Counterimmunoelectroosmophoresis for Detection of Neonatal Calf Diarrhea Coronavirus: Methodology and Comparison with Electron Microscopy

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Received for publication 22 May 1979

A counterimmunoelectroosmophoresis (CIE) technique is described for the detection of calf diarrhea coronavirus antigens in intestinal contents. The antibody reagent was prepared in rabbits against the Nebraska calf diarrhea coronavirus adapted to Vero cells and purified by density gradient centrifugation. The method was applied to intestinal contents of diarrheic normal calves and compared with electron microscopy (EM). Calf coronavirus antigens were detected in intestinal contents of 44% (21/48) of the diarrheic calves and 24% (4/17) of the normal calves. Two precipitin lines could be observed in the majority of the positive samples. When compared with EM, CIE detected more positive animals. In only two cases (2/20) CIE was negative despite the visualization of coronavirus particles by EM.

Diarrhea associated with the presence of coronavirus particles in feces of neonatal calves is now frequently observed and has been reported from various parts of the world (11, 14-16, 18, 19). Based on incidence data from two studies, coronavirus infections ranked second in importance in economic losses due to infectious calf diarrhea (5).

The detection of coronaviruses is presently done by electron microscopic (EM) examination of intestinal contents or feces, by in vitro cell culture, and by immunofluorescence staining of gut sections (3, 6-9, 12).

Immunofluorescence staining of colon sections (9, 11, 12) is the diagnostic method of choice for most laboratories. Unfortunately, this method can be used only following the death of the calves or by sacrificing a calf for diagnostic purposes. The efficiency of negative staining for EM examination is often disputable. Some workers reported that it permitted the frequent visualization of the virus in diarrheal feces (7), whereas others stated that the examination of the specimens often revealed a very small number of particles with morphology resembling that of coronaviruses but that the structures frequently were not sufficiently well defined to establish their identity. The surface projections in particular are often badly preserved (3). However, provided the samples contain a large number of virus particles, they can be rapidly identified (12, 16).

Although some bovine coronaviruses can readily multiply and induce cytopathic effects in FBK (9, 15), BEK-1 (6), Vero, and MDBK cells (S. Dea et al., manuscript submitted for publication), the cultivation of the virus appears difficult, and many passages are required to obtain satisfactory yields of infectious particles. However, in most instances the presence of the virus can be demonstrated on first passage through the use of EM and immunofluorescence procedures (6, 9, 15).

The purpose of the present work was to describe and compare with EM the use of counterimmunoelectroosmophoresis (CIE) for rapid detection of the coronaviruses associated with neonatal calf diarrhea.

MATERIALS AND METHODS

Source and preparation of intestinal samples. Jejunal and colonic contents were collected from 48 diarrheic calves within the first 5 to 6 h after the onset of diarrhea and from 17 healthy calves in 55 different herds around St. Hyacinthe, Quebec, Canada. All animals were examined clinically and autopsied; histopathological sections were made of the intestines, and their lumen contents were collected. Diarrheic animals showed intestinal lesions of bacterial or viral origin, whereas normal animals were asymptomatic and lacked intestinal lesions (M. Morin et al., Proceedings of the 2nd Symposium on Neonatal Diarrhea, Saskatoon, 1979, in press). All calves were between 1 and 21 days old when submitted for examination.

The intestinal contents of each animal were combined and diluted 1:5 in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH
8.0, sonicated for 30 s at 20 kilocycles per s, clarified by centrifugation at 5,000 × g for 30 min, and then filtered through membrane filters (Millipore Corp.) of 200-nm pore size.

Viruses used as controls. The FBK cell culture-adapted Nebraska calf diarrhea coronavirus (NCDC) (9) kindly supplied by C. A. Mebus, University of Nebraska, was adapted and propagated in Vero cells. The virus was used at Vero cell passage level 27. Infectious bovine rhinotracheitis (IBR), strain Colorado, and bovine enterovirus type II (BE-II) viruses, both adapted on Vero cells, and calf rotavirus supplied by C. A. Mebus and adapted on MDBK cells in our laboratory were used as controls.

Virus propagation. Confluent monolayers of Vero or MDBK cells were rinsed with Dulbecco phosphate-buffered saline (pH 7.0) and inoculated with 1 ml of infected cell extracts. After 1 h of viral adsorption at 37°C, the cultures were overlayed with Eagle minimal essential medium with Earle salt and glutamine (GIBCO) and incubated at 37°C in a 5% CO2 atmosphere. When maximal cytopathic effects occurred, infected cells and cell culture fluid were frozen and thawed three times and clarified and stored at −70°C. Viral infectivity was determined as described elsewhere (S. Dea et al., manuscript submitted for publication), and viral titers were expressed in 50% tissue culture infective doses (TCID50) according to the method of Reed and Muench (13).

