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Molecular Epidemiology of Rotavirus Diarrhea among Children and Adults in Nepal: Detection of G12 Strains with P[6] or P[8] and a G11P[25] Strain

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In anticipation of a rotavirus vaccine in Nepal, this study was undertaken to determine the distribution of the G and P serotypes and electropherotypes of rotaviruses in order to examine if there is any emerging serotype or unusual strain circulating in children and adults in Nepal. Of 1,315 diarrheal stool specimens, rotavirus was detected by an enzyme-linked immunosorbent assay in 116 (17%) of 666 patients less than 5 years of age, in 18 (7%) of 260 patients 5 to 14 years of age, and in 19 (5%) of 358 patients 15 years of age and older. Approximately 75% of rotavirus diarrhea occurred in children less than 5 years of age. Approximately 70% of rotaviruses found in each of the three age groups belonged to serotype G1P[8]. Interestingly, there were 29 (20%) G12 rotaviruses carrying either P[8] or P[6] and one (0.7%) G11 rotavirus carrying an unusual P[25] genotype. RNA polyacrylamide gel electrophoresis discriminated 19 strains (electropherotypes), among which there were three codominant strains carrying G1P[8] and long RNA patterns. Five electropherotypes were discriminated among G12 rotaviruses, all of which had long RNA patterns. The fact that 20% of rotaviruses were G12 strains carrying either P[8] or P[6] and had multiple electropherotypes suggest that G12 strains are not more rare strains but that they pose an emerging challenge to current and future vaccines. The presence of multiple strains as defined by electropherotypes suggests the richness of the rotavirus gene pool in Nepal, where unusual strains may continue to emerge.

Globally, each year, 2 million children are hospitalized (32) and 700,000 children die due to rotavirus diarrhea (8). The majority of these deaths occur in developing countries, although virtually all children experience rotavirus infection by the age of 3 to 5 years irrespective of whether they live in developing countries or developed countries (7, 8). Thus, it is clear that the standards of hygiene and sanitation available and practiced in developed countries are not sufficient to prevent the spread of rotavirus infection within the community. For these reasons, efforts are currently being made at various levels to accelerate the introduction of rotavirus vaccines into the countries where they are most needed (8). Recently, two live, attenuated rotavirus vaccines have gone through large-scale safety and efficacy trials, each involving more than 60,000 children in both developing and developed countries, and both vaccines have shown encouraging results (36, 46).

The genome of rotavirus comprises 11 segments of double-stranded RNA, which are encased within a triple-layered capsid. The outermost capsid of the virion is composed of two independent neutralization antigens, VP7 and VP4, which define the G serotype and the P serotype, respectively (8). Classification based on the VP7 sequence corresponds to serological classification, so the same numbers are assigned for both genotype and serotype. However, with respect to VP4, the classification defined by molecular methods does not completely agree with the classification defined by serological assays.

It is generally believed that serotype-specific immunity plays a role in protection against disease, so the epidemiology of G and P serotypes of circulating strains forms a critical knowledge base for the development and implementation of rotavirus vaccines (16, 38). For this purpose, regional rotavirus strain surveillance networks have been established in Africa (African Rotavirus Network) and in Asia (Asian Rotavirus Surveillance Network) (3, 43). In addition, the identification of electropherotypes provides complementary information on the genomic diversity of rotavirus strains circulating in the region, because the migration pattern of 11 segments of double-stranded RNA on a polyacrylamide gel helps define an individual strain of rotavirus (13, 25, 28, 29).

