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Original Article

Development of a Latex Agglutination Method for Diagnosis of Rotavirus Infections

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Abstract

Background: Rotavirus is a major cause of morbidity and mortality among children with gastroenteritis. Since the discovery of rotaviruses, several techniques have been used for their laboratory diagnosis; those included Electron Microscopy (EM) and enzyme immunoassay. These methods, however, are expensive and not readily available everywhere. We have developed a technique which can be used for routine diagnosis of rotavirus gastroenteritis.

Methods: Purified simian rotavirus, SA11, was injected into rabbits and the γ-globulin fraction of antisera was purified and used for coating of latex beads. The prepared sensitizied latex was then used for agglutination test on fecal samples. 94 stool samples from infants with acute gastroenteritis were tested by (EM), enzyme immunoassay and Latex Agglutination (LA) method.

Results: The sensitivity of enzyme immune assay and (LA) were 92.5% and 90%, respectively; the specificity of both tests was 98.1% as compared with (EM).

Conclusion: Latex Agglutination Test (LAT) is a simple and relatively inexpensive test which can be used for diagnosis of rotavirus gastroenteritis in diagnostic laboratories and health centers.

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Keywords ● Rotavirus ● gastroenteritis ● latex agglutination

Introduction

Human serotypes of group A rotavirus are a major cause of gastroenteritis in young children throughout the world.[1,2] Rotavirus gastroenteritis also occurs in older children and elderly populations.[3-5]

The laboratory diagnosis of rotavirus infections plays an important role in treatment of the disease and also control of its outbreaks.[6] Human serotypes of rotavirus do not grow readily in tissue culture and therefore, are difficult to be isolated from the clinical specimens.[6,7] The laboratory diagnosis of rotavirus infections relies on direct observation of virus by (EM) and/or detection of viral antigens by Enzyme-Linked Immunosorbent Assay (ELISA) and Radioimmunoassay (RIA).[7-10] These procedures, however, are technically demanding and require specialized equipments which limits their application.[6,11] Therefore, a rapid simple method for detection of rotavirus in stool specimens is desirable. The objective of this study was to develop a

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rapid agglutination method for detection of human rotavirus in fecal samples of children presenting with gastroenteritis.

Materials and Methods

Stool specimens

Stool samples were collected from 94 infants and children aged six months to five years who presented with acute gastroenteritis during the winter of 2003. Children were hospitalized with symptoms of vomiting, diarrhea and fever which lasted four to five days. Specimens were diluted 1:2 in Phosphate Buffer Saline (PBS) and clarified by centrifugation at 2000 rpm for 10 min. Supernatants were collected and stored at -70 ºC until used.[11,12]

Virus

The virus strain used in this study was simian SA-11 rotavirus which was passaged in a continuous African green monkey cell line (BSC-1). The virus was grown in cells using serum-free medium (DMEM, HiMedia, Iran) containing 0.3 g/L glutamine, 0.1 g/L pyruvate, 2.5 g/L sodium bicarbonate, 100 U/mL penicillin and 100 µg/mL streptomycin. Virus was treated with 4-5 µg/mL of trypsin and used as rotavirus seed.[8]

Virus Purification

Cells were infected with rotavirus at multiplicity of infection (moi) of 100 TCID50/cell. The infected cells were then harvested after 48 hrs. They were frozen thawed twice and clarified by centrifugation at 2000 rpm for 15 minutes using a clinical centrifuge. Virus was concentrated by ultracentrifugation (at 25,000 rpm for 2.5 h) on 40% sucrose cuchin and purified by ultracentrifugation (30,000 rpm for 3 h) in CsCl gradient.[3,6,13] The virus bands were collected and dialyzed against (PBS) at 4 ºC. Purified rotavirus was examined by negative staining EM. The upper band which contained double shelled rotaviruses was taken and dialyzed against phosphate buffer and stored until used.

Antiserum Preparation

Equal volumes of purified virus suspension and complete Freund's adjuvants were mixed and injected subcutaneously into rabbits. Protein concentration for each injection was approximately 200 µg. The rabbits were injected at weekly intervals for three weeks and a final booster dose was given after one month. Rabbits were bled 10 days later. The titers of pre-immunization and hyperimmune sera were determined by indirect immunofluorescent test.

Preparation of IgG

The γ-globulin fraction of antiserum was purified by ammonium-sulfate prcipitation at 45% saturation (v/v).[14] The precipitate was dialyzed against phosphate buffer (pH 7.5) for 24 hrs. The dialysate containing partially-purified γ-globulin was further purified by passing through an ion exchange column of Diethyl Amino Ethyl Cellulose. The column was eluted with 0.02 M phosphate buffer (pH 6.5). The γ-globulin fractions were further concentrated by polyethylene glycol through a dialysis bag and protein concentration was determined by measuring the optical density (OD) at wavelength of 280 nm.

