Human rotavirus antigen detection by enzyme-immunoassay with antisera against Nebraska calf diarrhoea virus

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SUMMARY A four-layer solid phase enzyme-immunoassay (EIA) with antisera against Nebraska calf diarrhoea virus (NCDV) as immunoreagents was developed to detect human rotavirus antigens from stool specimens of patients with acute rotavirus gastroenteritis. Polystyrene beads were used as the solid phase, guinea-pig and rabbit anti-NCDV immunoglobulin as the catching and secondary antibody, and peroxidase-conjugated swine anti-rabbit immunoglobulin as the indicator antibody. A comparison of the developed NCDV-EIA with an identical EIA, using antisera against human rotavirus (HRV-EIA) instead of NCDV antisera, was made with 216 stool specimens positive or negative for rotavirus. A complete agreement was obtained between the two methods provided that appropriate confirmatory tests were included. The developed NCDV-EIA was as sensitive and specific for rotavirus as the HRV-EIA, and it allowed the detection of both established rotavirus types 1 and 2 from stools with equal sensitivity. The difficulties in cultivating human rotavirus *in vitro* for immunisation and the relative ease of growing NCDV in widely-used continuous cell lines make NCDV a good alternative in the preparation of the highly specific and sensitive rotavirus antisera required in immunoassays, and facilitate the setting-up methods for the routine diagnosis of rotavirus gastroenteritis by EIA or RIA in diagnostic virus laboratories.

Rotavirus is the most important agent causing infantile gastroenteritis in hospitalised children.¹ Several methods have been used to detect rotavirus antigens from stool specimens²⁻⁴ but radioimmunoassay (RIA)⁵ and enzyme-immunoassay (EIA)⁶ seem to be the best alternatives when large numbers of specimens must be studied daily.⁷ Most of the EIA and RIA methods reported so far have used animal hyperimmune sera prepared against human rotavirus as the immunoreagents. Since all attempts to adapt human rotavirus in commonly-used cell lines have been largely unsuccessful,⁸ it has forced most laboratories to purify the human rotavirus used for immunisation from the stools of children with acute gastroenteritis⁷ or from animals infected with human rotavirus.⁶ Such cumbersome methods for the purification of immunising antigen cannot be easily applied on a large scale in most laboratories, and alternative methods are required. Animal rotaviruses, on the other hand, grow in many cell lines when small amounts of trypsin are added to the maintain-

ance medium.⁹ These animal rotaviruses also share a common group antigen with human rotavirus which cross reacts in serological tests.¹⁰ In the present report antisera against tissue-culture-adapted Nebraska calf diarrhoea virus (NCDV) were used as immunoreagents in EIA for the detection of human rotavirus antigens from the stool specimens of patients with acute rotavirus gastroenteritis. The results of the developed method were compared with those of an identical EIA but with the use of antisera against the human rotavirus as immunoreagents.

Materials and methods

VIRUS PURIFICATION

NCDV used as a positive control antigen in EIA and for immunisation was grown in LLC-MK2 cell cultures in the presence of trypsin and partially purified as reported earlier.¹⁰ This semipurified virus was further purified by two subsequent centrifugations in CsCl-gradients according to Petric *et al.*¹¹ The protein content was determined by the method of Lowry *et al.*¹² and was approximately 800 µg/ml of purified virus.

IMMUNISATIONS AND PREPARATION OF VIRAL IMMUNOREAGENTS

The immunising procedure for the preparation of rabbit and guinea-pig anti-NCDV hyperimmune sera is identical to the one described earlier for the preparation of anti-human rotavirus hyperimmune sera.⁷ The titres of both hyperimmune sera for NCDV were between one and two million as measured by EIA modified from RIA.¹⁰ The preparation of rabbit and guinea-pig immunoglobulins and purified IgG from the anti-NCDV and anti-human rotavirus hyperimmune sera, respectively, were done as described earlier.^{7 13}

