

An Immunoelectron Microscopic and Immunofluorescent Study on the Antigenic Relationship Between the Coronavirus-Like Agent, CV 777, and Several Coronaviruses

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With 2 Figures

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Summary

A possible antigenic relationship between the porcine enteropathogenic coronavirus-like agent (CVLA) and 6 known coronaviruses was examined by immunoelectron microscopy (IEM) and by immunofluorescence (IF). CVLA did not show cross reactivity with infectious bronchitis virus, transmissible gastroenteritis virus (TGEV), canine coronavirus (CCV) hemagglutinating encephalomyelitis virus (HEV), neonatal calf diarrhea coronavirus (NCDCV) or feline infectious peritonitis virus (FIPV). Antigenic relationship was detected by IEM between TGEV and CCV, NCDCV and HEV and by IF between TGEV and CCV, TGEV and FIPV, HEV and NCDCV.

Introduction

In the third report of the International Committee on Taxonomy of Viruses, the family Coronaviridae is cited to contain 5 definite members, 5 probable members and 2 possible members (8). The 12 species of this monogeneric family are grouped mainly on physico-chemical characteristics. They are pleomorphic enveloped particles, averaging 10 nm diameter, containing RNA and essential lipid. They all bear unique definite projections. The coronaviruses multiply in cytoplasm and mature by budding through endoplasmic reticulum (18). Some coronaviruses cause respiratory problems in birds and man, others are associated with enteritis in different species and some coronaviruses affect multiple organs (13). Antigenic relationships exist only between some members of this family (18).

In 1977, a coronavirus-like agent (CVLA) was isolated from an epizootic of diarrhea on Belgian swine breeding farms (14). Based on its morphologic characteristics, the CVLA was suggested to be a tentative member of the family Corona-

viridae (4). The CVLA was shown by serologic cross protection studies to differ from the two known porcine coronaviruses, transmissible gastroenteritis virus (TGEV) and hemagglutinating encephalomyelitis virus (HEV) (4, 14).

The purpose of the present report is to compare CVLA antigenically with 6 known coronaviruses. The demonstration of antigenic similarities to accepted coronaviruses would certainly contribute to definite classification of CVLA within the family Coronaviridae. This study was performed by means of immunoelectron microscopy (IEM) and immunofluorescence (IF). Both techniques have been used before to study serologic differences between virus species within a virus family (3a, 10, 20). The coronaviruses selected for the present study were TGEV, HEV, neonatal calf diarrhea coronavirus (NCDCV), canine coronavirus (CCV), avian infectious bronchitis virus (IBV) and feline infectious peritonitis virus (FIPV).

Materials and Methods

Immunoelectron Microscopy

Origin of Antigens

The CVLA was obtained by intestinal perfusion of a cesarean-derived colostrum-deprived (CDCD) piglet, previously inoculated orally with an intestinal homogenate containing the CV 777 isolate (4).

The cell culture-adapted Purdue strain of TGEV grown in SK-6 cells and the 11th cell culture passage of the VW 572 strain of HEV (12) grown in primary porcine kidney cells, were used as infected tissue culture fluid.

The Massachusetts-41 strain of IBV, in the form of allantoic fluid of inoculated embryonic eggs, was obtained from Dr. SPANOGHE, Laboratory of Avian Pathology, State University of Gent, Belgium.

NCDCV (Norden Laboratories vaccine strain) was grown in primary calf kidney cells, then concentrated 10-fold by precipitation with 40 per cent saturated ammonium sulphate.

The American Type Culture Collection strain 1-71 of CCV (2) was grown in secondary dog kidney (DK/2) cells.

FIPV was omitted from the antigens used for IEM as starting material with satisfactory morphology could not be obtained.

Preparation of Specific Antisera

A monospecific hyperimmune serum against the CV 777 isolate of CVLA was prepared in a CDCD pig. In the indirect fluorescent antibody technique using small intestinal cryostat sections of an experimentally inoculated pig, this antiserum had a titer of 1:900 (4).

Hyperimmune serum against a virulent Belgian strain of TGEV was raised in a conventionally reared pig. This hyperimmune serum had a virus neutralizing titer of 1:2560 when tested against the cell culture adapted Purdue strain of TGEV. This serum had no detectable antibodies against the CVLA and HEV when tested by the indirect fluorescent antibody technique and by the seroneutralization test respectively.

A monospecific hyperimmune serum against the VW 572 strain of HEV was prepared in a CDCD pig (1). This serum had a virus neutralizing titer of 1:12,288 in a microplate neutralization test with the cell culture adapted VW 572 strain grown in PK-15 cells (15).

