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Porcine epidemic diarrhea virus (CV 777) and feline infectious peritonitis virus (FIPV) are antigenically related

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Summary. Using gut sections from pigs infected with porcine epidemic diarrhea virus (strain CV 777) and ascitic fluid from cats which had succumbed to feline infectious peritonitis (FIP), a weak cross reaction was found by immunofluorescence. Its specificity was confirmed when detergent-treated purified CV 777 showed a prominent reaction with FIPV antibodies in ELISA; no reaction was obtained with intact virions, which indicated common determinants on an internal component of the particle. Antigenic cross-reactions at the nucleocapsid level were found in Western blot ELISA performed both ways (CV 777/FIPV antibodies; FIPV/CV 777 antibodies). In immunoprecipitation using [^{35}S]methionine labelled FIPV, anti-CV 777 sera recognized exclusively the nucleocapsid protein. The significance of these findings for the classification of coronaviruses is discussed.

Introduction

Porcine epidemic diarrhea is an acute viral disease in swine of all ages. Particles of the CV 777 strain of porcine epidemic diarrhea virus possess typical coronavirus morphology [2, 14] and morphopoiesis [4]. However, antigenic relationships between CV 777 and established coronaviruses have hitherto not been detected [15].

Coronaviruses show a characteristic pattern of structural polypeptides: a peplomer protein with apparent molecular weight (Mr) of 80,000 to 200,000, a nucleocapsid protein of Mr 50,000 to 60,000 and an envelope protein of Mr 20,000 to 35,000 [17, 18]. Recent studies from our laboratory have demonstrated that CV 777 is similar in this respect: it possesses a glycosylated surface protein of Mr 85,000 to 135,000, an envelope protein of Mr 20,000 to 32,000 and an unglycosylated RNA-binding protein of Mr 58,000 [5].

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Numerous studies have been done with the aim of detecting antigenic relationships between members of the family Coronaviridae. Most of this work employed tests such as serum neutralization [12], complement fixation [1, 12], gel diffusion [1], immunofluorescence (IFT) [12, 13, 15], enzyme linked immunosorbent assay (ELISA) [9] or, recently, immuno-electron microscopy (IEM) [15]. Using IEM and IFT, an antigenic relationship between CV 777 and several coronaviruses had not been found in a previous study [15]. In the present paper, the problem is re-examined with the aid of more sensitive techniques such as immunoblotting and immunoprecipitation. As will be shown, CV 777 is indeed antigenically related to a coronavirus at the nucleocapsid level.

Materials and methods

Antigen preparation

For IFT, frozen sections 4 µm thick were prepared from the jejunum of a cesarean-derived colostrum-deprived (CDCD) piglet which had been experimentally infected with the CV 777 isolate; frozen sections from the jejunum of a non-infected CDCD piglet were prepared in the same way to serve as a control.

The CV 777 antigen for the ELISA and immunoblot assay was prepared from faecal material collected from an experimentally infected CDCD piglet. Twenty ml of the faecal suspension was clarified by centrifugation at 10,000 × **g** for 30 minutes. The virus was then concentrated by centrifugation at 100,000 × **g** and 4 °C for 2 hours. The supernatant was discarded and the pellet was resuspended in 1 ml of TES buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl). After another centrifugation at 10,000 × **g** for 5 minutes, the virus-containing supernatant was saved and kept frozen until use.

Feline infectious peritonitis virus (FIPV) strain 79–1146 was grown in cells of the Norden Laboratories feline kidney (NLFK) line. For immunoblot assays, FIPV infected NLFK cell lysate was used, with mock infected lysate serving as a control. Monolayers of NLFK cells were inoculated with FIPV as described before [8]. After 7 to 8 hours, the medium was removed from the infected and mock-infected cells, respectively, and the monolayers were washed once with phosphate buffered saline (PBS, 0.1 M phosphate buffer pH 7.4, 0.15 M NaCl). The cells in a 75 cm^2 Costar flask were lysed by adding 2 ml of lysis buffer (0.5% Triton X-100, 0.5% naphthalene disulfonic acid-Na_2 and 0.14 M phenylmethyl sulfonyl fluoride in TES buffer: 0.01 M Tris · HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl), and the lysate were clarified by centrifugation at 10,000 × **g** for 2 minutes.

