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# Epidemiology of Rotavirus Electropherotypes Determined by a Simplified Diagnostic Technique with RNA Analysis

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**The incidence and RNA electropherotypes of rotavirus in stools or rectal swabs of children with diarrhea were studied for three rotavirus seasons (1981 through 1984) in Philadelphia, Pa. We used a simplified RNA analysis method involving polyacrylamide gel electrophoresis followed by silver staining. Phosphate-buffered saline suspensions of the stools and swab eluates were examined directly by polyacrylamide gel electrophoresis-silver staining analysis and enzyme-linked immunoadsorbent assay (Rotazyme; Abbott Laboratories); electron microscopy was performed on solid stool specimens. The RNA analysis results were compared with electron microscopy and enzyme-linked immunosorbent assay results and exhibited a sensitivity and specificity greater than or equal to that of electron microscopy or the enzyme-linked immunosorbent assay. Ten different electropherotypes were detected among the 68 rotavirus RNA-positive specimens examined over the 3-year study. The predominant electropherotype was different in each season. Our results indicate that the polyacrylamide gel electrophoresis-silver nitrate strain RNA analysis of simple unextracted stool suspensions is a uniquely useful diagnostic technique; it rapidly provides both a definitive positive result and immediate determination of the RNA electropherotype, which is of value for epidemiological study.**

Rotaviruses are known to be the single most important cause of acute gastroenteritis requiring hospitalization of infants and young children in the United States and in developing countries (14, 16). However, diagnosis by virus isolation is impractical because of the need to adapt human rotaviruses to cell culture by multiple passages. The most widely used diagnostic methods now are examination of stool by electron microscopy (EM) and by enzyme-linked immunoadsorbent assay (ELISA) (20, 34). EM examination is not readily applicable to all specimens, and electron microscopes are not universally available. The ELISA test may present difficulty in interpretation because of equivocal borderline readings; false-positive determinations have been observed by several investigators (4–6, 18, 26).

Although human rotaviruses can be assigned to four different serotypes by cross-neutralization tests (32, 33), the difficulty in cultivating clinical isolates, coupled with a lack of commercially available monospecific antisera, has made serotype assignment impractical in epidemiological studies. Therefore, the characteristic electrophoretic patterns of the segmented rotaviral double-stranded RNA genomes of different specimens have often been determined for use as specific markers of different virus populations. The procedure traditionally used for extraction of rotaviral RNA from stools is tedious and involves an overnight precipitation in many cases. Herring et al. (13) described an RNA preparation technique that involved phenol-chloroform extraction of the specimen without any prior purification. In this paper we describe an even more simplified extraction procedure, which together with ultrasensitive silver staining of polyacrylamide gels is capable of detecting rotaviral genome RNA segments in concentrations as low as 10 ng of viral RNA per 20-μl specimen.

This technique proved to be more sensitive in detecting rotavirus in human stools than either EM or ELISA procedures. False-positive results cannot occur, and only a single false-negative result was detected in studies of more than 100 specimens. We suggest that polyacrylamide gel electrophoresis-silver stain (PAGE-SS) analysis of phosphate-buffered saline (PBS)-extracted stools may represent a practical and unusually informative diagnostic test.

## MATERIALS AND METHODS

**Collection of specimens.** Stools and rectal swabs were acquired from all children admitted to the Children's Hospital of Philadelphia with a diagnosis of gastroenteritis or diarrhea and dehydration during January through May 1983 and January through April 1984. Additional samples were obtained when available from outpatient clients seen in the hospital's emergency ward. To compare electropherotypes prevalent in the 1981 through 1982 rotavirus season, 10 stools positive by the ELISA (Rotazyme; Abbott Laboratories) were obtained from the St. Christopher's Hospital for Children in Philadelphia. Stool and swab specimens were stored at −20°C before examination.

**Preparation of specimens for RNA analysis and ELISA.** Approximately 5 to 10% suspensions of stool were made in Dulbecco PBS. Rectal swabs contained in viral transfer medium were vortexed in the collection tube; rectal swabs without transfer medium were vortexed with 2.0 ml of PBS. Each of the various preparations was centrifuged at 1,000 × *g* for 10 min to remove sediment. Supernatant fluids were used for ELISA antigen testing and RNA gel electrophoresis.

