

## Human Rotavirus Detection by Agglutination of Antibody-Coated Erythrocytes

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We sensitized sheep erythrocytes (SRBC) with antibodies against human rotavirus strain Wa (SRBC-antiWa) and antibodies against calf rotavirus strain NCDV (SRBC-antiNCDV). These were readily agglutinated in the presence of homologous antigens, i.e., human rotavirus and calf rotavirus. By the hemagglutination of SRBC-antiWa and SRBC-antiNCDV (reverse passive hemagglutination [RPHA]), titration of rotavirus in extracts from feces of children suffering from diarrhea (61 specimens) was carried out. We found that the ratio of titers determined with SRBC-antiWa and SRBC-antiNCDV varied remarkably from specimen to specimen. This indicated that the antigenic determinants on human rotavirus in patients feces cross-react with antibodies against NCDV to varying extents. To express the cross-reactivity of human rotavirus with antibodies to NCDV, we propose a Wa/NCDV rotavirus index which can be calculated from the RPHA titer with SRBC-antiWa and SRBC-antiNCDV as follows:  $\text{Wa/NCDV rotavirus index} = (\text{antiWa-RPHA titer of specimen}/\text{antiWa-RPHA titer of NCDV})/(\text{antiNCDV-RPHA titer of specimen}/\text{antiNCDV-RPHA titer of NCDV})$ .

Rotavirus is the most common causative agent of infantile gastroenteritis in many countries (1, 4, 5, 7, 10, 12, 14-16). To detect rotavirus in fecal specimens, electron microscopy (EM) has been widely used (2, 4, 17). Recently, several serological methods have been developed to detect the virus easily and rapidly. These include enzyme immunoassay (21, 24), radioimmunoassay (8), counterimmunoelectrophoresis (7, 22), immune adherence hemagglutination (14), reverse passive hemagglutination (RPHA; 18), and latex agglutination (20). In some of these methods (6, 18-21), antibody to Nebraska calf diarrhoea virus (NCDV), which cross-reacts with human rotavirus (HRV; 11), has been used since HRV could not be grown in vitro whereas NCDV could. However, Yolken et al. (25) indicated that antisera to NCDV could not effectively detect small amounts of rotavirus in human stools. Furthermore, Zissis et al. (26) demonstrated that antiserum prepared against NCDV failed to react with some HRV. To avoid false-negative results due to failure of HRV to react with anti-NCDV, antiserum against HRV was recommended for use in detection of HRV in specimens. Previously, we demonstrated that sheep erythrocytes (SRBC) coated with anti-NCDV (SRBC-antiNCDV) could detect HRV in

patients' stools by hemagglutination of the indicator cells (18). Although the RPHA test with SRBC-antiNCDV showed a sensitivity comparable to that of EM, it might fail to detect HRV, which had no antigenic determinants reactive with NCDV. Therefore, we attempted to improve the RPHA method by using anti-HRV instead of anti-NCDV. Since, Wyatt et al. have succeeded in establishing an HRV strain, called the "Wa" strain (Wa) (24), to grow in vitro, we prepared antibody to Wa (anti-Wa) by immunizing guinea pigs with Wa and purified anti-Wa with a Wa-coupled Sepharose 4B column. With this purified antibody, we coated SRBC (SRBC-antiWa) for use in the RPHA test. The reactivities of this SRBC-antiWa and that of SRBC-antiNCDV in the RPHA test were compared with patients' feces.

### MATERIALS AND METHODS

**Fecal samples.** Fecal samples were obtained from infants with gastroenteritis admitted to Tachikawa Kyosai hospital and Takei Pediatric Clinic and were stored at  $-20^{\circ}\text{C}$  until used.

