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Predominant Human Rotavirus Genotype G1P[8] Infection in Infants and Children in Bangkok, Thailand

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Rotavirus is the main causative agent of acute infantile diarrhea worldwide, with the mortality rate in the developing world estimated to exceed 800,000 cases per year.[1] Since its discovery as a human pathogen in 1973, rotavirus has consistently been identified the most frequent cause of diarrhea among children below the age of 5 years.

SUMMARY Human rotavirus is the major etiologic agent of infantile diarrhea on a worldwide scale. In this study, rotaviruses were detected by reverse-transcription PCR in 42 of 83 stool specimens from children below the age of 3 years with acute diarrhea in Bangkok, Thailand, between November 1998 and August 1999. G and P types of all samples were characterized by restriction endonuclease analysis (REA) and multiplex PCR typing assay, respectively. Strain G1P[8] (76.1%) was the predominant type, followed by G1P[6] (2.4%). Strain G1 combined with mixed P[8]/P[6] was identified in 2 specimens (4.8%) and 7 untypeable G strains (16.7%) were observed. This information on the circulating G and P combinations should be useful for understanding the epidemiology of human rotavirus in Bangkok, Thailand.

Rotavirus, a member of the family Reoviridae, contains a genome of 11 distinct double-stranded RNA segments encoding 6 structural proteins and 5 non-structural proteins. Of those, the two outer capsid proteins, VP4 and VP7, elicit the production of neutralizing antibodies. VP7 is encoded by gene segments 7, 8 or 9 depending on the strain and thus designating the G (glycoprotein) serotypes.[2] Fourteen G serotypes (G1-G14) of group A rotavirus correlate with antigenic specificities of the VP7 glycoprotein. Ten of the 14 G serotypes have been associated with human infection, but only serotypes G1-G4 are major pathogens of acute diarrhea worldwide.[3] The P serotype is derived from VP4, the trypsin sensitive protein that enhances rotavirus infectivity. Recently, antisera or monoclonal antibodies (MAbs) raised to VP4 have been used to identify 12 P serotypes.[4] At present, 20 P genotypes have been described in rotaviruses of humans, cattle, swine, horses and other animals, based on differences of their amino acid sequences.[5]

In most studies to date, VP7 serotype G1 strains have been the most frequently detected serotype; however, in different endemic seasons, other serotypes may predominate at a given time in the same location.[6] Unusual G serotypes found only in animals may cause human diarrhea in some parts of the world, especially in developing countries.[7,8] Upon surveying rotavirus P genotypes, P [8] (P1A serotype) and P[4] (P1B serotype) have been shown to represent the 2 major P types in humans. Epidemiological data worldwide have demonstrated that

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four G-P genotype combinations, designated P[8]G1 (53%), P[8]G4 (14.3%), P[4]G2 (10.7%) and P[8] G3 (5.4%), are those most commonly identified in children with diarrhea.[9]

The purpose of the present study has been to determine the frequency and temporal distribution of human rotavirus G and P genotypes among children with acute gastroenteritis during one epidemic season (1998-1999) in Bangkok, Thailand. To that end, we have applied reverse-transcriptase polymerase chain reaction (RT-PCR) in order to detect rotavirus RNA in stool specimens. Restriction endonuclease analysis (REA) and multiplex PCR accomplished classification of G and P genotypes, respectively. The combination of G and P types was assessed for each specimen to determine rotavirus distribution in Bangkok.

MATERIALS AND METHODS

Clinical specimens and rotavirus diagnosis

Between November 1998 and August 1999, 83 stool specimens had been collected from 0-3-year old infants with acute gastroenteritis who had either reported to the Outpatient Department or been admitted to the short-term ward of the Pediatric Department, Chulalongkorn University and Hospital, Bangkok. Each sample was resuspended at a 10-20% concentration in phosphate buffered saline (PBS), followed by thorough mixing and centrifugation at 514 x *g* for 10 minutes. The supernatant was kept at -70°C for further molecular analysis.

RNA extraction

Viral RNA extraction was performed according to the method described by Chomczynski and Sacchi.[10] The RNA pellet was resuspended in 10 µl diethylpyrocarbonate (Dep-C) treated sterile water and directly used as a template for RT-PCR.

RT-PCR and restriction endonuclease analysis of the VP7 gene

Reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described.[11] The amplification reaction required 30 cycles comprising an initial cycle at 94°C for 3 minutes, denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, concluded by a final extension step at 72°C for 4 minutes. The primers employed for the PCR had been selected from the conserved region of the VP7 gene of human rotavirus type A.[12] The primer sequences were 5'-CCG TCT GGC TAA CG G TTA GCT-3' (nt. 21-41) for the sense primer and 5'-GGT CAC AT C GAA CAA TTC TAA-3' (nt. 1041-1059) for the anti-sense primer, respectively.

Stool obtained from the previous report[13] and known to be positive for rotavirus RNA and antigen served as the positive control and sterile water served as the negative control.

