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Exclusive asymptomatic neonatal infections by human rotavirus strains having subgroup I specificity and "long" RNA electropherotype

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Summary. A large number of stool specimes, of healthy new born infants, collected from various hospitals and clinics in Bangalore City, India, have been examined for the presence of asymptomatic rotaviral excretion. Out of 370 samples analysed during a three year period from 1988 to 1991, 133 specimens (36%) were positive for rotavirus RNA. All these asymptomatic neonatal strains, without exception, showed "long" RNA pattern, but subgroup I specificity. Serotype analysis by ELISA or by hybridization with serotype-specific probes indicated that these strains probably represent a new serotype in newborn children. We find an exclusive association of human rotaviruses having "long" RNA pattern and subgroup I specificity with asymptomatic neonatal infections in contrast to the earlier observations of association of such unusual strains with acute gastroenteritis in young children.

Introduction

Rotaviruses are the major causative agents of acute infectious diarrhea in infants and young children worldwide and represent one of the leading causes of infant morbidity and mortality in developing countries [20]. They also cause disease in adults and immunocompromised individuals and are widespread in many species of domestic and wild animals [20]. Rotavirus, whose genome consists of 11 double stranded RNA segments, belongs to the family *Reoviridae* which comprises of nonenveloped, icosahedral viruses with double shelled protein capsid and segmented, double stranded RNA genome [7]. Rotaviruses have

been classified into 7 groups, A to G, based on group-specific antigens detected primarily on VP 6 by immune electron microscopy and ELISA [31, 33]. Group A rotaviruses exhibit two commonly studied serologic specificities, i.e., serotype [17, 20] and subgroup [15, 38] and are subject to extensive studies as they constitute the majority of rotaviruses in man. The serotype specific antigenic sites involved in virus neutralization are found primarily on VP 7, the major outer capsid glycoprotein encoded by the 9th RNA segment [18, 30] but also on VP 4, the minor outer capsid protein encoded by segment 4 [18, 30]. Based on neutralization assays, Group A rotaviruses have been assigned to at least fourteen serotypes [4, 17, 20, 39, 42]. In contrast, the subgroup antigen resides in VP 6, the inner capsid protein encoded by the 6th segment of viral RNA, and at least 2 distinct specificities, subgroups I and II, have been identified [14, 15].

Besides identification of subgroup and serotype specificities, comparison of the migration patterns of viral genomic RNAs by polyacrylamide gel electrophoresis has been extensively used for preliminary characterization of rotaviral isolates [6, 16]. Although a great variety of migration patterns of RNA in different strains of rotavirus has been observed, all Group A rotaviruses can be classified into two distinctive groups exhibiting either "long" or "short" RNA pattern in which the migration of segment eleven is fast or slow, respectively [19, 20, 22]. Moreover, human rotaviral strains with serotype 2 specificity have short RNA electropherotypes and serotype 1, 3 and 4 strains have long RNA electropherotypes [27, 40]. Recently strains with a "super short" RNA pattern in which the 10th segment migrates slower than that of the "short" RNA pattern virus has been identified [1, 32]. It has been observed that the vast majority of human rotaviruses with Subgroup I specificity have a "short" or "supershort" RNA pattern where as those with Subgroup II specificity have "long" RNA pattern [1, 15, 19, 20, 26]. On the other hand, the great majority of animal strains appear to have a long RNA pattern but Subgroup I specificity [15, 20, 28]. In recent years several strains of human rotavirus have been isolated that exhibit Subgroup I specificity but "long" RNA pattern [12, 20, 28]. These unusual strains exhibit either a known or a new serotype specificity [39] and cause diarrhea in children. In some cases, these new human strains appear to be genetically related to rotavirus strains derived from animals such as cats and dogs [28].