Since natural infection with rotavirus does not provide complete protection against subsequent infections, older children and adults may repeatedly be infected, with some of these

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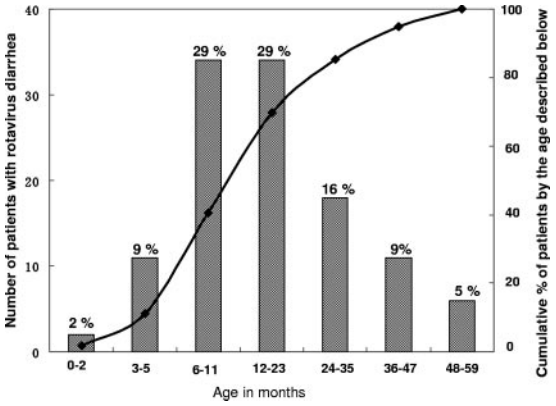

FIG. 1. The distribution of rotavirus diarrhea cases among children less than 5 years of age. Shaded columns represent the number of patients among the indicated age categories. The line indicated by "◆" shows the cumulative percentage of rotavirus-positive children by the indicated age.

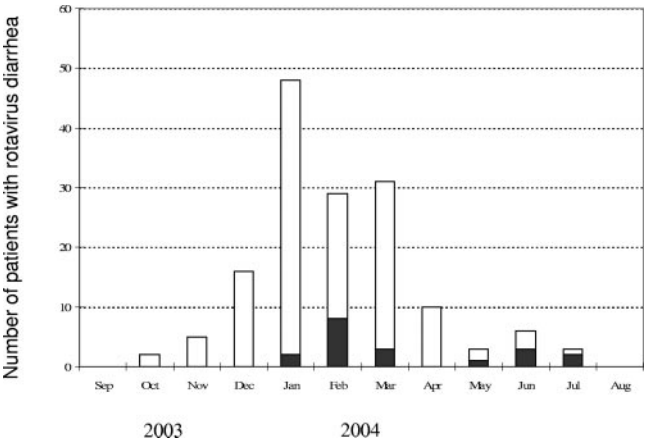

FIG. 2. Monthly occurrence of rotavirus diarrheal cases in children and adults in Nepal. The open column represents cases occurring in the group of patients less than 15 years of age, whereas the closed column represents cases occurring in the group of patients 15 years of age and older.

infections resulting in symptomatic disease. Based on a 4-year prospective study in a sentinel hospital in northern Japan, it was reported that rotavirus infection accounted for 10 to 15% of acute adult diarrhea cases encountered in the hospital (30). However, few studies have addressed the molecular epidemiology of rotavirus in adults, and knowledge in developing countries is completely lacking (1). Even though older children and adults may not escape rotavirus infections, it is conceivable that they have mounted certain levels of immunity against subsequent rotavirus infections based on previous exposures to rotavirus. Thus, characterization and comparison of rotaviruses circulating in different age groups may provide a key for understanding the spread of rotaviruses in the community.

Nepal is a landlocked mountainous country in Asia, with a very low per capita gross income. Available data show a high infant mortality rate (71 per 1,000 live births) (45) and a high child mortality rate due to diarrheal diseases (12). It is therefore likely that rotavirus is an important cause of childhood mortality. However, the literature on the epidemiology of rotavirus diarrhea in Nepal is scarce (26, 31, 39, 40), and molecular characterization of circulating strains has never been performed throughout a complete year.

The aim of this study was to determine the distribution of G and P serotypes and electropherotypes of rotaviruses circulating in children and adults in Nepal in order to examine if there is any emerging serotype or unusual strain in the region that may challenge the effectiveness of currently available rotavirus vaccines.

MATERIALS AND METHODS

Detection of rotavirus-positive stool specimens by ELISA. Stool specimens were collected from patients with acute diarrhea who were referred to the observation unit (outpatients, $n = 875$) and in the diarrheal ward (inpatients, $n = 30$) in Kanti Children's Hospital and from patients hospitalized for acute diarrhea in Sukra Raj Tropical and Infectious Disease Hospital (inpatients, $n = 410$), Kathmandu, Nepal, during a 1-year period between September 2003 and August 2004. A commercially available enzyme-linked immunosorbent assay (ELISA) kit (Rotaclone; Meridian Bioscience Inc., Cincinnati, OH) was used to detect group A rotavirus antigen. Rotavirus-positive specimens were partially purified by centrifugation through a 30% (wt/vol) sucrose cushion for 1 h at 50,000 rpm in a Beckman 100.2 rotor, and genomic RNAs were extracted with phenol chloroform and precipitated with ethanol.

