Rapid ELISA for the diagnosis of rotavirus

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Background & objectives: Rotavirus is the major cause of gastroenteritis in infants and young children all over the world. The objective of the study was to develop a rapid ELISA for the diagnosis of rotavirus infection in children hospitalised with diarrhoea.

Methods: Immune serum was raised in rabbits by inoculating semipurified rotavirus, SA-11 strain. Immunoglobulins were conjugated to horse radish peroxidase and a rapid ELISA for rotavirus diagnosis was developed. The rapid ELISA was compared with routine ELISA, developed earlier at NIV.

Results: Of the 155 faecal samples from patients with diarrhoea, 96 were positive by rapid ELISA and 95 in routine NIV ELISA. OD values were higher in rapid ELISA. The rapid ELISA takes only 4 h to complete.

Interpretation & conclusion: Rotavirus diagnosis by rapid ELISA is simple and easy to perform. This may lead to a significant reduction in the unnecessary usage of antibiotics, which cannot control infection due to rotavirus. This technology is being commercialized.

Key words Diagnosis - ELISA kit - rapid ELISA - rotavirus

Rotavirus infects almost all children by the age of five, both in the developing and developed countries. It has been estimated that per day, 370,000 episodes of rotavirus diarrhoea occur, 50,000 cases are hospitalised and 2000 die in the world. In India, about 20-30 per cent of hospitalised diarrhoea cases are caused by rotavirus. Clinically, rotavirus gastroenteritis is characterised by profuse diarrhoea, mild fever and vomiting, leading to mild to severe dehydration. The clinical manifestations of rotavirus diarrhoea alone are not sufficiently distinctive to permit diagnosis. Realising that the rotavirus causes severe diarrhoea, it is desirable to devise rapid, easy and cost effective methods for detection of rotavirus infection.

Of the various commercial ELISA kits available, Dakopatts kit, developed in Denmark was originally formulated for use in developed countries. This kit was sensitive, specific and could identify 98 per cent of positive specimens. The kit was modified at the request of WHO so that it was suitable for distribution to laboratories in developing countries. It was evaluated by WHO in six diagnostic laboratories in different countries. The sensitivity of the modified Dakopatts kit recorded by the WHO collaborating centre in England was 100 per cent. However, the high cost of the kit has hampered research on rotaviruses in developing countries. There is no commercially available Indian kit for rotavirus diagnosis.

In 1993, we developed an indigenous ELISA for rotavirus diagnosis. The test was compared with Dakopatts kit because the latter had been reported to be superior to other polyclonal ELISA kit and Flewitt et al had suggested that the kit was suitable for rotavirus diagnosis in etiological studies conducted by laboratories in developing countries. The NIV routine
ELISA was 100 per cent specific and sensitive. The test compared very well with the Dakopatts kit. The only drawback of the NIV routine ELISA is, the test requires at least 6 h against Dakopatts test, that needs 4 h.

The objective of the present study was to develop a rapid diagnostic test for rotavirus infection.

Material & Methods

Preparation of rotavirus stock and semi-purification of the virus: Simian rotavirus, SA-11 was grown in roller bottles using MA 104 cell line. The cell line was obtained from National Cell Culture Science, Pune. The virus was semi purified as per the procedure described earlier. The protein content of the virus stock was estimated by Lowry’s method.

Immunization of rabbit with SA-11 strain of rotavirus and collection of serum: Anti SA-11 antibodies were raised in rabbit by inoculating about 20 µg of viral protein/dose/rabbit. The first two doses were given intravenously at an interval of two weeks. Three weeks after the second dose, the third dose was given intramuscularly with Freunds incomplete adjuvant (Difco Labs, Detroit Michigan, USA). Post immunization serum was obtained on day 10 after the last dose of immunization. Before immunization, the rabbit was bled to get pre immunization serum, which was found to be negative for antibodies to SA-11. This was used as negative serum in ELISA.

Block titration of the rabbit serum: The dilution of the post immunization serum to be used in the ELISA was estimated by block titration as 1:10,000. Therefore, for the first coating of ELISA wells, 1:10,000 dilution of the rabbit post immunization serum and the same dilution of rabbit pre immunization serum was employed.

Preparation of anti-rotavirus serum horse radish peroxidase (HRP) conjugate: For conjugation of IgG to HRP, the method described by Wilson and Nakano was carried out. Bovine serum albumin at a concentration of 10 mg/ml was added to the conjugate, as a stabilizer. The conjugate was distributed in 1.0 ml aliquots and stored at -70°C.

