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Prevalence and Genotypic Distribution of Rotavirus in Thailand: A Multicenter Study

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Abstract. Rotavirus has been one of the major etiological agents causing severe diarrhea in infants and young children worldwide. In Thailand, rotavirus contributes to one-third of reported pediatric diarrheal cases. We studied stool samples from 1,709 children with acute gastroenteritis and 1,761 children with no reported gastroenteritis whose age ranged from 3 months to 5 years from four different regions in Thailand between March 2008 and August 2010. The samples were tested for the presence of rotavirus by real-time reverse transcription–polymerase chain reaction (RT-PCR) amplification of *vp6* gene and enzyme-linked immunosorbent assay. The positive samples were further characterized for their G and P genotypes (*vp7* and *vp4* genes) by conventional RT-PCR. From all four regions, 26.8% of cases and 1.6% of controls were positive for rotavirus, and G1P[8] was the most predominant genotype, followed by G2P[4], G3P[8], and G9P[8]. In addition, the uncommon genotypes including G1P[4], G1P[6], G2P[6], G2P[8], G4P[6], G9P[4], G9P[6], G12P[6], and G12P[8] were also detected at approximately 14% of all samples tested. Interestingly, G5P[19], a recombinant genotype between human and animal strains, and G1P7[5], a reassortant vaccine strain which is closely related to four human-bovine reassortant strains of RotaTeq™ vaccine, were detected in control samples. Data reported in this study will provide additional information on molecular epidemiology of rotavirus infection in Thailand before the impending national implementation of rotavirus vaccination program.

INTRODUCTION

Rotavirus is one of the major causes of diarrhea in infants and young children worldwide. The WHO estimated that rotavirus was responsible for more than 200,000 deaths among children younger than 5 years each year in 2010–2013.[1,2] Most of the mortality from rotavirus occurred in developing countries, especially in Asia and sub-Saharan Africa.[2] In Asia, the proportion of all diarrheal deaths due to rotavirus by region between 2000 and 2013 was 50.7–54.6% in Southeast Asia, 44.9–49.2% in Western Asia, 35.2–39.2% in Eastern Asia, and 34.1–38.6% in Southern Asia.[2]

The detection of rotavirus via real-time polymerase chain reaction (PCR) is often analyzed further for its G and P genotypes to yield molecular epidemiological information. Sequences of the two outer capsid proteins, VP4 and VP7, are used to classify rotavirus P and G genotypes, respectively.[3] Presently, 36 G genotypes and 51 P genotypes have been identified in humans and animals with various G- and P-genotype combinations.[4] The most common global human rotavirus genotypes associated with diarrhea are G1P[8], G2P[4], G3P[8], G4P[8], and G9[8].[5] These rotavirus genotype combinations are the major cause of human rotavirus infection in developed countries, causing greater than 90% of rotavirus gastroenteritis cases in children.[6] Uncommon G- and P-genotype combinations, which are the result of genetic reassortment between human and animal rotavirus strains, are prevalent in infected children in developing countries.[7,8] The WHO reported that 28.8–58% of rotavirus detected in 2013 were of uncommon genotypes in the Southeast Asia region, the Africa region, the eastern Mediterranean region, and the Region of the Americas.[9] The presently available rotavirus vaccines, RotaTeq® and Rotarix®, were developed from rotavirus genotypes G1–G4 in combination with P[5] and G1P[8], respectively.[10] The prevalence of uncommon rotavirus genotypes detected in different geographical regions of the world may warrant a vaccine that provides cross-protection or coverage against a broader group of genotype combinations.

In Thailand, rotavirus is a major cause of pediatric diarrheal cases contributing to one-third of reported diarrheal cases in children aged 6–11 months, with a peak incidence during the dry and cool season (October–February).[11] Genotype G9 was the most predominant genotype detected in Thailand in 2000–2002,[12–14] G2 in 2003, G1 in 2004–2009,[13–17] and G3 in 2009–2011.[17] The P genotype, P[8], predominated in 2000–2011, followed by P[4].[13–18] The most common rotavirus genotype combinations detected in Thailand are G1P[8], G9P[8], G2P[4], and G3P[8]. In addition, the uncommon genotypes G2P[8], G3P[3], G3P[9], G3P[10], G3P[19], G12P[6], and G12P[8] were also detected in 2000–2011.[19]

This study reports the prevalence and molecular epidemiology of rotavirus infections among children with and without acute gastroenteritis from a hospital-based surveillance in four different regions in Thailand in 2008–2010. This study provides useful epidemiological data regarding the circulating rotavirus genotypes before the implementation of a rotavirus vaccine in Thailand.

