

PREVALENCE OF GROUP A GENOTYPE HUMAN ROTAVIRUS AMONG CHILDREN WITH DIARRHEA IN THAILAND, 2009-2011

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Abstract. Rotavirus is the most common cause of severe diarrhea in infants and young children world-wide, with the highest fatality rate in developing countries. We investigated the presence and seasonal distribution of group A rotavirus infection among Thai children. The data will be used for vaccine development. Samples were collected from infants and children with acute gastroenteritis or diarrhea admitted to two hospitals between June 2009 and May 2011. Group A rotaviruses were detected in 250 (44.5%) of 562 specimens by RT-PCR. The most prevalent genotype was G3P[8] (60.4%) followed by G1P[8] (39.2%) and G2P[4] (0.4%). The specimens were subjected to phylogenetic analysis based on the VP7 and VP4 genes. We examined the rotavirus genotypes and compared them with data from the GenBank database.

Keywords: human rotavirus, prevalence, genotype, phylogenetic analysis

INTRODUCTION

Rotavirus is the most common cause of severe diarrhea in infants and children under age of 5 years world-wide, with the highest incidence in children aged range of 6-24 months. Disease frequency usually peaks during the cooler winter months (Hrdy, 1987; Palumbo *et al*, 2009; Tate *et al*, 2012). This infection causes approximately 527,000 deaths per year world-wide, with more than 85% of those deaths occurring

in developing countries in Africa and Asia (CDC, 2011). Rotaviruses belong to the Reoviridae family, genus *Rotavirus* and are classified into seven groups (A-G) on the basis of antigenic properties, with group A rotaviruses being the major cause of acute dehydration in children. The group A rotavirus genome consists of 11 segments of double-stranded RNA (dsRNA) encoding six structural viral proteins (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6) enclosed in a three layer protein capsid, consisting of a protein core, an inner protein capsid, and an outer protein capsid. The outer capsid VP7 and VP4 proteins, in particular, are involved in virus penetration into cells and induction of neutralizing antibodies. The characteristics of VP7 and VP4 serve to classify rotavi-

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ruses into G and P genotypes, respectively (Estes and Kapikian, 2007; Matthijnsens *et al*, 2008). To date, at least 35 G and 27 P genotypes have been identified, of which genotypes G1, G3, G4, and G9 (associated with P[8]) and G2 (associated with P[4]) represent the epidemiologically most important human rotavirus genotypes globally (Matthijnsens *et al*, 2009, 2011; da Silva *et al*, 2011; Yamamoto *et al*, 2011). These G/P-types are included in the two currently licensed rotavirus vaccines in Thailand. RotaTeq® (Rotavirus Vaccine, Live, Oral, Pentavalent, Merck, Darmstadt, Germany) contains five human-bovine reassortant rotavirus strains expressing the human serotypes G1, G2, G3, G4, and P1A[8], and Rotarix TM (Rotavirus Vaccine, Live, Oral, Monovalent, GlaxoSmithKline Biologicals) contains G1P1A[8] (Santos and Hoshino, 2005; Santosham *et al*, 2007).

Continuous surveillance of rotavirus types is essential to monitor for the emergence of new G/P combinations over time. The efficacy of the rotavirus vaccine depends on the predominant types causing infection in humans. Prior to vaccine use in a populations, it is essential to obtain epidemiological and molecular data regarding rotavirus infections in that country (Theamboonlers *et al*, 2008).

We explored the density and distribution of group A rotavirus in the feces of pediatric patients in Thailand presenting with acute gastroenteritis or diarrhea admitted to two hospitals between June 2009 and May 2011. Phylogenetic analysis was performed based on nucleotide sequences of the VP7 and VP4 genes detected in the group A rotaviruses.

MATERIALS AND METHODS

Specimen collection

The study protocol was approved by

the Ethics Committee, Ministry of Public Health and Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

A total of 562 stool specimens were collected from infants and children with acute gastroenteritis or diarrhea admitted to two hospitals in Thailand between June 2009 and May 2011. Five hundred twenty-one stool samples were collected from Chum Phae Hospital in Khon Kaen and 41 stool samples were collected from Chulalongkorn Hospital in Bangkok. The stool specimens were collected anonymously and stored at -70°C until examined.

Viral RNA extraction and RT-PCR

Fecal specimens were suspended in 10% phosphate-buffered saline (PBS), centrifuged at 3000 rpm for 10 minutes and the supernatant was collected for viral RNA extraction. Viral RNA was extracted using a RBC Viral Nucleic Acid Extraction Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's instructions. RT-PCR-based G- and P- genotyping assays were performed using genotype specific primers and conditions described previously (Khananurak *et al*, 2010).

Nucleotide sequencing

The PCR products were purified from agarose gel using a HiYield Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's instructions. Direct sequencing was performed by First BASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia).

