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Molecular Epidemiology of Human Rotaviruses in Melbourne, Australia, from 1973 to 1979, as Determined by Electrophoresis of Genome Ribonucleic Acid

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Rotaviruses contain a double-stranded ribonucleic acid genome consisting of 11 segments. Gel electrophoresis separates genome segments and allows identification of strain differences. This electrophoretic typing technique was applied to rotavirus specimens from 116 children and 72 newborn babies. Between 1973 and 1979, 17 different electropherotypes of rotavirus were observed in children with acute gastroenteritis. These electropherotypes showed a sequential pattern of appearance, with a limited number of electropherotypes present at any given time. By contrast, only two electropherotypes were identified from isolates from newborn babies in seven hospitals during 1975 to 1979. These two electropherotypes were very similar and were never identified in children with acute gastroenteritis. One of the neonatal electropherotypes was found in the nurseries of five different hospitals and persisted in one hospital for 4 years. Electrophoretic typing techniques can be applied routinely and reproducibly to small samples of feces and could prove to be of value in epidemiological studies of rotavirus infection.

Rotaviruses are now recognized as a major etiological agent of acute enteritis in many animal species, including humans (9, 12, 17). Many studies of their incidence and distribution have been undertaken, but traditional seroepidemiological surveys have been complicated by the close antigenic relatedness of various rotaviruses and the difficulty of growing cell cultures of strains isolated from humans (9, 12, 17). Although the existence of distinct human rotavirus serotypes has recently been recognized (8, 24, 29, 30), there is uncertainty as to the number of existing serotypes and, hence, their epidemiological significance. We were thus led to try an alternative, molecular approach to the epidemiology of human rotavirus types, using gel electrophoresis of their 11-segment, double-stranded ribonucleic acid (RNA) genomes for strain identification, as has been done retrospectively in studies of known serotypes of influenza virus (11, 18), orbiviruses (10), and reoviruses (13, 19).

Initially it was assumed that gel electrophoresis of genomes would provide a means of distinguishing rotaviruses obtained from different animal hosts (14, 20, 22), but subsequent studies revealed that genetic diversity also exists among isolates obtained from each animal species (4, 6, 15, 20, 21a, 22, 25-27). Recently, we reported the existence of at least eight different bovine rotavirus electropherotypes, of which four were found on one property during a single outbreak of disease (20), but little could be said about their distribution because of the limited number of samples available. Epidemiological interpretation of recent studies reporting the detection of two or three human rotavirus electropherotypes (4, 6, 7, 15) is similarly restricted by the small numbers of samples or the limited time periods which were investigated.

In this study we looked at the occurrence of human rotavirus electropherotypes in an urban area (Melbourne, Australia) during a 7-year interval. The results provide new information concerning the number of different electropherotypes of human rotavirus in this community, the apparent reproductive stability of electropherotypes, and the length of time for which an electropherotype may persist.

MATERIALS AND METHODS

Viruses. The 335 human rotavirus samples chosen for this study were obtained by extraction of single stools collected from children and neonates from various hospitals in Melbourne between 1973 and 1979. Fecal samples were prepared by homogenization and centrifugation and were all shown to contain rotavirus particles by electron microscopy (3, 20, 21). However, only 188 of these samples provided sufficient RNA for

this study.

The majority of samples tested originally comprised small volumes (10 to 100 μl) of material prepared for diagnostic electron microscopy (5). Their content of virus particles, as scored by electron microscopy at a magnification of ×20,000, ranged from + to +++, i.e., from only a few particles per grid square to 50 to 100 per visual field. Not surprisingly, it was generally found that samples of very small volume (<30 μl) or those containing very few particles failed to yield sufficient RNA for electropherotyping. In cases where more of the original fecal samples were still available, larger amounts (e.g., 1 g) were processed, and these often gave satisfactory results. Of the 188 usable samples, 116 were obtained from children aged between 3 months and 6 years admitted to two hospitals for treatment of acute gastroenteritis. These two hospitals serve most of the central urban area of Melbourne (population, 2.5×10^6).