Rotavirus G and P genotyping by reverse transcription-PCR (RT-PCR). G types were determined by using a method described previously by Gouvea et al. (17). For P types, the VP4 gene was amplified with con-2 and con-3 primers according to a method described previously by Gentsch et al. (15), and type-specific products were then obtained with specific typing primers described previously by Gunasena et al. (21). Those specimens that were amplified for their VP7 gene but that did not react with a single G-type-specific primer were recorded as G nontypeable. P nontypeable was defined in the same fashion.

Determination of G-nontypeable samples by RT-PCR based on deduced amino acids sequences in antigenic regions of VP7. For those samples that were G nontypeable by RT-PCR, a full-length VP7 gene was amplified with primers Beg9 and End9 using a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Cycle sequencing was performed with an ABI PRISM BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), and products were loaded onto an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). G types were determined by a comparison of deduced amino acid sequences of antigenic regions A, B, and C of the VP7 protein (14) with reference strains.

Polyacrylamide gel electrophoresis of rotavirus genomic RNA. Rotavirus genomic RNAs were separated on a 10% polyacrylamide gel by electrophoresis for 16 h at a constant current of 8 mA per gel in a Laemmli buffer system using an SE600 Ruby gel apparatus (GE Healthcare Bioscience [formerly Amersham Biosciences], Piscataway, NJ) as described previously (25, 28). Electropherotypes were determined by comparisons of the individual RNA migration patterns of genome segments on the gel. Each electropherotype was designated E1, E2, E3, etc.

RESULTS

Epidemiologic features of rotavirus diarrhea in Nepal. Of 1,315 stool specimens collected from patients with acute diarrhea, rotavirus was detected in 153 (12%) specimens by ELISA. Rotavirus was detected in 116 (17%) of 666 specimens collected from patients under 5 years of age, in 18 (7%) of 260 of patients 5 to 14 years of age, and in 19 (5%) of 385 of patients 15 years of age or older. When the distribution of cases among patients under 5 years of age was examined, 68% occurred in those between 3 months and 23 months of age, while only 2% occurred in the first 3 months of life (Fig. 1).

There was seasonal variation in the occurrence of rotavirus diarrhea, with peaks in January and March and no cases in August and September (Fig. 2). Thus, rotavirus diarrhea was

TABLE 1. Comparison of amino acids sequences in antigenic regions A, B, and C of the VP7 protein between Nepali strains and reference G12 strains or the reference G11 strain[a]

Strain	Antigenic region of VP7		
	A (aa 87–96)	B (aa 145–150)	C (aa 211–223)
Nepali G12 strains	SSVTTEITDP	QNSLAL	DVTTFEEVANAEK
CP1030	----------	------	-------------
CP727	----------	------	-------------
ISO5	----------	------	-------------
ISO2	----------	------	-------------
ISO1	----------	------	-------------
T152	----------	------	-------------
Se585	----------	------	-------------
L26	N---------	------	--A----------
Nepali G11 strain	NEAATQIADD	DGNSQL	DPTTFEEVASAEK
Dhaka 6	----------	------	-------I-----
A253	R---------	------	----------T
YM	H---------	------	-------------

[a] Shown are G12 strains (29 samples) and reference G12 strains. Nepali G11 strain and reference G11 strains of human (Dhaka 6) and porcine origin are also shown. aa, amino acids.

most prevalent in the dry season in Nepal. However, this seasonal pattern was less marked in patients with rotavirus diarrhea who were 15 years of age and older, and cases in this age group accounted for 46% of rotavirus cases that occurred during the rainy season (from May to July) (Fig. 2).