Sequence analysis

The nucleotide and deduced amino acid sequences were compared with those of reference strains available at the NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search

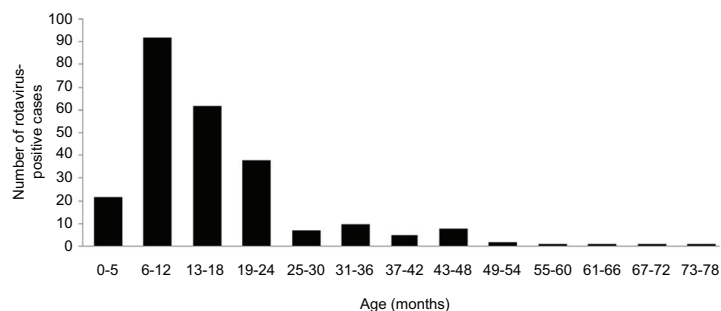


Fig 1—Rotavirus-positive patients distributed by age.

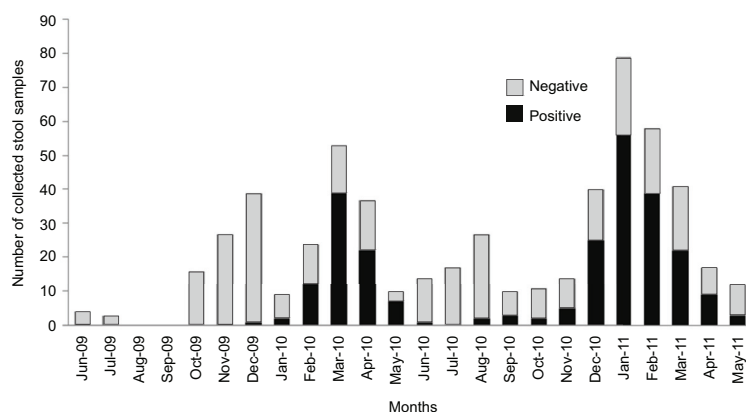


Fig 2—Seasonal patterns of rotavirus infection among infants and children hospitalized with diarrhea in Thailand between June 2009 and May 2011.

Tool) at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. The sequences were edited and assembled using the programs Chromas Lite, version 2.0 and SeqMan II (DNA STAR, Madison, WI).

Phylogenetic analysis

The nucleotide sequences of the reference rotavirus strains were retrieved from the NCBI GenBank database. Multiple sequence alignments were prepared using Clustal W, version 1.83. Phylogenetic trees were analyzed using MEGA software, version 4.1, based on the neighbor-joining method of measuring phylogenetic distances applying the Kimura two-parameter model.

Nucleotide sequence accession numbers

The nucleotide sequence data of the rotavirus strains were submitted to the GenBank database under accession numbers: JN705948-JN706443.

RESULTS

Prevalence of rotavirus

Of 562 stool specimens collected from infants and children hospitalized with diarrhea between June 2009 and May 2011, group A rotaviruses were detected by RT-PCR in 250 specimens (44.5%). The age distribution of rotavirus infected patients in this study ranged from 1 month to 8 years, with the highest rate of rotavirus-positive patients (36.8%; 92/250)

aged 6 and 12 months (Fig 1).

Seasonal distribution of rotavirus

After analysis, the data were divided into specimens obtained during two time periods: June 2009 to May 2010 and June 2010 to May 2011. Infected pediatric patients were found during December 2009 to March 2010 (when the highest infection rate was observed). The density of infection decreased slowly until May 2010.

During the second period (June 2010 to May 2011), rotavirus infections were found in children beginning in June 2010. The infection rate increased until January 2011, which was the peak of the infection during the second period. It subsequently

decreased until May 2011. The infection rates for both periods were higher during the winter months (Fig 2).

Distribution of G and P genotypes

The 250 rotavirus-positive specimens were characterized for G and P genotypes by BLAST. The majority of G genotypes detected were G3 (60.4%), followed by G1 (39.2%) and G2 (0.4%). For the P genotypes, P[8] was the most prevalent (99.6%), followed by P[4] (0.4%). With respect to the distribution of common G/P combination strains, G3P[8] (60.4%) was the predominant strain, followed by G1P[8] (39.2%) and G2P[4] (0.4%). The distribution of group A rotavirus genotypes from the two study hospital is shown in Table 1.

Nucleotide sequence and phylogenetic analysis of VP7 genes

Analysis of G1 strain. The examined feces samples yielded positive results for G1 98. When the genome was decoded and the nucleotides sequenced into the 5' and 3' groups, and the results were combined using the Seqman program to complete the genome. Eighty-two sample gene codes could be put together to complete gene VP7. It was 900 bp long, so to ensure precise results from the phylogenetic analysis group, the 82 samples were used. Based on the phylogenetic tree created for gene VP7 in genotype G1 (Fig 3a), G1 could be classified into 2 lineages: lineage I (sublineage Ic) and lineage II (sublineage IIc). Eighty-nine percent of samples (73/82) were classified into lineage II (sublineage IIc). The samples were obtained from both the Bangkok and Khon Kaen hospitals in 2010-2011. Nucleotide and amino acid similarities, when compared to other strains in lineage II, exceeded 95%. The lineage II (sublineage IIc) samples were similar to rotavirus strains found among humans

in Thailand (CU406-BK/09 and 86vp7w), Japan (7265 and 7014), Australia (CK00037 and CK00036), Cuba (Ha1, Ha5, Ha45 and Ha95), and the USA (2007719907), with nucleotide and amino acid similarities exceeding 98.2%. Only 11.0% of samples (9/82) were classified into lineage I (sublineage Ic) most of which were similar to strains found in Asia. The 2010 samples found in Khon Kaen were similar to strains found in the rest of Thailand (CU163-KK/07 and CU246-KK/08), Japan (91TA984/91), Taiwan (CH9), and China (PJ13/07), with nucleotide and amino acid similarities exceeding 96.1%.