Several studies have also indicated that although a majority of neonatal infections in humans are asymptomatic, symptomatic infections are also observed [5, 11, 20, 23, 25, 34, 37, 41]. All of the neonatal strains isolated to date exhibited antigenic specificity related to known human serotypes [13, 37]. Genetic and molecular biologic studies have indicated that asymptomatic neonatal strains contain unique gene 4 sequences that may be responsible for attenuation [8]. These studies indicated that neonatal asymptomatic strains may be genetically distinct from those causing diarrhea in older infants. Moreover, earlier studies have suggested that neonatal human rotavirus infection may confer

protection against subsequent development of diarrhea caused by the virus [3]. Based on the hypothesis that neonatal strains represent naturally attenuated viruses, several investigators have proposed that these neonatal strains be used as candidate vaccines [3, 20, 37, 41].

Although neonatal infections in India have been reported [23, 34], no knowledge on the molecular epidemiology of these viruses in different regions of the country is available. This work is undertaken to isolate and characterize the molecular nature of the rotaviruses that cause asymptomatic infections in new born infants in Bangalore, a major metropolitan city in southern India. During epidemiological studies of rotaviral infections in healthy infants in hospitals in Bangalore, several strains exhibiting Subgroup I specificity and "long" RNA electropherotype were observed. The neonatal infections caused by these unusual strains are exclusively asymptomatic in contrast to the observations in a north-eastern state of India that similar strains showing Subgroup I specificity and "long" RNA electropherotype are involved in acute diarrhea in young children [12]. In this report, we detail preliminary characterization of asymptomatic neonatal rotaviruses isolated in Bangalore, India from 1988 to 1991.

Materials and methods

Stool specimens and viruses

Human stool specimens from 2 to 30 day old healthy neonates were collected from pediatric wards of 6 major hospitals and clinics in Bangalore City, India. These samples were collected during a 3 year period between 1988 and 1991 during the months of November and March. Some samples were also collected in other months during this period.

Several tissue-culture adapted human and animal rotaviruses belonging to serotypes 1 to 6, 8 and 9 (strains KU or Wa, DS 1 or S 2, RRV, ST 3, OSU, NCDV or UK, 69 M and WI 61, respectively) were used as controls in serotyping and subgrouping ELISA. Reassortant viruses, DxRRV and DS 1 xRRV with serotype 1 and 2 specificity [24], respectively, were also used. These viruses were all grown in MA 104 cells in culture. Also stool extracts containing rotavirus strains of prototypes 1, 2, 3 and 4 have been used as controls in subgroup and serotype analysis. For example IS 2 is a sample containing serotype 2 virus with subgroup I specificity. These samples were collected from the same hospitals during the same periods as those for neonatal strains.

RNA extraction

A 20% suspension of the fecal samples was prepared in phosphate buffered saline and clarified by centrifugation at 4000 rpm for 15 min in a Sorval GSA rotor. The supernatants were further filtered through glass wool and were used for RNA extraction as well as determining subgoup and serotype specificities of the positive samples by enzyme-linked immunosorbent assay (ELISA) (see below). For RNA extraction, 0.4 ml of the clarified fecal sample was mixed with 0.05 ml of 10 × disrupting solution (10 mM Tris-HCl pH 6.8, 10 mM EDTA, 10 mM NaCl, 1% SDS), incubated15 min at 37 °C and extracted with phenol-chloroform. The mixture was centrifuged at 12,000 rpm for 10 min at 15 °C. The RNA was precipitated with ethanol and dissolved in 10 mM Tris-HCl pH 8.0 containing 0.1 mm EDTA.

Electrophoresis of rotavirus RNA

The rotaviral RNA in the stool samples was detected by polyacrylamide gel electrophoresis (PAGE) in 10% gels and staining the gel with silver nitrate as described previously [16]. Samples positive for rotaviral RNA were used for further characterization.

Monoclonal antibodies

For serotyping the rotaviral strains, the following VP 7-specific MAbs were used: Serotype 1, Mabs KU-4 and 5E8; serotype 2, MAbs S2-2G10 and 1C10; Serotype 3, MAbs YO-1E2 and 159; Serotype 4, MAb ST-2G7; Serotype 5, MAb 5B8; Serotype 6, MAb IC3. The VP 6-specific MAbs 255/60 and 631/9 which are specific for Subgroup I and Subgroup II, respectively, were used for determination of subgroup specificity [15]. The specificity of all the serotype specific MAbs and the VP 7 cross reactive MAb 129 were tested by serotyping ELISA with a panel of tissue culture-grown rotaviruses (mentioned above) belonging to serotypes 1 to 6, 8 and 9. The cross reactive MAb 129 was used to detect the presence of VP 7 [36]. The source, preparation, characterization and reactivities of the MAbs have been described by Noriega et al [29].