MATERIALS AND METHODS

Source of specimens. Stool samples were collected as part of regional surveillance for diarrhea etiology in Thai children aged 3–60 months. The surveillance was set up at major hospitals in four regions of Thailand: Chiang Rai (CR) in the northern region, Nakhon Ratchasima (Korat) (KR) in the northeastern region, Phitsanulok (PN) in the central region, and Surat Thani (SR) in the southern region: from March 2008 to August 2010 (Figure 1). Cases were inpatient or outpatient children who had three or more unformed stools per 24 hours

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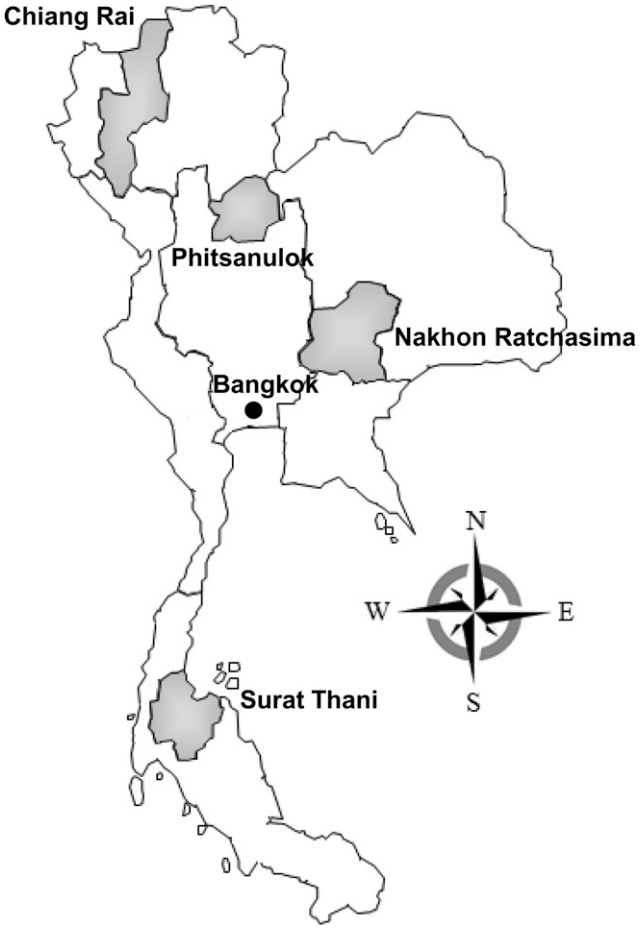

FIGURE 1. Location of the four regional sites used in this study: Chiang Rai Province in the northern region, Nakorn Ratchasima Province in the northeastern region, Phitsanulok Province in the central region, and Surat Thani Province in the southern region.

with at least one additional symptom (nausea, vomiting, abdominal pain, fatigue/lethargy, or fever). Controls were defined as children of the same age range who were seen or admitted at the same hospital with no history of diarrhea within the past 2 weeks. Samples were frozen at −70°C until processed. The study was approved by the ethical review committees of the Ministry of Public Health, Thailand, and the Walter Reed Army Institute of Research, Silver Spring, MD.

Detection of group a rotavirus. RNA were extracted from stool samples using NucliSens® Magnetic Extraction kit according to the manufacturer's instruction (bioMérieux, Lyon, France). The extracted RNA was detected by real-time reverse transcription–polymerase chain reaction (RT-PCR) to amplify the *vp6* gene as described previously.[20] Antigen detection of rotavirus by enzyme-linked immunosorbent assay (ELISA) using RIDASCREEN® Rotavirus EIA kit (R-Biopharm AG, Darmstadt, Germany) was also performed in parallel.

Genotyping of rotavirus by conventional RT-PCR. Samples that were positive by real-time RT-PCR and/or ELISA were further characterized for their G and P genotypes by conventional RT-PCR using a method described previously by Silapong et al.[20] Briefly, RNA was treated with DNase and was used as a template to generate complementary DNA fragments of *vp4* and *vp7* genes, which were then used as a template to amplify a smaller fragment for each gene. A size-specific band indicates each G and P genotype. Samples that did not generate a usable template by RT-PCR were characterized further by nested multiple PCR using various primer combinations.[20] The presence of a size-specific band indicates each G and P genotype. For samples that did not have identifiable band, *vp4* and *vp7* genes were amplified and cloned into pSC-A-amp/kan PCR Cloning Vector (StrataClone PCR Cloning Kit; Agilent Technologies, Santa Clara, CA) and sent to a commercial company for sequencing (Macrogen, Seoul, South Korea).