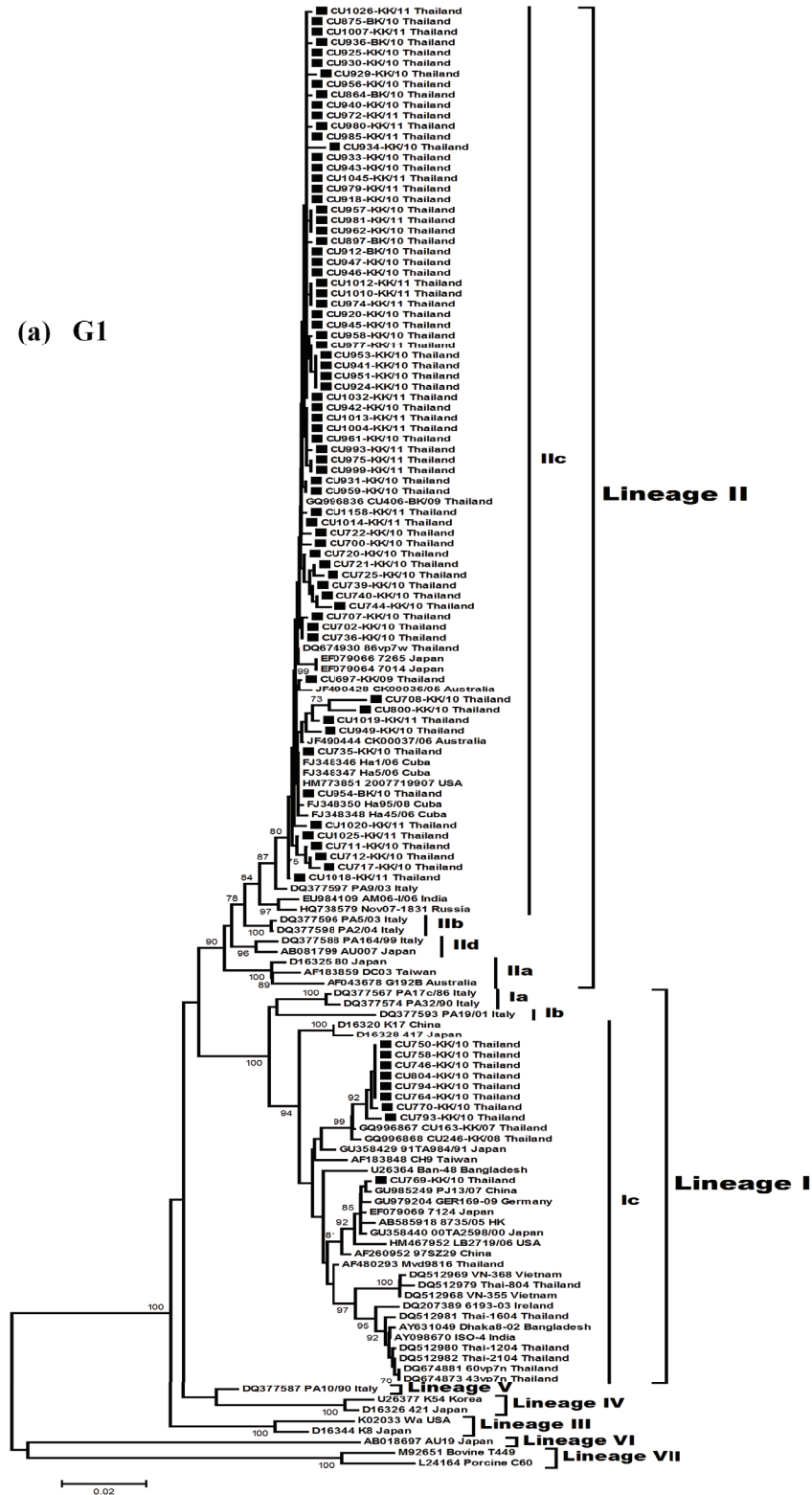
Analysis of G2 strain. Of the stool samples examined only one was positive for genotype G2: sample CU991-KK/11 from Khon Kaen Province in 2011. The phylogenetic tree (Fig 3b), shows the samples from lineage II (sublineage IIa) were similar to rotavirus KO-2 samples found in humans from Japan, with nucleotide and amino acid similarities of 99.9% and 97.0%, respectively.

Analysis of G3 strain. Analysis with a phylogenetic tree for genotype G3 (Fig 3c), found only one sample in lineage I (sublineage Ia) in Bangkok during 2010 (CU938-BK/10), the rest were from Khon Kaen during 2010-2011. All the samples were similar to rotavirus type E885 found among humans, from China and VN-374 from Vietnam, with nucleotide and amino acid similarities of 98.4-99.8% and 97.6-99.7%, respectively.