The 1039-bp PCR products, amplified from the VP7 gene, were analyzed by REA as previously described.[14] The three restriction endonucleases *Hae* III, *Sau*96 I and *BstY* I (New England, Biolabs) were individually used for digesting the amplified PCR products according to the manufacturer's specifications. The RFLP patterns thus obtained were compared with the REA profiles referenced for G serotype classification.[14]

RT-PCR and multiplex PCR analysis of the VP4 gene

RT-PCR of the VP4 gene was performed in a manner similar to that described for VP7 except for minor modifications in the PCR reaction mixture. The primers designed for the VP4 gene analysis have been reported by Gentsch *et al.*[15] The first primer pair, con 3 and con 2, was chosen for reverse transcription and the first PCR amplification as they correspond to a highly conserved region among human rotavirus strains of the VP4 genetic groups.

For typing the VP4 P genotype, the 876-bp DNA product from the first PCR was reamplified using a cocktail of primers that included the sense primer, con 3, and three group-specific anti-sense primers, 1T-1, 2T-1 and 3T-1 representative for P genotypes [4], [6] and [8], respectively. The PCR products were analyzed by electrophoresis in a 3.0% agarose gel with ethidium bromide added upon preparation. The DNA regions amplified by the specific primers were used for P genotype identification.

RESULTS

Employing RT-PCR analysis, a total of 83 stool samples was examined for human rotavirus. Forty-two samples (50.6%) have shown a positive result by RT-PCR and the full-length amplified VP7 gene was of the expected 1,039-bp size (Fig. 1).

Upon digestion of the VP7 gene with the respective restriction endonuclease, 83.3% (35/42) have shown the REA patterns designated as h1, s1 and b6 after incubation

with *Hae* III, *Sau*96 I and *Bst*Y I, respectively (Fig. 1). The h1s1b6 pattern represents strains with serotypes G 1, G 8 and G 12 of human rotavirus. Several samples displaying the predominant pattern in this study have been further classified into serotype G 1 by nucleotide sequence analysis (data not shown). Furthermore, 16.7% (7/42) of the rotavirus-positive specimens have displayed untypeable patterns whereas none of the other G-type REA patterns has been detected. All the nucleotide sequences established have been submitted to Genbank (accession nos. AF181863 and AF181864 for the predominant strains RCU-25 and RCU-37, respectively, and accession no. AF181865 for the untypeable strain RCU-55).

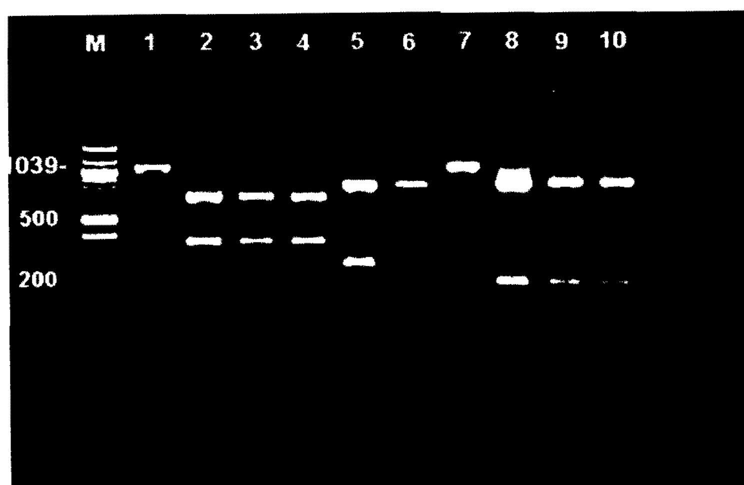

Fig. 1 REA profiles of rotavirus VP7 gene after 2% agarose gel electrophoresis. Full-length VP7 gene (lane 1); three rotavirus strains: RCU-25, RCU-37 and RCU-55 digested by *Hae* III (lanes 2-4), *Sau*96 I (lanes 5-7) and *BstY* I (lanes 8-10), respectively. Lane M, 100-bp DNA ladder (Promega Madison Wis).

Upon P typing by multiplex PCR, the specific PCR product sizes were 345, 267 and 483 bp for the P[8], P[6] and P[4] genotypes, respectively. The P[8] genotype was observed in 88.1% (37/42) whereas the P[6] genotype amounted to a mere 7.1% (3/42). Also, 2 samples (4.8%) constituted mixtures of both genotypes (Fig. 2) whereas the P[4] genotype could not be found in this study. All combinations of the G and P typing data possible have revealed G1P[8] as the predominant strain of human rotavirus infection in Bangkok in the course of 1998 to 1999 (Table 1).

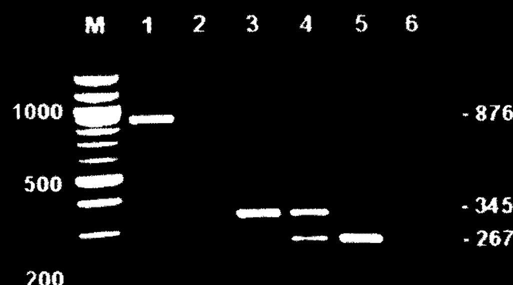

Fig. 2 P genotyping of rotavirus VP4 gene by multiplex PCR after 3% agarose gel electrophoresis. The 876-bp first-round PCR product (lane 1) was re-amplified using multiple semi-nested P-specific primers. The second-round PCR product of P[8] comprised 345 bp (lane 3) and of P[6] 267 bp (lane 5) whereas some specimens showed mixed P genotypes (lane 4). Lanes 2 and 6 are negative controls of both PCR rounds (water). Lane M, 100-bp DNA ladder (Promega Madison Wis).