**Preparation of SRBC-antiNCDV and SRBC-antiWa.** Anti-NCDV was purified from guinea pig antisera, using a column of Sepharose 4B conjugated with NCDV, and the antibody (immune adherence hemagglutination titer, 1,600) was fixed to SRBC as described previously to prepare SRBC-antiNCDV (18, 19). Wa was obtained through T. Konno from R. G. Wyatt and propagated in MA-104 cells. In cultivating

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Wa, 0.5  $\mu\text{g}$  of trypsin per ml was added to the maintenance medium. The culture fluid containing Wa was centrifuged at  $54,900 \times g$  for 4 h at  $4^\circ\text{C}$ , and the deposited virus was suspended in 0.001 M Tris-hydrochloride buffer containing 0.1 M NaCl (pH 7.2). The virus suspension was centrifuged in a sucrose density gradient (20 to 60%, wt/wt), and the purified Wa fraction was obtained as described for NCDV purification (20). Antiserum against Wa was prepared by immunizing guinea pigs by the same protocol described for producing antiserum against NCDV (18, 20). The antibody was purified by affinity chromatography on a column of Sepharose 4B conjugated with Wa. Antibody bound to the column was eluted with 3 M NaSCN, and the eluted antibody was dialyzed against saline. The purified anti-Wa (immune adherence hemagglutination titer, 1,600) was fixed to SRBC treated with tannic acid after glutaraldehyde treatment as described previously (18, 19).

**Preparation of specimens for RPHA tests.** One volume of feces was mixed with 9 volumes of a 5% suspension of glutaraldehyde-treated SRBC in 0.067 M phosphate-buffered saline, pH 7.2 (PBS), and the mixture was thoroughly homogenized. In this manner the fixed SRBC absorbed hemagglutinins to SRBC which interfered with results of the rotavirus RPHA. The homogenate was incubated at  $37^\circ\text{C}$  for 30 min and then centrifuged at 3,000 rpm for 20 min. To the supernatant fluid was added an equal volume of trifluorochloroethane to remove substances which might interfere with the sedimentation of SRBC-antiNCDV and SRBC-antiWa. The mixture was centrifuged at 3,000 rpm for 30 min, and the supernatant fluid was used as the specimen for RPHA tests.

**RPHA tests.** RPHA tests were carried out on a microtitration plate as described previously (18). Serial twofold dilutions of the specimens were made in quadruplicate in PBS containing 1.5% normal rabbit serum. In a dilution series for the test proper, 25  $\mu\text{l}$  of PBS-normal rabbit serum was added to each well, and in a corresponding set of dilutions, the same amount of anti-Wa or anti-NCDV (complement fixation titer, 160) was added as a blocking reagent to confirm the specificity of the RPHA. The mixtures were incubated for 1 h at  $37^\circ\text{C}$ , and then 25  $\mu\text{l}$  of a 0.6% suspension of SRBC-antiWa or SRBC-antiNCDV was added to each well. After shaking, the tray was kept at room temperature for 1 h or more and hemagglutination patterns were read. A fourfold or greater specific reduction of hemagglutination titer by the blocking test, with corresponding antibody, was regarded as an indication of a positive reaction.

**Isolation of rotavirus by tissue culture.** Isolation of HRV was carried out as described before (22). A 10% fecal suspension in Eagle minimum essential medium containing 1,000 U of penicillin and 1,000  $\mu\text{g}$  of streptomycin per ml was thoroughly mixed and centrifuged at 3,000 rpm for 30 min. The supernatant fluid was collected and centrifuged at 3,000 rpm for 30 min again. To the supernatant fluid was added an equal volume of 10  $\mu\text{g}$  of trypsin per ml, and the mixture was incubated at  $37^\circ\text{C}$  for 20 min, after which 0.1 ml of the trypsin-treated specimen was inoculated onto MA-104 cells grown in tubes (14 by 165 mm). After incubation at  $37^\circ\text{C}$  for 90 min, the inoculum was removed and cells were washed three times with PBS. The cells were cultivated in 1 ml of Eagle minimum essential

medium containing 0.5  $\mu\text{g}$  of trypsin per ml at  $37^\circ\text{C}$  in a roller drum for 7 days. Further passages were made at intervals of 7 days as follows. The cells together with culture medium in tubes were frozen and thawed three times, and 0.1 ml of the frozen and thawed material was inoculated to fresh MA-104 cells in the same manner as for the initial inoculation of fecal extract.

