DEVELOPMENT OF A SIMPLE AND RAPID LATEX TEST FOR ROTAVIRUS IN STOOL SAMPLE

Hideki Kohno, Tomoe Komoriya, Takuji Sakai and Susumi Ishii

College of Industrial Technology
Nihon University
Narashino, Chiba, JAPAN
h5kohno@cit.nihon-u.ac.jp

ABSTRACT

Recently, in order to detect infectious disease, a variety of immunoassay methods has been reported. Among them, more simple, rapid and cost-effective manual tests for infectious disease are needed. On the purpose of simple diagnosis of rotavirus infection, a rapid test against human rotavirus was developed. On the aim of preparation of the reagent, clinically isolated human rotavirus was cultured using MA-104 cells. Then, the cultured virus was purified by an ultracentrifugation method. The purified virus was inactivated with formaldehyde to use as antigen for immunization of rabbits. The antibody was purified and applied to sensitize the surface of latex particles to react with rotavirus in clinical diarrheal stools. The latex agglutination test showed that the sensitivity was 98.2% and the specificity was 94.8%, in comparison with electron microscopy as the gold standard. Furthermore, to measure the virus concentration in stool samples, an automated latex photometric immunoassay system (LPIA) was used, which has been developed for quantitative measurement of agglutination caused by immune complex formation. The range of the reactive viruses detected by the LPIA system was $3 \times 10^7$-10$^9$ virions/mL. The rotavirus-positive and rotavirus-negative subjects were clearly discriminated and measured. The results were in good accordance with electron microscope results. Those results showed that the latex agglutination test is the best tool for the simple and rapid detection of rotavirus in stool samples.

INTRODUCTION

Nowadays, immunoassays have advances greatly and it presents us an interesting history in rapid diagnosis of infectious disease. The first immunoassay, radioimmunoassay (RIA), was discovered in 1959 as a method of measuring human insulin for the diagnosis of diabetes (Yalow et al., 1959). Then, rather than using the biohazardous hot compound of 125I, a non-RIA called enzyme immunoassay (EIA) was developed. The non-RIA has become a great tool for immunoassay in laboratories (Nakane and Pierce, 1966; Engvall and Perlmann, 1971). However, EIA takes several hours to obtain results and is not the best way of rapidly testing for infectious disease, because many steps are needed in the procedure. Therefore, a simple and rapid assay has been required to indicate positive or negative results from clinical samples (Singer and Plotz, 1965; Kohno, 1985).

To obtain a quick result, we attempted to develop a rapid and simple testing method by using synthetic polystyrene latex particles, which had been sensitized with high-affinity purified
specific antibodies. The development of a long term stable latex particle reagent has enabled us to diagnose a variety of infectious diseases, such as rotavirus and adenovirus infections. More recently, an immunochromatography method has been introduced as a simple and rapid immunologic test. A comparison of immunoassay methods with others and their usefulness are shown (Fig. 1). Here, we describe a simple, rapid and cost-effective manual test for rotavirus infection.

<table>
<thead>
<tr>
<th>Principle</th>
<th>RIA</th>
<th>EIA</th>
<th>LA</th>
<th>Immunochromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3, T3-125</td>
<td>T3, T3-125</td>
<td>T3, T3-125</td>
<td>T3, T3-125</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Averaged sensitivity</th>
<th>10 pg/mL</th>
<th>100 pg/mL</th>
<th>1 ng/mL</th>
<th>1 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Depending on antibodies, solid phase, reaction time and condition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>18-24 h</td>
<td>2-5 h</td>
<td>3 min</td>
<td>5-10 min</td>
</tr>
</tbody>
</table>

Figure 1. Comparison of radioimmunoassay (RIA), enzyme immunoassay (EIA), latex agglutination (LA) and immunochromatography. Principle, averaged sensitivity, specificity and time to examine are indicated.

Rotavirus detection

Rotavirus is a major cause or infant gastroenteritis in winter. A simple and rapid method is greatly needed to detect viral antigen in stool samples. There are several methods to detect human rotaviruses. For example, electron microscopy (EM) is one of the reliable methods for detecting rotavirus antigen. In addition, an alternative method for rotavirus detection is RNA-polyacrylamide gel electrophoresis (RNA-PAGE) and the most popular method is EIA. As for the rapid detection of rotavirus antigen, the latex agglutination test seems to be the most powerful method (Kohno, 1989; Ushijima et al., 1986). There are several commercially available immunoassay kits for rotavirus. However, these kits were prepared on the basis of bovine rotavirus (NCDV strain) and simian rotavirus (SA-11). In this report, we isolated a rotavirus from a stool sample of a patient of nursing care house named ‘Odelia’. In our study, Odelia strain was determined to be serotype IV, subgroup II in characterization of rotavirus. This isolated strain was cultured in MA-104 cells. We separated the virus from the cultured supernatant and purified it by ultracentrifugation. The purified virus was used as an antigen to immunize rabbits by priming and boosting the process of immunization. The antibody IgG fraction was purified and modified by fragmentation by pepsin digestion, which modified the IgG fraction into the F(ab)2’ fraction to sensitize the latex beads. The purified antirotavirus antibody used to coat the surface of the polystyrene particles with a diameter of 0.22 µm. The antirotavirus antibody coated latex particles were applied to the extracted stool samples from patients. Here, we report on the clinical evaluation of the latex test by a manual procedure and by the automated latex photometric immunoassay (LPIA) system.
METHODS