**PAGE-SS technique.** The polyacrylamide gel technique for rotavirus detection has been employed by many investigators (13, 15, 25). The procedure used in this study is a modification of that described by Laemmli (19). The polyacrylamide gel consisted of 12% acrylamide with a stacking gel of 5% acrylamide. Gel plates were 16 by 19.5 $cm^2$ and were spaced 0.8 mm apart. In comparison to the recommended 1.5-mm-thick gel (15), we found the thinner gel to be

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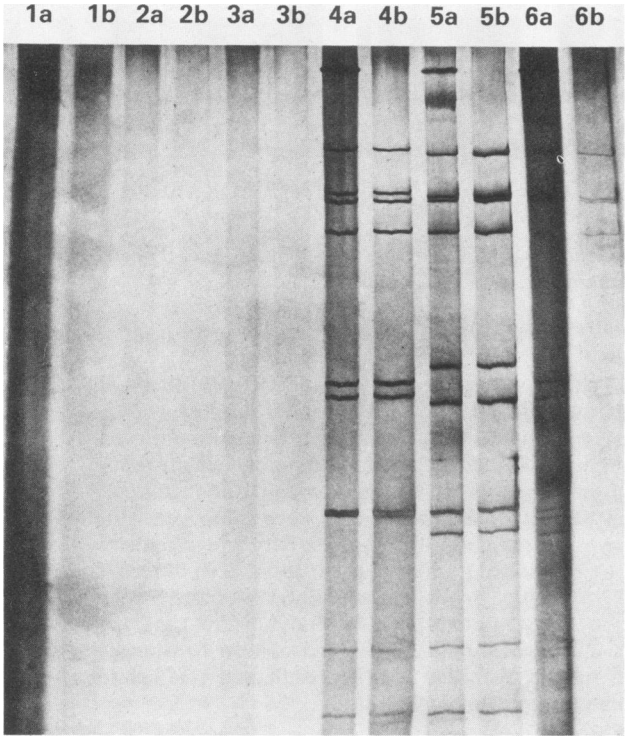


FIG. 1. Comparison of PBS-suspended versus phenol-chloroform-extracted stool specimens. Lanes 1 through 3 are stools that contained no detectable rotaviral RNA before (a) or after (b) extraction. Lanes 4 through 6 are stools that did contain rotaviral RNA before (a) and after (b) extraction. The gel is a 12% polyacrylamide gel that has been silver stained.

slightly more sensitive, apparently because of the more efficient uptake of the staining reagents.

To prepare the sample for loading on gel, 20 μl of the sample was added to 10 μl of reducing buffer consisting of 2% sodium dodecyl sulfate, 5 M urea, 20% glycerol, and 4% 2-mercaptoethanol in Tris-glycine buffer. This mixture was vortexed, heated at 68°C for 3 to 5 min, and vortexed again. The gel was run in a discontinuous Laemmli system (19) at 10 mA constant current for 18 h at room temperature. It was also possible to "rocket" the gel by running at 30 to 35 mA for 4 to 4.5 h with the same result.

After electrophoresis, the stacking gel was removed, and the resolving gel was stained with silver by a modification of the method of Herring et al. (13). All washes and soakings were performed in a gently shaken water bath at room temperature. The gel was washed three times for 10 min each in 100 ml of 5% acetic acid in 10% ethanol to remove sodium dodecyl sulfate. The gel was then soaked in 100 ml of 3.4 mM $HNO_3$ for 5 to 10 min. The nitric acid solution was poured off, 100 ml of 12 mM $AgNO_3$ was added, and the gel was allowed to soak in this solution for at least 30 min. The gel was then rinsed twice in 200- to 300-ml volumes of 0.28 M $Na_2CO_3$ in 2.2% (wt/vol) formaldehyde (developing solution). After the second rinse, the gel was soaked in the developing solution until discrete bands of double-stranded RNA appeared. It was occasionally necessary to remove the developing solution and add fresh solution to enhance staining. The gel could be stored in this solution; if desired, the developing solution could be removed, and ~100 ml 1% acetic acid could be added to stop further staining.

