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Characterization of rotavirus electropherotypes excreted by symptomatic and asymptomatic infants

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SUMMARY

Human rotavirus isolates from 1100 stool samples were analyzed by polyacrylamide gel electrophoresis, and 48 different migration patterns were detected. Heterogeneity in the migration of segment 10 was observed in both long and short electropherotypes in which three long and two short patterns were identified. In spite of these variations all short and long electropherotypes were subgrouped by enzyme immunoassay as subgroups I and II respectively. Mixed infections were detected in 17% of cases and the subgrouping correlated with the corresponding electropherotypes. The same electropherotypes were present in severe, mild and asymptomatic cases and no electropherotype was particularly associated with greater virulence. Furthermore, the electropherotypes isolated from nosocomial asymptomatic cases were the same as those detected from those admitted with severe diarrheoa. It seems unlikely that electropherotyping can be used to identify more virulent strains of rotavirus.

INTRODUCTION

Rotavirus has been identified as one of the most common aetiological agents of acute gastroenteritis in infants and newborns and also in several other animal species [1]. The virus particle contains at least six polypeptides forming a double-shelled icosahedral structure which surrounds a central core containing the viral genome [2, 3]. The genome has 11 double-stranded RNA segments with a characteristic migration pattern in polyacrylamide gels when separated by electrophoresis (PAGE) [4, 5]. The characterization of the electrophoretic migration pattern as an electropherotype may be used as a rapid and sensitive method both for viral identification and to identify different viral isolates based on variations in the migration of individual RNA segments [6, 7]. Based on the

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migration pattern of the two smallest RNA segments, rotaviruses have been classified into two groups with short and long electropherotypes, although variations in the electrophoretic mobility of the remaining segments have also been observed [8, 9].

The nature of the diversity in the electrophoretic migration of each segment remains unknown [10]. It has been suggested that it may correspond to changes either in the base composition or in the size of the RNA segments [11]. Previous reports have shown more than 38 different electropherotypes detected in a 2-year survey [5], and the same viral electropherotypes were detected during later years, suggesting that they represent stable viral variants [6]. Therefore, electropherotyping has became a useful epidemiological tool [7, 12, 13].

Rotaviruses have been classified into serotypes and subgroups by their antigenic structure [1]. Serotypes are defined by the surface antigens specified by two viral polypeptides: VP7, involved in recognition of the cell receptor, and VP4 related to viral penetration and to the haemagglutinin [14–16]. These antigens involved in neutralization have defined the nine different serotypes described so far [17, 18]. Several attempts have failed to show a clear relationship between electropherotypes and serotypes [9]. Another antigenic determinant, specified by a major viral polypeptide, VP6, which makes up the inner viral capsid defines the subgroups I and II [19].

The relationship between virulence and either electropherotypes or viral strains defined by antigenic markers of rotavirus has not been established. A study of this subject may identify naturally attenuated strains, and this could be useful in developing vaccine strains. At present several contradictory reports have suggested that newborns can be infected by rotavirus strains asymptomatically [20–22]. Therefore an analysis of the rotavirus strains isolated from patients of different age groups between 1 month and 12 months old with disease of different degrees of clinical severity may give more useful information on this subject than that obtained from newborns alone. In the study reported here, the relationship between electropherotypes and antigenic subgroup is analyzed on virus from stool specimens from hospitalized and ambulatory patients. It is a prospective study of rotavirus infection in hospital, acquired before or after admission, and in which the electropherotypes isolated from patients with severe, mild or asymptomatic infections have been correlated with the clinical manifestations of rotavirus infection.

MATERIALS AND METHODS

Patients

Rotavirus isolates obtained between April 1984 and January 1987 were studied. The samples were collected from 285 infants under 2 years old from the Roberto del Río Children's Hospital or from the out-patient clinic covering the same area. In one hospital ward for patients with acute diarrhoea, these rooms, each with seven cots, were monitored for rotavirus infection by RNA gel electrophoresis of stool samples obtained from all patients at admission and on every other day during their entire hospital stay [6]. Positive cases were followed up with daily testing until three consecutive samples were negative. Symptoms were recorded

Table 1. *Daily score for patients with diarrheoa**

	Parameter	Characteristics	Score
I	Stools*	Normal	0
		Soft	1
		Liquid	3
II	Vomit	No	0
		Only 1	0·5
		2 or more	1
III	Rectal temperature†	37·4 °C or less	0
		37·5 °C to 38·5 °C	0·5
		38·6 °C or more	1
IV	Dehydration	No	0
		Mild	1
		Severe	2
	Maximum score possible		7

* The characteristics of the stool were defined by the greatest alteration from normal during a 24-h period.