EIA PROCEDURE

The principle of the EIA using anti-NCDV immunoglobulins as immunoreagents (NCDV-EIA) is identical to the one described earlier for EIA using corresponding anti-human rotavirus antibody as immunoreagents (HRV-EIA).¹⁴ Stool specimens in 200 μ l volumes in a single 1/20 (vol/vol) dilution or serially diluted were pipetted into disposable polystyrene tubes and a polystyrene bead (Precision Plastic Ball Co, Chicago, USA) with adsorbed guinea pig anti-NCDV immunoglobulins (0.25 μ g/bead) was then added to each tube. After incubation at +37°C for one hour, the stool specimens were aspirated and the beads were washed twice with 5 ml of tap water. A 200 μ l volume of rabbit anti-NCDV immunoglobulin (4 μ g/ml) was then added to each tube and the beads were incubated at +37°C for one hour. The beads were then washed as described above and a 200 μ l volume of horse-radish peroxidase conjugated swine anti-rabbit immunoglobulins (Orion Diagnostica, Helsinki, Finland; 1/1000 dilution) was added to each tube. After one-hour incubation at +37°C the beads were washed as described above, changed to new tubes, and a 500 μ l volume of the substrate solution consisting of 3 mg/ml of o-Phenylenediamine (OPDA, Koch-Light Laboratories, Colnbrook, Bucks, England) in 0.1 M citrate-Na₂HPO₄ buffer, pH 5.5, and 10 μ l of 30% H₂O₂ per 15 ml of the same buffer, was added to each tube. After one-hour incubation at room temperature in the dark, the reaction was stopped by adding 500 μ l volume of 1 M HCL (Orion Diagnostica, Helsinki, Finland) to each tube. 500 μ l volumes of the reaction mixtures were then transferred to clean polystyrene cuvettes and the intensity of the colour measured at 492 nm using a FP-9 Analyzer (Lab-systems, Division of Finnpiptette Co, Finland). An absorbance value of two times the mean value obtained with the negative stools was taken as the cut-off level. This cut-off level was usually 0.5 and specimens with values at this level and above were considered positive provided that the confirmatory

test indicated a specific binding. The diluent (EIA diluent) in all steps except for the substrate solution was phosphate-buffered saline, containing 20% inactivated fetal calf serum, 2% Tween 20, and 10⁻⁴ M merthiolate.

EIA CONFIRMATORY TEST WITH ANTI-NCDV SERA

To test the specificity of the binding, a blocking test was performed on selected specimens. The test was done in the same way as described above except that anti-NCDV guinea-pig serum was added before the secondary antibody. The test was done as follows: 200 μ l volumes of the specimens were pipetted into three tubes, a bead with adsorbed anti-NCDV immunoglobulin was added to each of the three tubes, and the beads were incubated for one hour at +37°C. After the washing procedure a 200 μ l volume of anti-NCDV guinea-pig serum (1/2000 dilution) was added to the first tube and the same volume of non-immune guinea-pig serum (1/2000 dilution) to the other; to the third only dilution buffer was added. After the incubation at +37°C for one hour, a 200 μ l volume of rabbit anti-NCDV immunoglobulin (8 μ g/ml) was added to each of the three tubes (without removal of the previous 200 μ l sample) giving a total volume of 400 μ l. The beads were then incubated for one hour at +37°C. The rest of the test was performed as the assay proper. The test was considered positive if a 50% or greater decrease in absorbance values was observed with the bead incubated with anti-NCDV hyperimmune serum as compared to the bead incubated with normal guinea-pig serum or with dilution buffer.

HRV-EIA AND HRV CONFIRMATORY TEST

The EIA procedure for the detection of human rotavirus from the stool specimens using immune sera prepared against human rotavirus and the corresponding confirmatory test were done as described earlier.¹⁴

SPECIMENS

The material included in this study consisted of 98 rotavirus RIA-negative and 118 rotavirus RIA-positive specimens extensively studied earlier with both EIA and electron microscopy.^{7 14} Three type 1 and three type 2 rotavirus stools were kindly supplied by Dr T Johnsson, Department of Clinical Virology, University of Lund, Malmö General Hospital, Malmö, Sweden. Twelve other stool specimens positive for rotavirus in RIA but negative in electron microscopy were available from earlier studies.¹

SPECIMEN PREPARATION AND ELECTRON MICROSCOPY (EM)

A 10% suspension of the stool specimens was made in phosphate-buffered saline (pH 7.35) and clarified by low-speed centrifugation. The supernatant fluid was used directly to make EM grids, and for the EIA it was diluted 1/2 in the EIA diluent to make the final dilution 1/20. If an EIA-positive specimen was found negative by the first EM the supernatant was further centrifuged in a Spinco 50 rotor at 30 000 rpm for 90 minutes, and the pellet resuspended in a small volume of distilled water. The negative staining on carbon-coated grids was done with 2% phosphotungstic acid and the specimens were examined using a Siemens Elmiskope or a JEM 100 electron microscope at an instrumental magnification of $\times 30\,000$.