**EM.** Stool specimens were suspended in 1% ammonium acetate (~10%), and a drop was placed onto a carbon-Formvar-coated, 400-mesh grid. After blotting, the specimen was negatively stained by placing a drop of 2% phosphotungstic acid (pH 7.0) on the grid. After further blotting, the grid was air dried and examined under a Philips 201 electron microscope at 45,000×. Stools were considered negative when no virus particles were detected during 10 min of observation.

**ELISA for stool antigen.** The ELISA test was performed according to manufacturer's instructions and was read visually. A ± reading was considered a negative result. A + reading was considered borderline and was called positive if the + reading recurred upon reexamination.

## RESULTS

**Comparison of RNA extraction procedures.** The common practice for analysis of rotavirus RNA electropherotypes has been to first prepare suspensions of stool specimens in PBS, perform some type of purification of the virus, and then extract the viral nucleic acid by deproteinizing the suspensions with phenol-chloroform. This is followed by overnight precipitation with ethanol at −20°C (15). Although the purification and phenol-chloroform extraction technique has been successfully employed by several authors (8, 9, 15, 17, 21, 24, 25, 29–31), the necessity for purification and extraction was not reported. In our study, all stool specimens were suspended in PBS (5%, wt/vol), the sediments were pelleted by centrifugation, and the supernatant fluids were evaluated by the PAGE-SS technique. Rotavirus RNA was readily visualized in many such unextracted preparations (see below). For comparative purposes, six PBS suspensions that were positive for RNA (and EM and ELISA positive) and 12 randomly selected PBS suspensions that were negative for RNA (and EM and ELISA negative) were also extracted with phenol-chloroform. Both the extracted and nonextracted samples were then electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gels (Fig. 1). Two of six originally positive samples exhibited increased intensity of double-stranded RNA after extraction with phenol-chloroform, but none of these 12 originally negative samples exhibited the presence of rotaviral RNA after the extraction procedure.

**Comparison of EM, ELISA, and PAGE-SS in the 1983 study.** One hundred and one stool and swab specimens were collected in the 1983 study. Only 50 were suitable for examination by EM (sufficient solid stool was present in the sample to test on a grid). These specimens and 51 additional specimens considered unsuitable for EM testing were tested by the ELISA and PAGE-SS techniques (Table 1). Rotavirus virions were seen in 25 of 50 specimens observed with the

TABLE 1. Comparison of EM, ELISA, and PAGE rotavirus assays

| EM results | Total in group | Rotazyme$^+$, RNA$^+$ | Rotazyme$^+$, RNA$^-$ | Rotazyme$^-$, RNA$^+$ | Rotazyme$^-$, RNA$^-$ |
|---|---|---|---|---|---|
| Positive | 25 | 22 | 1[a] | 2 | 0 |
| Negative | 25 | 5 | 0 | 0 | 20 |
| Not done | 51 | 15 | 7[b] | 1 | 28 |

[a] Phenol-chloroform extraction of this specimen revealed the presence of RNA upon second PAGE-SS.

[b] Phenol-chloroform extraction of these specimens did not reveal any rotaviral RNA upon second PAGE-SS.

electron microscope. The Rotazyme ELISA test was positive in 28 of 50 specimens (positive result determined from color chart according to the manufacturer's recommendation). The segmented double-stranded RNA pattern of rotavirus was detected in 30 of 50 samples.

