibody to CV777 in serum was measured by an ELISA blocking assay, performed in a manner similar to the blocking assay used to control the specificity of the CV777 antigen ELISA. The same anti-CV777 globulin preparation, diluted 1/500, was used to coat the plates. Antigen for all assays was the standard CV777 preparation, diluted 1/20 in PBS supplemented with 2.5% fetal bovine serum and 0.05% Tween 80; this amount of antigen represented 16 E.U. Test sera were serially diluted in twofold series, starting from a 1/5 dilution, in PBS containing 0.5 M NaCl, 0.05% Tween 80 and 5% porcine negative control serum, and were added to the plates to which antigen had been bound. Diluent alone served as a control. The plates were incubated for 1 h at 37°C and thereafter overnight at 4°C before the conjugate was added. The anti-CV777 antibody, used for the preparation of this conjugate, was obtained from a pig other than that which provided the coating antibody in order to decrease the chance for aspecific reactions. The optimal dilution was 1/600 in test serum diluent. The amount of conjugate bound to each well was determined as above. A given dilution of test serum was considered to be positive if it reduced the absorbance by at least 50% when compared with the buffer control. Titers are expressed as the reciprocal of the highest positive dilution.

Electron microscopy

To compare the sensitivity of EM and ELISA for the detection of CVLA, the specimens of fecal material and intestinal contents obtained from experimentally infected CDCD piglets and experimental fattening pigs as described

in the section "Specimens from experimental pigs", were examined for coronavirus by EM. The samples were processed as described elsewhere (Pensaert and Debouck, 1978).

RESULTS

Reading procedure for the detection of CV777 antigen by ELISA

The absorbance profile obtained with serially diluted perfusate in the antigen assay is shown in Fig. 1A. All 25 control non-diarrheal fecal specimens, used to determine the limit between positive and negative absorbance values, showed values in the range of 0.00 to 0.05 and these values were not reduced in the blocking assay. The mean absorbance of 0.02 plus 3 times the standard deviation resulted in an absorbance value of 0.06. Therefore, an absorbance value of > 0.06 was considered evidence of the presence of CV777 antigen in a sample. Using this criterion, the standard CV777 antigen preparation in Fig. 1A was positive up to a dilution of 1/320. This preparation was, therefore, considered to contain 320 E.U.

Specificity of the ELISA for antigen detection

In the specificity tests all 23 fecal specimens from TGEV- or rotavirus positive pigs were ELISA negative for CV777 antigen.

Additionally, there was a good correlation between the ELISA results and clinical data in the experimentally inoculated swine. As shown in Table I, all preinfection fecal or intestinal samples, obtained from experimental piglets and fattening swine, were ELISA negative. All the fecal samples obtained during the incubation period were ELISA negative. However, all three samples of intestinal contents collected during the same period were ELISA positive. The twelve samples of intestinal contents and 13 of the 14 specimens of fecal material collected during the acute phase of diarrhea were ELISA positive.

TABLE I

Detection of CVLA antigen by ELISA in fecal samples and in samples of intestinal contents collected from pigs before and at different times after inoculation with CV777

Time of collection	Clinical signs at time of collection	No. positive / No. tested	
		Fecal material	Intestinal contents
Preinoculation	none	0/8	0/2
1 to 2 dpi[a]	none	0/7	3/3
2 to 5 dpi	severe diarrhea	13/14	12/12
6 to 8 dpi	recovery period	3/10	0/2

[a]dpi = days postinoculation; pigs were orally inoculated with 10^4 PID of CV777.

Only three out of ten fecal specimens and none of two samples of intestinal contents, obtained during the stage of recovery, were ELISA positive for CV777 antigen.

Of the 23 fecal samples, collected from naturally infected pigs of all ages, 18 were ELISA positive, all of which were obtained from diarrheic animals. The remaining five samples, in which no CVLA antigen could be demonstrated, were collected from animals that had convalesced for more than 1 week.

