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Enzyme-Linked Immunosorbent Assays Adapted for Serotyping of Human Rotavirus Strains

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Five enzyme-linked immunosorbent assay systems were adapted for serotyping human rotavirus strains and were compared with a sensitive complement fixation test in terms of specificity and sensitivity. The assays differed mainly with regard to the antibody systems involved in the double sandwich. Serotype differentiation of 34 rotavirus strains was achieved by determining a neutralization endpoint titer, either with a constant antiserum-varying antigen dilution method or vice versa. The procedure which proved to be highly specific and sensitive was one with two type-specific hyperimmune sera (enzyme-linked immunosorbent assay system 5) instead of only one, as in the four other systems.

Since human rotavirus strains produce no cytopathic effect in tissue culture, conventional neutralization tests cannot be used to study antigenic differences in the outer capsid layer (1). Recently, however, we were able to distinguish two serotypes of human rotavirus by complement fixation (CF) and immune electron microscopy (5). The latter technique is cumbersome and is not applicable to routine serotyping of large series of rotavirus-positive stools, whereas the former is not sensitive enough for specimens with a low antigen titer.

To study the epidemiology of rotavirus serotypes, we have modified the enzyme-linked immunosorbent assay (ELISA) procedure described by Yolken et al. (2) for the detection of this virus in stools.

MATERIALS AND METHODS

Specimens. During the winter of 1977 to 1978, we collected fecal specimens from infants and children with a diarrheal illness and also from neonates with or without gastroenteritis. All samples in which rotavirus was found by the ELISA technique for antigen detection (2) were stored at −20°C.

Preparation of hyperimmune sera. Type-specific immune sera (IS_1 and IS_2) were obtained by immunizing guinea pigs and specific pathogen-free (SPF) rabbits with purified rotavirus types 1 and 2 by the procedure previously described (5). Briefly, two rotavirus-positive stools for which the serotype had been established by CF, and which were known to contain numerous complete virions ($\geq 10^{10}$ particles per g of feces) were selected for purification. A 30% stool suspension (wt/vol) was prepared which was then centrifuged twice at low speed (1,000 × *g* for 10 min). The supernatant fluid was recovered and checked for rotavirus. Both preparations gave a titer ≥32 by the sensitive CF technique described below and showed 20 to 50 particles per grid square when examined with an electron microscope (negative staining) at a magnification of × 25,000.

A 0.5-ml volume of each of the specimens was subjected to rate zonal centrifugation through a 4.5 ml, 60 to 45 to 30% sucrose gradient at 100,000 × *g* for 2.5 h. The fractions with a density of 1.22 containing double-shelled rotavirus particles were collected and again checked by electron microscopy. There were no incomplete or broken rotaviruses observed in either preparation. After purification, 1 ml of each fraction with density 1.22 was mixed with 1 ml of Freund complete adjuvant and used for immunization.

Guinea pigs and SPF rabbits were pretested for the presence of rotavirus antibodies by an ELISA blocking assay (3). The majority (>90%) of a stock of guinea pigs bred in our own animal house, as well as the SPF rabbits, kindly provided by the Smith Kline R.I.T. Cie (B-1330 Rixensart, Belgium), did not have preexisting antibodies, at least at a serum dilution of 1:4. We could therefore easily select animals for immunization.

The animals received three subcutaneous injections, each of 0.6 ml, on days 0, 7, and 21. Two weeks after the last inoculation (day 35), the animals were bled by heart puncture. The sera thus obtained were designated as IS_1 (nonabsorbed; type-specific serum against human serotype 1) and IS_2 (nonabsorbed; type-specific serum against human serotype 2) and contained 1,024 CF type-specific antibody units, whereas both reacted with Nebraska calf diarrhea virus (NCDV) at a dilution of 128. Attempts were made to increase the specificity of the hyperimmune sera by first absorbing them with NCDV. A concentrated suspension was prepared from NCDV grown on BGM cells (Microbiological Associates, Bethesda, Md.) in the presence of trypsin (10 μg/ml of maintenance medium). These cells were previously shown to be an excellent host system (unpublished data) in which a clear-cut viral cytopathic effect appeared after 24 h. The infected culture was twice frozen and thawed after complete dissociation of the monolayer, which generally occurred within 2 or 3 days. After low-speed centrifugation (1,000 × *g* for 10 min) to eliminate the cellular debris, the supernatant fluid was subjected to ultracentrifugation (100,000 × *g* for 2.5 h). The pellet was then suspended in physiological saline to give a CF titer of 32 antigen units against a calf convalescent

serum, and 1 ml of each hyperimmune serum (IS_1 and IS_2), diluted to contain 64 type-specific antibody units, was mixed with 3 ml of the concentrated NCDV and incubated at 4°C overnight. The next day the mixture was layered on a 40% sucrose cushion (wt/vol) and subjected to high-speed centrifugation (100,000 × *g* for 2.5 h) to remove the rotavirus antigen-antibody complexes. This procedure yielded 4 ml of absorbed hyperimmune serum with a type-specific CF antibody titer of 1:16.