Cell culture of human rotavirus (Odelia strain)

MA-104 cells were incubated in Eagle’s minimal essential medium (MEM) with 10% fetal calf serum (FCS) in a 5% CO2 incubator at 37°C for 5 days. The incubated cells formed a monolayer sheet on the surface of the culture bottle. For maintenance of the rotavirus-infected cells, we used Eagle’s MEN with 10% tryptone phosphate broth, 0.05% yeast extract, 0.5% sodium glutamate, 0.1% glucose and 1 µg trypsin. The cells were incubated at pH 7.4. Rotavirus was incubated with infected MA-104 cells in Eagle’s MEN in addition to 10µg/mL trypsin. The procedure was as shown (Fig. 2).

Figure 2. Propagation of rotavirus. MEM, minimal essential medium; FCS, fetal calf serum.

Purification of rotavirus antigen

The harvested virus was purified as follows. The infected cells and the supernatants were frozen and thawed three times. Then, the fluid was centrifuged at 6,000g for 30 minutes. The supernatant was collected, put on the 30% sucrose cushion and centrifuged at 90,000g for 3 hours. The precipitate was dissolved and applied to the density gradient of CsCl and was ultracentrifuged at 150,000g for 18 hours. After ultracentrifugation, the viral band was collected directly from the centrifugation tube by syringe and then dialyzed (Fig. 3).
Preparation of rotavirus antibody

The virus inactivated in 0.1% formaldehyde solution at 4°C for 7 days and then dialyzed with PBS at pH 7.4. The protein concentration was adjusted to 2mg/mL. After that, 0.35mL of the antigen solution and 0.5 mL Freund’s adjuvant were mixed to make an emulsion. The antigen (1mg) was injected subcutaneously into the rabbits. Four weeks after the first injection, another 0.5mg antigen was immunized as booster. The serum was collected within 1 week after the second injection (Fig. 4).
**Preparation of antirotavirus latex reagent**

The antiserum was extracted with 33% ammonium sulfate. The purified immunoglobulin was coated on the surface of the latex beads. Briefly, 3mg immunoglobulin was added to 1% polystyrene latex bead emulsion under vigorously stirred conditions. The latex suspension was treated with 1% bovine serum albumin for 20 minutes. After the supernatant was discarded, 2mL 0.2mmol/L glycine-NaOH buffer at pH 8.2 was added. The process of preparation of the latex reagent is shown (Fig. 5).

Antirotavirus rabbit serum

Treated with 33% (NH₄)₂SO₄

IgG fraction

Dialysis

Sensitized to polystyrene latex particles with IgG fraction of F(ab)₂’ fraction

Antirotavirus latex reagent

Rotavirus positive: agglutination

Figure 5. Preparation of antirotavirus latex reagents
Latex agglutination test of paper slide

A stool suspension of approximately 10% was prepared. The supernatant was collected after centrifugation. The supernatant (100µL) and 50 µL reagent (one drop) were mixed and stirred on a paper slide for 3 min at room temperature. Then, agglutination of the latex was checked visually.

Measurement of latex agglutination by the LPIA system

An aliquot (40µL) of 0.25% latex solution and 250µL stabilizing buffer were added to 10µL of extracted sample and mixed. The agglutination process was monitored and calculated by computer and the results were available within 10 min. All of the process was carried out automatically. On the basis of a calibration curve, the antigen concentration was calculated. Precise information about the LPIA system has been reported previously (Figs. 6 and 7) (Kohno, 1992).

Figure 6. Schematic outline of the latex photometric immunoassay system

Figure 7. Calibration curve for the relative agglutination rate and the number of rotavirus virions
Enzyme immunoassay and electron microscopy

The EIA was carried out using commercially available reagents (Rotazyme II, Abbott, Chicago, IL, USA). The procedure was done according to manufacture’s instructions. For electron microscopic study, the virus was extracted with phosphate-buffered saline (PBS(-)) after ultracentrifugation. The diluted sample was put on the grid (H-400; Veco, Eerbeek, Holland). It was vaporized on the collodion membrane. The sample was negatively stained with 3% uranium oxide. The extracted viral sample was diluted tenfold. The number of viruses was counted in five meshes.