† The temperature recorded was the highest of the day.

daily for all of them using the scoring system outlined in Table 1 and correlated with the electropherotype of the virus in their stools. The study included 227 hospitalized cases from whom serial stool samples were obtained, 122 admissions for severe acute diarrhoea and 105 cases of nosocomial rotavirus infection. Severe diarrhoea was defined by dehydration and/or passage of liquid stools for longer than 4 days. Nosocomial infection was defined by the absence of rotavirus at admission and detection beyond the fourth day of hospitalization [23]. Mild diarrhoea associated with rotavirus infection was studied in a group of 57 ambulatory patients with diarrhoeal episodes lasting less than 4 days without dehydration and from whom more than three consecutive samples were obtained.

Electropherotyping of rotavirus genomic RNA

The stool samples were subjected to polyacrylamide gel electrophoresis as previously described [5]. Briefly, stool samples were diluted twofold in distilled water and then mixed with twice the initial volume of a 50 mM Tris-HCl buffer pH 8·0 containing 15 mM-NaCl, 8 mM-SDS. The mixture was extracted with phenol, ethanol precipitated overnight and then centrifuged [5]. The RNA pellet was resuspended in the same buffer as above but containing 20% glycerol and 1% β-mercaptoethanol and subjected to electrophoresis in a 7% polyacrylamide gel with a 3·5% stacking gel at constant current of 20 mA for 12 h with a Laemmli discontinuous buffer system as previously described [24]. Double-stranded segments of viral RNA were stained with silver nitrate. Final characterization of the electropherotypes to confirm small differences was done by mixing equal amounts of two samples of viral RNA and electrophoresing the mixture under the same conditions [25].

Antigenic subgrouping

An aliquot of the supernatant of the viral suspension in distilled water made for gel electrophoresis was used for antigenic subgrouping by enzyme immune assay (EIA) as previously described [15]. The monoclonal antibodies against both

antigenic subgroups were kindly provided by Dr J. Flores (Laboratory of Infectious Diseases, National Institutes of Health, USA).

RESULTS

Rotavirus genomic RNA isolated from approximately 1100 stool samples, obtained from different groups of patients, were analysed by polyacrylamide gel electrophoresis. In Fig. 1, examples of the electropherotypes of the isolates which had marked heterogeneity in the electrophoretic mobility of their RNA segments are shown. All the isolates could be grouped into long or short electropherotypes, although they showed differences in the mobility of some of the other segments. To define the variations in the electrophoretic mobility of the segments, the viral RNAs were coelectrophoresed with other isolates. As seen in Fig. 1, the RNA segments showed extensive variations in the mobility of all 11 segments. A summary of the different viral electropherotypes detected is shown in Fig. 2, where the mobility of the 11 viral RNA genes is analyzed by groups (I–IV) using a previously reported system [5, 7]. The first number indicates the mobility pattern of the first four and higher molecular weight segments (group I). The following numbers indicate the patterns of the segments in groups II and III. The mobility of the two smaller segments is indicated by letters (S or L) corresponding to short or long patterns. However, as shown in Fig. 1, the group IV segments are not constant and the variations found in these segments are indicated by a number. For example, two different types of short virus patterns were detected (S1 and S2) and three types of long pattern (L1, L2 and L3). When the mobility of all the segments from all the strains of rotavirus were analysed a total of 48 electrophoretic variants had been found.

Some of the 48 electropherotypes shown in Fig. 1 were detected throughout the study period while others, such as electropherotype L3, only appeared in brief outbreaks or were detected only once or twice. Furthermore, the high sensitivity and specificity of PAGE used for rotavirus detection would have also detected any non-group A rotaviruses or reoviruses, although neither were found in the present study [26].