**EM observations.** A 5-ml portion of fecal specimen prepared for RPHA tests as described above was centrifuged at 40,000 rpm for 2 h. The pellet was resuspended in 0.2 ml of distilled water and stained with 2% phosphotungstic acid to examine in a JEM-100B JEOL EM. To examine the morphology of Wa fractionated by a sucrose density gradient, 1 drop of each fraction was layered on a grid and dried at room temperature. The grid was washed with distilled water, and the specimen was stained with 2% phosphotungstic acid to examine by EM.

## RESULTS

**Sensitivity of SRBC-antiWa and SRBC-antiNCDV for detection of rotavirus particles.** SRBC-antiWa and SRBC-antiNCDV prepared as described above were evaluated for their sensitivity in detection of Wa and NCDV by determining minimum numbers of virus particles detectable by RPHA tests. SRBC-antiWa could detect Wa and NCDV at dilutions of 1:64,000 and 1:1,600, respectively (Table 1). SRBC-antiNCDV could detect Wa and NCDV at dilutions of 1:2,000 and 1:1,600, respectively. The number of virus particles in the original preparations of Wa and NCDV were determined by EM, using latex particles as a reference of counts as described previously (20). Wa and NCDV preparations contained  $10^{12}$  and  $4.3 \times 10^{10}$  virus particles, respectively (Table 1). Therefore, SRBC-antiWa could detect  $1.6 \times 10^7$  Wa particles per ml and  $2.7 \times 10^7$  NCDV particles per ml, whereas SRBC-antiNCDV could detect  $5.0 \times 10^8$  Wa particles per ml and  $2.7 \times 10^7$  NCDV particles per ml.

**Detection of HRV in feces by RPHA tests.** A total of 61 fecal specimens from gastroenteritis patients were examined by RPHA tests with both SRBC-antiWa and SRBC-antiNCDV. Of the 61 specimens, 49 agglutinated SRBC-antiWa, whereas only 29 specimens agglutinated

TABLE 1. Sensitivity of SRBC-antiWa and SRBC-antiNCDV for detection of virus particles

Virus	Virus particles/ml	RPHA titer with:	
		SRBC-antiWa	SRBC-antiNCDV
Wa	$1.0 \times 10^{12}$	64,000 ( $1.6 \times 10^7$ ) <sup>a</sup>	2,000 ( $5.0 \times 10^8$ )
NCDV	$4.3 \times 10^{10}$	1,600 ( $2.7 \times 10^7$ )	1,600 ( $2.7 \times 10^7$ )

<sup>a</sup> Values in the parentheses indicate the concentration of particles at the dilution indicated.

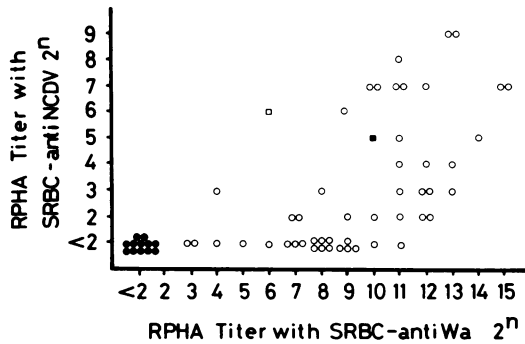


FIG. 1. Comparison of RPHA titers of HRV in feces against SRBC-antiWa and against SRBC-antiNCDV. Symbols: (■) standard Wa; (□) standard NCDV; (○) HRV-positive fecal extract by EM; (●) HRV-negative fecal extract by EM.