Results

The EIA results of NCDV antigen detection either with human rotavirus antisera (heterologous antisera) (HRV-EIA) or with NCDV antisera (homologous antisera) (NCDV-EIA) are shown in Fig. 1. The results indicate that both homologous and heterologous antisera easily detect NCDV antigen. The sensitivities of the NCDV-EIA and HRV-EIA for NCDV antigen were usually 3 and 10 ng/ml, respectively, but varied from 1 to 10 ng/ml of semipurified virus from test to test.

Fig. 1 Comparison of semipurified Nebraska calf diarrhoea virus (NCDV) antigen detection by EIA with human rotavirus antisera (a) and with NCDV antisera (b). The broken lines represent the cut-off values of two times the mean of the three buffer blanks.

Three type 1 and three type 2 stool specimens from children with acute rotavirus gastroenteritis were titrated in NCDV-EIA. It is evident from the results shown in Table 1 that both rotavirus types are detected by NCDV antisera and that NCDV-EIA is

not type specific. All three type 1 and type 2 stool specimens were also positive in HRV-EIA.

Table 2 shows the titrations of three rotavirus-positive and three rotavirus-negative stool specimens in NCDV-EIA and HRV-EIA. Each of the positive stool specimens was positive up to a dilution of 1/2000 or more in both assays and the individual cut-off points were approximately the same in each case for both assays. The absorbance values for the negative specimens were low in both assays.

Table 1 Detection of human rotavirus type 1 and 2 from stool specimens of children with acute rotavirus gastroenteritis by enzyme-immunoassay (EIA) with antisera against Nebraska calf diarrhoea virus (NCDV)

Specimen No	Rotavirus type	Dilution of specimen				
		1/20	1/200	1/2000	1/20 000	1/200 000
1	1	1.980*	1.485	0.383	0.233	0.117
2	1	1.624	0.834	0.352	0.103	0.087
3	1	2.108	1.651	0.449	0.168	0.111
4	2	1.592	1.280	0.477	0.170	0.106
5	2	1.339	0.994	0.293	0.102	0.101
6	2	2.153	2.025	1.460	0.651	0.169

*Absorbance at 492 nm.

Table 2 Comparison of rotavirus antigen detection with three positive (nos 1-3) and three negative (nos 4-6) stool specimens of children with acute gastroenteritis by enzyme-immunoassay (EIA) with antisera against either human rotavirus* or Nebraska calf diarrhoea virus (NCDV)†. The values represent absorbance measured at 492 nm

Specimen No	Dilution of specimen				
	1/20	1/200	1/2000	1/20 000	1/200 000
1	2.287*	1.598	0.542	0.278	0.225
	2.118†	1.380	0.372	0.190	0.119
2	2.227	1.985	1.122	0.381	0.193
	2.309	1.882	0.946	0.250	0.144
3	2.285	1.933	0.705	0.289	0.205
	2.155	1.599	0.503	0.503	0.161
4	0.195	0.196	NT	NT	NT
	0.119	0.123			
5	0.220	0.188	NT	NT	NT
	0.151	0.117			
6	0.235	0.204	NT	NT	NT
	0.123	0.125			

NT = not tested.

Twelve selected stool specimens were available which had been extensively studied by electron microscopy with negative results in spite of the concentration of specimens by ultracentrifugation. However, all these specimens were clearly positive in RIA and EIA with human rotavirus antisera. Now tested in NCDV-EIA all were positive and the bindings proved specific in NCDV-confirmatory tests.

Representative results of the NCDV-confirmatory test for two such specimens are shown in Table 3.

Ninety-eight rotavirus-negative and 118 rotavirus-positive stool specimens earlier studied in HRV-RIA and HRV-EIA were tested in NCDV-EIA (Fig. 2a and b). Out of these 98 RIA-negative stools, 95 were negative and 3 specimens were positive in HRV-EIA; in NCDV-EIA 97 specimens were negative and one was positive. Out of 118 rotavirus RIA-positive specimens 114 were positive in both EIA tests; two specimens were negative in HRV-EIA and three specimens were negative in NCDV-EIA.

Fig. 2 Distribution of absorbance values measured at 492 nm in enzyme-immunoassay (EIA) with human rotavirus (HRV) antisera (HRV-EIA) and with Nebraska calf diarrhoea virus (NCDV) antisera (NCDV-EIA) for 98 HRV-negative (a) and 118 HRV-positive (b) stool specimens from children with acute gastroenteritis. The broken lines represent the cut-off values.