The VP7 genes were successfully amplified in 142 (93%) of 153 rotavirus-positive specimens with RT-PCR. However, of these 142 samples, 30 were not typed with primers specific for G1 to G4, G8, or G9. When such VP7 amplicons were sequenced, it was found that 29 samples had completely identical amino acid sequences in antigenic regions A, B, and C of VP7. These 29 VP7 sequences were identified as G12 because they were identical to the amino acid sequences of reference G12 strains Se585, T152, ISO1, ISO2, ISO5, CP727, and CP1030 in antigenic regions A, B, and C (Table 1). The one remaining sample was typed as G11 because it had 100% amino acid sequence identity in antigenic regions A and B and 92% amino acid sequence identity in antigenic region C with strain Dhaka 6, the first human G11 strain available in the database (Table 1). Thus, of 153 rotavirus-positive specimens, 109 (77%) were typed as G1, 3 (2.1%) were typed as G2, 30 (20%) were typed as G12, and 1 (0.7%) was typed as G11 (Table 2).

For P types, 80% of the specimens were typed as P[8], 13% were typed as P[6], and 4.9% were nontypeable. When G and P types were combined, the majority (70%) of the specimens were G1P[8], while 20% were G12 with either P[6] or P[8] (Table 2). The P type of the G11 rotavirus was initially undetermined, but it was later typed as P[25] based on the result of the partial VP8* sequence. It was notable that the predominance of G1P[8] was observed for all three age groups, including older children and adults (Table 2). G12 strains were also found in all three age groups. On the other hand, P[6] was detected only in patients younger than 15 years of age (Table 2).

TABLE 2. Distribution G and P types according to age categories

G and P type	No. of rotavirus-positive specimens (%) for age group:			
	<5 yr (n = 112)	5–14 yr (n = 17)	>15 yr (n = 13)	All (n = 142)
G1P[8]	78 (70)	13 (76)	9 (69)	100 (71)
G1P[6]	3 (3)	0 (0)	0 (0)	3 (2)
G1PNT[a]	5 (4)	0 (0)	1 (8)	6 (4)
G2P[4]	1 (1)	2 (12)	0 (0)	3 (2)
G12P[8]	10 (9)	0 (0)	3 (23)	13 (9)
G12P[6]	14 (12)	2 (12)	0 (0)	16 (11)
G11P[25]	1 (1)	0 (0)	0 (0)	1 (1)

[a] NT, nontypeable.

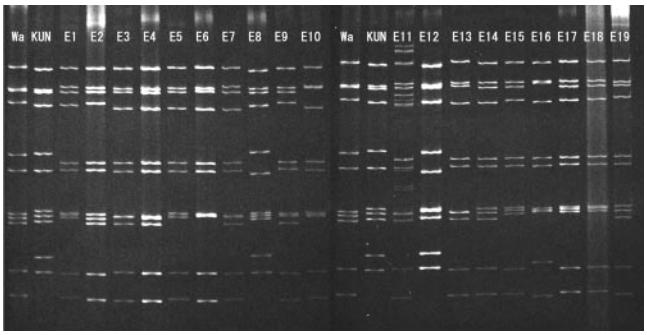

FIG. 3. Electropherotypes of rotavirus strains identified in Nepal during a 1-year period between September 2003 and August 2004. Electropherotypes named E3, E7, and E9 were codominant during the survey period. E1 and E18 viruses, both of which bear a G12P[6] specificity, look very similar on this panel, but it was observed in the original photograph as well as on another gel that segments 7 and 8 of E18 were clearly separated, whereas they were hardly so in E1. In addition, E3 and E4, both of which bear a G1P[8] specificity, look similar, but segments 7 and 8 were separated with a clear space between them when the genomic RNA was loaded slightly less on the gel (data not shown), thereby allocating a electropherotype number different from E3 whose segments 7 and 8 comigrated.