Nucleotide sequence and phylogenetic analysis of VP4 genes

Analysis of P[8] strain. Based on phylogenetic tree analysis of genotype P[8] (Fig 3d), all samples were classified into lineage III, comparison displayed nucleotide and amino acid similarities. Nearly all samples were similar to rotavirus among humans,

(a) G1



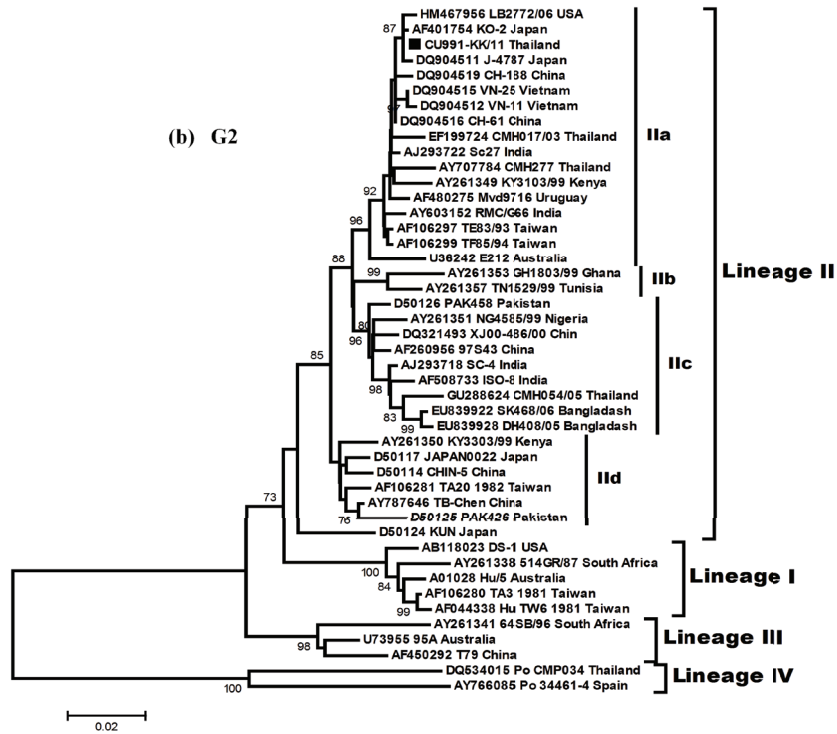


Fig 3–(Continued).

collected from Russia. Two samples were similar to strain BE00049 from Belgium, with nucleotide and amino acid similarities of 100%, but they were classified as the same type found in Russia.

Analysis of P[4] strain. Based on phylogenetic tree analysis of genotype P[4], only one sample was found from a patient in Khon Kaen in 2010 (Fig 3e). P[4] was classified in lineage V (sublineage Vb), similar to the rotavirus strain PSAL977-D from Brazil, with nucleotide and amino acid similarities of 99.0%.

DISCUSSION

According to RT-PCR studies of 562 samples derived from pediatric patients with diarrhea receiving treatment in both Chum Phae Hospital, Khon Kaen

and Chulalongkorn Hospital, Bangkok, from June 2009 to May 2011, 250 samples (44.5%) tested positive for group A rotavirus (46.3% and 22.0% from Chum Phae Hospital, Khon Kaen and Chulalongkorn Hospital, Bangkok, respectively). The difference in positivity between the two locations could be because children living in Bangkok might have better access to rotavirus vaccines than those living in rural areas.

In our study, rotavirus infection occurred primarily between December and March.

In our study rotavirus infections occurred primarily among children aged 6-12 months (36.8%). This was lower among children aged 13-18 months (24.8%), and 0-5 months (8.8%). A possible explanation for this could be passive im-