The study of 168 patients with diarrhoea from whom four or more sequential stool samples were available showed that 29 cases (17%) shed more than one electropherotype during one diarrhoeal episode. The clinical outcome of these mixed infections, based on our scoring system, did not differ from those cases where only a single electropherotype was present. Sixteen out of these 29 cases were children hospitalized for severe diarrhoea: 6 cases were admitted with mixed infection while 10 cases were super-infected after the third day in hospital. The remaining 13 patients were mild ambulatory cases of diarrhoea of whom 8 excreted mixed electropherotypes from the beginning and 5 after the third day of infection. These mixed infections included two different long electropherotypes, a long type with a short one or two different short types. Some representative examples of such mixed electrophoretypes are shown in Fig. 3.

The isolated viruses were analyzed by EIA using monoclonal antibodies against subgroups I and II with the results shown in Table 2. All 50 short electropherotypes reacted with the antibody to subgroup I and the 210 long ones

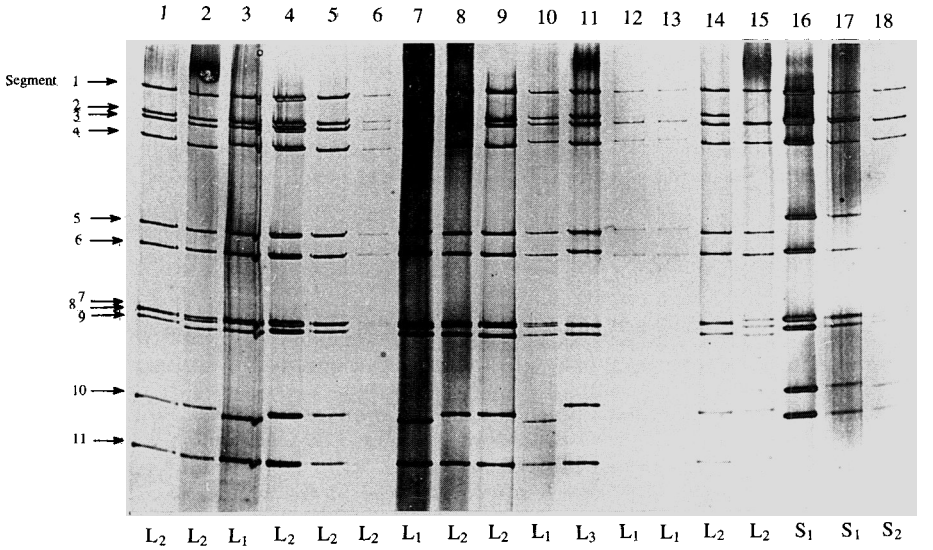

Fig. 1. Electropherotypes of human rotavirus. Genomic viral segments separated by polyacrylamide gel electrophoresis. Samples 1 to 15 are long electropherotypes and 16 to 18 short ones. The arrows indicate the 11 RNA gene segments. The type of long (L) and short (S) electropherotype is indicated by a number.

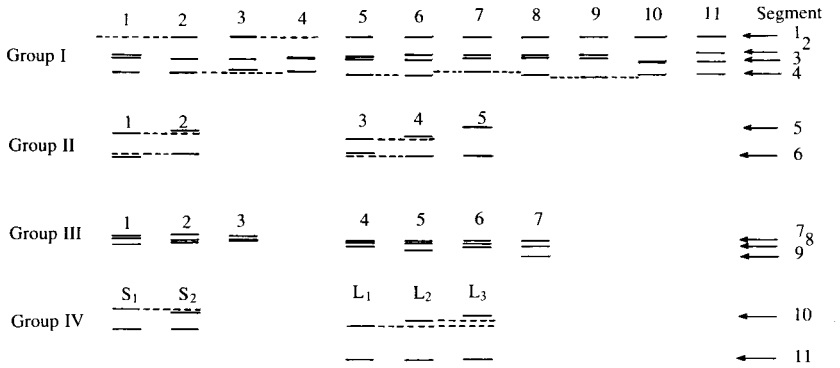

Fig. 2. Scheme of rotavirus electropherotypes isolated. Different mobility patterns are defined by groups (I–IV) and by a number within each group. Eleven different group I patterns were found, 5 group II, 7 Group II and 5 group IV.