SRBC-antiNCDV. The 29 specimens which agglutinated SRBC-antiNCDV all had the ability to agglutinate SRBC-antiWa. These results indicated that SRBC-antiWa could detect rotavirus which could not be detected with SRBC-antiNCDV. Figure 1 shows the relationship between the RPHA titers with SRBC-antiWa and those with SRBC-antiNCDV. The 20 specimens which did not agglutinate SRBC-antiNCDV showed RPHA titers of 8 to 2,048 with SRBC-antiWa.

EM examination for HRV was also carried out on the specimens. All specimens with HRV detectable by EM showed RPHA titers of more than 8 when examined with SRBC-antiWa.

**Wa/NCDV rotavirus index (Wa/NCDV index).** Since the ratio of RPHA titers with SRBC-antiWa and those with SRBC-antiNCDV varied remarkably from specimen to specimen (Fig. 1), we attempted to express the nature of rotavirus by the ratio of RPHA titers. For this purpose, the RPHA titer of a NCDV preparation with SRBC-antiWa and that with SRBC-antiNCDV were used as standards. The RPHA titer with SRBC-antiWa (antiWa-RPHA titer) of a test specimen was divided by the antiWa-RPHA titer of a standard NCDV. The RPHA titer with SRBC-antiNCDV (antiNCDV-RPHA titer) of the specimen was also divided by the antiNCDV-RPHA titer of a standard NCDV. Then the ratio of the former and the latter was tentatively designated as the Wa/NCDV index: 
$$\text{Wa/NCDV index} = \frac{(\text{antiWa-RPHA titer of specimen} / \text{antiWa-RPHA titer of NCDV})}{(\text{antiNCDV-RPHA titer of specimen} / \text{antiNCDV-RPHA titer of NCDV})}$$
 When a specimen did not have a detectable RPHA titer with SRBC-antiNCDV, the titer was considered to be 2 or less. With this index, the results shown in Fig. 1 were expressed in Fig. 2. As a control, cultured rotavirus Wa was also examined, and it showed

a Wa/NCDV index of 32. Most of specimens showed a Wa/NCDV index of more than 32, and the ratios ranged up to more than 2,048.

**Wa/NCDV index of intact and degraded Wa.** It was conceivable that HRV which failed to react with SRBC-antiNCDV might be digested or broken rotavirus which had lost antigens reactive with anti-NCDV antibodies. To examine this possibility, Wa was fractionated by sucrose density gradient ultracentrifugation to separate native and degraded viruses in the Wa preparation. Wa propagated in MA-104 cells was concentrated 100 times, and 0.5 ml of the concentrated virus suspension was layered on top of a 4.5-ml sucrose density gradient (20 to 60%, wt/wt) and centrifuged at 36,000 rpm for 90 min. Five-drop fractions were collected through a pinhole at the bottom of the gradient.

RPHA titers of each fraction are shown in Fig. 3. The titers with SRBC-antiWa were four times or more higher than those with SRBC-antiNCDV for all fractions except no. 18. In fraction 9 (Wa/NCDV index, 8), most viruses were complete particles with outer membrane, and in fraction 13 Wa/NCDV index, 32), incomplete particles without outer membrane were dominant as shown by EM (Fig. 4). Fraction 16 (Wa/NCDV index, 4) contained mostly disrupted virions. However, there was no fraction which agglutinated SRBC-antiWa without show-

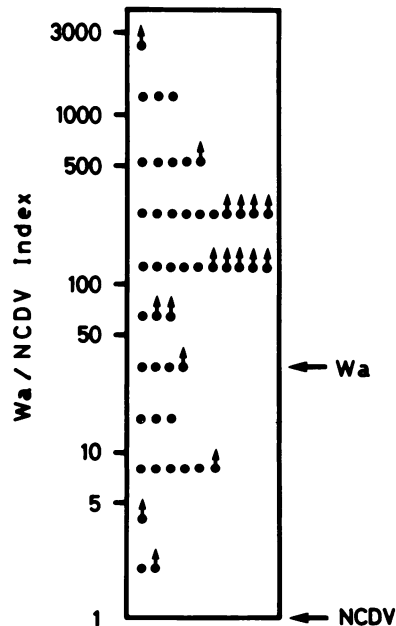


FIG. 2. Wa/NCDV indices of HRV in feces. (↑), Since RPHA with SRBC-antiNCDV was undetectable, the titer was tentatively regarded as 2 to calculate the index. Therefore, the actual index will be higher than the position indicated with this mark.