We also prepared a group-specific immune serum (IS) against human rotavirus by immunizing an SPF rabbit with the concentrated NCVD suspension mentioned above previously purified through a 60 to 45 to 30% sucrose gradient.

CF. The CF technique for detection of rotavirus in stool extracts with IS has been described elsewhere (6). For serotyping of human strains, we used in an earlier CF technique (5) 4 antibody units of each of the type-specific antisera (IS_1 and IS_2) as standardized against NCDV. However, on examination of large numbers of human rotavirus-positive stools, we observed less cross-reactivity with 4 CF antibody units of IS_1 and IS_2 as determined by chessboard titration against the homologous virus used for antiserum production. Therefore the latter procedure was chosen for the present experiments. The group-specific as well as the type-specific CF titer was expressed as the highest serial dilution of a 30% fecal suspension (wt/vol) in which rotavirus-antigen could be detected by a constant antibody level (4 units).

ELISA. To differentiate human rotavirus strains we modified the basic ELISA described by Yolken et al. (2). Based upon determining the endpoint titer of the hyperimmune sera against a constant amount of viral antigen, or vice versa, we developed five type-specific rotavirus ELISA methods. The origin and the concentrations of the antisera and the enzyme-labeled conjugates are shown in Table 1.

ELISA system 3 was in fact analogous to the one described by Yolken et al. (4) for an extensive epidemiological survey of human rotavirus types 1 and 2. The main difference between Yolken's system and ours was the origin of the antisera coated onto the wells of the microtiter plate (goat anti-human rotavirus instead of SPF rabbit anti-NCDV). However, we demonstrated (unpublished data) that this did not influence the typing results, because both species of antisera were used in the same range of specificity and sensitivity for detection of human rotavirus antigen. Therefore, the results obtained in this study with ELISA system 3 are comparable to those obtained in Yolken's system.

The ELISA antibody titer of each of the hyperimmune sera was predetermined by chessboard titration with three type 1 and type 2 reference strains of human rotavirus (previously typed by CF). Three control antigens were also used in this initial procedure. In each of the ELISA systems the test material was initially prepared as a 7.5% stool suspension, since rotavirus-negative specimens often reacted nonspecifically at higher concentrations (false-positives). For the same reason hyperimmune sera were not tested at dilutions lower than 1:64. The tests were run essentially in the same manner described previously by Yolken et al. (2), with serial dilutions of type-specific hyperimmune sera (ELISA 1, 2, and 5) or with serial rotavirus dilutions (ELISA 3 and 4). However, in our procedure we used peroxidase-labeled conjugates and 5-amino, 2-hydroxybenzoic acid as a substrate (E. Merck, Darmstadt, Germany).

Interpretation of the different assays was carried out as follows. For ELISA 1, 2, and 5, the neutralizing titer was expressed as the highest dilution of the type-specific antiserum (IS_1 and IS_2) able to detect a constant amount of antigen in a 7.5% stool suspension previously shown to be positive for rotavirus. For ELISA 3 and 4, the neutralizing titer was expressed as the highest dilution of a rotavirus-positive stool (starting from a 7.5% suspension) in which antigen was detectable with 4 ELISA antibody units of IS, IS_1, or IS_2.