Phylogenetic analyses. Nucleotide sequences of *vp4* (P genotype) and *vp7* (G genotype) genes were verified for consensus sequences using Sequencher software version 4.1.2 (Gene Codes Corporation, Ann Arbor, MI). A phylogenetic tree for each genotype was generated using neighbor joining with Kimura's two-parameter model with 1,000 bootstrap replicates in MEGA version 6.[21] Sequences of prototypes for each G and P genotype from GenBank were used as references.

Statistical analysis. The differences among proportions were analyzed by χ^2 test in IBM SPSS® Statistics version 22 (IBM Corporation, Armonk, NY).

RESULTS

A total of 3,470 stool samples (1,709 cases and 1,761 controls) were tested for rotavirus by real-time RT-PCR and/or ELISA. The results showed that rotavirus was positive in 486 samples (458 of cases, 26.8% and 28 of controls, 1.6%). Greater than 70% of children with rotavirus infection were younger than 2 years. Cases were most prevalent during the cooler months, specifically from January 2009 to March 2009, with a 52.0–69.0% detection rate (Figure 2). Rotavirus was detected more among hospitalized cases (34%) than outpatient cases (18%) ($P < 0.001$). Of the 458 cases in four regions, rotavirus was detected at the highest percentage in CR (37.1%; 198/533), followed by SR at 30.8% (111/360), KR at 24.1% (86/357), and PN at 13.7% (63/459). Rotavirus was detected every month in CR, located in the northern part of Thailand, where the weather is relatively cooler than that in the rest of the country.

All the rotavirus positive samples by real-time RT-PCR or ELISA were analyzed further for their G and P genotypes. G1 predominated in all regions (36.4–50.0%), followed by G2 (13.2–34.4%), except in the central region (PN), where G12 was the second most common genotype. Overall, G3, G9, and G12 were detected at 8.2%, 9.1%, and 5.8 %, respectively, whereas G4 was detected at 1%. There was no G4 detected in KR and SR sites, and G12 was not detected in SR. Eleven samples were non-typeable for G genotype (Table 1). Three common P genotypes—P[4], P[6], and P[8]—were detected at all four sites. P[8] was the most abundant P genotype detected at 67.5%, followed by P[4] and P[6] at 25.7% and 4.1%, respectively. There were 11 non-typeable P genotypes detected during the study (Table 1).

The distribution of rotavirus genotype combinations detected in the four regions of Thailand is shown in Table 2. The most prominent genotype combinations from all four sites were G1P[8] and G2P[4]. The uncommon genotype combinations—G1P[4], G1P7[5], G1P[6], G2P[6], G2P[8], G4P[6], G5P[19], G9P[4], G9P[6], G12P[6], and G12P[8]—were also detected at a combined rate of 13.4%. Sixteen samples (3.3%) were not-typeable for either or both genotypes.

