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Experience with an Enzyme Immunoassay for Serotyping Human Group A Rotaviruses

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An enzyme immunoassay utilizing neutralizing monoclonal antibodies to VP7 of four human group A rotavirus serotypes successfully typed rotaviruses in 71.4% (568 of 796) of fecal specimens. Sensitivity was enhanced by using homologous capture and detector antibodies. Serotyping was most successful with specimens stored for less than 3 years and containing 10^4 or more particles per ml.

It has been well established that diarrhea due to group A rotavirus is a worldwide problem and that development of an effective vaccine is a priority to control diarrheal rotavirus disease in young children (8). A simple, specific, and sensitive method to serotype rotavirus strains is needed in order to study epidemiology of serotypes in various communities and to appraise the efficacy of candidate rotavirus vaccines.

Currently, human and animal rotavirus strains are classified into serotypes on the basis of neutralization with polyclonal antisera. Although two outer capsid proteins of the virion, VP4 and VP7, are capable of eliciting neutralizing antibody (2), differences in VP7 correlate more closely to serotype (6, 10). We recently developed a serotyping enzyme immunoassay (EIA) utilizing monoclonal antibodies specific for the VP7 of each of the first four human serotypes to be identified (3, 6, 7). The specificities of the monoclonal antibodies have been validated in our laboratories (9). We report here an assessment of the coating antiserum required for maximum sensitivity of the serotyping assay and evaluate our success in assigning a serotype in relation to quantity of virus and to the influence of duration of storage of specimens.

The serotyping assay is based on a double-sandwich EIA method described elsewhere (7). Briefly, for each specimen to be tested, four wells of a microdilution plate were coated separately with rabbit hyperimmune antisera to each of the four rotavirus serotypes. Fecal specimens containing rotavirus were added, followed by serotype-specific mouse monoclonal antibodies. Bound monoclonal antibodies were detected by addition of horseradish peroxidase-conjugated anti-mouse immunoglobulins followed by substrate containing 3,3',5,5'-tetramethylbenzidine. All reagents were used at optimal concentrations.

The final format of the test was chosen after the effect of serotype specificity of the hyperimmune serum used as capture antiserum on the assay sensitivity was determined. Hyperimmune antisera were raised to rotaviruses of known serotype propagated in MA104 cells as described previously (1). Irrespective of the capture antiserum used, each tissue culture-adapted rotavirus reacted positively only in the wells containing the monoclonal antibody specific for its serotype. However, when capture antisera were produced against a serotype different from the detector monoclonal antibody, the optical density at 450 nm (OD_{450}) of the reaction was often 20 to 70% of the OD_{450} found when using homologous capture antisera (Table 1). In particular, serotype 2 and 4 rotaviruses were reliably detected only when homologous capture antiserum was employed. Serotype 1 and 3 viruses were reliably detected with capture antiserum produced to either serotype. Results with a mixture of capture antisera showed that the sensitivity of the test was lower, ranging from 44 to 101% of the homologous OD_{450}, and therefore all serotyping assays in our laboratory are now based on the use of homologous capture and detector antibodies. A serotype is assigned according to criteria already established (7). Specimens that react with a specific monoclonal antibody such that the OD reading (P) is greater than 0.1 OD units and at least two times the OD reading of nonreacting wells (N) (i.e., $P > 2N$) are assigned to that serotype. The color

TABLE 1. Effect of serotype specificity of the hyperimmune capture antiserum on the ability to serotype rotavirus strains by EIA

Virus tested (serotype)	% Homologous OD_{450}[a] for antiserum to virus (serotype):				
	RV-4 (1)	RV-5 (2)	RV-3 (3)	ST-3 (4)	Mixture[b]
RV-4, Wa, Ku (1)	**100**	41–75[c]	57–125	44–98	57–97
RV-5, DS-1, S2 (2)	20–48[d]	**100**	36–74[c]	36–65[d]	53–87
RV-3, P, Yo (3)	88–101	79–94	**100**	75–86	94–101
ST-3, VA70, Hosokawa (4)	39–50[c]	44–49[c]	52–62[d]	**100**	44–72[c]

[a] A homologous OD_{450} reading is that given when hyperimmune capture antisera and the detector monoclonal antibody used were raised to the same virus serotype. This is defined as 100% reaction and denoted in boldface type.
[b] Equal quantities of antiserum to each of the four serotypes were mixed to give a final concentration approximately equal to that used individually (7).
[c] One of the three tested viruses untypable with the given capture antiserum.
[d] All three viruses untypable with the given capture antiserum.

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change reaction is always read in a spectrophotometer, although positive reactions can be read by eye.

