THE INCIDENCE OF ROTAVIRUS A ISOLATES OF G GENOTYPE IN THAILAND IN 2002–2004

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Summary. – Rotaviruses are the leading cause of severe gastroenteritis among infants and young children worldwide. Between November 2002 and March 2004, 36 stool specimens of 108 children with acute diarrhea in Bangkok, Thailand were found positive for Rotavirus A (RV-A) by RT-PCR. The 36 isolates were subjected to genotyping by RFLP analysis and direct sequencing of a part of the gene for major outer capsid glycoprotein VP7. The sequences obtained were subjected to phylogenetic analysis. Among the isolates the genotypes G1 (5.6%), G2 (69.4 %) and G9 (25.0 %) were found. Comparison of these results with those of previous studies covering the period of 1982–1999 revealed a changing pattern of RV-A G genotypes and thus contributed to the understanding of RV-A epidemiology in Thailand. Any vaccine to be developed against this virus should target the G9 genotype as one of common global genotypes.

Key words: Rotavirus A; VP7; RFLP analysis; nucleotide sequencing; phylogenetic analysis; G genotype; Thailand

Introduction

Severe gastroenteritis in infants and young children results mostly from infection with RV-A. Each year, 600,000 to 870,000 children below the age of five years succumb to rotavirus diarrhea, mostly in developing countries where this disease accounts for 6% of all deaths (De Zoysa *et al*., 1985).

Rotaviruses are characteristic by icosahedral, non-enveloped virions containing 11 segments of a double-stranded RNA genome located inside a triple-layered capsid. The proteins VP1, VP2 and VP3 form the core region, while VP6, major glycoprotein VP7 and minor protein VP4 make up the capsid. As both VP7 and VP4 can induce neutralizing antibodies they can be used as the target for vaccine development. Taxonomically, rotaviruses (members of the *Rotavirus* genus) consist of 5 regular species, namely *Rotavirus A-E* and two tentative species, *Rotavirus F* and *G* (van Regenmortel *et al*., 2000). For RV-A, fourteen G and twenty P genotypes have been so far identified. Although global distribution has been demonstrated for four major G genotypes, G1, G2, G3 and G4 (Beards *et al*., 1989), recent studies have documented that also other genotypes are common in some regions of the world; e.g. G9 in India, Libya, Kenya, Cuba and Nigeria, G8 in Brazil, Malawi and Nigeria, and G5 and G10 in Brazil (Cunliffe *et al*., 2001; Gouvea *et al*., 1994; Ramachandran *et al*., 1996; Santos *et al*., 1998; Steele and Ivanoff, 2003). Although the G9 genotype has been found less frequently than the genotypes G1-G4, it has significantly contributed to the changes in the genotype pattern among the patients with diarrhea in India, Bangladesh and USA (Jain *et al*., 2001; Laird *et al*., 2003; (Cunliffe *et al*., 2002) .

A high frequency of uncommon and reassorted rotavirus isolates/strains in many countries has brought about extensive surveillance aimed at developing appropriate rotavirus vaccine. Rotavirus isolates/strains can be serotyped and genotyped on the basis of the outer capsid proteins that

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Abbreviations: MEIA = monoclonal antibody-based enzyme immunoassay; PAGE = polyacrylamide gel electrophoresis; RFLP = restriction fragment length polymorphism; RV-A = Rotavirus A

are the targets of neutralizing antibodies produced following natural infection. In this study, we used RT-PCR for highly sensitive and specific detection of RV-A in stool specimens from children with acute diarrhea. Thereupon, the RT-PCR products were subjected to genotyping by RFLP analysis, direct sequencing and phylogenetic analysis.

Materials and Methods

Specimens. The study protocol was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Children's parents were informed as to the study objective and their written consent was obtained before specimen collection. We collected 108 feces specimens from 71 male and 37 female children at 1.62 year mean age (the range from 2 months to 11 years) with acute diarrhea who lived in Bangkok, Thailand and had been admitted at the Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and Hospital, Bangkok, Thailand. The specimens were stored at -70°C until further examination.