Serotyping ELISA

Serotyping ELISA was done as previously described [35]. Briefly, Immulon II microtiter plates (Dynatech Laboratories, Inc., McLean, VA) were coated with appropriate dilutions of the MAbs from ascites in phosphate-buffered saline (PBS) containing 0.05% sodium azide (PBS-AZ), incubated overnight at 4 °C, washed twice with PBS-AZ and then blocked for 12–24 h at 4 °C, with 5% fetal bovine serum in PBS-AZ. All further washings and incubations were done in PBS-AZ and 5% fetal bovine serum in PBS-AZ respectively. The plates were then washed twice and incubated overnight with 0.05 ml of 1% clarified stool specimens. After washing four times, the plates were incubated with guinea pig hyperimmune anti-rhesus rotavirus serum for 1 h at 37 °C. After being washed 4 times, an alkaline phosphotase-anti-guinea pig immunoglobulin G conjugate (Kirkgaard and Perry Laboratories, Gaithersburg, MD) was added and incubated for 1h at 37 °C. The plates were then washed four times, the substrate (1 mg per ml of p-nitro-phenyl phosphate in 1 mm MgCl 2 - 1% diethanol-amine buffer, pH 9.8) was added to the plates and incubated at 37 °C until the A 410 of the controls was approximately 1.0. For each stool sample, two blanks of noncoated wells were also run. Tissue culture-grown rotavirus or rotavirus reassortants representing serotype 1 to 6, 8 and 9 were used as controls. To assign a specimen to a particular serotype the criterion that the A 410 value with a MAb should be higher than 0.2 and at least twice as high as the value corresponding to the other MAbs was used.

Serotyping by serotype-specific DNA probes

The rotavirus positive stool specimens were also subjected to serotype analysis by hybridization using serotype-specific DNA probes [9]. The RNA was extracted by the method described elsewhere in this section. These probes were kindly provided by Dr. Jorge Flores, NIH, Bethesda, U.S.A. The probes used are: Serotype 1, 80 E; Serotype 2, 35 B or 21 H; Serotype 3, 41 Q or 8 B; Serotype 4,19 B. Preparation of ^{32}P-labelled DNA probes by polymerase chain reaction (PCR) and hybridization of the dot blots were carried out as described earlier [9].

Subgrouping

To determine the subgroup specificity of the specimens, both subgrouping ELISA [15] and electropherotyping [16] were performed as previously described. Subgrouping ELISA

is similar to serotyping ELISA except that the plates were coated with subgroup specific MAbs 255/60 and 631/9 which are specific for Subgroup I and II, respectively.

Results

We have analyzed 320 stool specimens collected from 2 to 30 day old healthy infants during the winter months of November to February from 1988 to 1991. We have also examined 50 specimens collected in other months during the 3 year period. The presence of asymptomatic neonatal excretion of rotavirus was determined by electrophoresis of the RNA samples in polyacrylamide gels for 24 h. This electrophoretic analysis has revealed an 41% incidence of rotavirus infection during winter months and a 30% incidence of asymptomatic excretion in other seasons. Out of 370 samples analyzed, 133 samples showed the presence of rotavirus RNA (Table 1).