The remaining 72 specimens were from newborn babies (aged between 2 days and 2 weeks) with (43 specimens) or without (29 specimens) symptoms of gastroenteritis; the babies were housed in the nurseries of seven Melbourne obstetrical hospitals. The majority of specimens (50) were obtained between 1975 and 1978 from babies in one hospital. All babies had been delivered in the same labor ward and were transferred within 2 h of delivery to wards that were separately staffed (Table 1). Wards A to G housed babies "rooming in" with their mothers and provided routine postnatal care. Ward H housed babies separated from their mothers and provided special care. The remaining 22 specimens were from babies in six other obstetric hospital nurseries providing routine or special care and examined in 1975 (3 specimens), 1976 (1 specimen), 1977 (13 specimens), and 1979 (5 specimens).

Deproteinization of viral nucleic acid. Purified virus was disrupted with sodium dodecyl sulfate and deproteinized with phenol or a combination of phenol, chloroform, and isoamyl alcohol as described previously by Rodger and Holmes (20). Pelleted RNA was dissolved in 100 μl of Laemmli sample buffer (16) containing double-strength glycerol.

Polyacrylamide gel electrophoresis of RNA. Electrophoresis of deproteinized RNA was carried out in 10% polyacrylamide slab gels by using the discontinuous buffer system described by Laemmli (16) and used for RNA by Ramig et al. (19); electrophoresis was conducted for 4 h at room temperature as described by Rodger and Holmes (20). Gels were stained overnight with ethidium bromide at a concentration of 1 μg/ml before photography. Comparisons of different human rotavirus strains were made by mixing and then coelectrophoresing the RNAs. Aggregation did not appear to be a problem.

TABLE 1. *Sources of rotaviruses*[a]

Ward	No. of samples collected in yr			
	1975	1976	1977	1978
A	3			
B	2		4	1
C	4	1	2	
D			2	2
E	1			
F	6	1	3	1
G	2			
H	7	7	1	

[a] Samples were obtained from neonates in eight wards of a large obstetric hospital in Melbourne from 1975 to 1978. All samples were of electropherotype R.

RESULTS

Genetic diversity of the human rotavirus genome as shown by polyacrylamide gel electrophoresis. After extensive comparative studies of the samples by coelectrophoresis of similar and dissimilar genomes, 19 different electropherotypes were recognized. The electropherotypes are labeled A to S in accordance with a scheme previously proposed by Rodger and Holmes (20). Figure 1 was compiled from portions of various photographs of slab gels to illustrate the degree of genetic variability between the 19 electropherotypes. Seventeen electropherotypes (A to Q) were identified in rotavirus specimens from children with acute gastroenteritis. Two electropherotypes (R and S) were identified in specimens from neonates; the difference between these two electropherotypes was minimal (see below), and both differed markedly from all 17 electropherotypes from older children and adults (unpublished observation).

Validation of the genetic diversity of human rotavirus based on polyacrylamide gel electrophoresis. Identification of the 19 electropherotypes was based on the high resolving power of the Laemmli discontinuous buffer electrophoresis system that allows markedly different electropherotypes to be readily distinguished even on different slab gels. Strains producing similar genome patterns on the same or different gels must be coelectrophoresed before they can be considered identical. A list of coelectrophoretic comparisons between similar electropherotypes is given in Table 2. Cross-comparisons between all of the more obviously different electropherotypes were considered to be unnecessary and in a few cases were not possible because of lack of material.

The gels designated E, E + R, and R in Fig. 2 show the results of a typical electrophoretic comparison between two rotaviruses (E and R) with similar genome fractionation patterns. Before coelectrophoresis, differences in electrophoretic mobilities of "corresponding" segments (2 and 3 or 8 and 9) were anticipated (compare gels E and R), but only by actual coelectrophoresis (gel E + R) were the differences involving segments 2, 5, and 10 also detected. This example illustrates the absolute necessity of coelectrophoresing similar types before declaring them to