Tests were read by a spectrophotometer (Titertek Multiskan, Flow Laboratories) measuring the absorb-

TABLE 1. *ELISA systems adapted for the type-specific differentiation of human rotavirus strains*

ELISA system	Serum[a]		Conjugate[b]
	1 (amt)	2 (amt)	
1[c]	Rabbit anti-NCDV (4 U)	Guinea pig anti-rotavirus 1 and 2 (serial dilutions)	Rabbit anti-guinea pig IgG (4 U)
2[c]	Rabbit anti-NCDV (4 U)	Guinea pig anti-rotavirus 1 and 2, absorbed (serial dilutions)	Rabbit anti-guinea pig IgG (4 U)
5[c]	Rabbit anti-rotavirus 1 and 2 (4 U)	Guinea pig anti-rotavirus 1 and 2 (serial dilutions)	Rabbit anti-guinea pig IgG (4 U)
3[d]	Rabbit anti-NCDV (4 U)	Guinea pig anti-rotavirus 1 and 2 (4 U)	Rabbit anti-guinea pig IgG (4 U)
4[d]	Guinea pig anti-rotavirus 1 and 2 (4 U)	Rabbit anti-NCDV (4 U)	Swine anti-rabbit IgG (4 U)

[a] Serum 1, Hyperimmune serum coating the microplates; serum 2, hyperimmune serum added after incubation with rotavirus to be typed. U, ELISA antibody units.

[b] Conjugate, Hyperimmune serum labeled with peroxidase.

[c] These assays are based on a constant amount of antigen and serial dilutions of hyperimmune serum.

[d] These assays are based on a constant amount of hyperimmune serum and serial dilutions of antigen.

ance at a wavelength of 450 nm through the bottom of a microtiter plate (Cooke, U-shaped polyvinyl microtiter plates). The neutralizing titer was defined as the highest serial dilution with a positive-over-negative ratio greater than 2.0. The negative value was the mean absorbance of six human stools not containing rotavirus particles. The rabbit anti-guinea pig immunoglobulin G (IgG) conjugate was obtained commercially (Cappel Laboratories, Inc., Dynatech Produkte AG, Kloten, Switzerland), whereas the swine anti-rabbit IgG conjugate was kindly furnished by the Nationaal Instituut voor Dierengeneeskundig Onderzoek, Groeselenberg, Brussels, Belgium.

The five ELISA systems for type-specific rotavirus differentiation were compared with a sensitive CF test in terms of specificity and sensitivity.

In a first series of experiments, five untyped and two reference strains were simultaneously tested by CF and the ELISA 1, 2, and 3 systems. The two reference strains were those used for producing the type-specific hyperimmune sera.

In a second experiment, 12 rotavirus-positive stools were typed by CF and ELISA system 4, in which the endpoint titer was expressed as a serial antigen dilution. Initially, the antigen titer of the various strains was measured with IS to compare the relative sensitivity of the two techniques. Earlier studies had shown that low-titered preparations could not be typed by the CF procedure unless they were previously concentrated.

In further experiments attempts were made to serotype human rotavirus strains by ELISA system 5. Since the 12 strains used in the previous experiment were exhausted, another 17 rotavirus-positive stools were selected, 13 of which had a low group-specific antigen titer (CF titer <1).

RESULTS

Type-specific differentiation of five human rotavirus strains by CF and ELISA 1, 2, or 3 gave identical results (Table 2). Moreover, differences in neutralizing titer between homologous and heterologous antiserum were about the same in all tests, although slightly greater with the CF. Reading and interpreting the results, however, were much easier with the latter. The endpoint titer was very clear-cut by CF (Fig. 1), whereas cross-reactivity often occurred with the ELISA systems 1 and 3. Objective reading with the naked eye was therefore very difficult, although serotyping was easy to perform with a spectrophotometer. Initial absorption of the hyperimmune sera with NCDV facilitated reading the test, and the endpoint titer could easily be determined with the naked eye in ELISA system

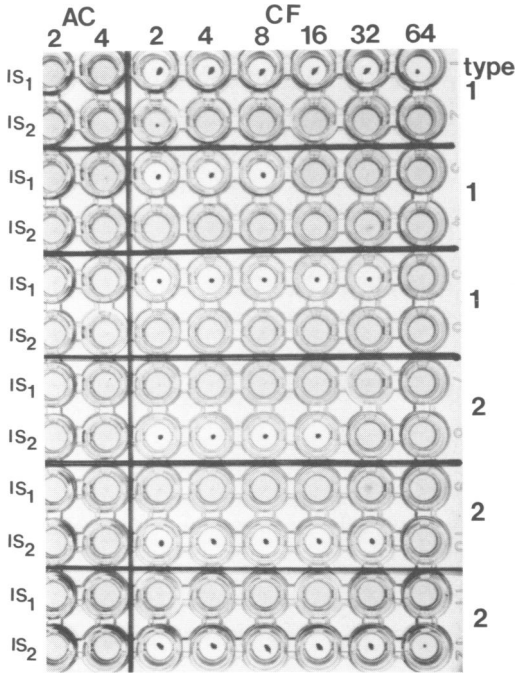

FIG. 1. *Serotyping of human rotavirus strains by CF. AC, Control of the anticomplementary activity of the stool; CF, CF antigen titer expressed as the highest dilution reciprocal of the rotavirus-positive stool which reacted with 4 CF antibody units of IS_1 or IS_2.*