All the strains, without exception, upon PAGE exhibited a "long" electrophoretic pattern when compared with that of a subgroup I, serotype 2 rotavirus strain of Indian origin (Fig. 1). Although all the isolates appeared grossly similar on these shorter electrophoretic runs, careful analysis revealed the presence of multiple electropherotypes as shown in Fig. 1. We observed circulation of more than one electropherotype in the same hospital/clinic. The presence of multiple distinct electropherotypes among the neonatal strains was further demonstrated by extended electrophoresis of RNA for 48 h. We have observed at least 10 distinct electropherotypes as shown in Fig. 2 among the strains, although several strains isolated from different hospitals/clinics exhibited nearly similar RNA pattern. Among the different electropherotypes differences in migration of majority of the segments of RNA were observed. Despite the subtle electrophoretic differences observed in general, the electropherotypes seemed somewhat related. For example in most cases, genes 2 and 3 migrated as close doublet. Also genes 7 and 8 migrated as a close pair separated from gene 9.

Subgrouping ELISA has, surprisingly, revealed Subgroup I specificity for

Table 1. Incidence of asymptomatic neonatal infections and characterization of the isolates

Number of samples analysed	370
Number positive for rotaviral RNA	133
Percent positive	36
Subgroup I + ve	133
Subgroup II + ve	–
Long Electropherotype	133
Short Electropherotype	–

Serotype specificity could not be determined either by ELISA or by hybridization with serotype specific DNA probes. Refer to Table 2 for details of ELISA results