Antisera

Anti-CV 777 hyperimmune sera had been prepared in CDCD piglets. The ascitic fluids used as a source of FIPV antibody (A 18, A 27, A 36) had been collected from naturally infected cats. A specified pathogen-free cat serum had been obtained from the Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands.

For direct blot-ELISA, the anti CV 777 hyperimmune serum was purified by standard ammonium sulphate precipitation and DEAE-Sephacel (Pharmacia Fine Chemicals AB, Uppsala, Sweden) chromatography; the purified immunoglobulins were conjugated with horseradish peroxidase (HRP, Boehringer Mannheim GmbH, Federal Republic of Germany) according to Wilson and Nakane [19].

Immunofluorescence test

The IFT was performed on frozen sections of CV 777-infected and non-infected jejunum samples. The tissues were fixed onto glass slides with precooled acetone at − 20 °C. After

3 cycles of washing with PBS (5 minutes each) the tissues were overlaid with 1 : 10 dilutions of pig antiserum and cat ascitic fluids, respectively, followed by incubation in a moist chamber at 37 °C for 45 minutes. After 3 more washes, the FITC-conjugated rabbit anti-swine IgG or rabbit anti-cat IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) diluted in PBS were pipetted onto the tissues and the slides were incubated as before and rinsed again. They were mounted with 90% glycerol in PBS and examined with a fluorescence microscope.

Enzyme linked immunosorbent assay

The ELISA was carried out in 96-well flat bottom microtiter plates (BIOREBA F). The wells were coated overnight at 4 °C with 100 µl-volumes of Triton X-100 (1%) pretreated and native CV 777 antigen (0.5–0.1 µg), respectively, diluted in coating buffer. After 3 cycles of washing with a 0.15 M NaCl solution containing 0.05% Tween 80, 100 µl-volumes of antiserum diluted in a buffer consisting of 0.15 M NaCl, 1 mM EDTA, 0.05 M Tris pH 7.4, 0.05% Tween 20 and 5% foetal calf serum (FCS) were pipetted into the wells, and the plates were incubated for 2 hours at 37 °C. They were then washed as described above and 100 µl-volumes of HRP-conjugated rabbit anti-swine IgG (Dakopatts, Glostrup, Denmark) or goat anti-cat IgG (Cappel Biomedical Inc., Malvern, PA, U.S.A.) were added. After an incubation period of 1 hour at 37 °C followed by another 3 cycles of washing, 200 µl of the substrate solution (ABTS, 2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]; Sigma, St. Louis, MO, U.S.A.) was pipetted into the wells. The reactions were read at 405 nm in a Titertek Multiscan Reader (Flow Laboratories, Irvine, Scotland).

Western blotting

The proteins in the respective antigen preparations were separated by electrophoresis in 12.5% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS-PAGE) and were electrophoretically transferred to nitrocellulose sheets (BA 85, 0.45 µm, Schleicher und Schuell, Dassel, Federal Republic of Germany) using a LKB NovaBlot Electrophoretic Transfer Unit (LKB-Produkter AB, Bromma, Sweden) at 0.8 mA/cm^2 for 1 hour in continuous transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% SDS and 20% methanol). After transfer, the nitrocellulose filters were blocked overnight at room temperature using gelaton buffer (0.5% pig skin gelatin, 0.1% Triton X-100 in PBS). The filters were incubated at 37 °C for 1 hour on a rocker platform with the antisera diluted in gelatin buffer. After 3 washings with gelatin buffer (15 minutes each at room temperature), the filters were incubated with HRP-conjugated anti-species IgG at 37 °C for another hour. They were then washed 3 times as above and once with PBS alone. The enzyme reaction was visualized by soaking the filters in freshly prepared substrate solution (0.5 mg/ml 3,3-diaminobenzidine tetrahydrochloride from Sigma, St. Louis, MO, U.S.A. and 0.075% H_2O_2 in PBS). The reactions were stopped by submerging the filters in 3% trichloroacetic acid.

Radioimmunoprecipitation

Mock- and FIPV-infected monolayers of NLFK cells in 25 cm^2 Costar flasks were labelled with [^{35}S]methionine starting at 6 hours after infection. The medium was removed, the monolayers were rinsed once with PBS and 2 ml of methionine-free Eagle's minimum essential medium (Gibco) supplemented with 2% foetal FCS and 60 µCi [^{35}S]methionine (specific activity, 1,440 Ci/mM; Amersham International) was added. After another hour of incubation, cell lysates (in 500 µl) were prepared as described above. Label incorporation was determined after precipitation with 5% TCA using a liquid scintillation counter.

