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# Rapid Serotyping of Human Rotavirus Strains by Solid-Phase Immune Electron Microscopy

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Nine cell culture-adapted, as well as 30 clinical, human rotavirus (HRV) strains from fecal extracts of children with primary HRV infection were typed by rapid solid-phase immune electron microscopy with protein A and absorbed DS-1 (HRV serotype 2), Wa (serotype 1), and VA70 (assumed serotype 3) rabbit immune sera. As a reference typing test for cell culture-adapted strains, the neutralization assay was used, whereas for noncultivatable strains typing was done for comparison, indirectly, based upon the differential neutralization reactivity of convalescent-phase serum samples from patients with primary HRV infection versus the three reference HRV serotypes. Typing results by solid-phase immune electron microscopy for all strains examined were in complete agreement with those obtained by the neutralization assay, both on cell culture-adapted strains with the three reference rabbit antisera and on three reference HRV strains with human convalescent-phase serum samples. Since adaptation to growth in cell cultures of clinical HRV strains from stool specimens is a time-consuming procedure and is often unsuccessful, solid-phase immune electron microscopy is preferred over the neutralization assay, giving results in about 16 h and also allowing typing of HRV strains from stool specimens low in virus particles. In addition, HRV strains reacting differently from the three reference serotypes may be easily selected by solid-phase immune electron microscopy for further characterization, as was the case for one strain in this study.

Human rotaviruses (HRV) are the etiological agents of a major part of acute nonbacterial gastroenteritis in infants and young children (2). Among HRV strains sharing a common group antigen with animal rotavirus (9, 20), two subgroups (subgroups 1 and 2) have been identified on the basis of both different electrophoretic mobility of RNA segments 10 and 11 (4, 15) and immunological tests, such as complement fixation (CF) (26, 28), enzyme-linked immunosorbent assay (ELISA) (25), and immune adherence hemagglutination (8). Subgroup 1 HRV strains seem to belong to a single serotype, whereas at least two distinct serotypes have been identified within subgroup 2 strains by neutralization (Nt) (1, 6, 21, 23), ELISA (19), and possibly CF (12).

Recently, solid-phase immune electron microscopy (SPIEM) with protein A has been used for HRV detection in fecal specimens (10, 13, 14). The same technique, which was originally developed for detecting plant viruses (17), has been employed successfully for identifying some selected human adenovirus serotypes (18).

In the present study, we developed a SPIEM method for rapid serotyping of HRV strains directly on stool specimens. The reference method for serotyping has been Nt on cell culture-adapted HRV strains, as previously reported (G. Gerna, M. Battaglia, G. Milanesi, N. Passarani, E. Percivalle, and E. Cattaneo, Infect. Immun. in press). Subgroup 1 serotypes closely related by Nt to strain DS-1 (23) are referred to as serotype 2 (DS-1-like) strains. Subgroup 2 strains reacting as Wa (24) are referred to as serotype 1 (Wa-like) strains, whereas subgroup 2 strains distinct from Wa and reacting similarly to our VA70 strain by Nt are tentatively referred to as serotype 3.

## MATERIALS AND METHODS

**Rotavirus strains.** Cell culture-adapted DS-1, Wa, and P HRV strains were kindly supplied by R. G. Wyatt, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), Bethesda, Md. The Lincoln strain of bovine rotavirus was obtained from the Institute of Agriculture and Natural Resources, University of Nebraska, Lincoln, Nebr. Our nine strains of cell culture-adapted HRV were propagated on MA-104 cells as described elsewhere (Gerna et al., in press). Thirty clinical HRV strains were obtained from stool specimens of infants and young children (aged 2 months to 2 years) admitted with a diagnosis of acute gastroenteritis to two different hospitals in northern Italy in 1981 to 1983. All patients had primary HRV infections, as diagnosed on the basis of seroconversion by ELISA on paired serum samples.