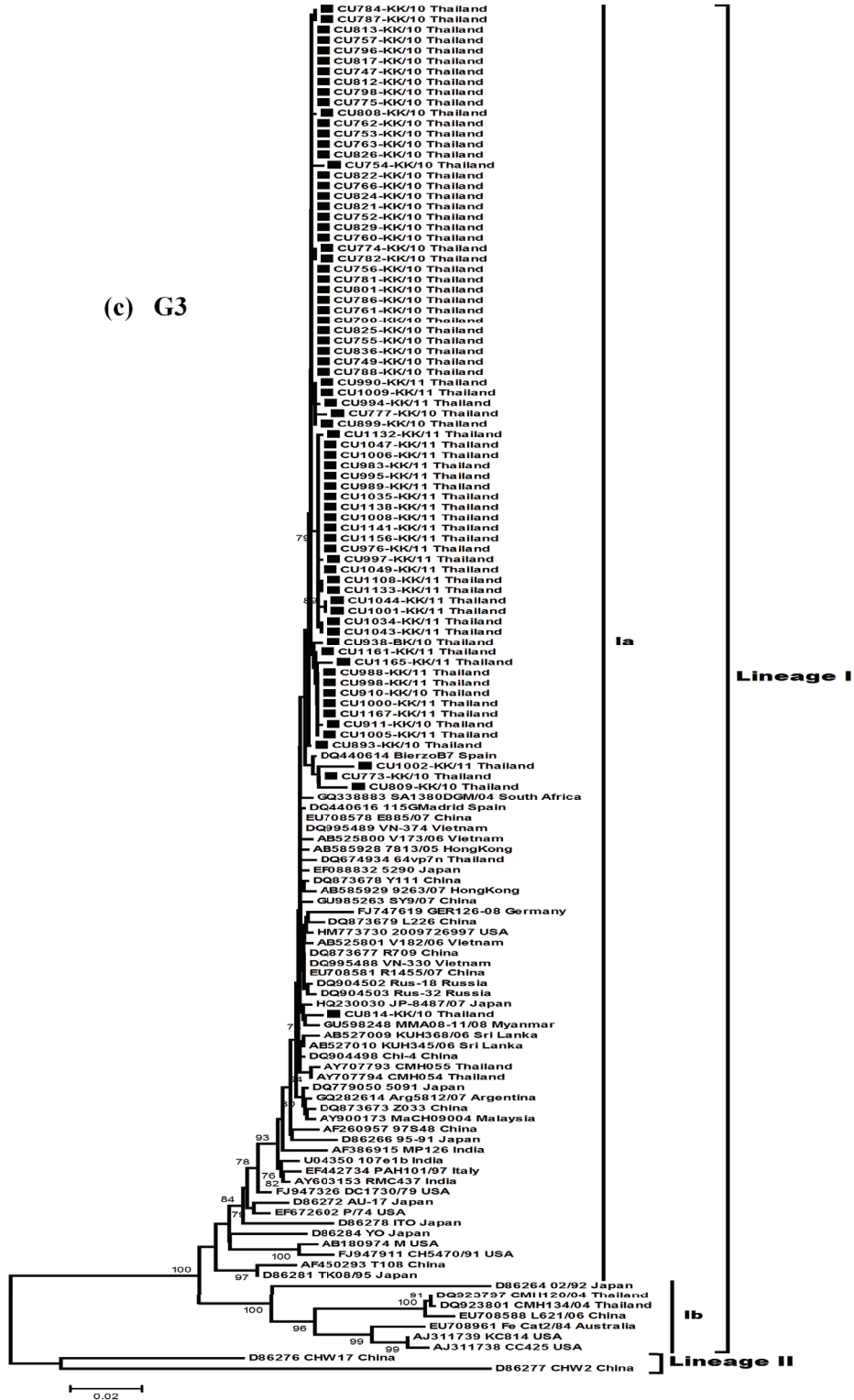


Fig 3-(Continued).