TABLE 2. *Comparison of the type-specific identification of human rotavirus strains by CF and by ELISA systems 1, 2, and 3*

Rotavirus strain reference	CF titer			Neutralizing titer						Rotavirus type
				ELISA 1		ELISA 2		ELISA 3		
	IS	IS_1	IS_2	IS_1	IS_2	IS_1	IS_2	IS_1	IS_2	
C 730	8	4	<4	4,096	1,024	1,024	256	64	16	1
C 731	64	<1	64	1,024	4,096	256	1,024	16	256	2
17491	32	<1	32	1,024	16,384	256	1,024	16	64	2
11568	8	<1	4	4,096	16,384	256	1,024	16	64	2
826/04	64	<1	64	1,024	16,384	1,024	4,096	16	256	2
8632[a]	32	32	8	4,096	1,024	1,024	64	256	16	1
706[a]	64	<1	64	1,024	16,384	1,024	4,096	16	256	2

[a] Strains 8632 and 706 are reference strains.

2. Unfortunately, type-specific antibodies were also removed by the absorption procedure, and the neutralizing antibody titer was 4 to 16 times lower with ELISA 2 than with ELISA 1.

Because the difficulty in reading results with the naked eye with ELISA systems 1 and 3 and the relative lack of sensitivity of the ELISA 2 assay, we ran comparative studies between CF and ELISA 4 (Table 3). The ELISA 4 system was more sensitive than CF for the detection of rotavirus antigen, but not for serotype differentiation. The CF technique detected 9 of the 12 strains (antigen titer, >1), and the ELISA 4 detected all 12. However, only the five strains with a CF titer >4 could be typed either with CF or with ELISA.

Type differentiation by visual analysis was much easier to perform with the ELISA 4 system than with the three preceding assays. Indeed, virtually no cross-reactivity was observed. However, for serotyping, sensitivity was not superior to that of the CF. In efforts to increase sensitivity, we developed an ELISA 5 system with two type-specific hyperimmune sera of different origin: the first one, produced in SPF rabbits, was coated onto the microtiter plate, and the second one, obtained from a guinea pig, was used to demonstrate the antigen bound by the former. Results are shown in Table 4. As would be expected, the type specificity of the four specimens containing the most rotavirus (CF titer >4) could easily be determined. In addition, excellent results were also obtained with low-titered preparations. Although the difference in neutralizing capacity of IS_1 and IS_2 was not always as great as that observed with the first four strains mentioned above, we did not encounter real problems in interpretation with lower-titered specimens. The specificity of the assay was sufficiently good that results of serotyping could be read without the use of a spectrophotometer (Fig. 2).

TABLE 4. *Comparison of the type-specific identification of human rotavirus strains by CF and by ELISA system 5*

Rotavirus strain reference	IS CF titer	Neutralizing titer (ELISA 5)		Rotavirus type
		IS_1	IS_2	
56618	64	1,024	<64	1
82804	32	1,024	16,384	2
3646	16	1,024	16,384	2
73841	8	1,024	16,384	2
56549	<1	1,024	64	1
62711	<1	256	1,024	2
82817	<1	64	<64	1
89406	<1	1,024	<64	1
C1957	<1	4,096	16,384	2
89483	<1	1,024	16,384	2
76732	<1	1,024	<64	1
73768[a]	<1	64	<64	1
73769[a]	<1	256	<64	1
C1944[a]	<1	1,024	<64	1
65270[a]	<1	1,024	<64	1
73770[a]	<1	1,024	<64	1
73830[a]	<1	4,096	64	1
8632[b]	32	1,024	<64	1
706[b]	64	1,024	16,384	2

[a] Strains originating from children in the same pediatric ward.

[b] Strains 8632 and 706 are reference strains.