Coating of latex

Latex beads (0.8 µ, DIFCO) were diluted 1:2 in 0.1 M glycine buffer (pH 8.2), and mixed with IgG solution at concentration of 200 µg/mL protein and incubated for 24 hrs. To saturate the nonspecific sites on latex beads glycine buffer containing 1% bovine serum albumin (BSA) was added with ratio of 2:1 (v/v). The suspension was then centrifuged at 2000 rpm for 15 minutes and the pellet was resuspended in the original volume of glycine buffer and stored at 4 ºC.

Latex Agglutination Test

Fifty µL of clarified stool suspensions were mixed with 50 µL of sensitized latex beads on a slide and placed on a lateral shaker. Latex beads were coated with various dilutions of IgG containing 200 µg/mL protein. Preimmune rabbit serum was used as the negative control. Agglutination reaction was evaluated after 3–5 min and was scored 1^+ to 4^+ according to the degree of latex clumping.

Rotavirus seed (TCID 50 = $10^{5.5}$/mL) was diluted up to 1:160. Agglutination test was carried out by mixing different dilutions of antigen with different titers of IgG. Different titers of rotavirus seed (TCID 50 = $10^{5.5}$/mL) was prepared and tested for agglutination. The titer of 1:16 of this suspension was used as the positive control.

Indirect Enzyme Immune Assay test

This test was carried out according to the manufacturer's instructions (Dakocytomation, Cambridgeshire, UK). Briefly, 100 µL of clarified stool samples or control was used. Then, 100 µL of peroxidase-conjugated antibody was added and the mixture was incubated at room temperature (RT) for 60 min. After washing the wells, 100 µL of the substrate was added and incubated at RT for 10 min. Then, 100 µL of stopping solution was added and absorbancy was read by an ELISA (Dakocytomation, Cambridgeshire, UK) reader.

Electron microscopy

Clarified stool specimens were negatively stained using 1% Phosphotungstic Acid (PTA) on 400 mesh copper grids. The grids were examined in a Zeiss EM10 electron microscope.

Comparison of various tests for detection of rotavirus in different dilutions of rotavirus

To compare the sensitivity of (LAT) with commercially-available IDEIA test, dilutions of rotavirus stocks with known titers ($10^{5.5}$ TCID 50/mL) were prepared. Parallel samples were tested by both methods.

Evaluation of LAT on clinical specimens

To determine the potential use of LAT for the diagnosis of rotavirus gastroenteritis, 94 specimens from children suffering from gastroenteritis were collected. These specimens were tested by the above-mentioned three methods—LAT, IDEIA and EM, the gold-standard method.

Results

Virus purification

Virus was purified by CsCl density gradient centrifugation as described earlier. The purity of virus band was examined by EM. We found that the upper band in the gradient tube contained double-shelled virus particles (fig 1) and the lower band contained single-shelled particles (fig 1). These preparations were apparently pure and free from the cell debris.

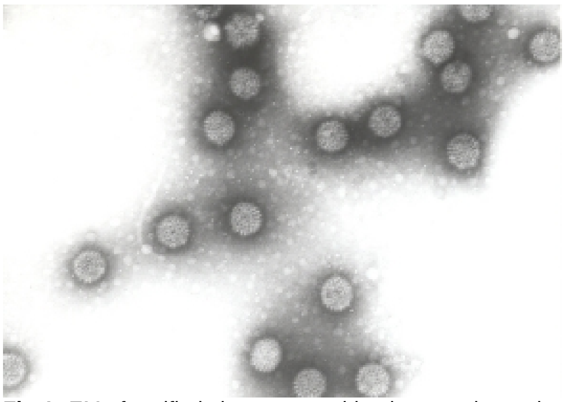

Fig 1: EM of purified virus prepared by the negative staining technique. Virus particles from the upper band of the tube are complete double-shelled rotavirus (a) (160,000×). The viruses from the lower band are single-shelled rotavirus (b) (130,000×).

Preparation of γ-globulin

Hyperimmune sera obtained from rabbits injected with purified virus were titrated by the immuno-fluorescent (IF) technique (fig 2). The titer of the antisera measured by IF, was 1:40 which showed clear staining of intracellular viral antigens. The γ-globulin fraction of antisera obtained by column chromatography was also titrated and its antibody potency was determined by IF test. At a dilution of 1:80 of γ-globulin, the solution contained 200 µg/mL protein and reacted positively with viral antigens. This preparation was used for coating latex particles.

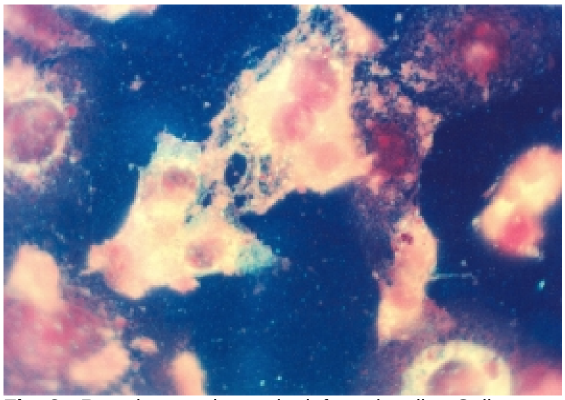

Fig 2: Rotavirus antigens in infected cells. Cells were stained by fluorescein-conjugated antibody using rotavirus-specific antisera.