with the antibody to subgroup II. These subgroup identities were not altered by variations of the short (S1 or S2) and long electropherotypes (L1 or L2 or L3), suggesting that there is a close relationship between short and long electropherotype and antigenic subgroups I and II respectively. Analysis of the mixed infections also confirms this observation. All the stools in which both a long and a short electropherotype were detected reacted with both subgroups' antibodies. Furthermore, in those cases who had sequential infections during which the electropherotype changed from long to short or from short to long in the course of the illness, a change to the corresponding antigenic subgroup was also observed, as shown in Fig. 3.

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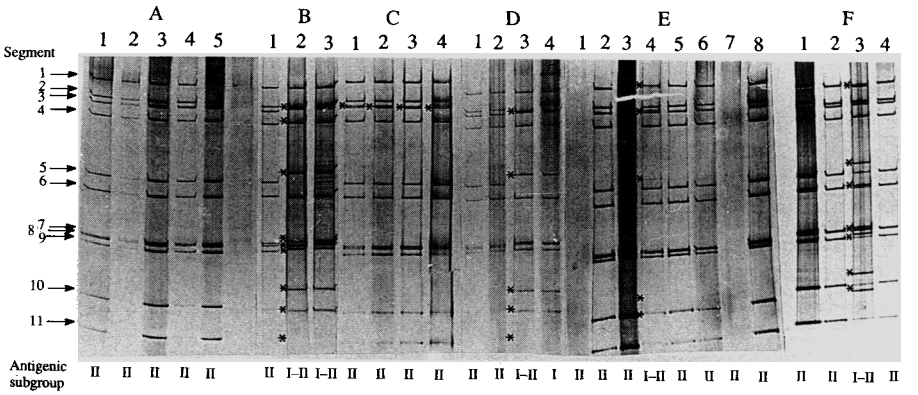

Fig. 3. Electropherotype and antigenic subgroup of mixed infections. Viral RNA was subjected to gel electrophoresis as indicated in Fig. 1. Six cases of mixed infection (A–F) are shown, and the number indicates the order of the sample. * indicates the presence of additional RNA segments for each case. At the bottom of the figure the antigenic subgroup for each sample is indicated.

Table 2. *Determination of antigenic subgroups of rotavirus isolates*

No.	Electropherotype	Antigenic subgroup 1	2
50	Short	50	0
210	Long	0	210
8*	Mixed infections	8	8

* Mixed infections of long and short electropherotypes.

Table 3. *Human rotavirus electropherotypes associated with different patients*

Electropherotypes	Hospitalized patients (n = 122)	Nosocomial infections (n = 105) Symptom.	Asympto.	Ambulatory patients (n = 57)
Long:				
645 L1	17	4	15	8
545 L1	13	1	7	3
635 L1	7	2	2	3
845 L2	10	2	8	3
835 L2	7	1	2	3
535 L2	6	2	3	1
945 L2	5	0	5	1
Others*	39	13	26	9
Short:				
121 L1	6	1	2	13
122 S1	3	3	1	9
421 S1	2	3	1	2
Others*	7	—	1	2

* Correspond to 39 different long and 6 short electropherotypes isolated only once.

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Any possible associations between certain electropherotypes and virulence was studied by comparing the electropherotypes obtained from those hospitalized with severe diarrhoea with those isolated from ambulatory patients with mild disease. One long electropherotype, designated 645L1, was the one most frequently isolated from hospitalized and ambulatory patients, indicating that it was associated with both severe and mild diarrhoea. Furthermore this particular electropherotype was also the type most frequently isolated from nosocomial infections, although 88% of them were asymptomatic. The same result was obtained for other long and short strains of rotavirus, as shown in Table 3, in which types, apparently virulent because they caused mild and severe diseases, were also frequently associated with asymptomatic nosocomial infections. There was a higher frequency of electropherotypes with long patterns than short ones. Since the number of short isolates is relatively small and their variety of electropherotypes is high, it is difficult to draw conclusions about their virulence.