A majority (22 of 30) of positive samples in the group subjected to EM study were detected by each of the three diagnostic techniques. However, five specimens were EM negative and RNA and Rotazyme positive. These are considered to be false EM negatives because of the definitive nature of the rotavirus RNA pattern. Two specimens that were EM and RNA positive and Rotazyme negative were considered to represent false-negative Rotazyme determinations. One specimen that was EM and Rotazyme positive and RNA negative appeared to represent a false PAGE-SS RNA determination; this specimen was not included in the original group of RNA-negative specimens tested for phenol-chloroform extraction utility. Repeated electrophoresis of this suspension after phenol-chloroform extraction revealed the presence of rotaviral RNA. These results indicated a false-negative diagnosis rate of 3.3% (1 of 30) by RNA analysis of nonextracted stool suspensions; this rate was reduced to 0% when stools were extracted. The false-negative diagnosis rate of direct EM in this study was 16.7% (5 of 30), and that of Rotazyme ELISA was 6.7% (2 to 30). The unique pattern of double-stranded RNA of rotavirus precludes a false-positive result by RNA electrophoresis.

Of the 51 specimens not suitable for EM examination, rotaviral RNA was detected in 16 specimens, whereas the Rotazyme test was positive in 22 specimens. A single RNA-positive specimen in this group was false-negative by ELISA test. Seven specimens were ELISA positive and negative for viral RNA when tested either nonextracted or after extraction with phenol-chloroform. In an effort to explain these RNA-negative Rotazyme-positive results (there were no examples of such discrepancies in the group containing an EM comparison), we performed a blocking test of the Rotazyme with a hyperimmune anti-rotavirus serum (12). Sufficient sample was available to test four specimens by blocking test; two specimens were confirmed as positive by this technique, and two were negative.

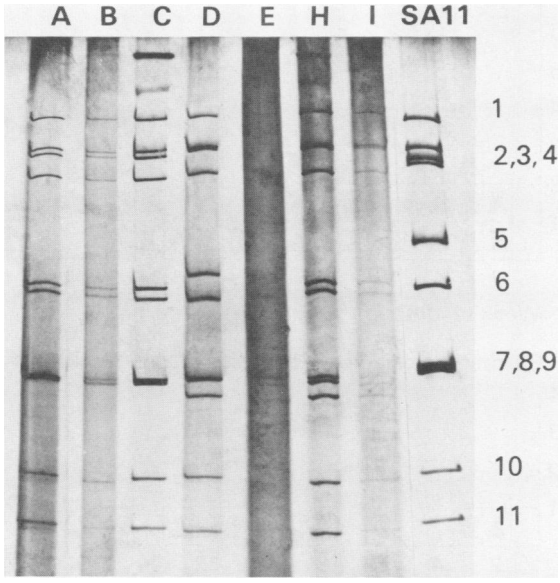


FIG. 2. Representative RNA electropherotypes of the 46 rotavirus stools observed in the January through May 1983 rotavirus season as seen in a 12% polyacrylamide gel after silver staining. Simian agent 11 (SA11) is included as a reference.

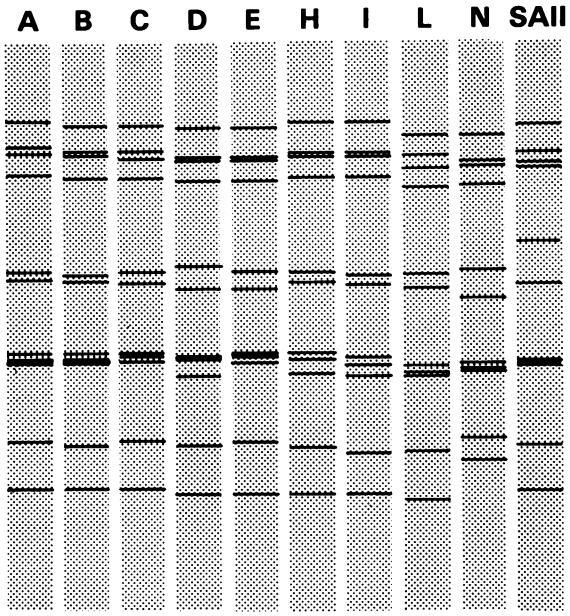


FIG. 3. Schematic representation of the representative RNA electropherotypes of the 68 rotavirus stools observed in the three rotavirus seasons (1981 through 1984) presented in this study. Simian agent 11 (SA11) is included as a reference.