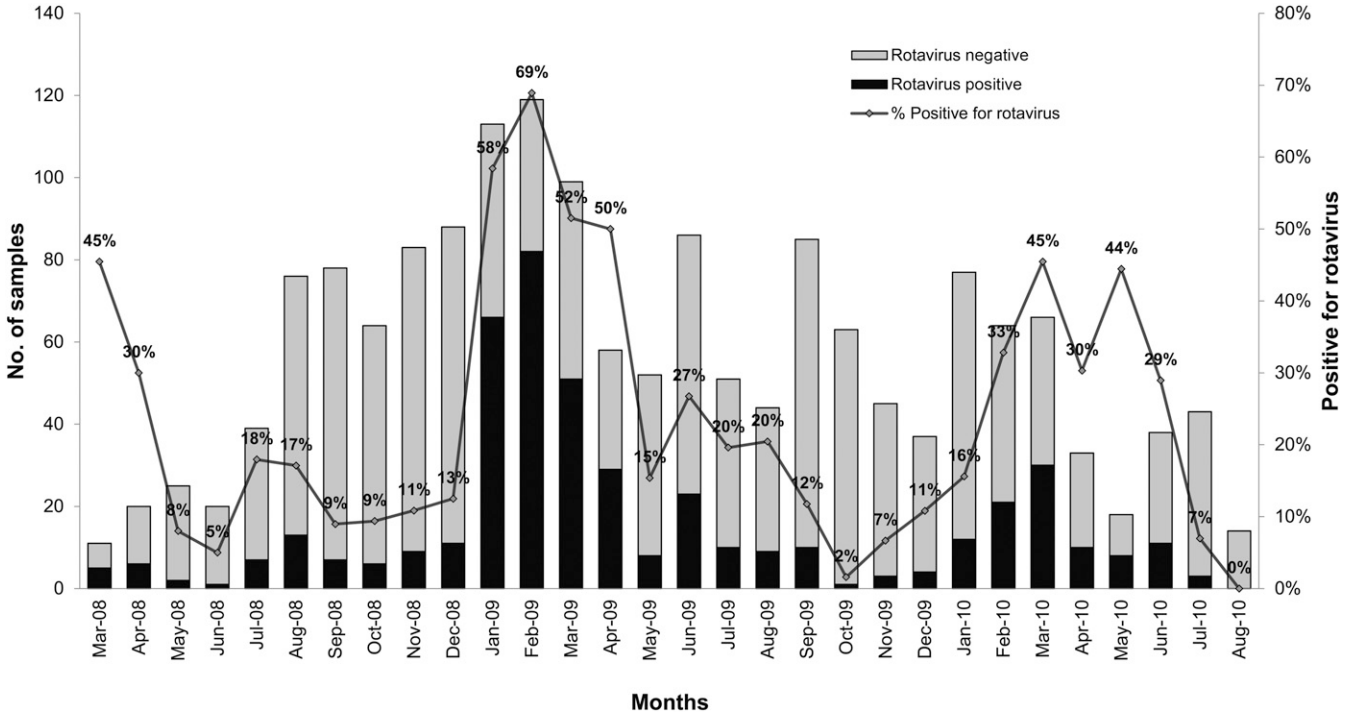

FIGURE 2. Distribution of rotavirus group A in young Thai children with acute diarrhea cases from four regions of Thailand by month from March 2008 to April 2010.

An annual analysis showed that G2P[4] was the most common genotype combination detected in 2008 in the north (CR), but G1P[8] became the most common genotype combination in the subsequent surveillance year (2009), except KR, where G2P[4] predominated. G1P[8] remained the major strain in the third surveillance year (2010), except at SR. An uncommon genotype, G12P[8], was the second most predominant genotype at PN in 2009 (Table 2).

Nucleotide sequences of the *vp7* gene (829 bp) showed 14 samples that clustered with the G genotypes: G1, G2, G3, G4, G5, and G9 (Figure 3A). Seven of the sequenced samples were clustered into sub-lineage Ic of lineage I of rotavirus G1, which includes strains from Asian countries such as Bangladesh, India, China, and Vietnam, with nucleotide identities ranging from 96.1% to 99.4%. In addition, one sample (SRC336-10) clustered into lineage III of rotavirus G1 which associated with P7[5], showing 100% identity to RotaTeq G1-reassortant vaccine strain containing G1, G2, G3, and G4 with P7[5].

Two samples from the north clustered into sub-lineage IIa of rotavirus G2. PNC372-10 and PN395-10 from the central part clustered into sub-lineage IIId of rotavirus G3 lineage III, and lineage V of rotavirus G4, respectively (Figure 3A). KRC276-09 from the northeastern region was clustered into lineage I of rotavirus G5 which was associated with P[19] and showed

TABLE 1

Distribution of G and P genotypes from rotavirus-positive stool samples in cases and controls by real-time reverse transcription–polymerase chain reaction and/or enzyme-linked immunosorbent assay in Thai children from March 2008 to April 2010

Genotype	No. of rotavirus strains (%)				
	CR site (*N* = 208)	KR site (*N* = 88)	PN site (*N* = 68)	SR site (*N* = 122)	Total (%) (*N* = 486)
G genotype					
G1	**104 (50.0)**	**32 (36.4)**	**30 (44.1)**	**61 (50.0)**	**227 (46.7)**
G2	56 (26.9)	23 (26.2)	9 (13.2)	42 (34.4)	130 (26.7)
G3	21 (10.1)	9 (10.2)	8 (11.8)	2 (1.6)	40 (8.2)
G4	4 (1.9)	0 (0.0)	1 (1.5)	0 (0.0)	5 (1.0)
G5	0 (0.0)	1 (1.1)	0 (0.0)	0 (0.0)	1 (0.2)
G9	9 (4.3)	14 (15.9)	7 (10.3)	14 (11.5)	44 (9.1)
G12	7 (3.4)	9 (10.2)	12 (17.6)	0 (0.0)	28 (5.8)
G-non-typeable	7 (3.4)	0 (0.0)	1 (1.5)	3 (2.5)	11 (2.3)
P genotype					
P[4]	55 (26.4)	20 (22.7)	7 (10.3)	43 (35.3)	125 (25.7)
P7[5]	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.8)	1 (0.2)
P[6]	4 (1.9)	14 (15.9)	2 (2.9)	0 (0.0)	20 (4.1)
P[8]	**147 (70.7)**	**53 (60.2)**	**56 (82.4)**	**72 (59.0)**	**328 (67.5)**
P[19]	0 (0.0)	1 (1.1)	0 (0.0)	0 (0.0)	1 (0.2)
P-non-typeable	2 (1.0)	0 (0.0)	3 (4.4)	6 (4.9)	11 (2.3)