Electron microscopy. Droplets of the clarified fecal contents were placed on a 200-mesh Formvar- and carbon-coated grids and allowed to remain on the grids for 30 s. Fluids were then blotted from the grids, and a droplet of distilled water was added for 10 s and blotted off. The specimen was stained for 10 s with 3% phosphotungstate acid (pH 7.0). The excess stain was blotted off. The grids were dried and then examined with a Philips 300 electron microscope operating at an accelerating potential of 80 kV.

Preparation of coronavirus antigen. Approximately 300 ml of NCDC-infected Vero cell culture fluids were frozen and thawed three times and centrifuged at 5,000 × g for 30 min at 4°C to remove coarse debris. The clarified suspension was then concentrated to a volume of 5 ml by ultrafiltration through an XM-100A Diaflo membrane. The concentrate was centrifuged at 78,600 × g for 2 h at 4°C. The supernatant fluid was discarded, and the pellet containing the virus particles was dispersed by sonication at 20 kilocycles per s for 30 s in 0.5 ml of 0.05 M Tris-hydrochloride buffer (pH 7.4). The viral suspension was then diluted in the same buffer up to 250 μg of protein per ml as determined by optical density at 280 nm and used for immunization.

Preparation of antiserum. Two New Zealand Albino rabbits (2 kg) were used for immunization. They were inoculated intramuscularly at four sites (0.5 ml per site) and in the footpads (0.5 ml) with a mixture of equal volumes of NCDC antigen and complete Freund adjuvant. After 20 days, the rabbits received an intravenous dose of 1.0 ml of antigen along with intramuscular injections (0.50 ml per site) consisting of a mixture of 1.0 ml of antigen and 1.0 ml of incomplete Freund adjuvant. On days 35 and 49, they were reinoculated intramuscularly as described previously. The animals were bled on day 63.

Thereafter, the antiserum was inactivated at 56°C for 30 min and then treated to eliminate unwanted nonspecific antibodies. A volume of 9 ml of the antisera was mixed with 1 ml of fetal calf serum and incubated at 4°C overnight. The precipitate formed was eliminated by centrifugation at 5,000 × g for 30 min. Then, 10 ml of the supernatant fluid was mixed with 1 g of bovine liver powder for 1 h and then clarified by centrifugation at 10,000 × g for 30 min. The antiserum was subsequently washed with Vero cells for 30 min, recovered, tested, and then used in serological tests. This coronavirus antiserum was shown to be free of reactivity against calf serum and bovine tissues by immunodiffusion and CIE.

Titration of neutralizing antibodies. Twofold dilutions of the antiserum (1:10 to 1:1,280) were made in 0.01 M phosphate-buffered saline (pH 7.2) supplemented with 0.1% bovine serum albumin. A 0.1-ml portion of a virus dilution containing 400 TCID50 was added to 0.1 ml of appropriate serum dilutions. The virus-serum mixtures were agitated vigorously and allowed to remain at 37°C for 1 h. Thereafter, each mixture was inoculated in 0.05-ml amounts into each of three wells of a 96-well Microtiter culture tray (Falcon Plastics, Oxnard, Calif.) plated with Vero cells. The cultures were examined for evidence of cytopathic effect after 4 to 5 days postinfection at 37°C. The titer was expressed as the reciprocal of the highest neutralizing serum dilution.

CIE. The CIE test was performed on Kodak projector slides (8.3 by 10.2 cm) coated with 13 ml of agarose in Tris-barbital buffer. After the gel had become firm at room temperature, two parallel rows of wells (4.0 mm in diameter) were punched 1 cm center to center. Anodal wells were filled with 15 μl of antiserum, and cathodal wells were filled with 15 μl of antigen. The antigens used were the clarified, infected cell culture fluids or intestinal contents obtained as described above and diluted in Tris-barbital buffer of the same molarity as that used to make up the agarose. Telfa nonadherent strips (Kendall, Chicago, Ill.) (4 by 10 cm) were used as wicks. Directly after electrophoresis, the slides were washed overnight at 0.85% NaCl and then immersed in 1% tannic acid. Thereafter, the agarose slides were examined with a high-beam illuminator for the presence of a precipitin line between antigen and antiserum wells.

RESULTS

Conditions for CIE. Some parameters of the CIE test were studied by using cell culture fluid from NCDC-infected Vero cells as the source of virus. A visible double precipitin line was obtained between antibody and antigen wells when we used as antibody the anti-NCDC serum supplied by C. A. Mebus and the rabbit anti-NCDC serum produced in our laboratory (Fig. 1). Both antisera possessed a neutralizing titer of 320. No precipitin lines were observed with our anti-NCDC serum against IBR, Nebraska calf rotavirus, and BE-II viruses.

Electrophoresis was conducted at 200, 150,
passage 27 of NCDC virus in Vero cells against our antiserum. Precipitin lines were noted with antigen dilutions up to 1:1,000. The endpoint dilution corresponded to $10^2\mathrm{TCID}_{50}$ per ml. No precipitin lines were observed with undiluted coronavirus suspensions when antiserum was diluted more than 1:4.