RT-PCR. Fecal specimens were diluted 1:10 in PBS, centrifuged, and the supernatants (50 µl) were used for RNA extraction by the guanidium-isothiocyanate method (Theamboonlers, 2002). The RNAs were reversely transcribed into cDNA using the primer End9 (5'-GGTCACATCATACAATTCTAATCTAAG-3', nt 1062–1036). The resulting cDNAs were amplified by PCR using Beg9 (5'-GGCTTTAAAAGAGAGAATTTCCGTCTGG-3', nt 1–28) (Gouvea *et al*., 1993) as the outer sense primer and End9 as the outer antisense primer. The amplicon consisted of a part of RV-A VP7 gene. The reaction was performed in a thermocycler (9600 Perkin Elmer Cetus, USA) as follows. Initial denaturation at 95°C for 3 mins was followed by 30 cycles of 95°C/1 min (denaturation), 60°C/1 min (primer annealing), and 72°C/1 min (extension) and final extension at 72°C for 10 mins. After electrophoresis in 2% agarose gel and staining with ethidium bromide the expected 1062 bp product was visualized on a UV transilluminator (Gel Doc 1000, BIO-RAD, USA).

RFLP analysis. cDNAs were purified using the Perfectprep Gel Cleanup Kit (Eppendorf, USA) according to the manufacturer's specifications. cDNA aliquotes (20 µl) were digested with 1 U (1 µl) of *Hae*III, *Sau*961 and *Bsty*I, yielding the *Hae*III fragments of 381 and 681 bp for G1 to G9, *Sau*961 fragments of 257 and 805 bp for G1, G3, G4 and G9, *Sau*961 fragments of 36, 221 and 805 bp for G2, *Bsty*I fragments of 154, 195 and 713 bp for G1, *Bsty*I fragments of 516 and 546 bp for G2, *Bsty*I fragments of 388 and 674 bp for G3; *Bsty*I fragments of 229 and 833 bp for G4, and *Bsty*I fragments of 195 and 867 bp for G9 (Gouvea *et al*., 1993). After 1 hr incubation at the respective temperature recommended for each enzyme, the fragments were electrophoresed in a 2% Nusieve-1% SeaKem agarose gel (FMC, USA).

Direct nucleotide sequencing. The PCR products separated by agarose gel electrophoresis were purified using the Perfectprep Gel Cleanup Kit (Eppendorf, USA) according to the manufacturer's specifications and reelectrophoresed in order to ascertain their purity. The concentration of the amplified DNA was assayed spectrophotometrically (A_{260} of 1.00 = 50 µg double-stranded DNA per ml). Each DNA (10–30 ng in 3–6 µl) was mixed with 4 µl of dye terminator, 2 µl of a DNA sequencing buffer and 3.2 pmoles of a specific primer in a final reaction volume of 20 µl in a thermocycler (9600 Perkin Elmer Cetus, USA). The amplification was performed according to the manufacturer's specifications (Big Dye Terminator V.3.1 Cycle Sequencing Ready Reaction, USA) using the primers Beg9 and End9 to amplify the particular DNA strand of interest. The PCR products were subjected to sequence analysis in an ABI Prism 310 Genetic Analyser.

Phylogenetic analysis. In order to determine the relationships between the genotypes of RV-A isolates obtained in this study and those of 38 others retrievable from GenBank, we generated an