All specimens with contradictory results between HRV-EIA and NCDV-EIA and all the results of rotavirus RIA-positive specimens with absorbance values < 1.5 and of rotavirus RIA-negative specimens with absorbance values > 0.5 were tested in appropriate confirmatory tests. The results of the NCDV

and HRV confirmatory tests proved the specificity of the binding with the rotavirus RIA-positive specimens, and showed that the positive reactions with the rotavirus RIA-negative specimens were non-specific in nature. The confirmatory tests also proved the specificity of the binding, in spite of the weakness of the reactions, of the four specimens with absorbance values slightly below the cut-off line in either or both EIA tests. Furthermore, three of these four specimens also contained characteristic rotavirus particles when studied in electron microscopy.

Discussion

The results of this study indicate that NCDV antisera can be used effectively as diagnostic reagents in rotavirus EIA instead of human rotavirus antisera. Complete agreement was obtained with the HRV-EIA and NCDV-EIA with rotavirus-positive and -negative specimens provided that appropriate confirmatory tests were included. The sensitivities of the assays were practically the same, and both tests detected small amounts of rotavirus antigens in stools which escaped detection by the electron microscope.

The NCDV used in this study for the production of immune sera in animals was adapted at first to grow in LLC-MK2 cells¹⁰ but can also be grown in other cell lines such as BSC-1⁹ and Vero cells. Since these cell lines are widely used in virus laboratories and also available commercially, the cultivation of NCDV for diagnostic purposes should not cause problems. The only special requirement of the NCDV is the need to add small amounts of trypsin to the maintenance medium.⁹

Electron microscopy of the purified immunising antigen showed that most of the viral particles were incomplete—that is, devoid of the outer capsid. It has

Table 3 Rotavirus confirmatory enzyme-immunoassay (EIA) test with antisera against Nebraska calf diarrhoea virus (NCDV) for testing the specificity of the binding in rotavirus EIA with four positive and one negative stool specimens from children with acute gastroenteritis

Specimen No	Electron microscopy	Absorbance* when specimen was incubated with dilution buffer	Absorbance* when specimen was incubated with hyperimmune guinea-pig serum	Absorbance* when specimen was incubated with preimmune guinea-pig serum	Interpretation† of the test
1	+	1.202	0.375	1.172	Positive
2	+	0.972	0.318	1.000	Positive
3	-	1.243	0.417	1.185	Positive
4	-	1.027	0.359	0.978	Positive
5	NT	0.290	0.095	0.106	Negative
NCDV 100 ng/ml		1.250	0.112	1.055	Positive

*Absorbance measured at 492 nm.

†Test considered positive if a 50% or greater decrease in absorbance values was observed with bead incubated with guinea-pig anti-NCDV hyperimmune serum as compared to bead incubated with nonimmune guinea-pig serum or with dilution buffer.

NT = not tested.

been shown earlier that the group antigen common to all the rotaviruses is located in the inner capsid of the incomplete virion, and that the type-specific antigens are located in the outer capsid of the complete virion.¹⁵ The fact that the human rotavirus infected faeces contain considerable amounts of the subunit of the internal components¹⁶ and that the immunising antigen contained mainly incomplete NCDV virions explains the effectiveness of the NCDV antisera in the detection of human rotavirus antigens in stool specimens.

The NCDV antisera have been used before to detect human rotavirus in stool specimens. Tufvesson and Johnsson⁴ found a 100% correlation between immunoelectro-osmophoresis and electron microscopy. Bishai *et al.*,¹⁷ on the other hand, found several electron microscopically-positive specimens negative by EIA using NCDV antisera as immunoreagents but positive by EIA using human rotavirus and simian rotavirus (SA11) antisera as immunoreagents. The discrepancy between the results of Bishai *et al.*¹⁷ and the results of this study might be explained by the fact that they used complete virus particles for the production of immune sera whereas incomplete virus particles were used in the present study.

The present NCDV-EIA cannot differentiate between the four rotavirus types 1, 2, 3, and 4.¹⁷⁻²¹ However, this is an advantage since the recent studies on the epidemiology of the two established rotavirus types 1 and 2^{22,23} have indicated that the clinical symptoms of the diseases caused by the different rotavirus types do not vary substantially. Thus, in routine diagnostic work, it is useful to be able to detect all the different rotavirus types with a single test.

RIA and EIA methods have been used in this laboratory, with excellent results, for more than eighteen months in the routine diagnosis of rotavirus and adenovirus gastroenteritis.¹⁴ However, the problem of obtaining sufficient amounts of immunising antigen for the production of rotavirus antisera limits the wider use of the HRV-EIA. The use of NCDV or other cell culture adapted rotaviruses such as SA-11 virus¹⁷ offer a clear-cut advantage over the human rotavirus in this respect and should facilitate the setting-up of sensitive and specific RIA or EIA methods for the diagnosis of rotavirus gastroenteritis in diagnostic virus laboratories.

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