**Antisera.** Immune sera against the reference strains DS-1 and Wa and against our nine cell culture-adapted strains were prepared both in guinea pigs and rabbits by using partially purified virus as described elsewhere (Gerna et al., in press). DS-1, Wa, and VA70 (assumed serotype 3) rabbit antisera were selected on the basis of their Nt specificities for absorption to increase the specificity of their SPIEM reactivity. First, the three antisera were absorbed at a dilution of 1:1,000 with purified EDTA-treated bovine rotavirus at a concentration of about 1.0 ml/20 μl of a virus preparation containing 100,000 virus particles per grid square, as determined by electron microscopy. Mixtures were incubated for 2 h at 37°C and overnight at 4°C. They were then centrifuged at 35,000 rpm for 90 min in an SW50.1 Beckman rotor to pellet the virus. At this point, the antisera were suitable for HRV subgrouping by SPIEM. Further absorption of the three antisera was necessary for serotyping and was done by incubation with the EDTA-treated Wa strain by the same procedure.

**SPIEM.** Carbon-Formvar-coated grids (400 mesh) were floated on 50-μl drops of protein A solution (25 μg/ml) for 10 min. After being washed with several drops of 0.1 M phosphate buffer (pH 7.0), the grids were floated for 20 min on 50-μl drops of each antiserum diluted 1:2,000, washed

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again, and then floated overnight at room temperature on 50-μl drops of stool extracts (30%, wt/vol) or crude cell culture HRV preparations. Grids were then rinsed with several drops of distilled water, and trapped virus particles were stained with a 2% aqueous solution of uranyl acetate. Twenty squares on each grid were examined for each preparation with a Philips EM 201 electron microscope at a magnification of ×45,000, and the average number of virus particles per grid square was determined. All specimens were read blindly and examined at least twice on different grids prepared at different times. The lower limit of virus particles established for SPIEM to be considered valid was five per grid square with the antiserum giving the highest reactivity.

**Nt.** The Nt test on cell culture-adapted HRV strains was performed by a previously described procedure (Gerna et al., in press). Briefly, equal volumes of trypsin-treated HRV suspension and serial twofold dilutions of type-specific antisera were incubated for 30 min at 37°C and then were inoculated into MA-104 cell monolayers on microtiter plates (0.1 ml per well). After 1 h of adsorption, serum-free medium 199 supplemented with 10% tryptose phosphate broth was added. After incubation at 37°C for 18 h, cells were fixed and stained by the indirect immunoperoxidase technique for the detection of nonneutralized virus according to a previously reported procedure for Nt testing of other viruses (5). The Nt titer was expressed as the reciprocal of the highest serum dilution giving 50% or more reduction in the number of stained cells as compared with control virus. A 16-fold difference in antibody titer was used to establish serotype difference.

**HRV subgrouping.** HRV were detected in infected cell culture media as well as in stool specimens by electron microscopy (3) and an indirect double-antibody sandwich ELISA similar to the ELISA system 5 proposed by Zissis and Lambert (27). Subgrouping was done serologically by CF and indirect double-antibody sandwich ELISA (27) by using subgroup 1- and subgroup 2-specific unabsorbed guinea pig and rabbit antisera. In addition, subgrouping could easily be done by SPIEM with DS-1 and Wa or VA70 rabbit antisera after absorption with single-shelled bovine rotavirus, as reported above. Initially, and periodically for cell culture-adapted HRV strains, subgrouping was checked by electrophoretic analysis of genomic RNA segments by using polyacrylamide gel electrophoresis (7).

## RESULTS

**Absorption of antisera.** Initially, the SPIEM technique was used for HRV detection in fecal specimens low in virus particles, and a mixture of the three type-specific unabsorbed rabbit antisera was employed. The number of virus particles trapped by antibody-coated grids was increased about 1,000 times, as compared with grids used for direct electron microscopy. A comparison of results by ELISA and SPIEM on several fecal extracts led to the conclusion that the ELISA was able to detect HRV antigens only when the sample contained at least one virus particle per grid square by SPIEM. Attempts to subgroup and serotype the three reference HRV serotypes by SPIEM by using the three homotypic unabsorbed antisera were unsuccessful because of the high degree of cross-reactivity. The first absorption with single-shelled bovine rotavirus gave antisera suitable for HRV subgrouping (Table 1). However, typing of the two subgroup 2 serotypes was still difficult to perform and often unreliable with these antisera (as documented by unreported data on several stool specimens). The second absorption with single-shelled Wa strain gave antisera sufficiently specific for typing.