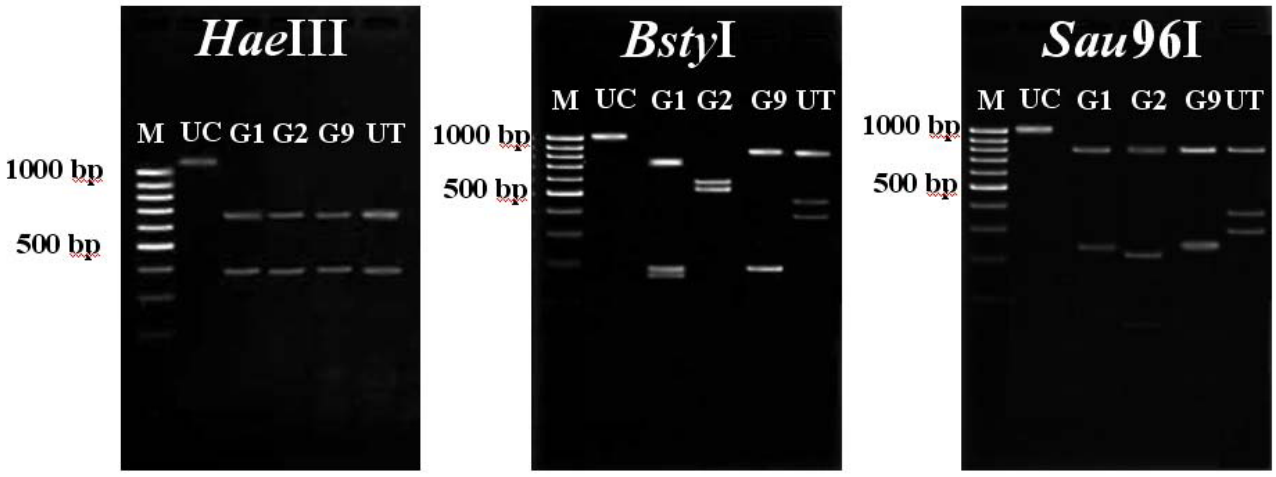

Fig. 1

RFLP analysis

Agarose gel electrophoresis. DNA size marker, 100 bp ladder (lane M); G1, G2 and G = genotypes; UC = uncut; UT = untypable.

Table 1 Epidemiology of G genotype of RV-A in Thailand in 1982–2004

Year	Typing method	No. of isolates tested	No. (%) of genotype						Reference
			G1	G2	G3	G4	G9	Untypable	
1982–83	MEIA	37	0 (0)	0 (0)	3 (8.1)	34 (91.9)	ND	–	Maneekarn *et al*. (2000)
1983–84	MEIA	33	7 (21.2)	5 (15.2)	0 (0)	21 (63.6)	ND	–	
1985–86	MEIA	226	176 (77.9)	39 (17.3)	0 (0)	11 (4.9)	ND	–	
1986–87	MEIA	106	80 (75.5)	15 (14.2)	0 (0)	11 (10.4)	ND	–	
1987–88	MEIA	25	11 (44.0)	12 (48.0)	0 (0)	2 (8.0)	ND	–	
1987–88	MEIA	101	32 (31.7)	39 (38.6)	0 (0)	0 (0)	ND	30	
1987–88	MEIA	178	38 (21.3)	35 (19.7)	0 (0)	1 (0.7)	ND	104	
1988–89	MEIA	67	30 (44.8)	6 (8.9)	0 (0)	3 (4.5)	ND	28	
1988–89	MEIA	82	36 (43.9)	6 (7.3)	0 (0)	7 (8.5)	ND	33	
1989	MEIA	148	17 (11.5)	30 (20.3)	7 (4.7)	14 (9.5)	ND	80	
1989–90	MEIA	82	36 (43.9)	16 (19.5)	3 (3.7)	0 (0)	ND	27	
1990–91	MEIA	77	18 (23.4)	19 (24.7)	20 (30)	1 (1.3)	ND	19	
1992	MEIA	251	55 (21.9)	84 (33.5)	3 (0.8)	4 (1.6)	ND	105	
1998–99	Seq	42	35 (83.3)	0 (0)	0 (0)	0 (0)	0	7	Noppornpanth *et al*. (2001)
2002–04	Seq	36	2 (5.6)	25 (69.4)	0 (0)	0 (0)	9(25)	0	This study

ND = not done. Seq = sequencing.

unrooted phylogenetic tree of nucleotide sequences comprising a part of the VP7 gene region. Multiple alignment and phylogenetic tree construction was done using the DNASTAR-5.x Program.

Results

Between November 2002 and March 2004, 36 children were diagnosed positive for RV-A RNA by RT-PCR. RFLP analysis of the PCR-amplified VP7 genes of the 36 RV-A isolates with *Hae*III, *Sau*96I and *Bsty*I yielded a genotype polymorphism (Fig. 1). Two isolates exhibited G1 (5.6%), twenty-two G2 (66.7%), nine G9 (25.0%) and one (2.8%) was untypeable. All the 36 isolates were sequenced for the 1062 bp VP7 gene region and together with 38 reference strains/isolates subjected to phylogenetic analysis (Fig. 2). We found out that of the 36 RV-A isolates 2 (5.6%) belonged to the genotype G1, 25 (69.4%) to G2 and 9 (25.0%) to G9. All the results obtained by sequencing correlated well with those from RFLP analysis except for one isolate that was untypable by RFLP. However, this isolate turned out to be G2 by phylogenetic analysis. The genotypes determined for 2002–2004 were compared with those for the previous two decades (Table 1).