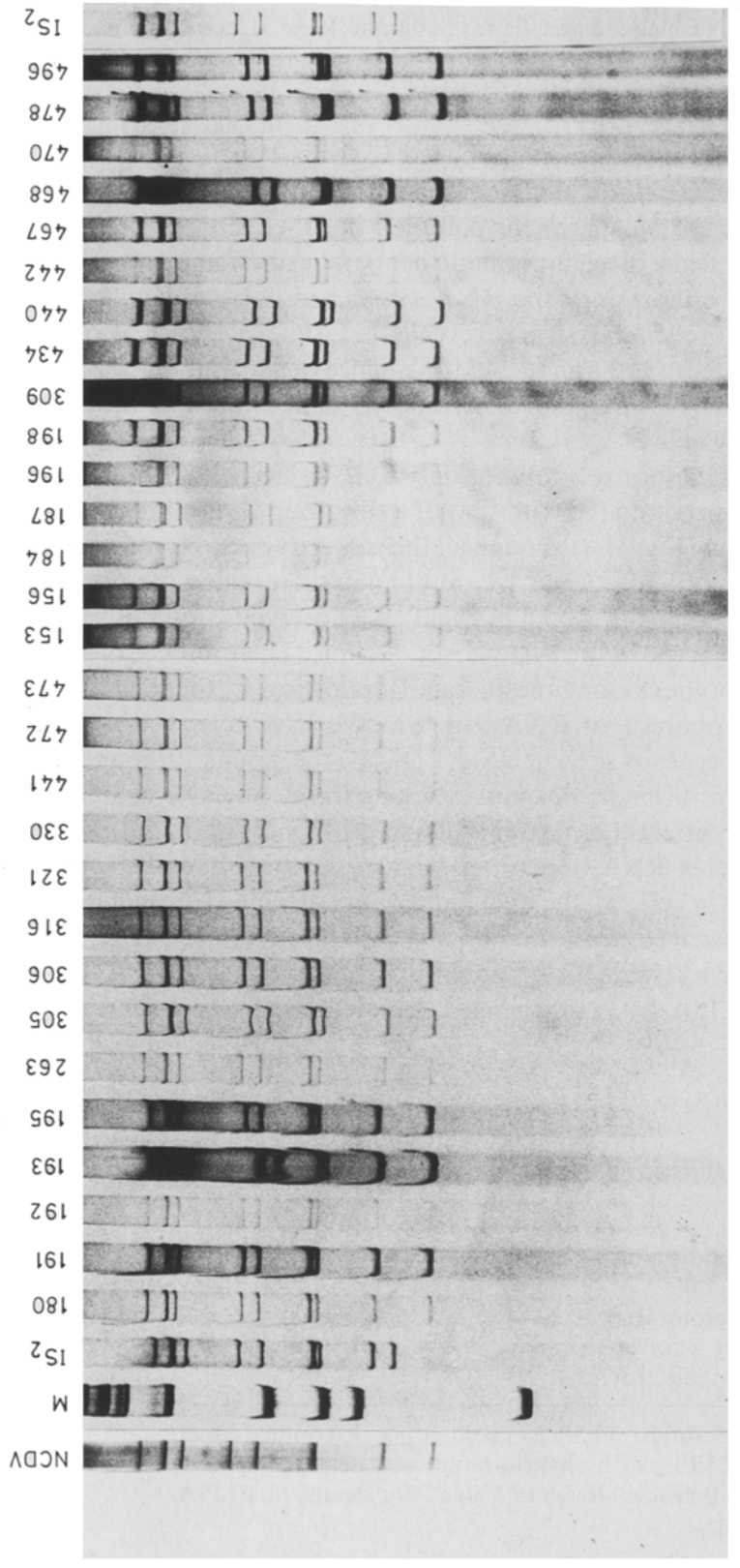

Fig. 1. RNA electrophoretic analysis of some of the positive asymptomatic neonatal isolates. RNA segments were separated by electrophoresis on 10 percent polyacrylamide gel for 24h and stained with silver nitrate. The numbers above the lane represent the code numbers of the samples collected from different hospitals. *H1* to *H4* Code names given to the hospitals. *M* DNA molecular weight standards containing Hind III digested bacteriophage λ DNA and Hae III digested φ × 174 RF DNA. *IS 2* An Indian rotavirus strain, isolated from a patient, exhibiting subgroup I and serotype 2 specificity and short RNA electropherotype

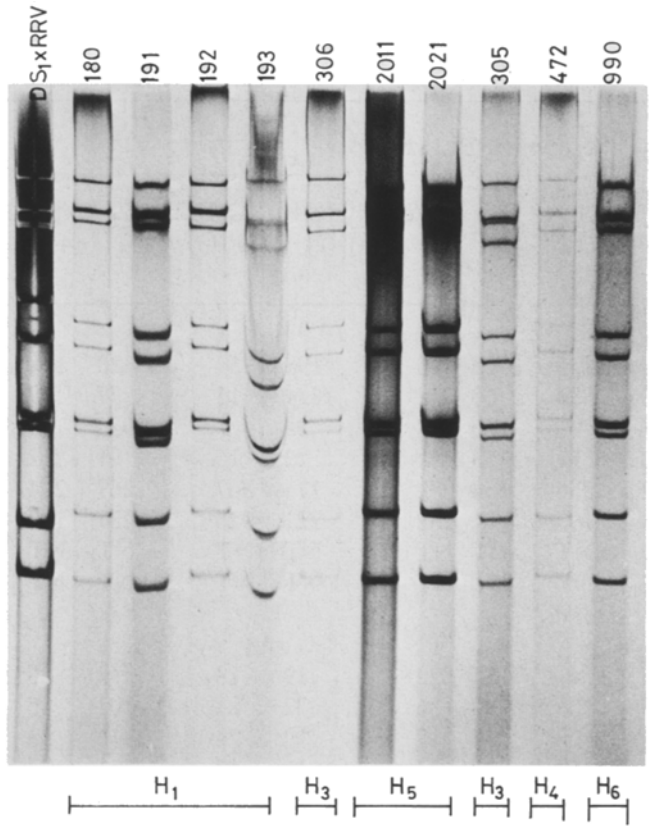

Fig. 2. Demonstration of distinct RNA electropherotypes among the neonatal strains by extended PAGE. RNA from isolates that showed observable variation in electrophoretic pattern earlier (Fig. 1) were subjected to electrophoresis for longer period (48 h). *H1* to *H6* Code names given to the hospitals

all the strains tested, without exception. All the specimens reacted strongly with subgroup I antibodies (Table 2). As controls, several prototype viruses exhibiting subgroup I or subgroup II specificity and different serotype specificities were included.