Chicken IBV antiserum was provided by Dr. SPANOGHE, Laboratory of Avian Pathology, State University of Gent, Belgium. This serum was raised in a SPF chicken and had a hemagglutinationinhibition titer of 1:256 when tested against the Massachusetts-41 strain.

The NCDCV (British isolate) antiserum was obtained from a gnotobiotic calf and had a titer of 1:1,600 in an indirect immunofluorescence test.

CCV convalescent serum was obtained from a normal dog in the open population. This serum had a titer of 1:200 in a microtiter neutralization test using 100 TCID₅₀ of CCV in DK/2 cells.

The IEM Test

Virus-containing fluids were clarified at 3000×g, at 4° C for 30 minutes. The supernatant was sonified for 30 seconds¹ and clarified again at 10,000×g, at 4° C for 10 minutes. Antisera were inactivated at 56° C for 30 minutes. One hundred µl of antigen was mixed with 100 µl of an appropriate serum dilution which was determined in the homologous systems. The mixture was held at 37° C for 1 hour and at 4° C overnight. One drop of the mixture was then placed on 200 mesh formvar-coated grids and stained with 2 percent K-phosphotungstate, pH 6.1. Grids were examined using a Zeiss EM 9 S-2 electron microscope. Micrographs were taken at an instrumental magnification of 28,000× and photographically enlarged to 84,000×.

Immunofluorescent Staining Technique

Preparation of the Antigens

Frozen sections from jejunum of a CDCD pig, experimentally infected with the CV 777 isolate, were used as the source of CVLA antigen (4). The preparation of the sections has been previously described (11).

Frozen sections from the jejunum of a piglet, naturally infected with TGEV, were used as the source of TGEV antigen. These sections were shown to be free from CVLA, HEV and rotavirus.

The VW 572 strain of HEV was cultivated in PK-15 cells grown on Leighton coverslips.

Primary calf kidney cell monolayers were grown in microtiter trays (Sterilin, England) and infected with NCDCV (Norden Laboratories). After 48 hours the cell sheets were fixed with 80 per cent acetone and used as antigen.

Coverslip cultures of DK/2 cells, infected with CCV 1-71 were harvested after 24 hours incubation.

Brain smears from FIPV infected mice were obtained from Dr. OSTERHAUS, National Institute of Public Health, Bilthoven, the Netherlands. They were used as the source of FIPV antigen. The propagation of FIPV in mouse brain has been previously described (9).

Due to non-specific staining reactions, the IBV system could not be used for the present immunofluorescent studies.

Source of Antibodies and Preparation of Conjugated Antisera

The globulin fraction of the hyperimmune sera against CVLA, TGEV and HEV, mentioned above, was conjugated with fluorescein isothiocyanate (FITC). Dilutions of the conjugated antisera were tested for optimal fluorescence in their homologous system. The optimal dilution, usually 1:20, was then used in the heterologous systems.

The antisera against NCDCV and CCV, mentioned above, were used in an indirect IF staining technique. In the homologous system, the NCDCV antiserum produced bright fluorescence at a dilution of 1:10 and the CCV antiserum at a dilution of 1:5. FITC conjugated anti-bovine and anti-dog globulins were produced in rabbits and obtained from Nordic Immunological Laboratories, Maidenhead, Berks, U.K.

The ascitic fluid from a cat naturally infected with FIPV was obtained from Dr. PASTORET, Laboratory of Virology, State University of Liège, Belgium. This ascitic fluid had a virus-neutralizing titer of > 6144 when tested against the cell culture adapted Purdue strain of TGEV. The globulin fraction of this fluid was conjugated with FITC². The undiluted conjugate produced bright fluorescence in FIPV—infected mouse brain smears.

¹ MSE Ultrasonic Disintegrator, Crawley, Sussex, England.

² Prepared by Drs. LEUNEN and BRONT, National Institute for Veterinary Research (NIDO), Brussels, Belgium.

Fluorescent Antibody Staining Procedure

Indirect fluorescent antibody staining was carried out as follows. Antigen-containing substrates were treated with the optimal dilution of antiserum. After an incubation period of 45 minutes in a moistened chamber at 37° C, the substrates were washed in 3 changes of phosphate buffered saline solution (PBS) for 10 minutes each. They were subsequently stained with the FITC conjugated antiglobulins. After an incubation time of 45 minutes at 37° C in a moistened chamber, the substrates were washed as described above. Finally they were rinsed in distilled water for 1 minute and dried in a warm air stream. All substrates were mounted with 90 per cent glycerol in PBS except for the microtiter trays which were read unmounted.

Direct fluorescent antibody staining was carried out by treating antigen-containing substrates with an optimum dilution of FITC-labelled antiviral serum as described above.