Molecular epidemiological analysis based on the comparison of electropherotypes and G and P types. Of 153 rotavirus-positive specimens analyzed by polyacrylamide gel electrophoresis, all 11 segments of genomic RNA were visualized in 57 (37%) specimens, which were classified into 19 electropherotypes (Fig. 3). The percentages of successful visualization of all 11 bands on the gel were different among the age groups, with 38% (44/116) in patients less than 5 years of age, 44% (8/18) in patients between 5 and 14 years of age, and 26% (5/19) in patients 15 years of age and older. This may suggest that less virus shedding occurred in the older age group, although the difference was not statistically significant. There were three codominant electropherotypes, named E3, E7, and E9, appearing in 11 (19%), 10 (18%), and 12 (21%) specimens, respectively (Table 3). No infection with more than one rotavirus strain was detected by RNA polyacrylamide gel electrophoresis. While this observation was unexpected, it does not entirely exclude the possibility of mixed infections, because the demonstration of more than 11 bands on the gel is an insensitive method of detecting mixed infections of more than one strain of rotavirus. Interestingly, there was one specimen that contained RNA patterns characteristic of reovirus as well as rotavirus (E11 in Fig. 3).

Each electropherotype corresponded to a single combination of G and P types, with an exception in which one specimen carrying E9 was typed as P nontypeable (Table 3). On the other hand, multiple electropherotypes existed within a single

TABLE 3. Rotavirus electropherotypes and G and P types according to age categories[a]

Electropherotype	G type	P type	No. of specimens for age group:			
			<5 yr	5–14 yr	>15 yr	All
E2	1	[8]	2	2		4
E3	1	[8]	6	4	1	11
E4	1	[8]	1			1
E7	1	[8]	8		2	10
E9	1	[8]	10	1		12
	1	NT	1			
E11	1	[8]	1			1
E13	1	[8]	1			1
E14	1	[8]	2			2
E19	1	[8]			*1*	*1*
E8	2	[4]	1			1
E12	2	[4]		*1*		*1*
E1	12	[6]	2			2
E15	12	[6]	1			1
E17	12	[6]	1			1
E18	12	[6]	1			1
E5	12	[8]	3			3
E6	12	[8]	1		1	2
E10	12	[8]	1			1
E16	11	[25]	1			1
Total			44	8	5	57

[a] Electropherotypes found in both the younger age group (under 5 years of age) and the older age groups (5 years of age and older) are underlined. Electropherotypes found only in the older age group are shown in italics. NT, nontypeable.

combination of G and P types (Table 3). Three codominant electropherotypes belonged to G1P[8], except one specimen that was G1P nontypeable, as mentioned above. All strains except G2P[4] carried a long RNA pattern, while all G2P[4] strains carried a short RNA pattern.

There was cocirculation of rotavirus strains with diverse electropherotypes over the 5-month period from December to April (Table 4). It was observed that despite the presence of many electropherotypes, there were not many different electropherotypes that were seen concurrently at any given period lasting more than 1 month. As to codominant strains, electropherotype E9 persisted the longest, for a 5-month period from January to May, followed by electropherotypes E3 and E7, which persisted during the same 4-months period, from December to March. Cocirculation of these three codominant strains was restricted in the period between January and March, which was the peak rotavirus season in Nepal. It deserves mention that the rotavirus strain bearing electropherotype E2 persisted the longest in the community, for at least 6 months, from October to March, although it never became dominant in the frequency of detection.

The distribution of electropherotypes in the three age groups is shown in Table 3. There were 17 electropherotypes in patients less than 5 years of age, 4 types in patients 5 to 14 years of age, and 4 types in patients 15 years of age or older. While the three codominant strains carrying electropherotypes E3, E7, and E9 had genotype G1P[8], their distribution among different age groups was different, and their frequency of detection was not necessarily proportionate to the number of rotavirus-positive specimens in each age group (Table 3). For example, electropherotype E9 seemed to be found more frequently in patients less than 5 years of age than in other age groups, and it was not found in patients 15 years of age and older. On the other hand, electropherotype E3 seemed to be disproportionately found in the older age groups.