The serotype specificity of none of these 133 strains involved in asymptomatic neonatal infections could be determined by serotyping ELISA using MAbs specific for serotype 1 to 6. As controls, we have used tissue culture-grown human, animal and reassortant viruses of known serotype specificity as well as stool samples containing rotavirus strains of serotypes 1, 2, 3 and 4. The reactivities of the different MAbs with neonatal strains were rather low and none showed an absorbance at 410 nm, twice that of the other (Table 2). To confirm the results obtained by ELISA, we have used the alternate method of serotype determination by hybridization of viral RNA containing dot blots with radiolabeled DNA probes specific for human serotypes 1, 2, 3 and 4. None of the samples hybridized to the DNA probes specific for serotypes 1, 2, 3 and 4 (data not shown), thus vindicating the negative results obtained by ELISA.

Table 2. Subgroup and serotype analysis of some representative asymptomatic strains as determined by ELISA with MAbs

Rotavirus strain	Subgrouping ELISA		Serotyping ELISA									
	255/60 (I)	631/9 (II)	KU-4 (1)	5E8 (1)	S2-2G10 (2)	IC 10 (2)	YO-1E2 (3)	159 (3)	ST-2G7 (4)	5B8 (5)	IC 3 (6)	129 (Cross reactive)
Prototype												
Wa	257	*2151*	*2452*	*2515*	290	198	254	320	198	230	176	641
IS 2	*2069*	210	84	60	*951*	*1124*	15	77	49	61	42	473
RRV	*2595*	308	91	35	48	35	*1077*	*2020*	44	132	106	764
ST 3	92	*2376*	15	53	17	23	16	21	*1852*	62	110	715
OSU	*1730*	142	254	154	271	174	367	269	244	*1815*	223	587
UK	*2314*	203	103	81	208	221	126	237	119	168	*1296*	512
69 M	*1948*	380	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
WI 61	290	*1782*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Asymptomatic strains												
I 195	*1420*	315	41	25	22	19	18	13	25	32	41	225
I 263	*976*	153	103	97	86	72	81	101	45	52	81	292
I 304	*1598*	168	28	41	17	19	24	16	23	9	42	193
I 473	*854*	105	50	37	35	46	57	31	27	39	40	169
I 990	*752*	96	12	19	28	16	23	25	10	21	22	148
I 2021	*527*	70	23	12	19	22	27	18	11	15	37	125

Data are shown as the average OD_{410} × 1,000 of 2 wells

NT Not tested

The results on only one representative isolate from each hospital are shown

The subgroup and serotype specificities of the strains are indicated by the corresponding OD_{410} values, in italics

Discussion

Rotaviruses are now considered to be the single most important causative agent of acute gastroenteritis in infants and young children [20]. However, during the past few years, rotaviral strains that do not cause apparent symptoms of diarrhea have been recovered from newborn infants. Asymptomatic infections are not caused by a single strain as each of the four major human rotavirus serotypes has been observed in both symptomatic and asymptomatic infections in infants and young children [8, 11, 37]. Although the neonatal asymptomatic strains might represent less virulent, attenuated strains, other host factors such as the lack of rotavirus receptors or the presence of maternal antibodies, might play a role in protection against the development of symptomatic infections in neonates.

Most of the epidemiological studies on human rotaviruses have identified a strong correlation between Subgroup I and II specificities and the "short"

and "long" viral RNA patterns, respectively [18, 22, 27, 40]. This relationship appears not to apply to all human rotaviruses as strains exhibiting "long" RNA pattern and Subgroup I specificity have been recovered from symptomatic diarrheal cases [12, 21, 25]. Several of the human isolates with "long" electropherotypes and Subgroup I specificity appear to resemble animal rotaviruses at the molecular level [28].

In the present study, we find an exclusive association of rotaviral strains having Subgroup I specificity but "long" RNA pattern with asymptomatic neonatal infections in the metropolitan city of Bangalore, India. Strains with similar electropherotypic phenotypes have been observed in hospitals/clinics that are separated by 5 to 10 kilometers and serve people of different social and economic status. These strains appear to have been in circulation for at least three years.