A total of 796 rotavirus-positive fecal specimens collected between 1973 and 1986 and stored at −70°C have been tested in this rotavirus serotyping EIA as part of en epidemiological survey (R. F. Bishop, L. E. Unicomb, and G. L. Barnes, manuscript in preparation). All were from children admitted to hospital for treatment of severe acute diarrhea. Rotavirus-positive specimens had been identified by electron microscopy (647) or by EIA (149). A total of 525 of the 796 specimens tested (66%) were successfully serotyped at the first attempt. Of the 285 specimens containing rotavirus that failed to react with any serotype-specific monoclonal antibodies, 199 samples were available in sufficient amount for further concentration with polyacrylamide hydrogel (Lyphogel; Gelman Sciences Inc.). Specimen (2 ml) was added to 0.2 g of Lyphogel and incubated at room temperature until the volume of the specimen was reduced to approximately 0.25 ml (11). The concentrated specimen was then retested in the EIA. A total of 43 of the 199 concentrated specimens (22%) could then be typed. Overall, the serotyping assay identified rotavirus serotypes in 71.4% (568 of 796) of rotavirus-positive fecal specimens, indicating that concentration of specimens had increased the sensitivity of the test by at least 5%.

The results in Table 2 show the success rate of assigning a serotype in 647 specimens where concentration of virions was assessed using electron microscopy. Results from electron microscopy indicated the concentration of rotavirus particles but not the proportion of intact and single-shelled particles. Only specimens typed without concentration are included. By using the chi-square test, we found a statistically significant interaction between the ability to determine a serotype and the number of virions observed in the specimen. Specimens containing approximately 10^5 virions per ml (1+) were less frequently typed than specimens with $\geq 10^6$ virions per ml (2 to 3+) ($P < 0.01$).

To further examine the sensitivity of the assay, serial twofold dilutions of cultivated rotavirus strains (containing at least 80% double-shelled particles) of each of the four major human serotypes were tested in the serotyping EIA. The minimum fluorescent cell-forming units (FFU) per milliliter of virus at which a serotype could be assigned was determined. The results showed that positive serotype results were given by RV-4 (serotype 1) at a concentration of 10^5 FFU/ml, by Wa (serotype 1) at a concentration of 3.5×10^4 FFU/ml, by RV-5 (serotype 2) at a concentration of 4.3×10^4 FFU/ml, by SA11 (serotype 3) at a concentration of 1.6×10^5 FFU/ml, and by ST-3 (serotype 4) at a concentration of 5.9×10^3 FFU/ml. RV-4, RV-5, and ST-3 rotaviruses are the strains against which the serotype 1, 2, and 4 typing monoclonal antibodies are derived, and these results probably represent the assay at the height of its sensitivity.

TABLE 2. Effect of number of rotavirus particles (as assessed by electron microscopy) on success of assigning a rotavirus serotype

EM grading[a] of virions	Total no. examined	Total no. (%) typed	No. (%) typed from serotype:			
			1	2	3	4
1+	278	151 (54)	94 (34)	12 (4)	21 (8)	20 (7)
2+	345	241 (70)	168 (49)	22 (6)	13 (4)	38 (11)
3+	24	20 (83)	13 (54)	2 (8)	1 (4)	4 (17)

[a] EM, Electron microscopy. EM grades are assigned arbitrarily, where 1+ is approximately 10^4 particles per ml, 2+ equals 10^6 to 10^7/ml, and 3+ is greater than 10^7/ml.

TABLE 3. Effect of length of storage of specimen on success of assigning a rotavirus serotype

Storage time (mo)	Total no. of specimens examined	Total no. (%) of viruses typed
0–6	168	114 (68)
6–12	87	59 (68)
13–36	142	104 (73)
>36	337	202 (60)

The age of the stool specimen at time of assay was examined with respect to success of assigning a serotype. In order to assess the short-term influence of storage on ability to serotype specimens, fecal specimens (shown to contain serotype 1 rotaviruses) were collected from four children on admission to hospital, divided into equal portions, and stored for 10 days or 1 month before serotyping by EIA. Storage of rotavirus at 4 or −70°C in feces for up to 1 month did not affect the ability to assign a serotype using the EIA (data not shown). The results in Table 3, however, show that there was a significantly lower success rate ($P < 0.01$) with specimens stored for more than 3 years using the chi-square test, therefore indicating that storage conditions affected serotyping success rates. Lack of sensitivity of the assay for some stool viruses is probably explained by the effects of collection and duration of storage on the integrity of the virions. Stool proteolytic enzymes, particularly trypsin, have been shown to affect particle integrity (4, 5).

In summary, we have found the reported EIA for serotyping human fecal rotaviruses to be easy to perform and appropriate for the testing of large numbers of specimens, including stored specimens. We have also shown that the rate of successful determination of rotavirus serotype is dependent on virion concentration and length of storage of stools. Use of this test should further refine epidemiological studies of rotavirus serotypes causing severe disease in children and be of value in assessing efficacy of candidate rotavirus vaccines used in field trials.

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