The ELISA positive samples had absorbance values in the range of 0.07 to 0.58 (median 0.25). After blocking by incubation of the specimens with CV777 antiserum, the absorbance values were reduced in every instance by more than 50%, ranging from −0.01 to 0.10 (median 0.04).
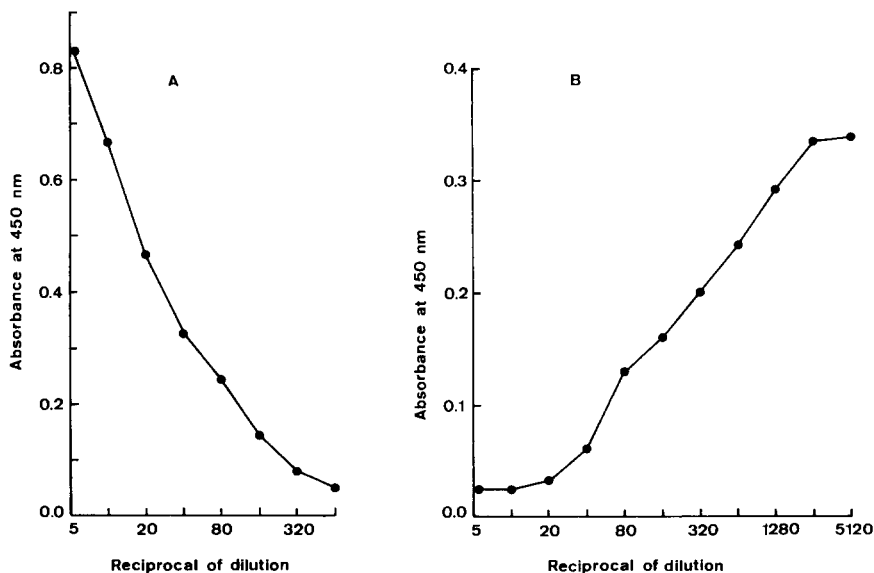

Fig. 1. ELISA absorbance values at 450 nm obtained with: (A) serial dilutions of intestinal perfusate; (B) serial dilutions of a porcine serum, the buffer control measuring 0.35.

Sensitivity of the ELISA for antigen detection compared to that of EM

As shown in Table II, the experimental piglets Nos. 4, 5, 6 and 7 were found to excrete the coronavirus-like agent in their feces both by EM and ELISA. In the remaining piglets, Nos. 1, 2 and 3, virus shedding was detected by ELISA only. In total, nine fecal specimens found negative for coronavirus-like particles by EM were ELISA positive, whereas only one EM positive specimen. was negative by ELISA. All the samples collected during the acute stage of diarrhea at 4 days after inoculation, when coronavirus was regularly but not always detected by EM, were ELISA positive. Towards the end of the diarrhea (6 and 7 days after inoculation) some EM negative samples were still ELISA positive.

TABLE II

Detection of CV777 by EM and ELISA in fecal samples from seven experimentally inoculated piglets

Piglet No.	Coronavirus detected by ELISA / EM							
	Days after inoculation							
	0	1	3	4	5	6	7	8
1	−/−ᵃ	−/−		+/−	+/−	−/−	−/−	
2	−/−	−/−	+/−	+/−			−/−	−/−
3	−/−	−/−		+/−	+/−	+/−	−/−	
4	−/−	−/−		+/+				
5	−/−	−/−		+/+				
6	−/−	−/−		+/+	−/−	−/−	+/−	
7	−/−	−/+	+/+	+/+			+/−	−/−

ᵃ+, coronavirus detected; −, coronavirus not detected.

The fecal samples of the two experimentally inoculated fattening pigs were negative for CVLA by EM, but positive by ELISA.

In intestinal contents of experimentally infected piglets, CVLA was detected at various times after inoculation by both methods. In all three specimens, collected during the incubation period, CVLA was detected by both EM and ELISA. Nine out of twelve specimens, obtained during the stage of diarrhea, were also positive by both methods. However, the three remaining specimens, obtained on day 2, 3 and 4 after inoculation respectively, were negative for coronavirus by EM, but positive by ELISA. Finally, the two samples collected at the time of recovery were negative both by EM and ELISA.

ELISA blocking assay for the detection of antibody to CV777

The absorbance profile obtained with a serially diluted porcine serum sample in the ELISA blocking test is shown in Fig. 1 B. By comparison with the buffer control, having an absorbance value of 0.35, the titer of this sample was estimated to be 160.