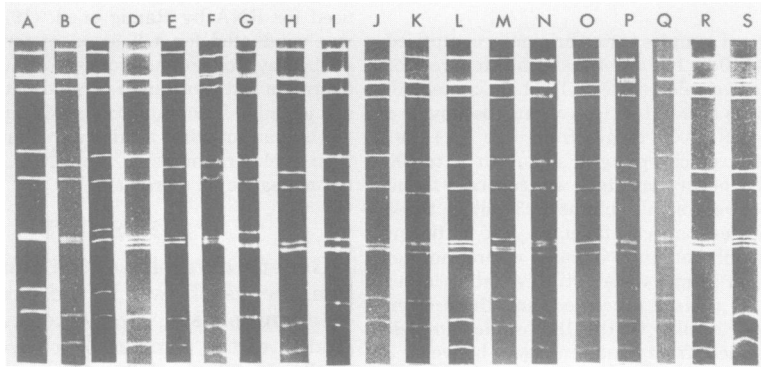

FIG. 1. *Human rotavirus electropherotypes A to S. The figure was compiled from portions of photographs taken of several polyacrylamide gels. This accounts for apparent similarities and differences that are not real (see text). Migration was from top to bottom. Although alphabetical designations were used to distinguish the electropherotypes encountered in this study, it is not intended that they should represent reference types. In view of the limited samples available and the current inability to cultivate most human rotavirus strains, it is not possible at present to set up a collection of reference types since identification of electropherotypes cannot be made without access to RNA samples for direct comparison (coelectrophoresis).*

TABLE 2. *Segment variations between various human rotavirus isolates as determined by coelectrophoresis of genome RNA*

Electropherotypes	Segment variations										Total no. of variations
A plus J	1			4	5		7		9	10	11	7
C plus E	1	2		4	5		7			10	11	7
C plus I					5		7			10	11	3
D plus K	1			4		6		8	9	10	11	7
D plus L								8				1
E plus H				4		6			9			3
E plus I					5		7					2
E plus M	1	2		4	5	6	7	8		10	11	9
E plus R	1	2			5			8		10		5
F plus R	1			4	5	6						4
G plus J	1			4	5		7					4
G plus M	1			4	5		7	8	9	10	11	8
I plus M	1	2		4			7	8		10		6
I plus R		2		4	5		7	8		10	11	7
J plus L	1		3		5		7	8	9	10	11	8
J plus S	1	2		4	5		7	8	9	10		8
K plus O	1			4		6	7		9	10	11	7
L plus M	1			4		6		8		10	11	6
L plus S					5				9			2
M plus O	1			4	5	6	7	8	9	10	11	9
R plus M		2		4	5		7	8		10	11	7
R plus S								8				1

be identical or different.

The gels designated R, R + I, and I in Fig. 2 show the results of a comparison made between two rotaviruses (R and I) with quite different electrophoretic patterns, especially in the region of segments 10 and 11. Coelectrophoresis (gel R + I) revealed differences in the relative mobilities of segments 2, 4, 5, 7, 8, 10, and 11 and possibly 3. It is interesting to note that segment 10 of electropherotype R (Fig. 2) comigrated with segment 11 of electropherotype I. Whether segments 10 and 11 of type I are both of lower mobility than segments 10 and 11 of type R or whether the apparent segment 11 of type I and other "short" rotavirus genomes corresponds to the apparent segment 10 of a "long" genome is unknown. Hybridization experiments which should answer this question are currently under way.

When sufficient material was available, frac-

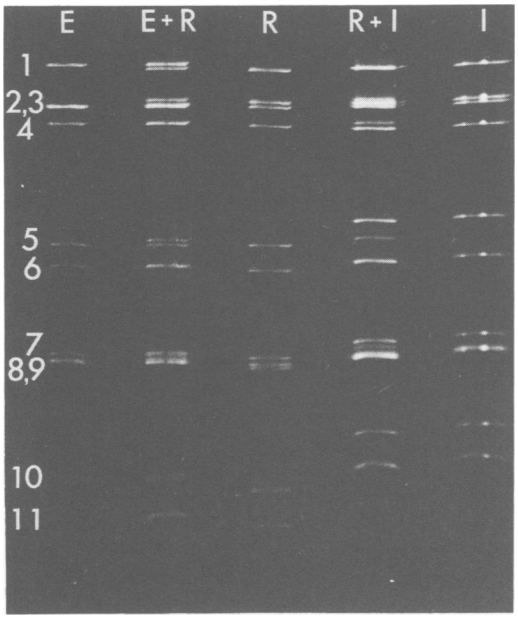

FIG. 2. *Comparison of human rotaviruses by RNA gel electrophoresis. Gels contained electropherotypes E, E plus R, R, R plus I, and I, as indicated. Migration was from top to bottom, and segments are numbered from the top from largest to smallest.*