Latex sensitization and standardization

Nonspecific agglutination was seen at dilution 1:2 (100 µg/mL protein) of IgG-coated beads. The nonspecific agglutination of coated latex beads could be eliminated by diluting the IgG before sensitization of latex. The optimum dilution of IgG for antigen detection was 1:4 which corresponded to 50 µg/mL protein.

Comparison of various tests for detection of rotavirus in different dilutions of rotavirus

Test results at different virus concentrations are shown in table 1. At a dilution of 1:32 of the virus, both tests showed positive results. At dilution of 1:64, which was the end-point, IDEIA test was positive but LAT had borderline results. At this dilution, viral particles could be observed by EM.

Table 1: Comparison of various tests for detection of rotavirus at various dilutions of rotavirus.

Reciprocal of Titer	ELISA	LA	EM
1	+	+	+
2	+	+	+
4	+	+	+
8	+	+	+
16	+	+	+
32	+	+	+
64	+	+/–	+
128	+/–	–	+
256	–	–	–

Rotavirus stock with titer ($10^{5.5}$ TCID 50/mL) was diluted up to 1:256. Samples were tested simultaneously by ELISA, LA and EM.

Evaluation of LAT on clinical specimens

Fig 3 shows a positive stool sample showing presence of rotavirus particles. table 2 shows that out of 94 specimens tested, 40 were positive by EM, 39 by IDEIA and 38 by LAT. The sensitivity of IDEIA and LA tests were therefore, 92.5% and 90%, respectively; the specificity of the both tests was 98.1%.

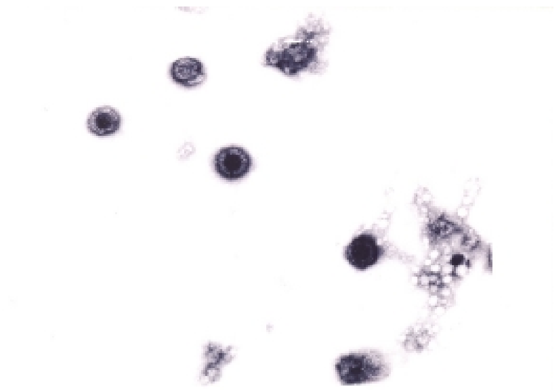

Fig 3: EM of a positive stool sample by negative staining technique showing the presence of rotavirus particles.

Table 2: Comparison of EM, IDEIA and LA tests for detection of rotavirus in stool specimens collected from 94 children with diarrhea.

Method	Positive	Negative
EM	40	54
IDEIA	39	55
LA	38	56

The supernatants of clarified specimens were used for the tests. EM was carried out as the gold standard method.

Discussion

Several methods have been used for diagnosis of viral gastroenteritis.[8,9,15] EM is the gold standard for detection of rotavirus[8-10] This method, however, requires expensive equipments and skilled personnel which is not readily available in many laboratories. Commercial IDEIA kit which is based on ELISA has been used by some investigators for the diagnosis of rotavirus infections in Iran.[16,17] This method is rather expensive and requires several hours to be completed. LA is a rapid method which can be performed even in physician's office and comparatively is inexpensive. We used SA11strain of the virus because it has a group-specific antigen with human rotavirus and its antibody can be used for diagnosis of human rotavirus gastroenteritis.

The most important factor for specificity of LAT was to use purified antigen for antibody preparation.

We purified rotavirus and confirmed its purity by EM. Care was taken to use proper positive and negative controls to detect false reactions. Using concentrated IgG at a dilution of 1:2 for coating, was found to give false positive. At lower concentrations, this problem was eliminated. The possible cause of false-negative results could be the lack of adequate sensitivity to detect low number of virus particles which could be observed by EM. When LAT was concurrently used with EM and IDEIA methods, for detection of rotavirus in clinical specimens, we found that the sensitivity and specificity of LAT was quite high and acceptable comparing to IDEIA. Although these values are slightly lower than those of IDEIA, considering the simplicity and the relative low cost of LAT, it can be used in many laboratories and health centers for diagnosis of rotavirus gastroenteritis. Although we did not use commercial (LA) kits, previous studies have shown that the specificity and sensitivity of commercial kits were 99.5% and 81.7%, respectively.[11]

Conclusion

LAT can be used for diagnosis of rotavirus gastroenteritis. This method is non-expensive and simple to perform as compared to IDEIA and EM. Its sensitivity and specificity is good and comparable with those of IDEIA.

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