For RIP, 12 µl of [^{35}S]methionine-labelled FIPV or mock infected cell lysate containing approximately 200,000 cpm was diluted with 100 µl lysis buffer and mixed with 3 µl-volumes

of undiluted serum or ascitic fluid. Immune complexes were allowed to form at room temperature for 1 hour, and subsequently KCl (final concentration 0.5 M) and 25 µl of a 10% suspension of formaldehyde-fixed *Staphylococcus aureus* cells were added. After incubation for 45 minutes at room temperature, the immune complexes were pelleted at 10,000 × **g**, washed 3 times with TES buffer containing 0.1% Triton X-100 and resuspended in Laemmli sample buffer (60 mM Tris · HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol). The proteins were analyzed in 12.5% gels by SDS-PAGE and fluorography as described previously [16].

Results

Both pig anti CV 777 hyperimmune serum and cat FIP ascitic fluids showed fluorescence in CV 777 infected epithelial cells of the small intestinal villi. With heterologous antibody, the fluorescence was much weaker than with homologous serum; however, distribution of fluorescent cells in the tissue was identical to the positive control (results not shown). No fluorescence was seen when pig antiserum or FIP ascitic fluids were tested on normal gut sections or when a negative pig serum and an SPF cat serum were tested on CV 777-infected sections (Table 1).

Since these results suggested a cross-reaction between FIPV antibodies and CV 777 antigen, we attempted to confirm the reaction by ELISA. In order to detect possible cross-reactions also at the level of internal virion proteins, CV 777 was disrupted with Triton X-100 and the results were compared with those obtained with intact virus. The positive/negative (P/N) ratios were calculated by dividing the absorbance readings obtained with the positive sera or ascitic fluids (P) by the values obtained with the negative sera (N).

A quotient of 1.0 would mean that no specific reaction has occurred. We obtained P/N ratios in the homologous reaction of 3.1 ± 0.0 for disintegrated virus and of 2.0 ± 0.0 for the native CV 777 preparation, respectively. An average P/N ratio of 2.5 ± 0.7 ($P < 0.01$) was found when FIP ascitic fluid A 27 had reacted with Triton X-100 treated CV 777 whereas a ratio of 0.9 ± 0.2 was

Table 1. Heterologous immunofluorescence in CV 777 infected intestinal epithelium cells

Frozen sections	Positive pig anti CV 777 serum	Negative pig serum	Cat FIP ascitic fluids			SPF cat serum
			A 27	A 36	A 38	
CV 777-infected gut	+ + +	−	+	+	+	−
non-infected gut	−	−	−	−	−	−

+ + + Strong fluorescence
+ Weak fluorescence
− No fluorescence

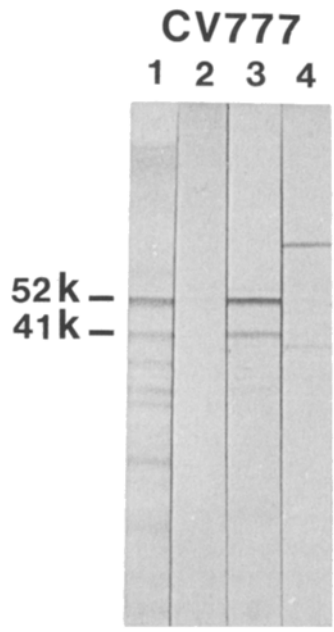

Fig. 1. Western blots of CV 777 antigen reacted with pig anti-CV 777 hyperimmune serum (*1*), pre-immunization pig serum (*2*), cat FIP ascitic fluid (*3*) and SPF cat serum (*4*). Pig sera were used at a 1 : 100 dilution, cat materials at 1 : 20

obtained with native CV 777 antigen. We concluded from these observations that the heterologous reaction involved internal components of CV 777.