tionation patterns identical to those obtained originally could be successfully reproduced. Once extracted from the fecal sample, the RNA did not alter in electrophoretic mobility upon storage, and reproducible results could be obtained even after storage for 15 months. However, occasionally RNA preparations could be completely degraded after storage for a few weeks at −20°C or even at −70°C. Such preparations migrated as a smear rather than as discrete segments upon electrophoresis.

Retarded migration of all segments of an electropherotype in comparison with that of the other types was occasionally observed (Fig. 2, type I). The cause of this phenomenon is not known, but it could be eliminated by purification of virus from feces before phenol extraction.

Molecular epidemiology of human rotaviruses in Melbourne from 1973 to 1979. Figure 3 illustrates the occurrence of electropherotypes when arranged according to the date of collection of the sample. The number of samples examined at a particular time does not necessarily reflect the incidence of diarrhea in the community at that time, so, to serve as a general guide, a graph illustrating the total number of children admitted per month with acute gastroenteritis (bacterial and nonbacterial) to the two hospitals has been superimposed on Figure 3. When the results are shown in this manner it is apparent that the 17 types (A to Q) from children did not occur at random throughout the seven years (1973 to 1979). Instead, they showed a sequential pattern of appearance, with a limited number of electropherotypes present at any given time. One electropherotype was usually predominant in any given period, often in combination with less common types. For example, from December 1973 to December 1975, electropherotype E was predominant, with types A, B, C, D, F, and G being detected less frequently. There was no apparent difference in electropherotypes present in children admitted to the two hospitals during the same period.

Commonly encountered rotavirus electropherotypes, e.g., E and M, did not appear to change annually. Both were readily detectable over 2 years, including winters when the incidence of rotavirus gastroenteritis was at its peak in Melbourne (5). It will be interesting to see if electropherotype M persists for a third winter in 1980. It must be stressed that the samples examined were from children admitted to hospital with acute gastroenteritis and may not reflect the possible occurrence of rotavirus strains producing mild or asymptomatic infections in the community.

It is interesting to note that electropherotypes E and M, the two most commonly detected types from children aged between 3 months and 6 years, were found to be strikingly dissimilar when compared by coelectrophoresis, as variations in the mobilities of nine corresponding segments were detected (Table 2). Although common electropherotypes were often superseded by dissimilar types, this was not always the case. For example, electropherotype E was followed by electropherotype H (detected once in the survey), and although the two types differed in the mobilities of three segments (4, 6, and 9), the variations were very slight indeed. Similarly, the relatively common electropherotype D was followed by type L (also detected only once) in which the only difference involved a slight variation in the relative mobilities of segment 8. The absence of samples during 1976 makes it impossible to determine the incidence of both type H and L before this date. Melbourne appeared to be relatively free of rotavirus gastroenteritis cases requiring admission to the hospital from January 1976 through until June 1977 (Fig. 3). This may indicate that electropherotypes H and L, and possibly other undetected types present in the community that were closely related to types D and E, produced asymptomatic or relatively mild infections not requiring hospital admission during this period.