When the two older age groups were combined and the age groups were redefined as the younger age group (under 5 years

TABLE 4. Monthly distribution of electropherotypes

Genotype	Electropherotype	No. of specimens[a]											
		Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.
G1 codominant	E3				3	4	3	1					
	E7				1	6		3					
	E9					1	2	7	1	1			
G1 other than codominant	E2		1			2		1					
	E4			1									
	E11					1							
	E13						1						
	E14						2						
	E19										1		
G2	E8					1							
	E12					1							
G12P[6]	E1		1		1								
	E6				1		1						
	E15								1				
	E17								1				
	E18										1		
G12P[8]	E5				2	1							
	E10					1							
G11P[25]	E16								1				

[a] The numbers of patients in the peak month of codominant electropherotypes are underlined.

of age) and the older age group (5 years of age and older), five electropherotypes were found in both age groups, and they accounted for 68% of electropherotyped specimens. While 12 electropherotypes (28.5%) were found only in the younger age groups, there were only two electropherotypes (3.5%) unique to the older age group. A caveat here is, as stated above, that there was a smaller percentage (26%) of successful visualization of all 11 segments on the gel in the age group of 15 years and older than in the age groups of less than 5 years and 5 to 14 years (38 to 44%). Therefore, the possibility that the electropherotypic diversity in the older age group was more diverse than was observed in this study cannot be excluded.

DISCUSSION

Infants and young children under 2 years of age are most vulnerable to rotavirus infection that often results in severe diarrhea and dehydration, causing hospitalization and deaths. This age group has been the primary target of protection with rotavirus vaccines, and large-scale efficacy and safety trials of two major rotavirus vaccines have demonstrated the usefulness of these vaccines in developing countries (36, 46). The first dose of these vaccines are to be given between 6 and 12 weeks of age, and since the efficacy after the first dose alone has not been established, infants at this age period, i.e., those who are less than 3 months of age, will not be protected by the vaccine. Therefore, a detailed description of age groups that are most affected by rotavirus diarrhea will inform the optimum time for rotavirus vaccination. In Nepal, while only 2% of rotavirus diarrhea occurred before 3 months of age, the majority (68%) occurred between 3 and 23 months of age (Fig. 1). These data will support the introduction of rotavirus vaccines in Nepal according to the current schedule of the expanded program on immunization (6, 10, and 14 weeks).

While the majority of rotavirus diarrhea occurred in children less than 5 years of age, rotavirus diarrhea was not restricted to this age group; it occurred in 5 to 7% of diarrheal cases of older children and adults in Nepal. It bears repeating that patients aged 15 years and older were hospitalized patients, meaning that they had severe diarrhea and dehydration. Thus, this study confirmed and extended previous observations that rotavirus plays an important etiological role in acute diarrhea in older children and adults (1, 30).

The majority (73%) of the genotypes of rotavirus strains recovered from older children and adults were G1P[8], the relative frequency of which was almost identical to that of this genotype among children less than 5 years of age (70%) (Table 2). This observation may be in contrast to data from many previous reports in which rotavirus gastroenteritis outbreaks affecting older children and adults were attributed to infection with G2P[4] strains (18, 27). When G1P[8] rotaviruses found in different age groups were further analyzed by electropherotypes, it appeared that the detection of one codominant strain, E9, deviated toward the younger age group, whereas the detection of the other codominant strain, E3, deviated toward the older age group (Table 3). Thus, a hypothesis can be postulated that there are some antigenic differences even within the same G1P[8] strains that may affect the immunity conferred by prior rotavirus infections. However, the existence of such differences in the induction of protective immunity has not been systematically explored, except in the case of genotype-specific neutralization profiles within G9 rotaviruses (22).