Discussion

In the studies conducted prior to 1992, MEIA and PAGE have been applied to serotyping of rotaviruses (Laird *et al*., 2003). Since the introduction of RT-PCR genotyping has become feasible. Some studies have claimed that serotyping harbors limitations as to sample storage and collection and correct interpretation of cluster patterns (Steele and Ivanoff, 2003).

Nowadays, RT-PCR, RFLP analysis and direct sequencing constitute the most specific and sensitive methods of genotyping.

In genotyping the 36 RV-A isolates from 2002–2004 from Thailand by RFLP analysis and sequencing we found out that the genotypes determined by these two methods were identical with the exception of one isolate that was untypable by RFLP but assessed as G2 by sequencing. In 2002–2004, the predominant genotype was G2 (69.4%), whileG1 and G9 represented 5.6% and 25.0%, respectively. Between November 1998 and August 1999, the predominant RV-A in Bangkok, Thailand was of the G1 genotype (83.3%) based on RFLP analysis and direct sequencing (Noppornpanth *et al*., 2001). Comparing the periods 1998–1999 and 2002–2004 in Thailand, the genotypes G2 and G9 increased, while the genotype G1 decreased; the predominance moved from G1 to G2, respectively.

A prevalent change of the pattern of RV-A G genotype during 2002–2004 in Bangkok, Thailand consisted of an increase of G9 and G2 and a decrease of G1, although G1 remained the prevailing genotype.

The G9 genotype was unexpectedly predominating in Japan in Sapporo (62.5%) and Tokyo (52.9%) in 1998–1999 and in Saga (78.4%) in 1999–2000 (Zhou *et al*., 2003). The incidence of the genotypes G2, G3 ad G4 was low in 1996–1999, but the relative prevalence of G2 increased in the course of 1999–2000 in Tokyo, Maizuru, Osaka and Saga (12–27%) (Zhou *et al*., 2003).

In 1982–84, 1987–88, 1990–91 the prevalent genotypes in Thailand were G4, G2 and G3, respectively. Since 1983 G9 has increasingly advanced to the third prevailing genotype with regard to its recent continuing emergence on a worldwide scale (Maneekarn and Ushijima, 2000; Armah *et al*., 2003; Laird, 2003; Donck *et al*., 2003).