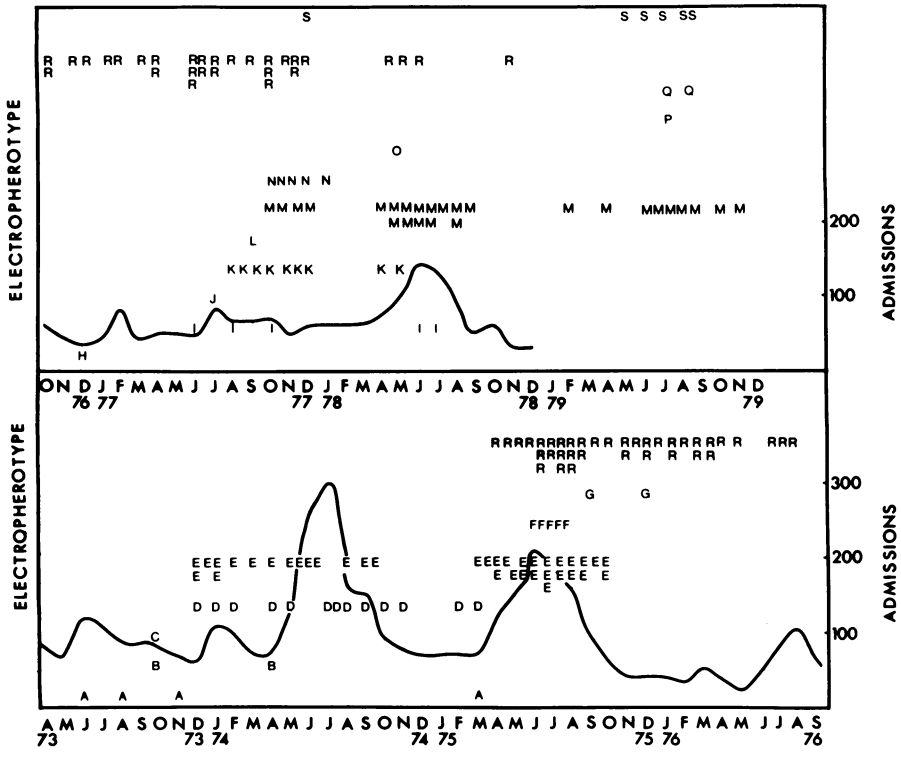

FIG. 3. *Occurrence of human rotavirus electropherotypes obtained from children admitted to two Melbourne hospitals from May 1973 to November 1979. The graph shows the total number of children admitted to these hospitals with acute gastroenteritis (bacterial and nonbacterial).*

The four most commonly detected electropherotypes, D, E, K, and M, were each first detected in the mid-spring or early summer (October to December) following a winter (June to August) peak of highest incidence. In three of the four cases each was the most commonly detected agent the following winter. The significance of this observation is discussed below.

Neonatal rotaviruses—electropherotypes R and S. Rotavirus electropherotypes R and S were detected only in the stools of neonates and, as determined by coelectrophoresis, appeared to be very closely related. The only variation in electrophoretic mobility involved segment 8. Neither of these electropherotypes was detected in older children admitted to hospital with acute gastroenteritis.

Type R was detected in 1975 when the problem of rotavirus diarrhea in neonates was first recognized (2). It was detected in both symptomatic and asymptomatic neonates and was distributed throughout the eight separate wards of one hospital (Table 1). Type R persisted in ward G of this hospital without apparent change for 4 years and was located in other wards at some time during 1975 to 1979. Type R was also detected in stools from neonates in the nurseries of four other obstetrical hospitals during 1975 to 1978. In one of these hospitals it was recovered from samples from consecutive outbreaks of diarrhea among neonates and from asymptomatic neonates during the 6-week interval between the outbreaks.

Electropherotype S was detected in neonates from two other Melbourne obstetrical hospitals, in November 1977 in one and from May to August 1979 in the other. Type R was previously detected in the latter hospital in 1976.

DISCUSSION

In Melbourne, a succession of at least 19 different human rotavirus electropherotypes has occurred since 1973. Seventeen of these types were found in children with acute gastroenteritis. This observation indicates a constantly changing population of rotaviruses in the community, which, by analogy with influenza viruses, may reflect the occurrence of both antigenic shift and drift (28). The results presented here confirm and extend those reported by Espejo et al. (6, 7) and by Croxson and Bellamy (4), who detected both predominant and less

common strains of human rotavirus in shorter-term surveys in Mexico City and Auckland (New Zealand), respectively. In agreement with Espejo et al. (6, 7), we observed that particular rotavirus electropherotypes may persist for 12 months or, as in our case, even over 2 years. However, we obtained no evidence that rotavirus electropherotypes cycle over a 3- or 4-year period as suggested by Kalica et al. (15) in a survey of eight samples.