Comparative study of commercially available latex kits

We used two commercially available latex reagent kits, Slidex (Bio Merieux, Macy-1’Etolle, France) and Rotalex (Orion, Espoo, Finland) for our comparative study. All rests were performed according to the instructions of the manufactures.

RNA polyacrylamide gel electrophoresis

Briefly, the 10% stool suspension in PBS was homogenized and centrifuged at 6,000g for 30 minutes. Supernatant (2mL) was collected and was put on the cushion of 30% sucrose. The tube was centrifuged at 100,000g for 3 hours. The precipitate was collected and suspended in 0.5mL 0.4mol/L NaCl. After the addition of an equal volume of 10% SDS, the extraction of RNA was carried out with phenolchloroform. The precipitate was dissolved in 1mL PBS(-) and the same amount of chloroform containing 4% isoamylalcohol. After centrifugation, the upper layer was collected and electrophoresed in a 10% polyacrylamide gel. The gel was stained with silver solution.

RESULTS AND DISCUSSION

In our findings (Honna el al., 1990), 50% of infants under 2 years of age suffer from severe diarrhea caused by rotavirus infection. We isolated human rotavirus from a patient’s stool and named it the ‘Odelia strain’. The virus was used as an antigen to prepare antibody for the development of rapid detection latex kit for rotavirus. One hundred and nine patients were investigated in a comparative study between electron microscopy and our rapid diagnostic latex reagent. Furthermore, we compared our latex reagent (Rota-check, Tokyo, Japan) with the other commercially available latex test and an EIA test kit (Rotazyme-II) for sensitivity and specificity (Table 1). The comparison of the latex agglutination test and EM showed that results from both are in agreement. The sensitivity of the latex agglutination test was equal to that of the EIA test kit.

A number of 307 stool samples were examined by electron microscopy and Rota-check. The cases of (±), treated with delipidation reagent, were found to be positive. The final results showed good correlation between the alternative methods. The EM method showed positive results with 113 cases and negative 194 cases, whereas the latex test showed 112 positive cases. Sensitivity and specificity was 98.2% and 94.8%, respectively (Table 2).
In order to measure the rotavirus concentration in stool samples, we used the LPIA system (Model L-100, Mitsubishi Chemical, Tokyo, Japan; Fig. 6). The calibration curve for standard materials was prepared using a known concentration of virus solution, which was counted by EM. Based on the standard material, we found that the range of virus measurable by the LPIA system was between $3 \times 10^7$ and $10^9$ virions/mL (Fig. 7).

The results of three methods, EM, RNA-PAGE and LPIA, were compared. This comparative study revealed that $10^8$ virions/mL was the cut-off value for positivity and negativity both by microscopy and RNA-PAGE. We found that $10^8$ virions/mL was the clinically significant value of rotavirus infection to be detected by the LPIA system. In all 94 samples the results correlated between the two alternative methods. This LPIA measurement is rather quick, with results within 10 min, and it is effective in an emergency test to know the concentration of virus (Fig. 8).

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive</th>
<th>Negative</th>
<th>Equivocal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elecron Microscopy</td>
<td>57 (52.3%)</td>
<td>52 (47.7%)</td>
<td></td>
</tr>
<tr>
<td>Rota-check (our latex test)</td>
<td>60 (55.6%)</td>
<td>47 (43.1%)</td>
<td>2 (1.8%)</td>
</tr>
<tr>
<td>Slidex</td>
<td>62 (56.9%)</td>
<td>44 (40.3%)</td>
<td>3 (2.8%)</td>
</tr>
<tr>
<td>Rotalex</td>
<td>57 (52.3%)</td>
<td>49 (45.0%)</td>
<td>3 (2.8%)</td>
</tr>
<tr>
<td>Rotazyme-II</td>
<td>55 (32.1%)</td>
<td>54 (49.5%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Comparison of the latex agglutination test, electron microscopy and ELISA

<table>
<thead>
<tr>
<th>Rota-check</th>
<th>Electron microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>95</td>
</tr>
<tr>
<td>±</td>
<td>17</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

| Total      | 113                 |

Table 2. Comparison of the latex agglutination test and electron microscopy
CONCLUSION

We developed a latex agglutination test based on the immunoassay to detect the clinically isolated Odelia strain (human rotavirus, serotype IV, subgroup II). From the development of the latex agglutination test and clinical investigation, we understood the fact as follows.

1. Our latex test was evaluated as an alternative method to EM and RNA-PAGE.
2. The latex test is available for rapid detection of rotavirus.
3. To measure rotavirus in stool samples, the LPIA system was applicable for automatic measurement.
4. In the process of development of a rapid and simple test for rotavirus infection, we feel strongly that we need more rapid testing for effective therapeutic treatment, especially in infantile infectious diseases..

REFERENCES