CR = Chiang Rai; KR = Nakorn Rachasrima (Korat); PN = Phitsanulok; SR = Surat Thani. Bold values indicate genotypes identified with the highest percentage at each site.

TABLE 2
Distribution of detected rotavirus strains in Thai children from four regions of Thailand, 2008–2010

	CR (*N* = 208)			KR (*N* = 88)			PN (*N* = 68)			SR (*N* = 122)			
Genotypes	2008 (%) (*N* = 40)	2009 (%) (*N* = 128)	2010 (%) (*N* = 40)	2008 (%) (*N* = 14)	2009 (%) (*N* = 40)	2010 (%) (*N* = 34)	2008 (%) (*N* = 3)	2009 (%) (*N* = 37)	2010 (%) (*N* = 28)	2008 (%) (*N* = 11)	2009 (%) (*N* = 108)	2010 (%) (*N* = 3)	Total (%) *N* = 486
Common genotypes													
G1P[8]	1 (2.5)	**70 (54.7)**	**28 (70.0)**	2 (14.3)	5 (12.5)	**21 (61.8)**	**2 (66.7)**	**16 (43.2)**	**12 (42.9)**	**9 (81.8)**	**48 (44.4)**	–	214 (44.0)
G2P[4]	**34 (85.0)**	15 (11.7)	–	2 (14.3)	**16 (40.0)**	–	1 (33.3)	6 (16.2)	–	1 (9.1)	38 (35.2)	–	113 (23.3)
G3P[8]	–	17 (13.3)	4 (10.0)	–	3 (7.5)	6 (17.6)	–	1 (2.7)	7 (25.0)	1 (9.1)	–	1 (33.3)	40 (8.2)
G4P[8]	–	2 (1.6)	–	–	–	–	–	–	–	–	–	–	2 (0.4)
G9P[8]	2 (5.0)	4 (3.1)	3 (7.5)	**5 (35.7)**	8 (20.0)	–	–	1 (2.7)	3 (10.7)	–	10 (9.3)	–	36 (7.4)
Uncommon genotypes*													
G1P[4]	–	4 (3.1)	–	–	2 (5.0)	–	–	–	–	–	2 (1.9)	–	8 (1.7)
G1P7[5]	–	–	–	–	–	–	–	–	–	–	–	1 (33.3)	1 (0.2)
G1P[6]	–	1 (0.8)	–	–	–	2 (5.9)	–	–	–	–	–	–	3 (0.6)
G2P[6]	–	–	–	1 (7.1)	1 (2.5)	1 (2.9)	–	–	–	–	–	–	3 (0.6)
G2P[8]	1 (2.5)	4 (3.1)	2 (5.0)	–	2 (5.0)	–	–	2 (5.4)	–	–	3 (2.8)	–	14 (2.9)
G4P[6]	1 (2.5)	1 (0.8)	–	–	–	–	–	–	1 (3.6)	–	–	–	3 (0.6)
G5P[19]	–	–	–	–	1 (2.5)	–	–	–	–	–	–	–	1 (0.2)
G9P[4]	–	–	–	–	–	–	–	–	–	–	2 (1.9)	–	2 (0.4)
G9P[6]	–	–	–	–	–	1 (2.9)	–	1 (2.7)	–	–	–	–	2 (0.4)
G12P[6]	1 (2.5)	–	–	4 (28.6)	1 (2.5)	3 (8.8)	–	–	–	–	–	–	9 (1.9)
G12P[8]	–	3 (2.3)	3 (7.5)	–	1 (2.5)	–	–	10 (27.0)	2 (7.1)	–	–	–	19 (3.9)
Non-typeables													
G1 PNT	–	–	–	–	–	–	–	–	–	–	1 (0.9)	–	1 (0.2)
G9 PNT	–	–	–	–	–	–	–	–	2 (7.1)	–	1 (0.9)	1 (33.3)	4 (0.8)
GNT P[4]	–	2 (1.6)	–	–	–	–	–	–	–	–	–	–	2 (0.4)
GNT P[8]	–	3 (2.3)	–	–	–	–	–	–	–	–	–	–	3 (0.6)
GNT PNT	–	2 (1.6)	–	–	–	–	–	–	1 (3.6)	–	3 (2.8)	–	6 (1.2)