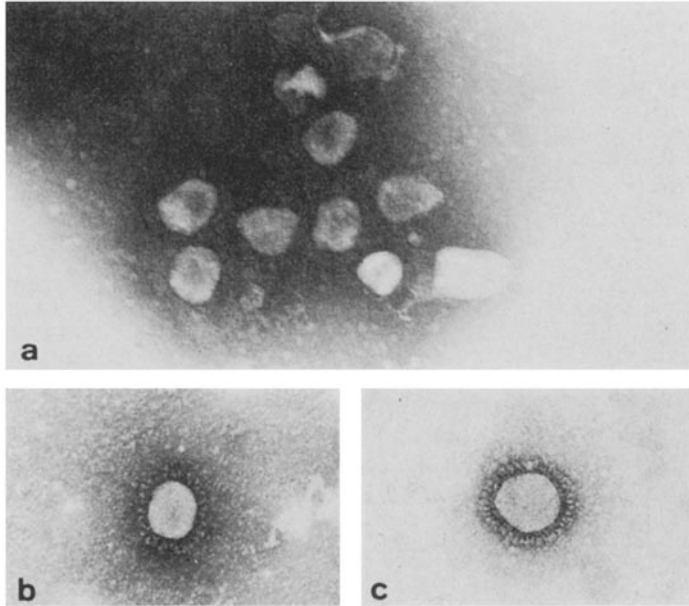


Fig. 1. Immunoelectron microscopy: A positive homologous and two negative heterologous reactions. *a* CV 777 treated with pig anti-CVLA serum 1:10, *b* CV 777 treated with pig anti-HEV serum 1:100, *c* HEV, strain VW 572 treated with pig anti-CVLA serum 1:10

Results

Immunoelectron Microscopy

The results of the IEM are shown in Table 1. Immune aggregates were observed in all homologous systems. They were recognized by aggregation of widely spaced virus particles, surrounded by a fuzzy rim of antibodies. Figure 1 shows an example of a positive homologous and two negative heterologous reactions. CVLA did not show detectable antigenic cross-reactivity with IBV, TGEV, CCV, HEV or NCDCV. TGEV antiserum coated CCV antigen but not vice versa. An antigenic relationship was observed between NCDCV and HEV (Fig. 2).

Table 1. *Antigenic relationship between CVLA and 5 known coronaviruses examined by immunoelectron microscopy*

Antigens	Antiserum against					
	CVLA	IBV	TGEV	CCV	HEV	NCDCV
CVLA	+	-	-	-	-	-
IBV	-	+	-	-	-	-
TGEV	-	-	+	-	-	-
CCV	-	-	+	+	-	-
HEV	-	-	-	-	+	+
NCDCV	-	-	-	-	+	+