One of the stool specimens giving discrepant results contained a sufficient amount of material to warrant purification to facilitate visualization of rotaviral RNA upon analysis of the purified virus. However, the cesium chloride gradient of the purported rotavirus did not yield any visible bands, nor did a PAGE-SS analysis of the pelleted fractions of the gradient reveal any rotaviral genome.

**Rotavirus electropherotypes.** From January to May 1983, 101 stool specimens and rectal swabs were collected from children under the age of 2 years with nonbacterial gastroenteritis diagnosed at Children's Hospital. Eight different electropherotypes were seen in the 46 rotavirus RNA-positive samples; seven of these patterns are shown in Fig. 2. The eighth pattern (designated M) represents a single aberrant isolate from an immunodeficient child discussed separately below.

A continuing survey of stool specimens from patients with gastroenteritis examined during the 1984 rotavirus season revealed 14 RNA-positive samples. A previously unrecognized electropherotype, L (Fig. 3), predominated in this series, and the D and E types appeared intermittently.

For a retrospective evaluation of the 1981 through 1982 rotavirus season, eight Rotazyme-positive stool specimens were obtained from St. Christopher's Hospital for Children. Rotaviral RNA detected in these specimens by the PAGE-SS technique was predominantly of an electropherotype pattern designated D in our laboratory (Fig. 2). Only one "short" RNA pattern, one with a slower migration of segments 10 and 11, was detected among these eight RNA-positive samples (designated type N) (Fig. 3).

The single aberrant M electropherotype observed during the 1983 study represented a rotavirus strain isolated from a child admitted to Children's Hospital with severe combined immunodeficiency syndrome. Stool suspensions prepared

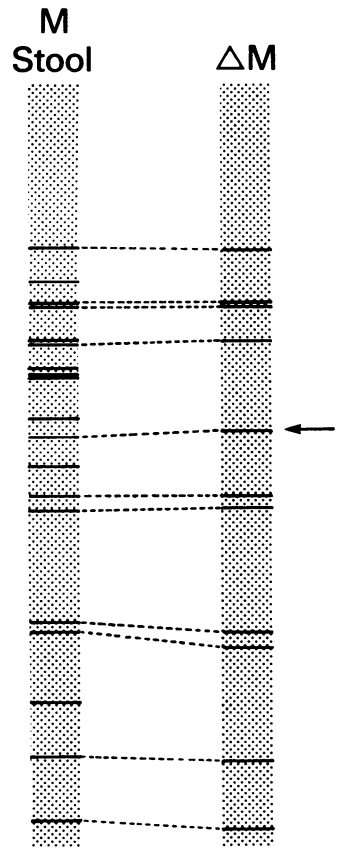


FIG. 4. Schematic representation of the M electropherotype isolated from the stools of a child with severe combined immunodeficiency syndrome. The ΔM pattern represents that of the tissue culture-adapted M rotavirus. The arrow indicates the double-stranded RNA band in a location atypical for human rotavirus.

from this child exhibited a unique RNA pattern containing 19 bands. This pattern of 19 bands was repeatedly observed in 17 stool specimens obtained over a period of 3 months. Rotavirus from this patient was adapted to growth in cell culture; this virus (ΔM pattern, Fig. 4) contains the rotavirus-characteristic number of 11 gene segments, although only 2 segments are observed in the normal 7, 8, 9 gene cluster (see Fig. 2), and a segment with an atypical location between the usual location of segments 4 and 5 is observed (arrow, Fig. 4). Rotavirus possessing the alternate RNA species seen in the M pattern has not yet been cultivated. Similar abnormal rotavirus RNA patterns have recently been reported by Pedley et al. (22) in immunodeficient children. It was suggested that extra double-stranded RNA bands are the result of formation of covalently linked concatemers among the 11 rotavirus RNA segments. A side-by-side comparison of the M (stool) and ΔM (cultivated virus) electropherotypes indicated that additional segments may indeed be "uncut" genomes or aggregates of certain segments.