TABLE 1. Serotyping of three HRV reference strains by SPIEM

| Absorption step | Rabbit immune serum | Double-shelled virus particles trapped per grid square by SPIEM[a] | | |
|---|---|---|---|---|
| | | DS-1 | Wa | VA70 |
| None | DS-1 | 35 (2.5) | 31 (1.0) | 23 (1.0) |
| | Wa | 20 (1.4) | 160 (5.1) | 30 (1.3) |
| | VA70 | 14 (1.0) | 86 (2.7) | 60 (2.6) |
| First[b] | DS-1 | 24 (4.8) | 4 (1.0) | 2 (1.0) |
| | Wa | 5 (1.0) | 170 (42.5) | 28 (14.0) |
| | VA70 | 11 (2.2) | 90 (22.5) | 65 (32.5) |
| Second[c] | DS-1 | 13 (13.0) | 2 (1.0) | 1 (1.0) |
| | Wa | 1 (1.0) | 41 (20.5) | 7 (7.0) |
| | VA70 | 1 (1.0) | 6 (3.0) | 49 (49.0) |

[a] Values represent the average number obtained in three countings performed at 1-month intervals on three different grid preparations of the same specimen; the lower limit established for a valid SPIEM typing was five virus particles per grid square with the antiserum giving the highest reactivity. Numbers in parentheses represent for each strain the ratio between the number of virus particles trapped by the indicated antiserum and the number of particles trapped by the antiserum showing the lowest reactivity.
[b] Single-shelled bovine rotavirus, Lincoln strain.
[c] Single-shelled Wa strain.

**Serotyping by SPIEM of reference and cell culture-adapted strains.** By using DS-1, Wa, and VA70 rabbit antisera for the capture of homotypic strains, the number of virus particles trapped per grid square by each antiserum depended upon the absorption step, i.e., the increased type specificity of antisera (Table 1). The degree of cross-reactivity by SPIEM was determined for each HRV strain by comparison of the three ratios obtained by dividing the number of virus particles captured by each antiserum by the number of particles captured by the antiserum showing the lowest reactivity. Thus, for strain DS-1, the ratios of reactivity with DS-1, Wa, and VA70 antiserum were 2.5, 1.4, and 1.0, respectively, with unabsorbed antisera, but the ratios shifted to 4.8, 1.0, and 2.2 after the first absorption and 13.0, 1.0, and 1.0 after the second absorption. For strain Wa, ratios of 1.0, 5.1, and 2.7 with unabsorbed antisera became 1.0, 42.5, and 22.5 and 1.0, 20.5, and 3.0 after final absorption. For strain VA70, ratios of 1.0, 1.3, and 2.6 with unabsorbed antisera changed to 1.0, 14.0, and 32.5 and finally to 1.0, 7.0, and 49.0 (Fig. 1). On the basis of these preliminary findings, typing by SPIEM was done in parallel on our nine cell culture-adapted HRV strains (whose serotype had previously been determined by Nt) and their respective original stool specimens. In addition, the three HRV strains obtained from NIH were typed. There was complete agreement between SPIEM and Nt results (Table 2). However, typing could be performed by SPIEM both on cell culture-adapted strains and on fecal specimens, whereas typing by Nt could be performed only on cell-adapted virus preparations since in several stool specimens the amount of virus growing in MA-104 cells was too low to allow reliable Nt tests. The cross-reactivity of serotype 2 HRV strains with the other two serotypes was very low, whereas it was greater between serotypes 1 and 3. However, the homotypic reactivity was consistently at least more than three times greater than the heterotypic reactivity. Thus, problems in typing by SPIEM were never encountered, except for NIH HRV strain P, which could be typed