ROTAVIRUS GENOTYPE IN THAI CHILDREN WITH DIARRHEA

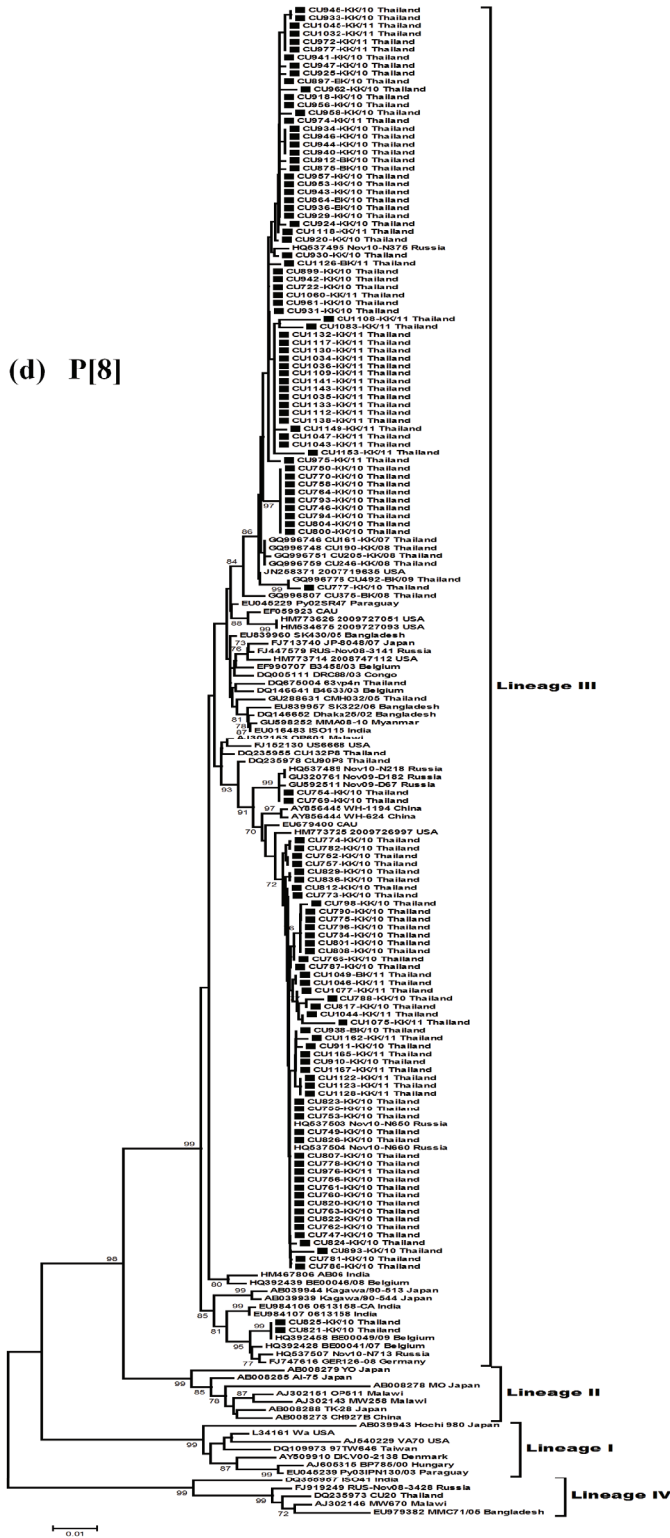


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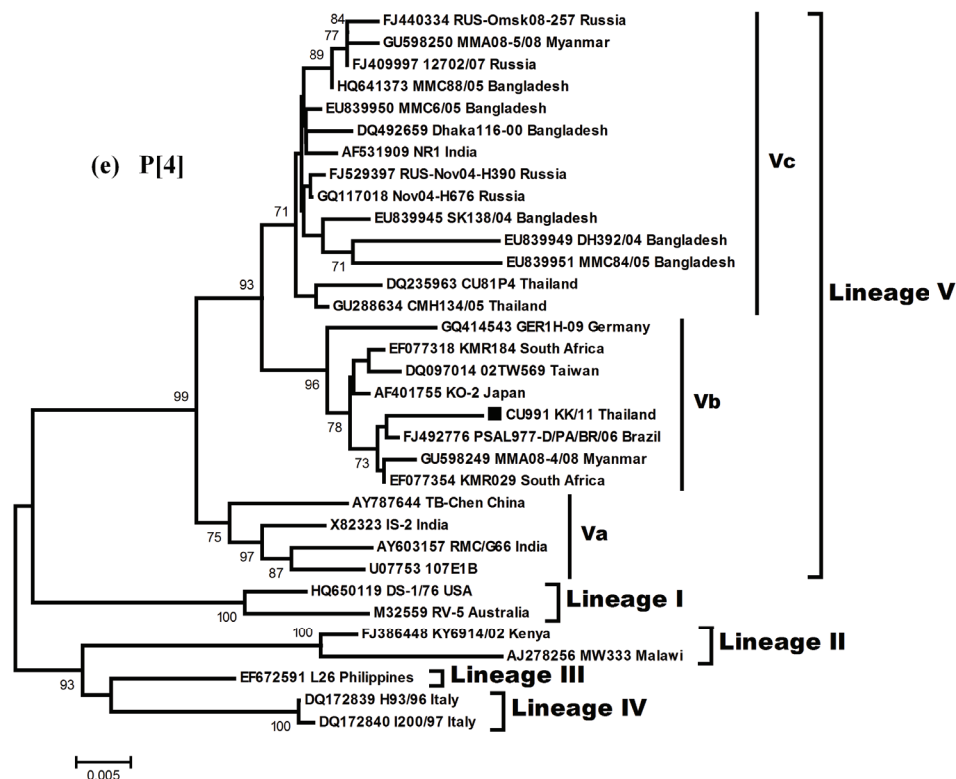


Fig 3–Phylogenetic analysis of nucleotide sequence of the VP7 gene in (a) G1, (b) G2, (c) G3, and VP4 gene among (d) P[8] and (e) P[4] rotavirus strains from Thailand between June 2009 and May 2011. ■ indicates G- and P-type rotavirus strains in this study. Reference sequences were obtained from the GenBank database. Percent bootstrap support was indicated by the values at each node, and values below 70 were omitted. The bar indicates the variation scale.

munity obtained from the mother at birth or through breast milk.

The most prevalent G-genotype was G3 (60.4%) followed by G1 (39.2%) and G2 (0.4%). The most prevalent P-genotype was P[8] (99.6%), followed by P[4] (0.4%). On binary classification the G-P combination had 3 patterns; G3P[8] was the most common (60.4%), followed by G1P[8] (39.2%) and G2P[4] (0.4%). When compared to previous studies (Table 2) the genotype of rotaviruses in Thailand have changed over time. The G1 genotype, a common strain in Thailand, was found throughout the year in this study with a high incidence rate, including the year

this research has focused on; however, the incidence was low during 1999–2003. Genotype G2, compared to 2007–2009, was much lower in this study (0.4%). The G3 genotype had a lower incidence years ago, a peak during 2004–2005, but during 2009–2011, the incidence rate increased again, exceeding that of G1.