The sensitivity and specificity of the ELISA blocking assay in detecting a sero-conversion to CV777 infection was first established by testing pre- and post-inoculation sera obtained from experimentally infected piglets and fattening swine. As shown in Table III, upper part, all preinfection and early postinfection sera from the CDCD piglets were negative (titer < 5). Starting from 43 days after inoculation, the ELISA blocking assay detected CV777 antibodies in all the piglet sera and titers ranged from 40 to 1280.

Of the seven conventional experimental fattening pigs (Table III, lower part), three animals had no detectable antibody titers in their preinfection

TABLE III

Prevalence and titers of CV777 antibodies in sera collected from four piglets (up) and seven fattening pigs (down) at different times after inoculation with CV777, as determined by ELISA blocking assay

Time of collection	ELISA seropositive animals		
	No./No. tested	Mean titer	Range of titer[a]
Preinoculation	0/4	—	—
15 dpi.[b]	0/4	—	—
43 dpi	4/4	60	40— 80
73 dpi	4/4	240	80— 640
81 dpi	4/4	460	80—1280
Preinoculation	4/7	16	5— 40
7 dpi	7/7	1486	160— 2560
14 dpi	7/7	4023	1280—10240
21 dpi	7/7	777	320— 1280
29 dpi	7/7	366	320— 640
41 dpi	7/7	366	160— 640

[a]titers expressed as the reciprocal of the highest dilution with absorbance $\leqslant 50\%$ of the control.
[b]dpi = days postinoculation.

sera, but in the remaining animals preinfection titers varying from 5 to 40 were found. Significant antibody rises ($\geqslant$ fourfold rise in antibody titer) were detected in the sera from the latter animals 7 days after inoculation, when the ELISA blocking titers ranged from 160 to 2560. The highest titers were found on day 14 after inoculation.

The results of the serological examination of the pigs of all ages naturally infected in the field are given in Table IV. Fourteen sera were collected between 5 and 14 days after the onset of the diarrheal outbreak and were ELISA negative. All the sera except 3, collected 45 days or later after the start of the disease, contained antibodies detectable by ELISA blocking. The titers of these positive sera varied from 10 to 160.

Finally, the specificity of the ELISA blocking assay was indicated by the negative results obtained with convalescent and hyperimmune antisera directed towards other viruses, including TGEV, rotavirus and HEV.

DISCUSSION

The results obtained with intestinal contents and fecal material of experimentally and naturally infected swine demonstrate the efficacy of ELISA for the detection of CVLA antigen. The specificity of the test was proven by the results on control material containing other viruses. Further evidence

TABLE IV

ELISA detection of CV777 antibodies in sera of swine of all ages, naturally affected in the field with PED

Days after start of diarrheal outbreak	ELISA seropositive animals		
	No./No. tested	Mean titer	Range of titer[a]
5	0/8	—	—
14	0/6	—	—
30	8/25	68	5—160
45	10/12	44	10—160
60	8/8	26	10— 80
90	6/6	23	10— 40
120	5/6	48	10— 80
180	5/5	60	20—160

[a]titers expressed as the reciprocal of the highest dilution with absorbance $\leqslant 50\%$ of the control.

of the specificity is obtained by the correlation of the ELISA results with the clinical data: virus shedding is detected with high consistency during the acute phase of disease, but much less consistently during the incubation period and during the recovery phase. The ELISA results on samples collected during the latter two periods appear to depend upon the type of material tested. All samples of intestinal contents collected the first and second day after inoculation were positive, whereas all fecal samples collected 1 day after inoculation were negative. During the phase of recovery on the contrary, some fecal samples were positive, while all intestinal samples were negative. It is likely that this is a reflection of the time delay between the production and release of virus progeny in the intestine and its excretion in the feces. However, as only a small number of specimens have been tested, more extensive examination is needed to corroborate the present findings.