CR = Chiang Rai; GNT = non-typeable G-genotype; KR = Nakorn Rachasrima (Korat); PN = Phitsanulok; SR = Surat Thani; PNT = on-typeable P-genotype. Bold values indicate genotypes identified with the highest percentage in a given year.
* Uncommon genotypes were classified based on the definition provided by the WHO.[9]

92–92.4% sequence similarity to strains CMP178 and 206 which were detected in piglets in Chiang Mai.[22] Last, SR142-09 from the south was clustered into lineage III of rotavirus G9, which is similar to strains isolated from Asia, Europe, and the United States (Figure 3A).

Phylogenetic analysis of the *vp4* gene (640 bp) clustered 12 samples into different P genotypes: P[4], P7[5], P[6], P[8] and P[19] (Figure 3B). Eight samples were clustered into lineage III of rotavirus P[8], which is the most common P genotype. These samples had sequence similarity ranging from 96.9% to 100% to strains isolated from Belgium, Japan, Russia, and Thailand. Rotavirus P[4] (SRC189-09) and P[6] (PNC203-09) samples were clustered into lineage V and lineage I of their respective genotypes. Both samples had sequence similarity (93–97.8%) to strains isolated from Asian countries.

DISCUSSION

This study reports the molecular epidemiology of rotavirus from 2008 to 2010 in young Thai children from four study sites located in northern, northeastern, central, and southern parts of Thailand, representing the major geographic regions (Figure 1). Rotavirus was responsible for a quarter of children with acute gastroenteritis, whereas only a small percentage of rotavirus was detected among children without gastroenteritis symptoms.

Epidemiological surveillance of rotavirus by several groups in Thailand has shown a shift in the predominant genotype combinations over the years, starting with G9P[8] during 2000–2002,[13,14] G2P[4] in 2003,[14] G1P[8] during 2004–2009,[14–16,18,23] and G3P[8] during 2009–2011.[17] However, most of the studies only focused on one geographical region which might not be completely representative of the shift in strains and circulating strains in other regions of Thailand during the same period. Moreover, predominant genotypes of rotavirus may differ by population studied, such as hospitalized cases versus outpatient diarrhea cases. Certain genotypes, for example, G9, have been reported to be associated with more severe diseases.[20] In this study, G1P[8] was the most common genotype detected between 2009 and 2010. However, analysis based on geography revealed a slightly different trend of the predominating rotavirus strains as G2P[4] predominated during the first and second years at CR and KR sites (Table 2), which further indicates the cycling of the predominating genotype. In addition, G4 was detected at 1.0%, which is lower than 5.3% reported by Jiraphongsa et al.,[12] whereas other similar studies have no reported G4 genotypes in Thailand between 2002 and 2011.[13–18,23] The phylogenetic analysis of rotavirus strains into lineages/sublineages of G1, G2, G3, G9, P[4], P[6], and P[8] (Figure 3A and B) shows that rotavirus genotypes in Thailand are similar to genotypes found in other geographical regions,[15,17,24–27] including Europe, America, and Asia

The detection of 1.6% of rotavirus in control samples is similar to data reported by Mullick et al. (2014), where 2% of asymptomatic Indian children were rotavirus positive.[28] Previous studies have demonstrated that the fecal excretion by asymptomatic persons may be a source of infection for susceptible person.[29,30] However, information on asymptomatic rotavirus infection is rarely reported. Interestingly, G5P[19] from non-diarrhea controls may have derived from a close association between human and piglets (Figure 3), as there have been reports of G3P[19] and G5P[13] from piglet