G3 is a common strain found every year in China (Fang *et al*, 2005; Trinh *et al*, 2007; Wang *et al*, 2007, 2009). During 2005–2006, G3 was found on the increase in Vietnam and Myanmar (Nelson *et al*, 2009). In our study, analyzed samples could be classified into lineage I (sublineage a), similar to strains collected from

Table 1

Distribution of group A rotavirus G/P combination strains among infants and children with diarrhea in Thailand, June 2009 to May 2011.

| Area | No. of collected stool samples | No. of rotavirus positive cases | No. of G/P combination strains (%) | | |
|-----------|--------------------------------|---------------------------------|------------------------------------|---------|------------|
| | | | G1P[8] | G2P[4] | G3P[8] |
| Bangkok | 41 | 9 | 7 (77.8) | - | 2 (22.3) |
| Khon Kaen | 521 | 241 | 91 (37.8) | 1 (0.4) | 149 (61.8) |
| Total | 562 | 250 | 98 (39.2) | 1 (0.4) | 151 (60.4) |

South Africa, the USA, Europe, and many strains from Asia, including China, Thailand, Vietnam, Myanmar, Hong Kong, and Japan. When comparing nucleotide and amino acid samples to other strains in the same lineage, all the samples were found to be similar to the human rotavirus strain E885, found in China in 2007, and strain VN-374, the most common strain found in Vietnam, with homologies of the samples from the aforementioned countries exceeding 98.4% for nucleotides and 97.6% for amino acids. According to the phylogenetic tree and the topography of the countries, G3 rotavirus may have spread to Thailand from China. The virus could have been imported by Chinese tourists or immigrants entering through Myanmar or Vietnam.

All P[8] samples, the most common in the world, were classified into lineage III. These samples are similar to strains from Russia, Thailand, the USA, and Belgium. The majority of samples were similar to human rotavirus from Russia in 2009 and 2010. This may indicate transmission of P[8] rotavirus from Russia to China. The G3 strains found in our study were combined with P[8]. Most of the samples had nucleotides similar to the strain from China. It may have been caused by a reassortment of genes between two strains

of rotavirus from Russia, since China and Russia share a border. G1P[8] is a common strain found from continental Europe, America, Asia and Australia. As for P[4] combined with G2, the results of the phylogenetic analysis of genes VP7 and VP4 showed they did not match. The nucleotide sequence of gene VP7 was similar to strains found in Japan and the VP4 gene was similar to strains found in Brazil, which may be attributed to gene reassortment between two rotavirus strains from different areas.

The results have been arranged by phylogenetic analysis of the VP7 and VP4 genes. This study examined rotavirus infections and compared them to nucleotides from other countries in the GenBank database. The genotypes in these samples included G1, G2, G3, P[4], and P[8]. Besides being common strains throughout the world, and in Thailand, they had characteristics classified in the phylogenetic tree. These included homologies similar to the aforementioned genotypes from the countries the disease had originated from. Genotypes G1 and P[8] are the most widespread in continental Europe, America, Asia and Australia. In our study, G1 and P[8] were similar to the genotypes found in the aforementioned countries. G2, G3 and P[4] are the most widespread geno-

Table 2
Distribution of G genotypes among infants and children hospitalized with diarrhea in Thailand during 1993-2011.