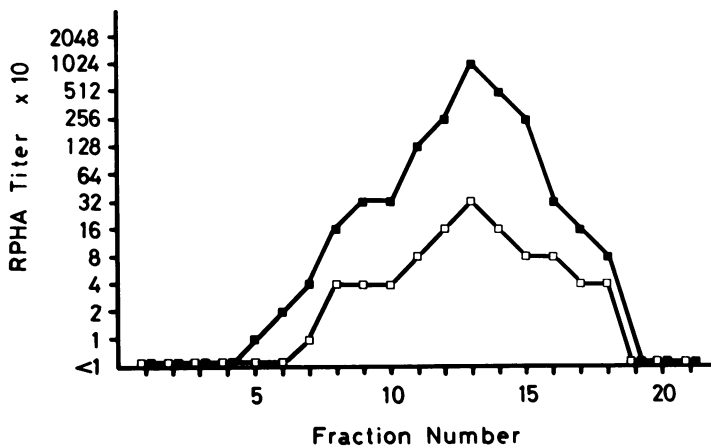


FIG. 3. Fractionation of Wa by sucrose density gradient ultracentrifugation. RPHA titers with SRBC-antiWa (■) and SRBC-antiNCDV (□) were examined on each fraction. Morphology of virus particles examined by EM on fractions 9, 13, and 16 are shown in Fig. 4.

ing reactivity with SRBC-antiNCDV. This indicated that in the course of degradation HRV did not lose reactivity with anti-NCDV while retaining reactivity with anti-Wa.

**Effect of partial purification of virus particles on Wa/NCDV index.** It was conceivable that some agents in fecal extracts such as degraded viral particles or gastrointestinal antibodies might influence the Wa/NCDV index. To exclude this possibility, we compared RPHA titers of fecal extracts and virus from the fecal extract partially purified on a sucrose cushion. The partially purified virus fractions were suspended to their original volume of fecal extract. Essentially no difference was noted between the partially purified virus fraction and the original fecal extracts in RPHA titers or in Wa/NCDV indices (Table 2). Furthermore, the Wa/NCDV indices of specimens obtained at different time from the same patients were essentially the same. Taken together, these findings suggest that Wa/NCDV indices will be characteristic of each specimen from a patient.

**Isolation of rotavirus from feces by tissue culture.** Six specimens from gastroenteritis patients were used to attempt to isolate HRV in tissue culture. One strain (no. 17) of HRV was successfully isolated (Table 3). By EM examination, numerous virus particles were observed in the culture fluid. The Wa/NCDV index of the no. 17 HRV was over 256 before inoculation and 128 after three passages in cell culture, and the values were different from those of NCDV and Wa.

## DISCUSSION

Previously, we reported that the RPHA test for HRV detection was a simple method with

high sensitivity and satisfactory reproducibility as compared with EM (18). For the RPHA test, only a microtitration set (microtiter plate and dilution loops) is required equipment, and the procedure is simple enough to be performed in clinical laboratories. Therefore, the method can be recommended for use in countries where clinical laboratories are not well equipped and where the diagnosis of HRV infection is seriously needed.