**Epidemic distribution of rotavirus electropherotypes.** The epidemic pattern of rotavirus infection seen during 3 consecutive years of study is pictured in Fig. 5. The predominant electropherotype in the 1983 season was designated type A. It was seen in 65% of the cases and was detected throughout the season. Two of these type A isolates have been adapted to growth in cell culture; one has been determined to be a human serotype 3 virus. The other electropherotypes, designated B, C, D, E, H, and I, were seen less frequently and occurred sporadically in the 5 months of study. Isolates of electropherotype D and E have been adapted to cell culture and determined to be human serotype 1. Attempts at correlating electropherotype of rotavirus with age, length of illness, number of diarrheal stools, or location of residence showed no association.

It is interesting to note that the predominant electropherotype A in the 1983 study was not seen in either the 1982 or 1984 rotavirus seasons in this area. Conversely, the D electropherotype was the only strain detected in all 3 years of study.

## DISCUSSION

Many investigators have previously studied the electropherotype of the double-stranded RNA segments purified and extracted with phenol-chloroform from rotavirus-infected stools (7, 11, 15, 23–25, 29, 30). In a single previous report of PAGE-SS analysis of nonextracted rotavirus RNA, the virions were nevertheless purified from the stool suspension before analysis (7).

The sensitivity of our simplified technique was 24 of 25 (96%) in the 1983 study of stools positive for rotavirus by EM observation. The single false-negative sample revealed rotaviral RNA after extraction. The simplified PAGE-SS

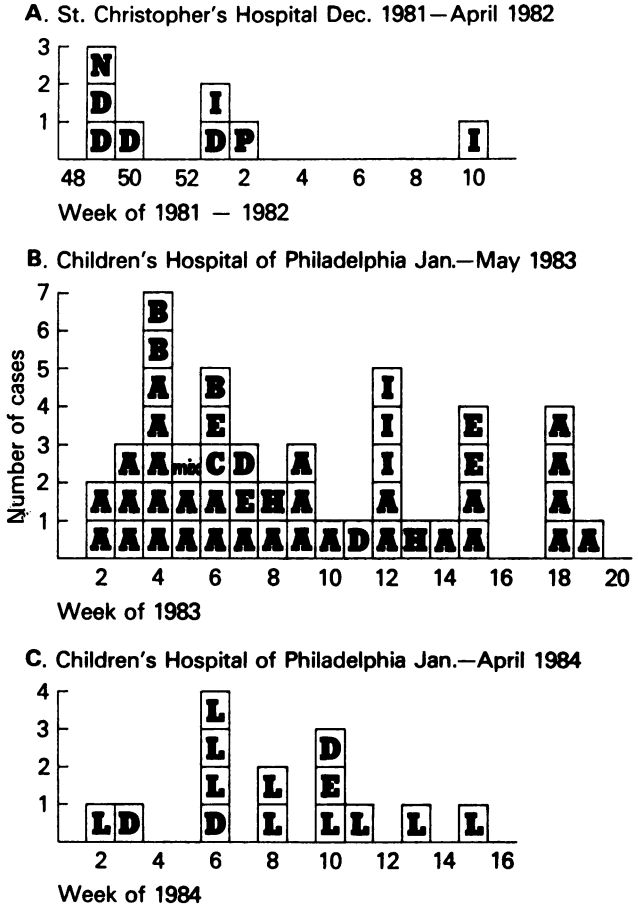


FIG. 5. Distribution of rotavirus electropherotypes during 3 years of observation in Philadelphia children's hospitals as determined by RNA analysis of 68 stool specimens.

method detected rotavirus RNA in five samples in which virus was not detected by EM study and in two samples that were rotavirus-negative by the ELISA (Rotazyme) test. The PAGE-SS method is thus slightly more sensitive than EM observation and equal to the ELISA test.

Our method was capable of detecting rotavirus in many samples too small to be usable for EM examination, many of which represented rectal swabs only. The Rotazyme ELISA kit requires a 200-μl sample per test and up to 1 ml if confirmatory blocking tests are performed. The PAGE-SS rotavirus RNA analysis technique uses a 20-μl sample, a clear advantage when limited volumes of sample are available. A comparison of detection limits for RNA PAGE-SS and Rotazyme with a tissue-culture adapted laboratory strain of rotavirus revealed a 2-fold higher sensitivity for the Rotazyme analysis with a 10-fold larger sample volume. A very small specimen may contain minute amounts of viral nucleic acid, but the unique banding pattern of the rotaviral genome allows for a reliable positive result even when only the larger segments of the genome are visible upon silver staining of the gel.