Specimens: During 1990-97, a total of 1,272 faecal specimens were collected from hospitalised children suffering from acute diarrhoea. The samples were stored at -70°C for further studies. Of the 1,272 specimens, 334 were positive for rotavirus by NIV routine ELISA. We tested 96 rotavirus positive specimens i.e., 95 faecal samples and one positive control (SA-11) virus was grown in MA-104 cell line. Sixty two rotavirus negative specimens including 60 faecal specimens and 2 negative controls (phosphate buffered saline and extract of uninfected MA-104 cell line) were tested by NIV routine ELISA as well as NIV rapid ELISA.

Sample preparation: Approximately 10 per cent wt/vol stool suspension was prepared in 10 mM phosphate buffer saline (PBS, pH 7.2) containing 0.01 M calcium chloride. It was centrifuged at 1777 G (MSE coolspin-2, Rotor: 34115 - 613 fixed angle) for 20 min at 4°C. Before use, in ELISA test, 375 µl of specimen was added to 125 µl of sample diluent [2.5% (wt/vol) skimmed milk (Hindustan Lever, Mumbai) in 10 mM PBS-T, which is PBS containing 0.05% (vol/vol) Tween 20 (SRL, Mumbai)].

The same sample diluent was also used for diluting the conjugate.

Procedure for NIV rapid ELISA: The tests were performed employing Immulon 2, Removawell strips/plates (Dynatech, Virginia, USA). The washing buffer employed was 10 mM PBS-T.

ELISA plate was coated with 100 µl/well of pre and post immunization sera diluted in 10 mM PBS solution at 1:10,000 and marked as “-” and “+” respectively. The plate was incubated overnight at 4°C, washed 5 times and 200 µl of blocking reagent [1.2% BSA (wt/vol) in washing buffer] was added to all the wells. The plate was incubated at least for 4 h at 4°C, washed 5 times and stored at -70°C. The coated plates could be used at least for 2 months.

Assay procedure: 100 µl of each sample to be tested, was added to two wells of ELISA plate, coated with pre and post immunization sera. Positive and negative controls were also included in each test. Plates were incubated in a humid chamber at 37°C for 2 h and washed 5 times. The rotavirus antigen was detected by adding 100 µg/well of HRP conjugated to anti SA-11 antibody at a dilution of 1:500. The plate was incubated for 90 min at 37°C in a humidified chamber, washed 5 times...
and 100µl of the O-phenylene diamine (OPD)/ tetramethyl-
benzidine (TMB) substrate was added to each well. The
plate was incubated for 10 to 15 min at room temperature
and the reaction was stopped by adding 100 µl/well of
4N sulphuric acid in case of OPD (Sigma, USA) and 1 M
phosphoric acid in case of TMB (Kirkegaard and
Perry Laboratories, Maryland, USA). The results were
read visually or with the aid of ELISA reader (BIO-
TEK Instruments Inc., Vermont, USA) at 492 nm in
case of OPD and at 450 nm in case of TMB substrate.

The rapid ELISA was compared with the NIV routine
ELISA for rotavirus diagnosis developed. This process
has since been patented in India.

Results

A total of 158 specimens, including one positive control
and two negative controls were tested simultaneously in
NIV routine ELISA and NIV rapid ELISA and the
results were compared. The reactions to positive and
negative samples could be clearly differentiated in both the
tests.

Out of 155 faecal specimens tested, 96 specimens
were positive in NIV rapid ELISA, whereas 95 were
positive in NIV routine ELISA. The OD value range for
positive and negative specimens in NIV rapid ELISA
are shown in Table I. The minimum T/C value for positive
specimen was 2.222 whereas maximum T/C value for
negative specimen was 1.906. From this the value 2.0
was deduced, as the minimum T/C value required for
the specimen to be considered positive. The T minus C
(T-C) value in NIV routine ELISA for a sample to be
called positive is 0.1. The minimum T/C value for positive
specimen was 2.213 whereas maximum T/C value for
negative specimen was 2.167 (Table II). From this, the
value 2.2 is deduced as the minimum T/C value required
for a specimen to be considered positive. In earlier
studies\(^7\) also, the minimum T/C value was 2.2.

Table I. Data for positive and negative specimens by NIV rapid ELISA

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<thead>
<tr>
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<th>Rapid ELISA-ve</th>
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<th>Rapid ELISA+ve</th>
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<tr>
<td></td>
<td>T-C</td>
<td>T/C</td>
<td>T-C</td>
<td>T/C</td>
</tr>
<tr>
<td>n</td>
<td>58</td>
<td>59</td>
<td>97</td>
<td>96</td>
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<tr>
<td>Mean</td>
<td>0.007</td>
<td>1.106</td>
<td>1.227</td>
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<tr>
<td>Median</td>
<td>0.008</td>
<td>1.104</td>
<td>0.889</td>
<td>9.637</td>
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<td>Range Min.</td>
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<tr>
<td>Max.</td>
<td>0.051</td>
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Table II. Data for positive and negative specimens by NIV routine ELISA