The sensitivity of ELISA compares favourably to that of EM. More fecal and intestinal specimens from experimentally infected pigs were positive by ELISA than by EM. ELISA is more reliable than EM as shown by the finding that during the acute phase of diarrhea, virus excretion could be demonstrated in the feces of all experimentally infected piglets by ELISA but not by EM. The finding that viral antigen is still occasionally detected by ELISA in feces towards the end of diarrhea, but not by EM, further substantiates the previous statement. For use as a diagnostic tool ELISA is to be preferred above EM because it is a specific test for CVLA, whereas EM would have to be followed by a confirmatory test such as IEM (Debouck et al., 1981b).

In order to detect CVLA antigen in fecal and intestinal suspensions the standard "double antibody sandwich" ELISA, as originally described by Ellens and De Leeuw (1977) for bovine rotavirus, has been modified in this study by incubating the specimens with porcine serum free of antibody to

CV777, prior to determination of the extent of conjugate binding. This was required to eliminate false-positive results that otherwise would have been encountered during this study in 15 of a total of 42 fecal specimens. Similar experiences have been reported with solid-phase immunoassays for detection of human rotavirus (Yolken et al., 1977; Cukor et al., 1978; Brandt et al., 1981) and Norwalk enteritis virus (Greenberg et al., 1978) in feces. As suggested by Brandt et al. (1981), these false-positive reactions may be due to the presence in the test samples of intestinal bacteria or their products, which bind nonspecifically to the antibodies used as reagents in ELISA.

The ELISA blocking assay, using crude virus-containing intestinal perfusate as antigen, is an efficient assay for the detection of an antibody response to CVLA, as shown by the clear-cut seroconversion found in sera of experimentally and naturally infected swine. The specificity of the assay is demonstrated because the monospecific sera directed against other viruses failed to react in the CV777 ELISA blocking assay. The finding that in sera of experimental piglets and of naturally infected pigs of all ages, antibodies to CV777 were only detectable by ELISA at 6 weeks after the onset of diarrhea, may be caused by a low sensitivity of the ELISA blocking assay or by a weak immunogenicity of CVLA antigen. The high antibody titers found in sera of experimental fattening pigs, very soon after inoculation, probably represent a secondary response because several animals had a low titer prior to inoculation.

To date, the routine detection of CVLA antigen in diarrheal feces and its antibody in sera from recovered swine in field outbreaks has been hampered by the lack of suitable diagnostic and serological techniques. This was related to the inability to cultivate the agent in vitro. Attempts to isolate CV777 from intestinal perfusates in porcine organ cultures and in cultures of primary and continuous cells, usually of porcine but also of bovine, simian and human origin, have failed. Pancreatic enzymes, used for treatment of cell cultures or inoculum, remained ineffective as a means to promote viral growth (Callebaut and Debouck, 1981). The development of an ELISA will, therefore, facilitate further study of this virus. Extensive epizootiological research now becomes possible, and will reveal the importance of the coronavirus-like agent as a cause of PED in field outbreaks of enteritis in pigs of all ages.

REFERENCES

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

Callebaut, P.E. and Debouck, P., 1981. Some characteristics of a new porcine coronavirus and detection of antigen and antibody by ELISA. In: Abstracts Fifth International Congress of Virology, August 2—7, 1981, Strassbourg, France. Imprimerie Centrale Commerciale, Paris, p. 420.

Cukor, G., Berry, M.K. and Blacklow, N.R., 1978. Simplified radioimmunoassay for detection of human rotavirus in stools. J. Infect. Dis., 138: 906—910.

Debouck, P. and Pensaert, M., 1979. Experimental infection of pigs with Belgian isolates of the porcine rotavirus. Zentralbl. Veterinärmed. Reihe B, 26: 517—526.

Debouck, P. and Pensaert, M., 1980. Experimental infection of pigs with a new porcine enteric coronavirus, CV777. Am. J. Vet. Res., 41: 219—223.

Debouck, P., Pensaert, M. and Coussement, W., 1981a. The pathogenesis of an enteric infection in pigs, experimentally induced by the coronavirus-like agent, CV777. Vet. Microbiol., 6: 157—165.

Debouck, P., Callebaut, P. and Pensaert, M., 1981b. The diagnosis of coronavirus-like agent (CVLA) diarrhea in suckling pigs. In: P.W. De Leeuw and P.A.M. Guinée (Editors), Laboratory Diagnosis in Neonatal Calf and Pig Diarrhoea. Current Topics in Veterinary Medicine and Animal Science, Vol. 13. Nijhoff, The Hague, The Netherlands, pp. 59—61.