DISCUSSION

At present the mechanism of virulence of rotavirus strain is not well understood, although some reports have indicated that their virulence may be specified by gene segment 4 [12, 16]. We have carefully studied different viral strains, defined by their electropherotype, which could be associated with different degrees of diarrhoeal severity, and particularly in patients from whom sequential stool samples could be obtained. So far such electrophoretyping has provided a useful tool for diagnosis and epidemiological surveys, although the molecular basis for the different electropherotypes remains unknown despite the fact that several viral genes from several rotavirus strains have now been sequenced [21, 27, 28].

For this study stool samples were collected over a period of 3 years and 48 viral electropherotype were found by RNA gel electrophoresis. Detailed analysis reveals that there were variations of the electrophoretic mobility of segments 10 and 11 in both long and short electropherotypes. For example, three different types of long patterns (L1, L2 and L3) and two of short pattern (S1 and S2) were defined. The major difference between the long patterns was associated with the electrophoretic mobility of segment 10 rather than segment 11, and similar results were obtained for the short ones. The electrophoretic mobility of the other nine genes also showed considerable variation among long and short electropherotypes as previously reported [5, 7, 13]. It was found that the long electropherotypes of the L1 and L2 pattern were detected more frequently as seen in Table 3. Most of the electropherotypes found in this study were identical to those detected in previous surveys but others, such as L3, seemed to be new [5, 22]. This could be explained by a longer survey period and a higher number of positive samples in the present study. This may also be because some electropherotypes were detected only in outbreaks and were not found otherwise. To explain this it will be necessary to understand how different types arise in nature.

The detection of several types of migration pattern in genes 10 and 11 did not affect the subgrouping of these strains. Those with long or short patterns belonged to the corresponding antigenic subgroups I or II, which are associated with protein VP6 coded by gene 6. The exact relationship between antigenic subgroups

and long or short electropherotypes remains unclear but as shown in Table 2, there is a complete correlation between both markers. This remained true even when dual isolates with both short and long electropherotypes were present at the same time; they showed the expected antigenicity of both subgroups. This was also observed when serial stool samples from the same patient were analyzed and changes in the electropherotype from long to short or from short to long were detected; there was a corresponding change to the other antigenic subgroup (Fig. 3). These results let us conclude that it is possible to determine the antigenic subgroup of an isolate by analyzing the viral electropherotype.

As previously indicated, the electropherotyping of rotaviruses allowed us to study the relationship between particular viral strains and the clinical characteristics of the associated diarrhoea. Viral isolates were grouped by the severity of the disease: severe diarrhoea, mild diarrhoea from ambulatory patients and those with nosocomial infections. The results showed that the same electropherotypes were present in all three groups, including nosocomial infections where most of the patients remained asymptomatic. Furthermore, no electropherotype causing only severe diarrhoea was detected. This was also supported by the finding that mixed infections did not develop into more severe cases of diarrhoea even if the electropherotypes were the same as those isolated from infants with severe infections hospitalized on the same ward. Mixed rotavirus infections were first detected studying these nosocomial cases and were explained as superinfections from exposure of the infants to a highly contaminated environment, represented by hospital wards for children with diarrhoea [25]. However, the detection of mixed electropherotypes from the onset of the disease in community-acquired cases with mild or severe diarrhoea means that mixed rotavirus infections are not uncommon in nature and they do not have a more severe outcome when compared with single infections. Therefore it is possible to argue that host factors rather than variations in the virulence of viral strains may account for the asymptomatic outcome of most rotaviral infections [23]. The electropherotypes detected in mixed infections corresponded to those more frequently isolated at this period, suggesting that no viral strain is predominantly responsible for this situation and the presence of a particular viral electropherotype was probably because it was the most abundant one in the environment.

Asymptomatic excretion of rotavirus was originally thought to be unique to newborns [20], but later it was also reported in older children [22, 23]. The results shown in Table 3 confirm any viral electropherotype in any age group can produce symptomless infection.

In conclusion, our results, using electropherotypes as epidemiological markers, show that a wide variety of rotavirus strains were detected. The rotavirus types more frequently isolated produced single or mixed infections with clinical manifestations ranging from severe diarrhoea with dehydration to completely asymptomatic cases. No relationship between virulence and electropherotype was observed.

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