DISCUSSION

In the present study, we have investigated the prevalence of G and P genotype distribution of human rotavirus among children with acute diarrhea between November 1998 and August 1999 in Bangkok, Thailand. RT-PCR, an efficient method with high specificity and sensitivity, combined with

REA and multiplex PCR have been applied for rotavirus classification. Based on our data, cases positive for rotavirus infection have been detected in 50.6% (42/83) of children with acute gastroenteritis. Based on a previous investigation performed in Thailand, rotavirus infection could be identified among only 18% of childhood diarrhea cases.[16] However, the high rate of rotavirus infection observed in this study may be due to the stool samples having been collected from young children and infants during the epidemic season. Among those samples positive for rotavirus, the G1P[8] serotype (76.1%) was the predominant one, followed by G1P [6] (2.4%) and finally, 2 samples (4.8%) of the G1 type combined with mixed P[8]/P[6] strains. Furthermore, 5 untypeable G strains (11.9%) showed combinations with P[8] whereas the 2 additional ones (4.8%) displayed the P[6] genotype. The presence of a large number of G1P[8] is not unexpected since this serotype has been identified as the one most commonly distributed worldwide.[9] Distinctive yearly changes of rotavirus distribution in Thailand have been observed by polyacrylamide gel electrophoresis analysis, serotype specific monoclonal antibodies and RT-PCR. Previous data on the respective serotypes of human rotavirus dominant during 1982-1991 are depicted in Table 2. However, with epidemiological data lacking for the period after 1991, the present report might have important implications for future vaccine strategies.

Table 1 G- and P- genotype combinations of human rotavirus strains detected in Bangkok, Thailand, between November 1998 and August 1999

Number of VP4 genotype(s) (%)	Number of VP7 serotypes (%)		
	G 1 strain	Untypeable strain	Total
P[8]	32 (76.1)	5 (11.9)	37 (88.1)
P[6]	1 (2.4)	2 (4.8)	3 (7.1)
P[8]/P[6]	2 (4.8)	-	2 (4.8)
Total	35 (83.3)	7 (16.7)	42 (100)

Table 2 Epidemiology of human rotavirus in Thailand during 1982-1991

Duration	Prevalent rotavirus strain	References
1982 – 1983	G 4 serotype	17
1983 – 1984	G 4 serotype	18
1985 – 1987	G 1 serotype	17
1987 – 1988	G 2 serotype	18
1988 – 1989	G 1 serotype	19
1989 – 1990	G 1 serotype	19
1990 – 1991	G 3 serotype	19

Rotavirus has genetically evolved employing two major mechanisms: genetic drift resulting from successive intragenic mutations and genetic shift as a consequence of gene reassortment between different strains.[3] Based on the more common observation of genetic shift, the untypeable G pattern encountered in this study may have been caused by a reassortment mechanism as has been reported in a previous study on human and animal rotaviruses.[20] This mechanism would produce the new G serotypes not identifiable by the technique applied and hence, requires further analysis by sequencing. However, with the RCU-55 (accession no. AF181865) analysis we have met the limits of REA for G serotype classification. This sample was identified as an untypeable G type as *Sau*96I (Fig. 1, lane 7) could not cleave its VP7 gene. After nucleotide sequencing analysis, the data have been compared with other rotavirus sequences stored in Genbank and hence, could be defined as the G1 serotype. We detected a point mutation abolishing the restriction site of *Sau*96I (data not shown). From this we can infer that RCU-55 might have been subject to genetic drift and thus, REA might lose its applicability in cases of spontaneous point mutations.

Additional limiting factors of the present study have been the restriction of the serotype distribution studied to one endemic season

in one city of Thailand. Hence, further studies performed in other regions of Thailand will be required to continue serotype characterization and thus determine whether the current serotype distribution is widespread in the country and whether the serotype pattern changes with time.

In fact, the RRV tetravalent reassortant vaccine previously evaluated in clinical trials has proven its protective efficacy against serotypes G1-G4.[21] However, prompted by reports of unusually large numbers of infants having developed bowel obstruction associated with intussusception after receiving this new rotavirus vaccine in July 1999 the CDC recommended parents and health care providers that they postpone its use as a precautionary measure[22]. At the same time, the manufacturer in consultation with the FDA voluntarily ceased distribution of the vaccine and in mid October, withdrew the vaccine from the US market. Once a rotavirus vaccine proven safe and efficacious has been developed the present study may support its introduction in Thailand. However, additional long-term surveys conducted as part of a strain surveillance system will be required to monitor the strains circulating in this area.

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