Regarding the practical significance of antigenic differences accumulated over time on the VP7 protein, many previous vaccine trails provide evidence that the immunity conferred by an older G1 strain gave good clinical protection against severe diarrhea caused by more recent G1 strains circulating at the time of clinical trials. For example, (i) the G1 component of a tetravalent rhesus-human reassortant rotavirus vaccine (i.e., the VP7 of strain D isolated in the United States in 1974) (47) provided 88% protection against severe diarrhea caused by prevailing G1 viruses during a phase 3 clinical trial in 1994 to 1995 in Venezuela (33) (i.e., the 1974 G1 virus versus the G1 virus from 1994 to 1995); (ii) a G1 monovalent vaccine (Rotarix) derived from strain 89-12, isolated in the United States in 1988 (2), gave 90.8% protection against severe diarrhea caused by prevailing G1 viruses during a phase 3 clinical trial in 2003 to 2004 in 11 Latin American countries and Finland (36) (i.e., 1988 G1 virus versus the G1 virus from 2003 to 2004); and (iii) the G1 component of a pentavalent human-bovine vaccine (RotaTeq) (i.e., the VP7 of strain WI79 isolated in the United States in 1983) (6) provided 95.1% protection against severe diarrhea caused by prevailing G1 viruses during a phase 3 clinical trial from 2001 to 2004 in 11 countries (46) (i.e., 1983 G1 virus versus the G1 virus from 2001 to 2004).

A notable feature of the distribution of G and P genotypes in Nepal disclosed in this study was the greater dominance of G1P[8] and the emergence of G12 in combination with P[8] or P[6] as the second most common G type (20%). While the first human rotavirus strains carrying G12 were isolated in the Philippines in 1990 (44), G12 strains were reported in the United States (20) and Thailand (34) in 2002. There is an increasing number of G12 strains being reported in the literature, e.g., from India in 2003 (11), from Korea in 2002 to 2003 (24), from Japan in 2004 (41), and from Argentina in 2004 (5). Whereas these G12 strains were all from sporadic cases, there are more recent reports from India and Argentina showing that G12 strains are emerging as a significant proportion of rotaviruses causing diarrhea in children (4, 37). In Kolkata and Berhampur, India, from January 2003 to April 2005, the G1 genotype was the most predominant genotype (54%), followed by G2 (23%) and G12 (17%). The G12 viruses in that study were detected in association with P[4], P[8], and P[6] (37). Similarly, during the period between 1999 and 2003 in Buenos Aires, Argentina, the relative frequency of various G types was G4 (44.5%), followed by G1 (40.3%), G12 (6.7%), G9 (1.7%), and G2 (1.7%). In that study, G12 was found in association with either P[9] or a nontypeable P type (4). Taken together with our observation that G12 was found in combination with P[8] or P[6], it is tempting to speculate that G12 strains have evolved such that they are capable of spreading more frequently between humans by gaining the VP4 protein gene of the P[4], P[8], or P[6] specificity, which are common P genotypes found in human rotaviruses.

Of further interest was the detection of a G11 rotavirus in combination with P[25]. The human G11P[25] strain was first detected in Bangladesh in 2005 (35), so the detection in Nepal may imply that such unusual strains are on the increase in the Ganges region.

In the present study, P[6] strains were found only in the age

group under 15 years old, and its relative frequency was higher in Nepal than in other countries, except those in Africa (9, 10, 38, 43). Historically, P[6] strains were detected in asymptomatic neonates (23), but later studies established that rotaviruses with P[6] have also been recovered from children with diarrhea (19, 42). Continuous surveillance is needed to address the question of whether such P[6] rotaviruses will increase and spread to other age groups.

Genetic variability as revealed by the presence of multiple electropherotypes within the population is far greater in this study than, for example, in the study previously undertaken in Honjo, Japan, where the surveyed population was approximately 120,000 people (25). One major difference in the two settings was the size of the cohort in which rotavirus transmission was maintained. Kathmandu is the capital of Nepal with a population of approximately 1 million people with frequent communications with other areas of the country and thus has a much greater potential to maintain diverse strains of rotavirus during a limited time. This certainly favors the chance of coinfection with two or more strains, leading to the emergence of viral reassortants. Continuous rotavirus surveillance in Nepal is warranted to understand the mechanisms by which strain variability occurs under natural conditions.

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