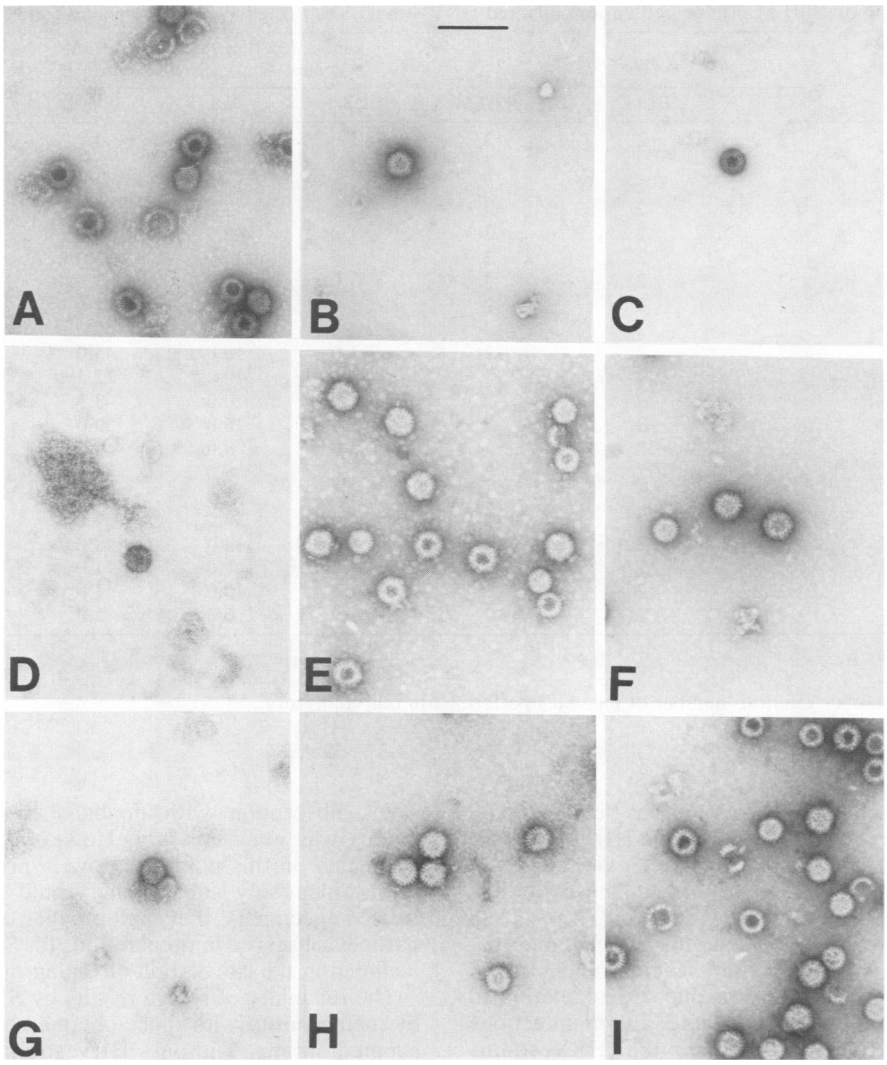


FIG. 1. Typing of the reference HRV strains by SPIEM with absorbed antisera, showing trapping of HRV strains DS-1 (A through C), Wa (D through F), and VA70 (G through I) by rabbit antisera against DS-1 (A, D, and G), Wa (B, E, and H), and VA70 (C, F, and I). Bar, 200 nm (same magnification for all pictures).

neither by Nt (absent or poor reactivity with all three type-specific antisera) nor by SPIEM. This strain, which belongs to subgroup 2, showed a similar fair reactivity with both serotype 1 and 3 antisera by SPIEM. For all strains, the subgroup was determined by CF, ELISA, and SPIEM (with subgroup 1 and subgroup 2 rabbit antisera which were made subgroup specific by absorption with only single-shelled bovine rotavirus).

**Serotyping of fecal HRV strains.** In 30 cases of primary HRV infection, serotyping was done directly on stool extracts by SPIEM. Of the 30 HRV strains, 2 were typed as serotype 2 (DS-1-like), 17 as serotype 1 (Wa-like), and 11 as serotype 3 (VA70-like). These cases were selected on the basis of the previous finding that convalescent-phase serum specimens from young patients with primary HRV infection possess a typical Nt reactivity which allows identification of the infecting serotype. A few examples of the Nt reactivity of convalescent-phase serum specimens from infants with primary HRV infection are reported in Table 3. Typing of the actual infecting HRV strain by SPIEM directly on stool extracts and by Nt indirectly on convalescent-phase serum specimens gave identical results. In convalescent-phase serum samples from patients with serotype 3 infection, no significant difference in antibody titer to strains Wa and VA70 was observed, whereas in animal immune sera, a 64-fold difference was found.