The presence of a number of electropherotypes at any time within a single city raises the possibility of dual infections which could result in new strains arising by reassortment. It may be more than a coincidence that the predominant electropherotypes E and M first appeared after periods during which a variety of transient types were present (Fig. 3). New strains might also result from reassortment between rotavirus strains from different animals. Rotaviruses of bovine (20, 27) and equine (21a) origin have been shown to have quite diverse genomes.

The wide range of vertebrates in which rotaviruses are found and the fact that cross-species transmission has been demonstrated suggest that there could be opportunities for genetic exchange between viruses infecting different animal species, including humans (9, 12, 17). The majority of samples examined in this study were obtained from children admitted to the hospital with acute gastroenteritis and may not be representative of strains causing mild or asymptomatic infections in the community. However, a few samples of electropherotypes I and M were obtained from symptomatic adults not requiring hospitalization. Thus, the rotavirus electropherotypes found in this study may, in fact, be representative of those causing less severe infections in the general community.

In contrast with the diversity and variability observed in electropherotypes from children and adults, rotavirus specimens from neonates were restricted to two electropherotypes, one of which persisted for 4 years. One possible interpretation of the results from neonates is that a community strain of rotavirus was introduced into one or more of the Melbourne obstetric hospital nurseries before 1975. It is possible that this occurred before 1973 since it was not detected in the survey of community strains collected from 1973 onward. Once the virus was introduced into a nursery, conditions would favor its survival with the constant availability of newborn babies as susceptible hosts. The homogeneity of the strains from hospital to hospital might be due to simultaneous introduction of a predominant community strain into all nurseries or due to cross-infection from nursery to nursery via visiting medical staff or transferred babies. These hypotheses leave unexplained the observation that rotavirus infection in at least one nursery is seasonal, with a winter peak reflecting the community incidence of infection (3). It was thought that the seasonal incidence of infection in the controlled climate of a special care nursery must be due to introduction of rotavirus strains via visitors and hospital staff, but the electrophoretic analyses show that this was not the case.

The persistence of the neonate electropherotype R for at least 4 years provides a good indication of the apparent genetic stability of the rotavirus genome upon passage. Similar stability has been observed in the electropherotype of the cell culture-adapted SA11 rotavirus which has been passaged repeatedly in our laboratory for 4 years.

The relationship between electropherotype and serotype is yet to be established, as the RNA segment(s) coding for the type-specific immunogen(s) is unknown, although some progress has been made with coding assignments (23). It seems probable that, as has been shown with myxoviruses and reoviruses, gel electrophoresis of the rotavirus genome will reveal heterogeneity within serotypes, thus providing more detailed epidemiological information than serotyping alone (11, 13). The advantage of electrophoresis is illustrated when comparisons are made between the neonate electropherotype (R) and others (H, L, and O) obtained from the general community. These four electropherotypes were all serotyped by enzyme-linked immunosorbent assay as type 2 rotavirus (R. H. Yolken, personal communication). However, in view of the distribution and distinct nature of the neonate electropherotype R, the classification is misleading in that it suggested that the neonate strain was the same as those obtained in the general community at the same time. Clearly then, assignment of rotaviruses to one of the few established serotypes, especially to the most common one (1), provides much less epidemiological information than does electropherotyping.

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ADDENDUM IN PROOF

A number of the rotavirus samples included in this study were serotyped by R. H. Yolken, whom we thank for his assistance. When the results were correlated, it was apparent that the eight samples identified as ELISA (enzyme-linked immunosorbent assay) serotype 1 had "short" gel patterns (seven of

electropherotype M and one of type K), whereas those of ELISA serotype 2 (three of electropherotype R and one each of types H, L, and O) produced "long" patterns. It thus appears possible that the ELISA serotype depends on the products of genome segment 10 or 11, and the gel pattern can be used to predict the ELISA serotype.

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