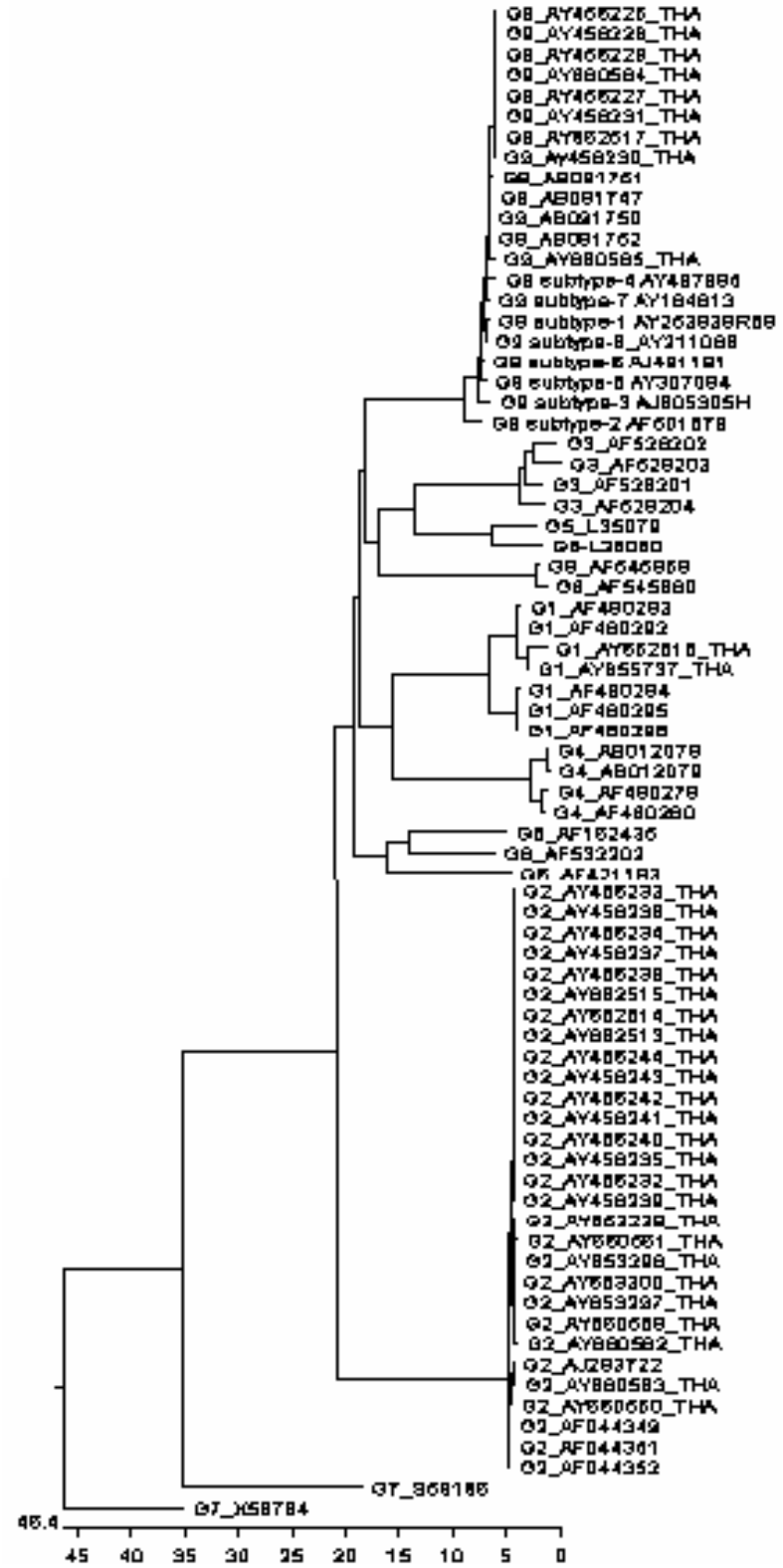

Fig. 2

Phylogenic tree of RV-A isolates of G genotype based on a part of VP7 gene

The four-year survey on rotavirus infection conducted between 1996 and 2000 revealed the changing G genotype distribution pattern in Japan with a decrease of G1 and increase of G9 and G2 (Zhou *et al*., 2003). This result was consistent with the findings of other epidemiology studies, which have indicated that unusual genotypes have been emerging as global strains since 1992 (Gouvea and Santos, 1999; Santos *et al*., 1998; Ramachandran *et al*., 1996; Cunliffe *et al*., 2001; Gouvea *et al*., 1994). The increased incidence of the G9 genotype in 1998–1999 should be considered a re-emergence following an eclipse since the mid 1980s. Coinciding with a global emergence, the results from Thailand in 1996–1997 indicated that the prevalence of the G9 genotype recently increased. This could either be due to the fact that G9 was able to escape detection due to searching for the genotypes G1-G4 only. In addition, the data obtained in each region over consecutive years have shown that the G9 genotype did not prevail for more than one year, while the G1 genotype predominated continuously. It is not clear why G1 shows a more or less stabile incidence pattern and G9 occurs in a single peak pattern. It might be useful to compare the levels of serum antibodies against rotaviruses in children in regions with G9 prevailing and those in regions with G9 non-prevailing or absent (Zhou *et al*., 2003).

Understanding the differences between the epidemiological features of *Rotaviruses* in developing and developed countries is essential for designing optimal *Rotavirus* immunization programs. This and previous studies have demonstrated that vaccines being developed should target the G9 strains and that G9 should be included as one of the common global genotypes.

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