## DISCUSSION

To date, serotyping of HRV strains has been done by Nt on cell culture-adapted strains. However, the adaptation of HRV strains from stool specimens to growth on cell cultures is a very tedious and laborious procedure and is often unsuccessful (24). When successful, it may take several weeks to obtain virus yields sufficient for Nt typing. In addition, in our experience as in that of others, subgroup 2 strains are much more readily grown in cell cultures than subgroup 1 strains are. Successful serotyping of HRV strains by Nt by direct use of fecal extracts has been reported (1), but in our experience the technique was feasible only on high-titer specimens. Thus, so far, the only Nt assay exten-

TABLE 2. Typing by SPIEM of nine cell culture-adapted HRV strains, their original stool specimens, and three NIH strains

| HRV strain (origin) | HRV subgroup by: | | | Ratios of SPIEM reactivity of HRV strains with typing antiserum[a]: | | | HRV serotype by: | |
|---|---|---|---|---|---|---|---|---|
| | CF | ELISA | SPIEM | DS-1 | Wa | VA70 | SPIEM | Nt |
| VA20, MA/20 (stool) | 2 | 2 | 2 | 1.0 | 10.0 | 3.2 | 1 | 1 |
| | 2 | 2 | 2 | 1.0 | 16.0 | 3.3 | 1 | ND[b] |
| VA66, MA/16 (stool) | 2 | 2 | 2 | 1.0 | 12.0 | 4.0 | 1 | 1 |
| | 2 | 2 | 2 | 1.0 | 12.0 | 2.5 | 1 | ND |
| VA70, MA/14 (stool) | 2 | 2 | 2 | 1.0 | 9.0 | 50.0 | 3 | 3 |
| | 2 | 2 | 2 | 1.0 | 10.0 | 55.0 | 3 | ND |
| VA73, MA/16 (stool) | 2 | 2 | 2 | 1.0 | 15.0 | 3.5 | 1 | 1 |
| | 2 | 2 | 2 | 1.0 | 18.0 | 4.0 | 1 | ND |
| VA75, MA/16 (stool) | 2 | 2 | 2 | 1.0 | 10.0 | 55.0 | 3 | 3 |
| | 2 | 2 | 2 | 1.0 | 10.0 | 45.0 | 3 | ND |
| VA78, MA/13 (stool) | 2 | 2 | 2 | 1.0 | 10.0 | 2.5 | 1 | 1 |
| | 2 | 2 | 2 | 1.0 | 10.0 | 2.5 | 1 | ND |
| VA79, MA/18 (stool) | 2 | 2 | 2 | 1.0 | 10.0 | 50.0 | 3 | 3 |
| | 2 | 2 | 2 | 1.0 | 8.0 | 50.0 | 3 | ND |
| PV5249, MA/17 (stool) | 2 | 2 | 2 | 1.0 | 8.0 | 30.0 | 3 | 3 |
| | 2 | 2 | 2 | 1.0 | 7.0 | 28.0 | 3 | ND |
| PV5257, MA/15 (stool) | 2 | 2 | 2 | 1.0 | 10.0 | 35.0 | 3 | 3 |
| | 2 | 2 | 2 | 1.0 | 12.0 | 40.0 | 3 | ND |
| DS-1, MA/5[c] | 1 | 1 | 1 | 14.0 | 1.0 | 1.0 | 2 | 2 |
| Wa, MA/22[c] | 2 | 2 | 2 | 1.0 | 18.0 | 3.0 | 1 | 1 |
| P, MA/4[c] | 2 | 2 | 2 | 1.0 | 6.0 | 5.0 | NT[d] | NT[d] |

[a] See Table 1, footnote *a*.
[b] ND, Not done.
[c] Indicated passage number refers to number of passages performed in our laboratory.
[d] NT, Not typable.

sively used for serotyping of HRV strains has been based on the reassortant technique introduced by the NIH group (23) to make cultivatable HRV strains which otherwise could not be propagated in cell cultures. Recently, Japanese workers succeeded in propagating several HRV strains (11, 16, 22). In our laboratory, several subgroup 2 strains have been propagated (Gerna et al., in press), but several other strains failed to grow. By the SPIEM technique, no adaptation to cell cultures of HRV strains was required, and no infectious virus was necessary for typing. In addition, HRV strains from low-titer fecal specimens could be easily typed.

Sequential absorptions of the three type-specific antisera with single-shelled bovine rotavirus and strain Wa removed group and subgroup reactivity, thus giving antisera suitable for subgrouping after the first absorption and for serotyping after the second absorption. The proportion of single-shelled virus particles captured by unabsorbed antisera was greater than 50%, whereas it was only 10% after final absorption. Thus, absorption with double-shelled heterotypic HRV strains was not necessary. However, for comparison, experiments on this point are now in progress in our laboratory. Since only subgrouping could be done on EDTA-treated specimens, the small number of single-shelled virus particles observed in preparations for SPIEM typing was not included in the calculation of typing ratios.