The subgroup specificity of rotaviruses is determined by VP 6, the product of gene segment 6 and the apparent association of subgroup specificity with short or "long" RNA pattern exhibited by Segment 11 in most human rotavirus strains is not understood. The strains showing "long" RNA pattern and Subgroup I specificity might arise due to genetic reassortment in humans between human and animal or human and human rotaviruses. Although such reassortments in humans have not been directly demonstrated such strains can be generated in vitro by mixed infection in cell culture [24]. The fact that the serotype specificity of none of the present strains could be determined using a battery of type-specific MAbs indicates somewhat that these strains are more likely of animal origin.

Identification of extremely large number of these unusual rotavirus strains in humans indicates that these strains may be quite common in India. Recently, it has been reported that similar virus strains exhibiting long electropherotype and subgroup I specificity are involved in acute diarrhea in young children in the eastern Indian state of Tripura [12]. The serotype specificity of these strains is not yet clear. In contrast, it is interesting to note that the strains identified in the present study are exclusively involved in asymptomatic infections in neonates. It is not known, at present, whether these strains can cause disease in children upon infection and retain their unique serotype specificity. To date, all known neonatal strains identified belonged to serotypes 1 to 4 and no subgroup I human rotaviral strains showing long RNA pattern have been identified in neonates. In our study, no known human serotype rotavirus could be detected in the large number of neonatal strains isolated. It is unlikely that these specimens contained inadequate VP 7 since the reactivity of the subgroup and serotype specific Mabs including the cross reactive MAb 129 with majority of the strains is comparable to the values obtained with stool samples, containing the prototype strains, collected from young children suffering from gastroenteritis from the same hospitals during the same period. For example, the values obtained for the stool sample containing the serotype 2 strain IS 2, are comparable to those of majority of neonatal strains (Table 2). The values obtained

for stool samples containing other prototype strains are also similar to those of neonatal strains (data not shown). Albeit, the values are lower compared with the cell culture grown prototype strains. However, at least three isolates (I 195, I 263, I 473) appear to have more than enough VP 7 reactivity to be detectable by typing ELISA if they contained a type 1, 2, 3, 4, 5 or 6 strains. Also we could detect intact double shelled virus particles under transmission electron microscope (data not shown). The fact that virus-positive stool samples collected from the same hospitals at the same time from patients suffering from gastroenteritis were serotypable suggests that the low-reactivity of these neonatal strains with type-specific MAbs was not due to the loss of integrity of the virus structure. The results obtained by ELISA are also in accordance with those observed by hybridization of viral RNAs using serotype-specific DNA probes.

In preliminary studies we produced hyperimmune sera to one of the asymptomatic strains. This sera did not neutralize the serotype 12 human rotavirus strain L 26, that also exhibits long RNA profile and subgroup I specificity, at a dilution of 1/500 (S. Urasawa, pers. comm.). However, definitive proof that these new born strains do not represent known human serotypes must await cultivation and reciprocal neutralization assays or amino acid sequence analysis of VP 7. These strains might be of animal origin and might represent a serotype that has not been described so far in neonates. Genogroup analysis [28] of these strains has to be performed to know whether these strains are related to known animal viruses.

Our results also indicate that even though all the strains exhibit long RNA pattern and Subgroup I specificity, some strains exhibited distinct variations in the migration pattern of the genomic RNA, with majority of the strains showing grossly similar RNA pattern. At least 10 distinct RNA migration patterns have been observed. These differences in migration are observed in majority of the gene segments. Moreover, more than one electropherotype has been found to circulate within the same hospital. Electropherotypic variations in strains of a single human rotavirus serotype have been observed earlier [2, 10]. The fact that all these strains appear to share a unique serotype as well as the same subgroup specificity and grossly similar, though not identical, electropherotypes suggests that these strains are highly related and probably are all derived from a similar progenitor virus. The origin of this progenitor strain (animal or human) must await further studies. Asymptomatic human neonatal strains have been shown to share highly related gene 4 sequences that are different from those of symptomatic strains [8] and it will be of interest to know the extent of relatedness of gene 4 of these strains with that of others. Production of polyclonal and monoclonal antibodies against some of these strains would be necessary for antigenic charcterization of these strains. Sequence determination of genes 4 and 9 would define the degree of antigenic relatedness among the different strains.

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