In a group of 51 samples not adequate for EM examination, seven (14%) did not yield visible RNA by the PAGE-SS technique despite the fact that they were ELISA positive. Purification, rotavirus protein analysis, and extraction with phenol-chloroform failed to reveal any detectable rotavirus. Retesting of four of these specimens with an ELISA blocking test employing hyperimmune antirotavirus serum confirmed a positive result in only two. Therefore ELISA-positive and RNA-negative determinations may represent false-positive ELISA results in some cases; the alternative is possible that in the course of rotavirus infection, abortive viral replication may occur leading to excretion of viral protein detected by the ELISA test unaccompanied by complete virions containing viral RNA. False-positive reactions with Rotazyme analysis have previously been reported by other investigators (4–6, 18, 26).

Our technique appears to be ideal in terms of 100% specificity and good sensitivity. EM requires expensive equipment and an experienced operator. The Rotazyme ELISA is expensive, and no confirmatory method for suspicious positive results is included in the kit. In terms of adaptability to the clinical laboratory, the PAGE-SS technique is a useful, reliable, and sensitive technique for diagnostic use when a small amount of specimen is available, when small or large numbers of samples are analyzed, and when technician time and cost of supplies are factors.

Unfortunately, the potential use of electrophoresis of rotaviral RNA to determine the antigenic serotype of the infecting strain is not feasible at this time. To date, the only consistently demonstrated correlation occurs between the "short" migration pattern of segments 10 and 11 and the serotype 2, subgroup 1 strain of human rotavirus (27, 32). In this laboratory we are currently attempting to cultivate the short rotavirus from the 1982 St. Christopher's specimens to confirm this correlation.

We have successfully adapted rotaviruses with the A, D, and E electropherotypes to growth in tissue culture; and we have tentatively determined that the D and E patterns represent serotype 1, and the A pattern represents serotype 3 (unpublished data). These results suggest a predominant role for serotype 3 rotavirus in the etiology of an unusually severe and explosive rotavirus diarrhea outbreak that appeared in Philadelphia in January 1983.

Both hospitals represented in our study are referral pediatric hospitals that attract patients from throughout the Philadelphia metropolitan area. Thus, data from either may presumably be compared. Although the numbers are limited for two of the three rotavirus seasons compared, they do suggest that a single distinct electropherotype of rotavirus predominated in the community in each of three successive years, with other electropherotypes appearing concomitantly in lesser incidence. This observation of a predominant electropherotype in a given rotavirus season is in agreement with results of similar studies reported from Melbourne (24), Alice Springs (Australia) (28), Mexico City (9), Palermo (3), Tokyo (31), Yogyakarta (Indonesia) (2), Papua New Guinea (1), Scotland (10), Paris and Dijon (21), Saiseikan (Japan) (17), and Bangui (Central African Republic) (11). The predominance of the long electropherotype (subgroup 2) has also been universally observed in recent years, with the exception of a single outbreak of subgroup 1 short electropherotype rotavirus gastroenteritis in Papua New Guinea (1). The simplicity of the rapid technique described in this report should facilitate the rapid detection of the emergence of new rotavirus electropherotypes and the analysis of their epidemiological significance.

The usefulness of a technique that simultaneously diagnoses and identifies the strain of rotavirus infection becomes apparent as the search for a suitable vaccine candidate continues. Such rapid determinations of electropherotype will be especially invaluable in evaluating naturally occurring rotavirus infections and shedding of vaccine virus after live rotavirus vaccine administration. Despite the apparent absence of a relationship between serotype and electropherotype of rotaviruses, the PAGE-SS technique should also prove to be extremely useful in following the incidence of rotavirus disease in a community and in tracking nosocomial infections in a hospital, clinic, or nursery. This simplified technique affords a viable alternative to the clinical laboratory in need of a rapid, reliable, and informative method for diagnosis of rotavirus disease.

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