As a next step, immunoblotting experiments were designed to identify the viral subunit responsible for the cross reaction. Figure 1 shows the Western blot-ELISA of CV 777 proteins with homologous and heterologous antibodies. When homologous antiserum was used, prominent bands were seen at positions corresponding to Mr of 52,000 and 41,000; they probably relate to the RNA-binding protein of Mr 58,000 found in 15% gels [5] and its degradation product. At the same positions bands were seen in the lane with FIP ascitic fluid was used. At these Mr locations, bands were absent in the lanes where a pre-immunization pig serum or a SPF cat serum had been used.

In the second series of experiments, we studied the inverse reaction, i.e. FIPV antigen with homologous and CV 777 antibodies (Fig. 2). Using FIP ascitic fluid, a strong band with a calculated Mr of 43,000 and three additional bands between Mr 25,000 and 30,000 (representing the nucleocapsid and envelope proteins, respectively, of FIPV) [7, 8] can be seen. When an anti CV 777 hyperimmune serum is used, a band is seen occupying the same place as the FIPV nucleocapsid protein. Porcine epdidemic diarrhea virus immune sera from Belgium and the Netherlands gave identical patterns (results not shown). The specificity of the reaction was confirmed by the empty appearance of the lanes where negative cat and pig serum had been used. No reaction was found at the nucleocapsid protein position when blots of a mock infected NLFK cell lysate had been incubated with anti-CV 777 antibodies.

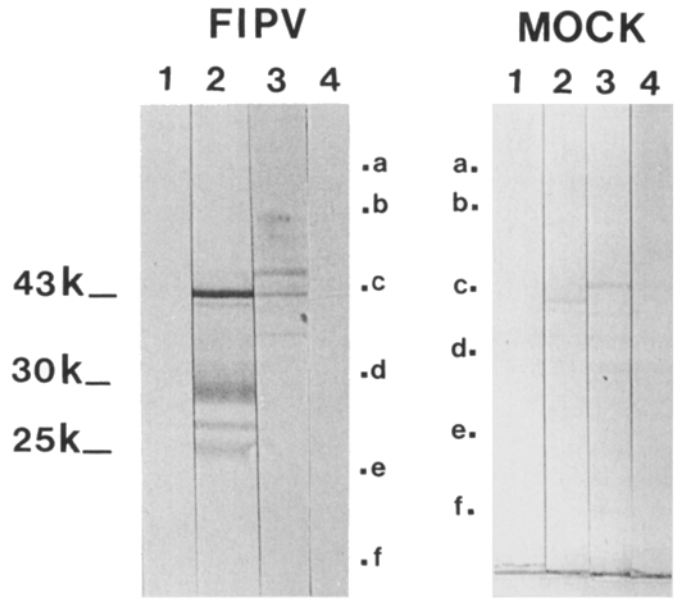

Fig. 2. Western blots of FIPV infected and mock-infected NLFK cell lysates reacted with an SPF cat serum (*1*), FIP ascitic fluid (*2*), pig anti-CV 777 hyperimmune serum (*3*) and pre-immunization pig serum (*4*). The positions of the marker proteins in both gels are indicated by dots; apparent molecular weights are 94,000 (*a*), 67,000 (*b*), 43,000 (*c*), 30,000 (*d*), 20,100 (*e*) and 14,400 (*f*). Cat materials were used at a dilution of 1 : 400, pig sera at 1 : 50

Immunoblotting would not detect reactions with conformation-dependent antigenic determinants. To show the reaction between homologous or heterologous antibodies and FIPV antigens under non-denaturing condition, immunoprecipitation was employed using [^{35}S]methionine labelled infected and non-infected cell lysates. The inverse experiment was not performed since CV 777 virus cannot be grown and labelled in culture. As shown in figure 3, results of the RIP confirmed the cross-reaction between pig anti-CV 777 sera and the 43,000 protein of FIPV found in Western blots; the homologous reaction reveals the typical pattern of FIPV structural polypeptides with Mr of 210,000 (peplomer), 45,000 (nucleocapsid) and 25,000 to 32,000 (envelope). No precipitation was seen in this Mr range using a negative pig serum or a mock infected lysate.