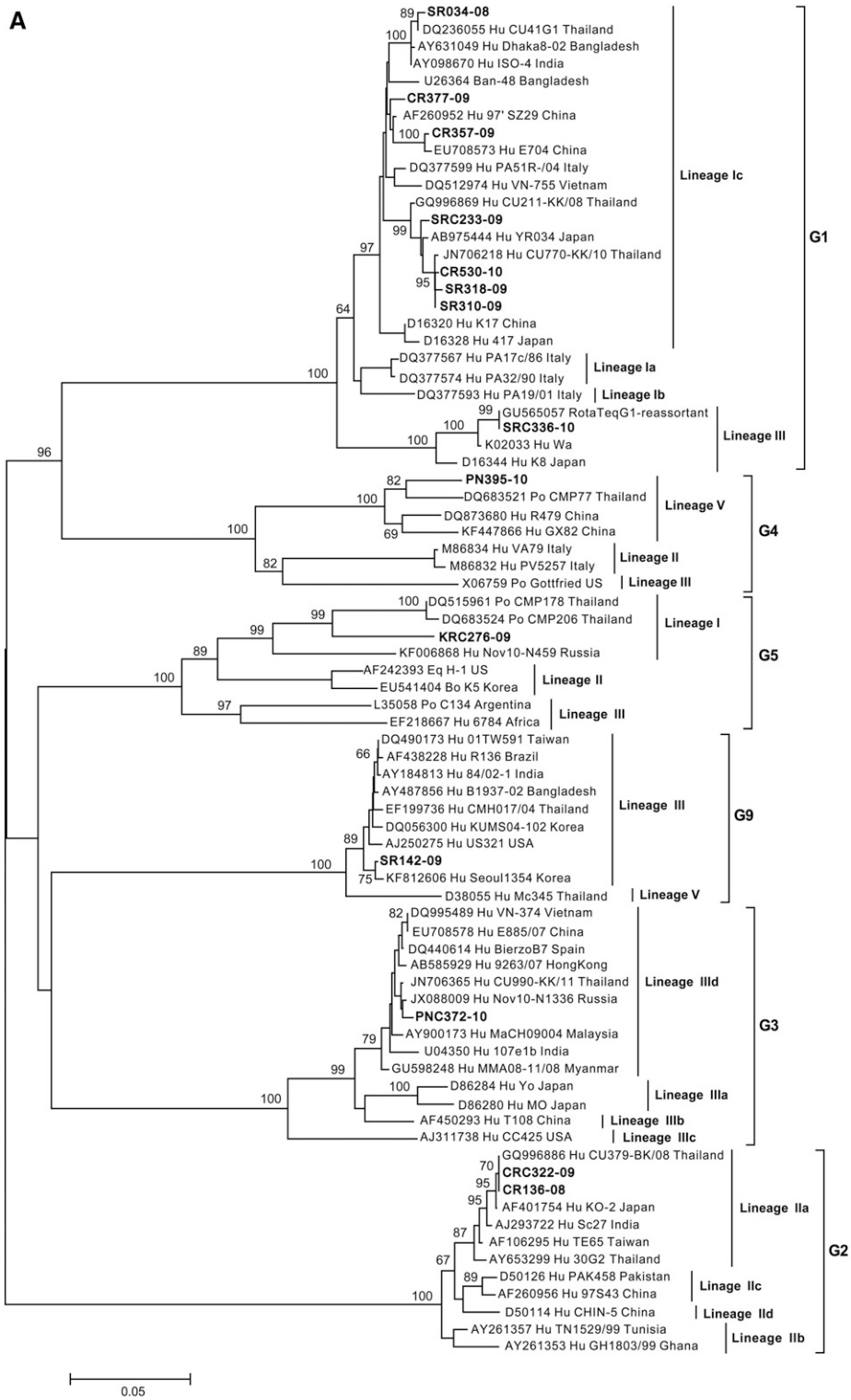

FIGURE 3. Neighbor-joining phylogenetic tree generated from partially sequenced (**A**) *vp7* gene (G genotype) and (**B**) *vp4* gene (P genotype). Representative sequence prototypes for each genotype were used in the analysis, and accession numbers are listed next to the name. Numbers at nodes indicate bootstrap values.

stool in Chiang Mai in 2000–2001 and 2008, respectively.[31,32] These results indicate a possible recombination between human and animal rotavirus strains, demonstrating the diversity of circulating strains in Thailand. This study is the first to report rotavirus G5P[19] in Thailand from an asymptomatic patient. This study was a passive surveillance and was limited by the small sample size; however, the surveillance was conducted at multiple regional sites representing all of the four main regions in Thailand, which provides a wider range of rotavirus epidemiological pattern.