TABLE 3. *Comparison of the type-specific identification of human rotavirus strains by CF and by ELISA system 4*

Rotavirus strain reference	CF titer			Neutralizing titer ELISA 4			Rotavirus type
	IS	IS_1	IS_2	IS	IS_1	IS_2	
B 1351	8	8	<1	64	64	<4	1
16402	16	16	4	64	64	<4	1
5786	8	8	<1	64	16	<4	1
4748	16	<1	16	64	<4	16	2
169	16	<1	8	64	4	64	2
C 1316	4	<1	<1	4	<4	<4	?
16 C	4	<1	<1	4	<4	<4	?
37882	4	<1	<1	4	<4	<4	?
37883	4	<1	<1	4	<4	<4	?
439	<1	<1	<1	4	<4	<4	?
10 A	<1	<1	<1	4	<4	<4	?
16 D	<1	<1	<1	4	<4	<4	?
8632[a]	32	32	8	256	64	<4	1
706[a]	64	<1	64	256	4	64	2

[a] Strains 8632 and 706 are reference strains.

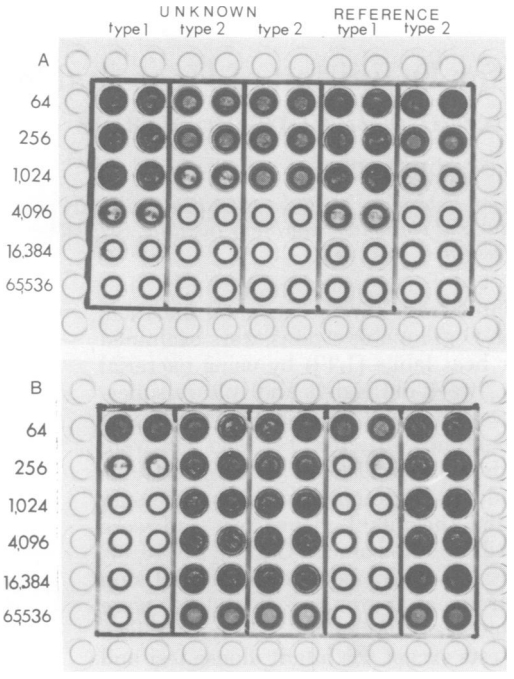

FIG. 2. *Serotyping of human rotavirus strains with ELISA system 5. (A) Numbers on the left indicate serial dilutions of IS_1 (guinea pig) on plates coated with IS_1 (rabbit). (B) Numbers on the left indicate serial dilutions of IS_2 (guinea pig) on plates coated with IS_2 (rabbit). Each strain was tested in duplicate, i.e., two wells were used for each dilution of IS_1 and IS_2.*

DISCUSSION

The ELISA systems 1, 2, and 3 showed the same degree of specificity in differentiating between human rotavirus types 1 and 2. However, reading results of the ELISA 1 and 3 systems with the naked eye was very difficult due to the occurrence of cross-reactions. This disadvantage could be overcome by initially absorbing the hyperimmune sera with tissue culture-grown NCDV (ELISA system 2) but at the cost of a loss of sensitivity of the test.

High specificity was obtained with ELISA 4, but this test showed no obvious advantages over the sensitive CF technique, since both methods showed the same level of sensitivity with regard to serotyping.

In ELISA systems 1 to 4, one group-specific and one type-specific antisera were used. When the antibody sandwich consisted of two type-specific hyperimmune sera (ELISA system 5), both the sensitivity and the ease of visual reading of the typing procedure were much increased. For this reason we advocate the use of this last assay for studying the epidemiology of infections with rotavirus types 1 and 2 in humans.

Since our first survey (4), we have typed, by means of the ELISA 5 assay, 330 human rotavirus strains obtained over a period of 10 months (from August 1978 to May 1979) from children with gastroenteritis living in the Brussels area. This study confirmed our previous serotyping results in Belgium (4), i.e., approximately 15% of the isolates belonged to type 1 and 85% belonged to type 2. Only one specimen could not be classified as one serotype or the other. Whether this strain might represent another rotavirus type is uncertain, because the specimen contained only a low level of virus as demonstrated by the basic ELISA for antigen detection (2). Thus, a low level of type-specific antigen could also be incriminated for its lack of reactivity in the ELISA system 5.

Recently, we also analyzed 33 strains from a maternity unit in Brussels in which 60% of the newborns became infected with rotavirus during May 1979. All the specimens could be differentiated with the ELISA system 5, and serotyping revealed that 100% of the infections were due to type 2.

Finally, the following observation is noteworthy: the origin of the six type 1 strains shown in Table 4 was checked retrospectively and it was found that they all originated from children with a nosocomial rotavirus infection who had been hospitalized at nearly the same time in the same pediatric ward.

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