Ellens, D.J. and De Leeuw, P.W., 1977. Enzyme-linked immunosorbent assay for diagnosis of rotavirus infections in calves. J. Clin. Microbiol., 6: 530—532.

Ellens, D.J., Van Balken, J.A.M. and De Leeuw, P.W., 1978a. Diagnosis of bovine coronavirus infections with hemadsorption-elution-hemagglutination assay (HEHA) and with enzyme-linked immunosorbent assay (Elisa). In: S.D. Acres, A.J. Forman and H. Fast (Editors), Proceedings Second International Symposium on Neonatal Diarrhoea, October 5, 1978, University of Saskatchewan, Canada. Veterinary Infectious Disease Organization, Sask., pp. 321—330.

Ellens, D.J., de Leeuw, P.W., Straver, P.J. and Van Balken, J.A.M., 1978 b. Comparison of five diagnostic methods for the detection of rotavirus antigens in calf faeces. Med. Microbiol. Immunol., 166: 157—163.

Greenberg, H.B., Wyatt, R.G., Valdesuso, J., Kalica, A.R., London, W.T., Chanock, R.M. and Kapikian, A.Z., 1978. Solid-phase microtiter radioimmunoassay for detection of the Norwalk strain of acute nonbacterial epidemic gastroenteritis virus and its antibodies. J. Med. Virol., 2: 97—108.

Kraaijeveld, C.A., Madge, M.H. and Macnaughton, M.R., 1980a. Enzyme-linked immunosorbent assay for coronaviruses HCV 229 and MHV 3. J. Gen. Virol., 49: 83—89.

Kraaijeveld, C.A., Reed, S.E. and Macnaughton, M.R., 1980b. Enzyme-linked immunosorbent assay for detection of antibody in volunteers experimentally infected with human coronavirus strain 229 E. J. Clin. Microbiol., 12: 493—497.

Osterhaus, A., Kroon, A. and Wirahadiredja, R., 1979. ELISA for the serology of FIP virus. Vet. Q., 1: 59—62.

Pensaert, M.B., 1981. Porcine epidemic diarrhea. In: A.D. Leman, R.D. Glock, W.L. Mengeling, R.H.C. Penny, E. Scholl and B. Straw (Editors), Diseases of Swine, 5th Edition. Iowa State University Press, Ames, IA, pp. 344—346.

Pensaert, M.B. and Callebaut, P.E., 1974. Characteristics of a coronavirus causing vomiting and wasting in pigs. Arch. Gesamte Virusforsch., 44: 35—50.

Pensaert, M.B. and Debouck, P., 1978. A new coronavirus-like particle associated with diarrhea in swine. Arch. Virol., 58: 243—247.

Pensaert, M.B., Debouck, P. and Reynolds, D.J., 1981. An immunoelectron microscopic and immunofluorescent study on the antigenic relationship between the coronavirus-like agent, CV777, and several coronaviruses. Arch. Virol., 68: 45—52.

Peters, R.L., Collins, M.J., O'Beirne, A.J., Howton, P.A., Hourihan, S.L. and Thomas, S.F., 1979. Enzyme-linked immunosorbent assay for detection of antibodies to murine hepatitis virus. J. Clin. Microbiol., 10: 595—597.

Purcell, R.H., Wong, D.C., Alter, H.J. and Holland, P.V., 1973. Microtiter solid-phase radioimmunoassay for hepatitis B antigen. Appl. Microbiol., 26: 478—484.

Wilson, B. and Nakane, P.K., 1978. Recent developments in the periodate method of con-jugating horseradish peroxidase (HRPO) to antibodies. In: W. Knapp, K. Holubar and G. Wick (Editors), Immunofluorescence and Related Staining Techniques. Proc. VIth Int. Conf., Vienna, 1978. Elsevier/North Holland Biomedical Press, Amsterdam/New York, pp. 215—224.

Yolken, R., Wyatt, R.G. and Kapikian, A.Z., 1977. ELISA for rotavirus. Lancet, 2: 819.