Comparison of EM and CIE for detecting coronavirus. Typical coronavirus particles with an average diameter of 120 nm and surrounded by bulbous or petal-shaped projections 13 to 17 nm long were visualized by EM in 16 (33%) of 48 diarrheal intestinal contents and in 4 (24%) of 17 normal intestinal contents (Fig. 2).

As shown in Table 1, nine diarrheic calves were singly infected by coronavirus, whereas three others had only rotaviruses. Five calves showed mixed infection by coronavirus and rotavirus, one calf showed infection with coronavirus and reovirus, and another one showed infection with coronavirus and picornavirus. A total of 29 diarrheic calves were free of any virus by this technique.

Four and two of the healthy calves were singly infected with coronavirus or with picornavirus, respectively. The other 11 calves were free of any viral particles as examined by EM.

As shown in Table 1, 21 (44%) of 48 clarified...
intestinal contents of diarrheic calves and 4 (24%) of 17 intestinal contents from normal calves contained coronavirus antigens as detected by CIE. No antigenic reaction occurred with the intestinal contents shown to contain only rotavirus or only picornavirus by EM. More than half of the samples that were positive for coronaviruses by this technique were positive only after staining with 1% tannic acid as described above.

As reported in Table 1, CIE was positive on all intestinal contents shown to contain coronavirus by EM with the exception of one diarrheic and one normal calf. Coronavirus antigens were detected by CIE in 6 of the 29 intestinal contents from diarrheic calves in which no viral agents were seen by EM. From the 11 normal intestinal contents that were free of any virus particles as determined by EM, in only one instance did we observe a positive reaction by CIE.

These results indicate that CIE could detect more coronavirus-positive intestinal contents than could EM. However, a negative result obtained by CIE must be verified by EM.

Based on the combined use of EM and CIE, 46% (22/48) of the diarrheic calves and 30% (5/17) of the healthy calves studied had coronaviruses in their intestinal contents.

**DISCUSSION**

Two precipitin lines were observed in the majority of the positive samples, indicating that, as with other members of the coronavirus group, the bovine coronavirus possesses more than one precipitating antigen (1, 2, 4, 10, 17).

As applied to intestinal contents, the reaction seemed specific for coronavirus since no immunoprecipitate could be detected with the contents shown to contain viruses other than coronavirus by EM. It is worth stressing the fact that two intestinal contents were negative by CIE although they were positive by EM. This fact could be explained by a technical failure of CIE or observer error by EM. However, it could also suggest the existence of more than one antigenic variant among bovine coronaviruses.

CIE provided a reliable, simple, and fast method for large-scale screening of intestinal contents for coronavirus. However, before monospecific antisera to all known bovine coronaviruses are available, it is advisable to further examine the CIE-negative samples by EM.

Unexpectedly, coronavirus particles and antigens were detected by EM and/or CIE in 30% of the healthy calves. To our knowledge no asymptomatic coronavirus shedding has been reported in this species. However, since all calves were subjected to necropsy, we do not know whether the healthy calves were infected with coronavirus but had not yet developed diarrhea. Moreover, no pathological lesions were seen in these normal calves after a postmortem examination (M. Morin et al., Proceedings of the 2nd Symposium on Neonatal Diarrhea, Saskatoon, 1979, in press). Samples collected from other regions in Quebec over a long period of time are needed to confirm and to generalize this finding. If this is correct, the significance of a high percentage of asymptomatic calves containing such high levels of coronavirus needs to be elucidated to obtain the correct picture of the epidemiology and the mechanism of the onset of the diarrhea associated with coronaviruses. It would appear necessary to identify the factors modifying the clinical effects of coronavirus infections.

**ACKNOWLEDGMENTS**

The technical assistance of Francine Breton is gratefully acknowledged.

This study was supported by the Conseil de Recherches et de Services Agricoles du Quebec (Canada) grant no. MMV 75-604.

**LITERATURE CITED**


**Table 1. Comparison of EM and CIE for detection of coronavirus in intestinal contents of diarrheic and normal calves**

<table>
<thead>
<tr>
<th>Virus seen by EM</th>
<th>CIE</th>
<th>Normal calves</th>
<th>CIE</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Coronavirus (9)*</td>
<td>9</td>
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<td></td>
</tr>
<tr>
<td>Rotavirus (3)</td>
<td>0</td>
<td>3</td>
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<tr>
<td>Coronavirus and</td>
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</tr>
<tr>
<td>rotavirus (5)</td>
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<td>0</td>
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</tr>
<tr>
<td>Coronavirus</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>reovirus (1)</td>
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<td></td>
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</tr>
<tr>
<td>No virus observed</td>
<td></td>
<td>(29)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate the number of samples with the indicated virus(es).

* Number of samples in which no viruses could be observed by EM.