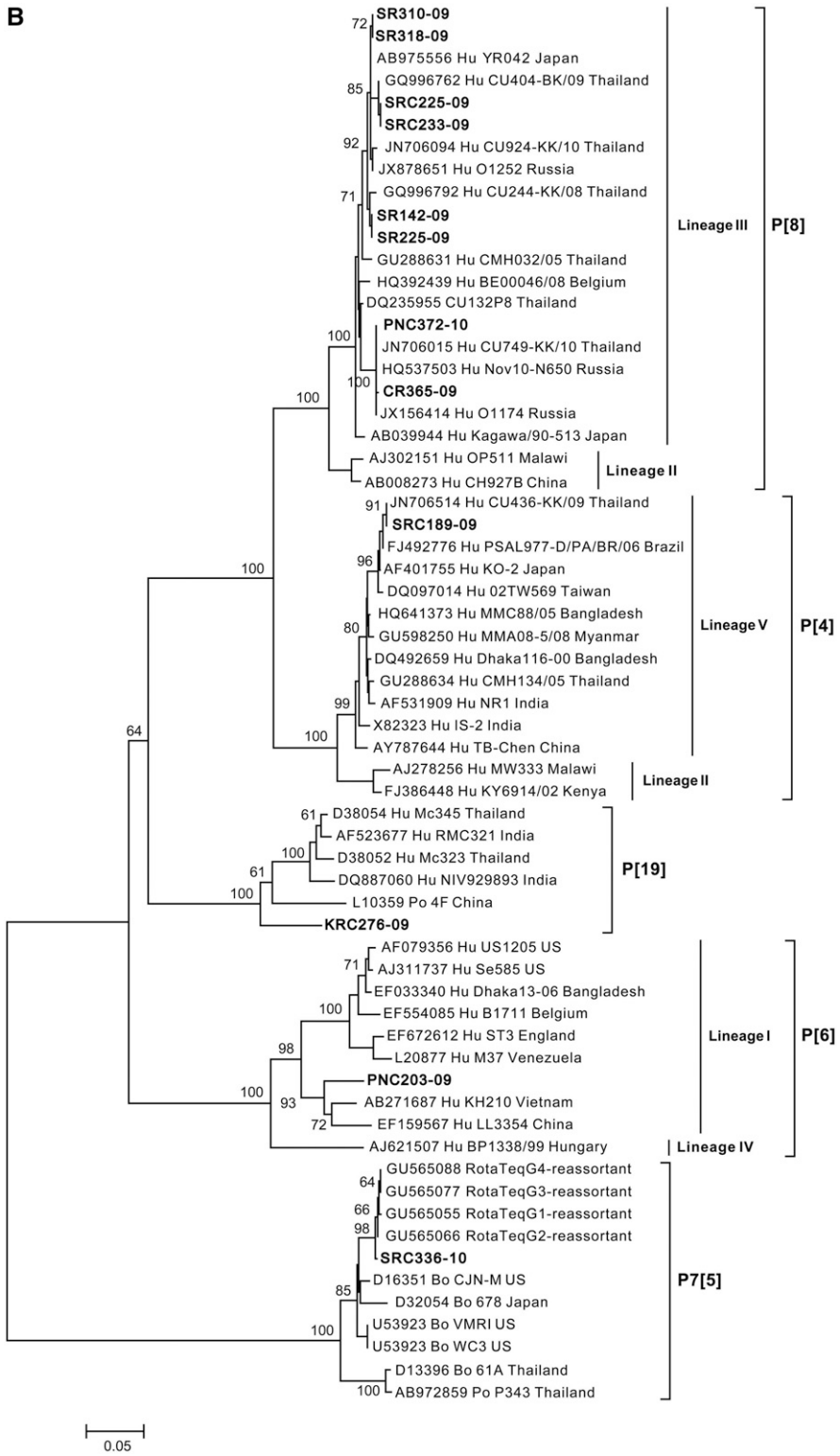

FIGURE 3. (Continued).

The WHO reported that five common genotypes (G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]) cause nearly 100% of rotavirus infection in developed countries, whereas uncommon genotypes cause 10–35% of rotavirus infection in developing countries.[9] The percentage of uncommon rotavirus genotypes (13.4%) reported in this study is consistent with the reported trend.[9] Uncommon genotypes found in this study—G1P7[5], G5P[19], G9P[4], and G9P[6]—add to the existing list of uncommon genotypes reported to circulate in Thailand.[19,33,34] The presence of uncommon genotypes and the regular shifting of predominant genotypes may contribute to the likelihood of uncommon genotypes to become

common global genotypes.[5,7] This will have an important implication for the development of an effective vaccine with genotype cross protection against rotavirus in the future, specifically in Thailand.

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