In previous RPHA tests we used SRBC-antiNCDV as indicator cells of hemagglutination. However, Yolken et al. (25) indicated that antisera to NCDV could not effectively detect small amounts of rotavirus in human feces, and Zissis et al. (26) demonstrated that some HRV failed to cross-react with antiserum to NCDV. Therefore, we attempted to replace SRBC-antiNCDV with SRBC-antiHRV for RPHA tests. Antibody to HRV was prepared by immunizing guinea pigs with the Wa strain of HRV which Wyatt et al. (24) adapted to in vitro growth in MA-104 cells. Anti-Wa was used to prepare SRBC-antiWa by the same procedure used for preparation of SRBC-antiNCDV (18, 19). To examine the effectiveness of substituting SRBC-antiNCDV with SRBC-antiWa for RPHA tests, we determined HRV titers in RPHA with SRBC-antiWa and SRBC-antiNCDV on fecal specimens of 61 patients with infantile diarrhea.

In this study, SRBC-antiWa was considerably more efficient for the detection of HRV than was SRBC-antiNCDV. Of 49 specimens positive by SRBC-antiWa, only 29 were also positive with SRBC-antiNCDV. These 20 specimens which were missed were not false positive as each was also positive by EM. These findings differed from our previous report that SRBC-antiNCDV

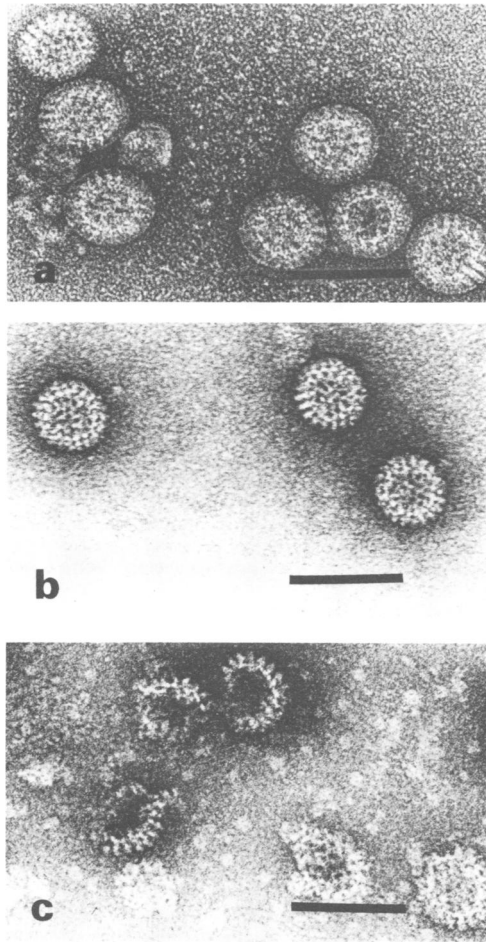


FIG. 4. Electron micrographs of fractionated Wa. Bar, 100 nm. (a) Fraction 9: mainly complete particles with outer membranes were observed. (b) Fraction 13: mainly incomplete particles without outer membranes were observed. (c) Fraction 16: disrupted particles were dominant.

was more efficient than EM for the detection of HRV. The reason for this discrepancy may be due to the use of different specimens in the two studies. The specimens used in the previous study may have contained prevailing HRV strains which were more reactive with SRBC-antiNCDV.

Since the ratio of RPHA titers with SRBC-antiWa and SRBC-antiNCDV varied significantly (Fig. 1), we adopted a Wa/NCDV index to express the ratio of RPHA titers determined with SRBC-antiWa and with SRBC-antiNCDV. The ratio of RPHA titers of the specimen with SRBC-antiWa and with SRBC-antiNCDV was divided by that ratio of NCDV to obtain a Wa/NCDV index. This index expresses the ratio

of RPHA titer with SRBC-antiWa to that with SRBC-antiNCDV in comparison with the ratio of a standard NCDV. The standard NCDV corrects the reactivity of SRBC-antiWa and that of SRBC-antiNCDV, which may differ from lot to lot of these indicator cells.