<table>
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<th>Routine ELISA+ve</th>
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<tbody>
<tr>
<td></td>
<td>T-C</td>
<td>T/C</td>
<td>T-C</td>
<td>T/C</td>
</tr>
<tr>
<td>n</td>
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<td>60</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td>Mean</td>
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<tr>
<td>Median</td>
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<td>0.793</td>
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<td>Range Min.</td>
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<td>2.213</td>
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<tr>
<td>Max.</td>
<td>0.085</td>
<td>2.167</td>
<td>2.343</td>
<td>22.300</td>
</tr>
</tbody>
</table>
Fig. 1. NIV rapid ELISA and NIV routine ELISA. OD values = test well (T) - control well (C).

Fig. 2. NIV rapid ELISA and NIV routine ELISA. OD values = test well (T)/ control well (C).
Criteria for positive NIV rapid ELISA are (i) the absorbance of the test well minus the absorbance of the negative control well (T-C) should be greater than 0.1 and (ii) the absorbance of the test well divided by the absorbance of the negative control well (T/C) should be greater than 2.0. Figs 1 and 2 represent T-C and T/C values respectively for all the 158 specimens including 155 faecal specimens, one positive control and two negative controls in NIV routine ELISA and NIV rapid ELISA. It is evident that T-C and T/C values for strongly positive samples, (i.e., T-C values above 1.0, and T/C values above 5.0) are much higher in NIV rapid ELISA as compared to NIV routine ELISA. Whereas, NIV routine ELISA showed slightly higher ODs for weakly positive specimens. Another advantage of the rapid ELISA is that the time required for the test is much shorter (4 h) compared to the NIV routine ELISA which takes one day, and can be completed within 6 h by reducing sample incubation time to 2 h.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) for T-C were 97.94, 96.55, 97.94 and 96.55 and for T/C 97.89, 95.0, 96.88 and 96.61 respectively. These values have been calculated taking NIV routine ELISA as the Gold standard.

**Discussion**

Anti SA-11 rabbit serum was used as coating antibody, as well as for preparing conjugate for rapid ELISA. It has been shown that SA-11 antigen is superior to the Nebraska Calf Diarrhoea Virus (NCDV) antiserum for the detection of rotavirus in human stool by counterimmuno electrophoresis\(^\text{10}\).

The results of the present study show that the NIV rapid ELISA for the diagnosis of rotavirus infection is highly specific and sensitive. The test is rapid and can be carried out within 4 h. Besides diagnosis of rotavirus infection, the test can also detect rotavirus in tissue culture stocks. During rotavirus isolation in tissue culture we have used this test to check whether rotavirus is growing (unpublished observation). Besides, the NIV rapid ELISA has potential for testing environmental samples for the presence of rotavirus and may also be useful for testing antiviral drugs against rotavirus.

Only one sample, which was negative in NIV routine ELISA was positive in the rapid test. This was not due to non-specificity, because the rapid test includes negative control for each faecal specimen. The rapid ELISA also shows higher OD values as compared to the NIV routine ELISA. This sample was tested by RNA-PAGE and was confirmed as positive for rotavirus.

In the routine ELISA higher OD values (both T-C and T/C) were seen in the positive samples in the below 1 range for T-C and below 5 range for T/C. The reason for this pattern is not known. It could probably be the time factor. In NIV routine ELISA incubation with faecal samples is overnight whereas in NIV rapid ELISA it is for 2 h.

Regarding the conjugate, which was indigenously prepared, we obtained about 15.0 ml of conjugate by using 2.0 ml of anti SA-11 serum. About 2500 samples can be tested in one ml of conjugate. Several lots of conjugate can be prepared. Thus the cost of production is low. Therefore, this rapid ELISA has good potential for commercialisation.

ELISA plates coated with pre and post immunization sera can be prepared and kept ready to use. If the procedure is followed as per instructions, with a week’s training, technicians at the hospitals can carry out this test for the diagnosis of rotavirus infection. Due to the short testing time, we recommend that stool samples from children hospitalised with diarrhoea should be tested for rotavirus infection. The results can be noted down visually also. It is not possible to distinguish diarrhoea caused by rotavirus clinically\(^\text{11}\). Children testing positive for rotavirus infection need not be given any antibiotic unless use of antibiotics is indicated for other bacterial infections. Use of anti-microbials adds to the cost of treatment, risks adverse reactions and enhances the development of resistant bacteria.

This rapid ELISA test developed at NIV is easy to perform, has high sensitivity and specificity and would be cost effective as a large number of faecal samples could be tested using indigenously developed reagents.

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References


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