+ = presence of immune aggregates

- = presence of single, non antibody coated virus particles

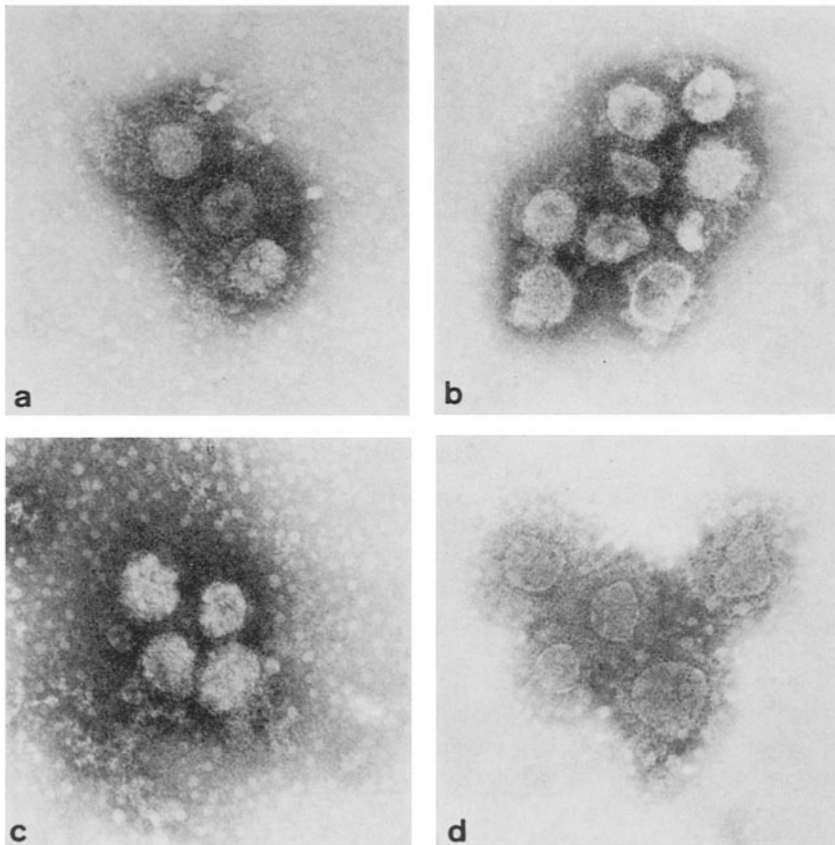


Fig. 2. Antigenic relationship between HEV and NCDCV established by immunoelectron microscopy. *a* HEV, strain VW 572 treated with pig anti-HEV serum 1:100, *b* HEV, strain VW 572 treated with calf anti-NCDCV serum 1:10, *c* NCDCV, Norden Laboratories vaccine strain, treated with pig anti-HEV serum 1:100, *d* NCDCV, Norden Laboratories vaccine strain, treated with calf anti-NCDCV serum 1:10. Note that the antibody coating is stronger in the homologous systems than in the heterologous systems

Immunofluorescence

The results of the immunofluorescence tests are shown in Table 2. Conjugated antiserum to CVLA reacted only with the homologous antigen, and CVLA viral antigen was not detected by any of the other antisera. Antigenic cross-reactivity was shown between TGEV and CCV and between TGEV and FIPV. Antiserum to TGEV reacted with CCV, but did not show detectable antigenic cross-reactivity with FIPV. On the other side, antiserum to CCV and to FIPV both reacted with TGEV. The antigenic relation between CCV and FIPV was not studied. A "two-way" antigenic relationship between HEV and NCDCV was also detected.

Table 2. *Antigenic relationship between CVLA and 5 known coronaviruses examined by immunofluorescence*

Viral Substrates	Antiserum against					
	CVLA	TGEV	CCV	FIPV	HEV	NCDCV
CVLA	+	-	-	-	-	-
TGEV	-	+	+	+	-	-
CCV	-	+	+	NT	-	-
FIPV	-	-	NT	+	-	NT
HEV	-	-	-	-	+	+
NCDCV	-	-	-	NT	+	+

+ = positive
 - = negative
 NT = not tested

Discussion

The earlier results in which CVLA was found to be antigenically unrelated to the 2 known porcine coronaviruses, TGEV and HEV (4, 14), are hereby confirmed. The present study did not reveal any evidence for a "one-way" or "two-way" cross-reactivity between the CVLA and 4 non-porcine coronaviruses.

Since CVLA cannot be cultivated in *in vitro* systems, only serologic tests such as IEM and IF could be used for examining cross-reactivity with coronaviruses. The results of the present study are expressed either as positive or negative because only one particular serum dilution was used in each of the tests. The dilution of the serum was kept as low as possible in order to assure a maximal degree of sensitivity. Even a low degree of cross-reactivity would, therefore, most likely have been detected.

The present results suggests that CVLA may represent a serologically distinct coronavirus species. Such a feature is not unique within the family Coronaviridae since the prototype species, IBV, does not appear to be related to other coronaviruses (3, 18).

Since the present study did not reveal further evidence for a more definite classification of the CVLA, its morphological appearance remains the only feature for a tentative grouping within the coronavirus family.

The existence of an antigenic relationship between HEV and NCDCV has earlier been reported using virus neutralization (17), IF (10) and enzyme-linked immunosorbent assay (ELISA) (5). It is further corroborated by the present work using IEM.

The immunofluorescent cross reactivity of TGEV antiserum with CCV, reported by PEDERSEN *et al.* (10), is confirmed by the present study. The relationship between these 2 viruses can also be shown by IEM. In contrast to the findings of PEDERSEN *et al.* (10), CCV antiserum was found in the present experiments to cross-react in the indirect IF with TGEV. While the existence of different serotypes of CCV cannot be ruled out, other points should be taken in consideration in trying to explain these contradictory results. The antibody titer of the serum used may be important, particularly when the antigenic relationship between 2 coronaviruses is examined. Similar observations were made by REYNOLDS and GARWES (16) in examining the TGEV-FIPV relationship. Furthermore, it cannot be excluded that the dog serum, used in the present study, contained homologous TGE antibodies. The serum had been randomly collected. It is known that dogs can be naturally infected with TGEV (7).

The present finding in which the dog serum, used in the IF test, failed to aggregate TGEV in the IEM test is somewhat unexpected and is difficult to explain. In heterologous IEM test, hyperimmune sera may be necessary to obtain positive reactions. The antibody concentration in the convalescent dog serum, used in the present study may have been too low to establish aggregation of TGEV.

The results of our IF studies comparing FIPV with TGEV, are in agreement with the "one way" antigenic relationship existing between these 2 viruses as earlier reported (6, 19). Indeed, TGEV antiserum failed to react with FIPV. The "two way" antigenic cross reactivity between FIPV and TGEV, described by PEDERSEN *et al.* (10), could not be confirmed in the present studies.

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