With this index, we differentiated HRV specimens having a large amount of antigens cross-reactive with antiWa and those having little of the cross-reactive antigen with Wa. The indices varied from 2 to more than 2,048, and 49.2% of the specimens showed an index of 128 or more. It was considered that the variation in indices might have been related to the extent of degradation of rotavirus. However, this might not be the case, since none of the fractions of Wa showed Wa/NCDV indices over 32 after sucrose density gradient ultracentrifugation, which separated intact virus and degraded virions. The lack of effect of viral degradation on Wa/NCDV indices was supported by the findings that fecal specimens obtained on different days from each patient showed essentially the same index. Furthermore, RPHA titers of fecal extracts and of viral fractions prepared by ultracentrifugation on a sucrose cushion were shown to be essentially the same, indicating that the Wa/NCDV index did not differ before and after purification of HRV (Table 2).

Analysis of antigenic determinants responsible for the Wa/NCDV index remains to be done. However, RPHA tests with both SRBC-antiWa and SRBC-antiNCDV can be easily performed even in undeveloped countries, and a tentative Wa/NCDV index might be useful for rough classification of HRV in epidemiological studies

TABLE 2. Effect of partial purification of virus particles on Wa/NCDV index

Sample	Days after onset of disease	RPHA titer with:				Wa/NCDV index	
		SRBC-antiWa		SRBC-antiNCDV		Ex-tract	Puri-fied
		Ex-tract <sup>a</sup>	Puri-fied <sup>b</sup>	Ex-tract	Puri-fied		
1	3	8,192	8,192	512	512	16	16
	4	8,192	4,096	512	256	16	16
2	6	2,048	2,048	256	256	8	8
	7	1,024	2,048	128	128	8	16
3	3	512	256	64	32	8	8
	3	1,024	512	128	64	8	8

<sup>a</sup> Fecal extract.

<sup>b</sup> Partially purified virus fraction. Fecal extracts were centrifuged on a sucrose cushion (30%, wt/wt) at 45,000 rpm for 60 min, and pellets were suspended to the original volume of each fecal extract.

TABLE 3. RPHA titers of culture fluids of HRV-inoculated cells

Sample	Inoculum		RPHA titer of culture fluid							
			Original		1st passage		2nd passage		3rd passage	
	Wa <sup>a</sup>	NCDV <sup>b</sup>	Wa	NCDV	Wa	NCDV	Wa	NCDV	Wa	NCDV
2	8	2	<2	<2	<2	<2	NT <sup>c</sup>	NT	NT	NT
6	32	<2	<2	<2	<2	<2	NT	NT	NT	NT
14	2,048	8	<2	<2	<2	<2	NT	NT	NT	NT
17	512	<2	8	<2	4	<2	16	<2	256	2
20	1,024	124	<2	<2	<2	<2	NT	NT	NT	NT
25	256	2	<2	<2	<2	<2	NT	NT	NT	NT

<sup>a</sup> RPHA titer was estimated with SRBC-antiWa.

<sup>b</sup> RPHA titer was estimated with SRBC-antiNCDV.

<sup>c</sup> NT, Not tested.

in south Asia or Africa, where HRV infection is a serious medical problem.

Recently, Kapikian et al. (9) have classified rotaviruses into subgroups 1 and 2 by immune adherence hemagglutination and enzyme-linked immunosorbent assay. Since the Wa/NCDV index of NCDV (subgroup 1) was calculated as 1 and the index of Wa (subgroup 2) was calculated as 32, our index is in keeping with the classification of subgroups 1 and 2. However, our index does not separate subgroups since subgroup-specific antibody was not used in our present system. Subgroup-specific antisera such as those Kapikian et al. have developed by using group-specific postinfection antiserum from gnotobiotic calves are not generally available at this time. However, our index may contribute a simple method to determine the antigenic characteristics of HRV epidemic in the field. Replacement of the polyclonal antibodies to HRV and NCDV used in this study with monoclonal antibodies may improve our index so that it will be able to identify subgroups.

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