Discussion

Porcine epidemic diarrhea virus, strain CV 777 has been tentatively classified as a member of the family Coronaviridae based on its morphologic, morphogenetic and physico-chemical properties [2, 4 14]. Characteristics of the viral structural polypeptides supported this taxonomic proposal [5]. We now present evidence for an antigenic relatedness between CV 777 and FIPV which provides a decisive argument in favor of the classification of CV 777 as a coronavirus: in Figs. 1 to 3, a two-way cross reaction is shown between the nucleocapsid

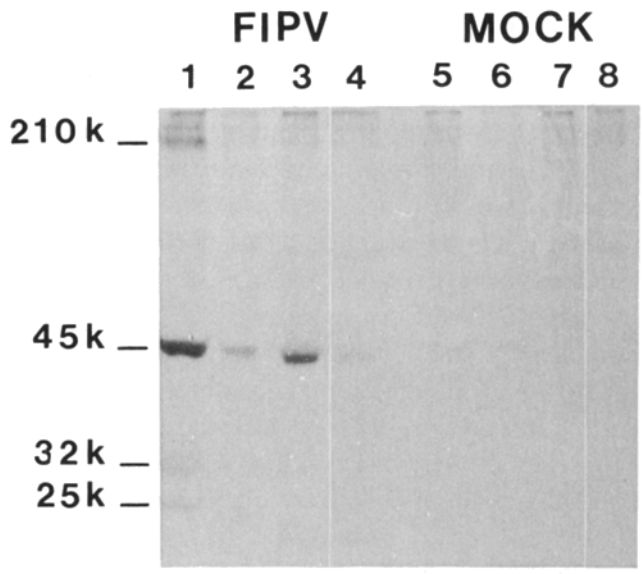

Fig. 3. Immunoprecipitation using [^{35}S]methionine-labelled FIPV infected cell lysate (*1–4*) and mock infected lysate (*5–8*) with cat FIP ascitic fluid (*1* and *5*) and pig anti-CV 777 serum (*3* and *7*); a SPF cat serum (*2* and *6*) and a pre-immunization pig serum (*4* and *8*) served as controls

proteins of both viruses. No antigenic relationship could be detected between the viral envelope (E 1) and peplomer (E 2) proteins of FIPV and CV 777.

At the present time, coronaviruses are assigned to four antigenic clusters. In 1978, Pedersen et al. studied eight mammalian coronaviruses and established two groups on the basis of cross-reactivities by immunofluorescence; the authors found that viruses within one group were antigenically unrelated to members of the second group [13]. We have confirmed the finding of cross reactions within one group, namely between transmissible gastroenteritis virus (TGEV) of swine, FIPV and canine enteric coronavirus, and showed that common determinants are present on all three structural polypeptides [7]. Surprisingly, another study at the virion subunit level revealed an antigenic relatedness between mouse hepatitis virus type 3 (MHV) and human coronavirus (HCV) 229 E which had been assigned to different clusters [6]. Also, the HCV 229 E nucleocapsid protein can be detected by antisera directed against TGE, MHV and hemagglutinating encehalomyelitis virus (HEV) of swine [21]. We have obtained preliminary evidence in immunoblotting assays that MHV strain A 59 and avian infectious bronchitis virus (IBV) strain M 41 cross-react at the nucleoprotein level [Niesters 1986, unpublished observations], as do also FIPV and HEV [Yaling 1987, unpublished observations]. These observations, together with the finding that amino acid sequences are conserved between coronaviruses from different antigenic clusters [3], indicate that common antigenic determinants may exist which are suitable for the identification of many, if not all, members of the family.

We have reported earlier that antigenic cross-reactions between CV 777 and other coronaviruses could not be detected by IFT [15]. Using ELISA, immunoblotting and RIP in the present study, however, we did find a cross reaction of CV 777 with FIPV. Upon re-examination, a reaction was also seen by IFT, which—when assessed by itself—would have appeared too weak to be considered as specific. Several explanations can be offered for the discrepancies obtained with the different techniques. The selected polyclonal antiserum may be crucial. It has been demonstrated that, e.g., FIP ascitic fluids may lack antibodies against one or two of the three viral proteins [8]. Since a cross reaction may exist at the level of only one of them—as found in this study—the antigenic relationship may thus escape detection. Cross-reactivities may vary with different serological variants of the viruses compared (e.g., MHV and HCV); the existence of antigenic differences between FIP viruses should also be taken into account [12, 13]. Due to its low sensitivity, the fluorescent antibody technique is not ideal for the examination of viral cross reactivities and contradictory results have indeed been obtained [1, 13, 15, 20]. In view of the high frequency of mutation and recombination of coronaviruses, the establishment of antigenic clusters within the family may be of only limited value.

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