The reliability of typing results by SPIEM was confirmed by their identity with those obtained by Nt on cell culture-adapted strains. The only HRV strain which could not be typed by SPIEM was strain P from NIH, which could not be typed by Nt either since it was unneutralized or poorly neutralized by all of the three type-specific reference antisera. This strain, belonging to subgroup 2, might be a candidate serotype 4. Results of SPIEM typing on other 30 HRV strains which were not adapted to cell cultures were compared with those obtained indirectly by the study of the differential Nt reactivity of convalescent-phase serum sam-

TABLE 3. Comparison of indirect HRV typing by differential Nt reactivity of convalescent-phase serum samples versus different HRV serotypes and direct typing by SPIEM of fecal extracts in six representative cases of primary HRV infection

| Patient | Nt antibody titer versus HRV strain: | | | | Serotype[a] | Subgroup[b] | HRV serotype by: | |
|---|---|---|---|---|---|---|---|---|
| | DS-1 | Wa | VA-70 | P | | | SPIEM | Nt[c] |
| VA25 | 160 | 40 | <40 | <40 | 2 | 1 | 2 | ND[d] |
| VA29 | 160 | 40 | <40 | <40 | 2 | 1 | 2 | ND |
| VA26 | <40 | 320 | <40 | 40 | 1 | 2 | 1 | 1 |
| VA66 | <40 | 320 | <40 | 40 | 1 | 2 | 1 | 1 |
| VA70 | <40 | 320 | 320 | 40 | 3 | 2 | 3 | 3 |
| VA75 | <40 | 320 | 640 | 40 | 3 | 2 | 3 | 3 |

[a] Determined by Nt on convalescent-phase serum samples.
[b] Rotavirus subgroup of each strain detected in stool was determined by CF, ELISA, and SPIEM (see Table 2).
[c] HRV serotype was determined by Nt on Ma-104 cell culture-adapted strains from the respective stool specimens by using DS-1, Wa, and VA70 reference rabbit immune sera. A difference in antibody titer of 16-fold or greater was used to establish serotype difference.
[d] ND, Not done.

ples from infants with primary HRV infection against the three reference serotypes. Again, results were identical by the direct SPIEM and indirect Nt typing methods. On this basis, we now routinely type new HRV strains directly in fecal extracts by SPIEM, looking for possible strains with atypical SPIEM reactivity.

Recently, an ELISA method for HRV typing has been reported (19). However, extensive cross-absorption of the three type-specific antisera from two different animal species with purified HRV strains was necessary, and low titer HRV specimens might be difficult to type. In addition, a CF test has been proposed for detecting HRV subgroups, but it is not clear whether the three HRV subgroups represent the three known serotypes, whose type-specific antigens may react also in a CF system (12). In addition, for CF typing, high-titer specimens free from anticomplementary activity are needed.

In conclusion, the SPIEM technique represents the most rapid and simple technique for typing clinical HRV strains and is a promising tool for the study of the epidemiology of HRV serotypes. It needs only protein A and partially absorbed antisera from a single animal species for HRV serotyping directly on fecal specimens. In addition, by using antisera to the two HRV subgroups which were made subgroup specific by absorbing their common group antigen reactivity, SPIEM could be used for subgrouping directly on fecal specimens. Finally, using a pool of the three (or more) unabsorbed type-specific antisera, we found that SPIEM was an extremely sensitive method for HRV detection, as previously reported by others (10, 13, 14).

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## ADDENDUM

After this paper was submitted, R. G. Wyatt, H. D. James, Jr., A. L. Pittman, Y. Hoshino, H. B. Greenberg, A. R. Kalica, J. Flores, and A. Z. Kapikian (J. Clin. Microbiol. **18**:310–317, 1983) identified strain P as serotype 3 and a new HRV strain (ST no. 4) as serotype 4. Thus, according to their numbering system, our VA70 strain (reported in our paper as serotype 3) could be designated as serotype 4 if it reacted as ST no. 4 did, or it could be designated as a new serotype if it reacted differently.

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