| Year | Area | No. of positive rotavirus | No. of G genotype (%) | | | | | | | | | | References | |
|-----------|--|---------------------------|-----------------------|------------|-----------|-----------|---------|-----------|-----|--------------|-----------|---|------------|-------------------------------------|
| | | | G1 | G2 | G3 | G4 | G8 | G9 | G12 | Unclassified | | | | |
| 1993-1994 | Bangkok, Chiang Mai, Nakhon Phanom, Songkhla | 368 | 316 (85.9) | 7 (1.9) | 0 | 11 (3.0) | 0 | 0 | 0 | 0 | 0 | 0 | 34 (9.2) | Pongsuwanma <i>et al</i> , 2010 |
| 1994-1995 | Bangkok | 99 | 79 (79.8) | 4 (4.0) | 0 | 4 (4.0) | 0 | 1 (1.0) | 0 | 0 | 0 | 0 | 11 (11.1) | Pongsuwanma <i>et al</i> , 2010 |
| 1995-1996 | Bangkok | 31 | 26 (83.9) | 4 (12.9) | 0 | 1 (3.2) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Pongsuwanma <i>et al</i> , 2010 |
| 1996-1997 | Bangkok | 51 | 32 (62.8) | 9 (17.6) | 0 | 3 (5.9) | 0 | 0 | 0 | 0 | 0 | 0 | 7 (13.7) | Pongsuwanma <i>et al</i> , 2010 |
| 1997-1998 | Nakhon Phanom | 29 | 26 (89.7) | 1 (3.4) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 (6.9) | Pongsuwanma <i>et al</i> , 2010 |
| 1998-1999 | Nakhon Phanom | 101 | 91 (90.1) | 2 (2.0) | 0 | 0 | 0 | 0 | 0 | 0 | 1 (1.0) | 0 | 7 (6.9) | Pongsuwanma <i>et al</i> , 2010 |
| 1998-1999 | Bangkok | 42 | 35 (83.3) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 (16.7) | Noppornpanthet <i>et al</i> , 2001 |
| 1999-2000 | Bangkok | 14 | 1 (7.1) | 6 (42.9) | 0 | 0 | 0 | 0 | 0 | 5 (35.7) | 0 | 0 | 2 (14.3) | Pongsuwanma <i>et al</i> , 2010 |
| 2000-2001 | Nakhon Phanom | 53 | 3 (5.7) | 0 | 1 (1.9) | 3 (5.7) | 0 | 0 | 0 | 42 (79.2) | 0 | 0 | 4 (7.6) | Pongsuwanma <i>et al</i> , 2010 |
| 2001-2002 | Nong Khai, Chanthaburi, Tak, Songkhla, Sa Kaeo | 356 | 4 (1.1) | 0 | 1 (0.3) | 57 (16.0) | 0 | 0 | 0 | 294 (82.6) | 0 | 0 | 0 | Pongsuwanma <i>et al</i> , 2010 |
| 2002-2003 | Nong Khai, Chanthaburi, Tak, Songkhla, Sa Kaeo | 428 | 2 (0.5) | 153 (35.8) | 0 | 2 (0.5) | 0 | 0 | 0 | 210 (49.1) | 0 | 0 | 61 (14.3) | Pongsuwanma <i>et al</i> , 2010 |
| 2003-2004 | Nong Khai, Chanthaburi, Tak, Songkhla | 174 | 107 (61.5) | 14 (8.1) | 0 | 6 (3.5) | 1 (0.6) | 18 (10.3) | 0 | 0 | 0 | 0 | 28 (16.1) | Pongsuwanma <i>et al</i> , 2010 |
| 2002-2004 | Bangkok | 36 | 2 (5.6) | 25 (69.4) | 0 | 0 | 0 | 0 | 0 | 9 (25.0) | 0 | 0 | 0 | Theamboonlerset <i>et al</i> , 2005 |
| 2004-2005 | Nong Khai, Chanthaburi, Tak Bangkok, Buri Ram | 109 | 36 (33.0) | 5 (4.6) | 24 (22.0) | 2 (1.8) | 0 | 0 | 0 | 2 (1.8) | 0 | 0 | 38 (34.9) | Pongsuwanma <i>et al</i> , 2010 |
| 2004-2005 | Nong Khai, Chanthaburi, Tak, Songkhla | 51 | 36 (70.6) | 4 (7.8) | 0 | 0 | 0 | 0 | 0 | 11 (21.6) | 0 | 0 | 0 | Theamboonlerset <i>et al</i> , 2008 |
| 2005-2006 | Bangkok, Buri Ram | 428 | 361 (84.4) | 6 (1.4) | 6 (1.4) | 0 | 0 | 0 | 0 | 5 (1.2) | 0 | 0 | 50 (11.7) | Pongsuwanma <i>et al</i> , 2010 |
| 2005-2006 | Nong Khai, Chanthaburi, Tak, Songkhla | 75 | 72 (96.0) | 0 | 2 (2.6) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 (1.4) | Theamboonlerset <i>et al</i> , 2008 |
| 2006-2007 | Bangkok, Khon Kaen, Tak, Nakhon Ratchasima | 289 | 120 (41.5) | 21 (7.2) | 6 (2.1) | 0 | 0 | 0 | 0 | 2 (0.1) | 6 (2.1) | 0 | 134 (46.4) | Pongsuwanma <i>et al</i> , 2010 |
| 2007-2008 | Bangkok, Khon Kaen, | 83 | 38 (45.8) | 8 (9.6) | 1 (1.2) | 0 | 0 | 0 | 0 | 35 (42.2) | 1 (1.2) | 0 | 0 | Khananurak <i>et al</i> , 2010 |
| 2008-2009 | Tak, Nakhon Ratchasima | 70 | 37 (52.9) | 22 (31.4) | 1 (1.4) | 0 | 0 | 0 | 0 | 0 | 10 (14.3) | 0 | 0 | Khananurak <i>et al</i> , 2010 |
| 2009-2010 | Bangkok, Khon Kaen | 83 | 27 (32.5) | 0 | 56 (67.5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | This study |
| 2010-2011 | Bangkok, Khon Kaen | 167 | 71 (42.5) | 1 (0.6) | 95 (56.9) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | This study |

types in Asia and America. G3 is the most widespread in China, while G2 and P[4] are the most widespread in Bangladesh, India, and Brazil (Aung *et al*, 2010).

In conclusion, our study found a higher prevalence of rotavirus infection than the previous studies (2007-2009). The results have been arranged by phylogenetic analysis of the VP7 and VP4 genes. This study examined rotavirus infections and compared them to nucleotides from other countries stored in the GenBank database. The genotypes in these samples included G1, G2, G3, P[4] and P[8]. Besides being common strains throughout the world and in Thailand, they had characteristics classified in the phylogenetic tree. These included homologies to other genotypes from countries where the disease originated. The current vaccines are sufficient to protect people from infection by these genotypes. However, the epidemiology of rotavirus should be monitored continuously due to genetic variation. Spread of rotavirus can lead to new genotypes. Environmental changes can contribute to virus evolution. Epidemiology studies and virus characterization are needed for virus control and vaccine development.

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