

PHYLOGENETIC ANALYSIS OF HUMAN ROTAVIRUS IN MYANMAR : DETECTION OF INDIAN-BANGLADESHI G1/G2 LINEAGES, CHINESE G3 LINEAGE AND OP354-LIKE P[8] LINEAGE (P[8]B SUBTYPE)

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Abstract. As a first phylogenetic study of human rotavirus in Myanmar, VP7 and VP8* gene sequences of 5 group A human rotaviruses detected in children in Yangon City were determined and analyzed for their relatedness to rotavirus strains reported in other countries. VP7 genes of the two G1P[8] strains and the two G2P[4] strains clustered phylogenetically with those of Indian-Bangladeshi lineages with extremely high sequence identities. In contrast, a G3P[8] strain exhibited a close relatedness of VP7 gene to G3 rotaviruses currently prevailing in China, which had been referred to as a new variant G3 rotavirus. While VP8* genes of P[4] and P[8] strains clustered with those of Indian and Bangladeshi strains, only the G1 strain was grouped into a rare P[8] subtype, *ie*, P[8]b (OP354-like P[8]) with close relatedness to the P[8]b strains in eastern India and Thailand. The coexistence in Myanmar of G1/G2 and G3 rotaviruses, which are virtually identical to those predominating in India/Bangladesh and China, respectively, suggests the spread of these predominant rotaviruses from the two regions into Myanmar.

Key word: rotavirus, phylogenetic study, gene sequence, Myanmar

INTRODUCTION

Rotavirus is the leading cause of severe acute diarrhea among infants and young children worldwide, resulting in an estimated 527,000 deaths annually (Parashar *et al*, 2009). Rotavirus comprises the genus *Rotavirus* within the family Reoviridae, and has a genome consisting of 11 segments of double-stranded RNA.

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Rotavirus is a nonenveloped particle composed of 3 concentric layers, namely, outer capsid, inner capsid and core (Estes and Kapikian, 2007). Based on antigenicity of the inner capsid protein VP6, rotaviruses are classified into at least 7 species, namely, group A-G. While 3 groups (A-C) are known to be diarrheal pathogens in humans, group A rotaviruses have been most commonly associated with infections in children (Estes and Kapikian, 2007).

The outer capsid of rotavirus particle consists of 2 structural proteins, VP7 and VP4, which have neutralization antigens and define G and P serotypes of group A

rotavirus, respectively. Based on the sequence diversity of VP7 and VP4 genes, group A rotaviruses are discriminated into G genotype (G type) and P genotype (P type), respectively. VP8*, an N-terminal portion of trypsin cleavage products of VP4, is highly divergent among rotaviruses and is associated with P types (Hoshino and Kapikian, 1994). Among the 24 G types and 33 P types of group A rotaviruses classified so far (Maes *et al*, 2009), 5 G types (G1-G4, G9) and 3 P types (P[4], P[6], P[8]) account for most of the G/P types of human rotaviruses detected globally.

A large number of epidemiologic studies on human rotavirus conducted to date revealed that the predominant G/P types are different depending on countries or regions, and change by years or seasons (Bányai *et al*, 2004; Reidy *et al*, 2005; Santos and Hoshino, 2005; Lennon *et al*, 2008). Epidemiological surveillances of the G/P types have been significant in monitoring the impact of rotavirus vaccines, which have been introduced in many countries (Widdowson *et al*, 2009). Furthermore, phylogenetic analyses of VP7 genes and other rotavirus protein genes have elucidated the origins of virus strains as well as individual gene segments and regional or global expansion of specific rotavirus clones (Rahman *et al*, 2007b; Wang *et al*, 2009). Within a single G type, different VP7 gene lineages may be associated with subtle differences in antigenic composition of the VP7 protein, and such difference might influence the efficacy of rotavirus vaccines (Santos and Hoshino, 2005).

In Myanmar, a high disease burden of rotavirus infection among children was reported in a study conducted in Yangon, in which rotavirus was identified in 53% of stool specimens from diarrheal diseases and up to 10% of all hospitalization of children was attributable to rotavirus infection

(Moe *et al*, 2005). Rotavirus G/P types in Myanmar was described in only one study, with G1P[8] being the most common genotype in 2004-2005 (Moe *et al*, 2009). However, phylogenetic analysis of rotavirus strains has never been performed. The present study is the first phylogenetic analysis of human group A rotaviruses conducted in Myanmar in order to characterize them at the molecular level and elucidate their relatedness to rotaviruses distributed to other countries.

MATERIALS AND METHODS

Rotavirus samples and genotyping

Viral dsRNA was extracted from 400 µl of 10% stool suspensions using QIAamp Viral RNA Mini kit (Qiagen Science, MD, USA). G and P types, and VP6 and NSP4 genotypes were determined by RT-PCR with the primers reported previously (Gentsch *et al*, 1992; Taniguchi *et al*, 1992; Wang *et al*, 2007). VP7 and VP8* genes of 5 group A rotavirus specimens were successfully amplified while sufficient amounts of viral RNA were not available from other specimens. Stool specimens were collected from children in January and February 2008. Rotavirus strains in these specimens were designated as MMA08-4, MMA08-5, MMA08-8, MMA08-10, and MMA08-11.

Phylogenetic analysis

Full-length VP7 gene and VP8*-encoding region of VP4 gene were sequenced directly from RT-PCR products amplified from these genes. RT-PCR was performed using primers C1 and C2 for VP7 gene (Taniguchi *et al*, 1992), and primers Con3 and Con2 for VP8* gene (Gentsch *et al*, 1992). PCR amplicons were purified using Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, WI). Sequencing reaction was performed with fluorescent

dideoxy chain termination chemistry using BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). DNA sequence was determined using ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). GENETYX-Win Version 5.1 (Software Development, Tokyo, Japan) was used to perform pairwise alignments and to calculate sequence identity of viral genes from different strains. Phylogenetic analysis was performed with MEGA software version 4.1 based on the neighbor-joining method. Phylogenetic distances were measured by Kimura two-parameter model, and phylogenetic trees were supported statistically by bootstrapping with 1,000 replicates. Nucleotide sequences of VP7 and VP8* genes for the 5 group A rotaviruses determined in the present study were deposited in GenBank database under accession numbers GU598244-GU598253.

RESULTS

The 5 rotavirus strains analyzed were assigned to 3 genotypes, namely, G1P[8] (MMA08-8 and MMA08-10), G2P[4] (MMA08-4 and MMA08-5), and G3P[8] (MMA08-11)(data not shown). Three strains, MMA08-8 and MMA08-10, and MMA08-11, belonged to VP6 genotype II (I1) and NSP4 genotype B (E1), while the strains MMA08-4 and MMA08-5 were assigned to VP6 genotype I (I2) and NSP4 genotype A (E2).

VP7 genes of G1 strains, MMA08-8 and MMA08-10, showed 99.1% sequence identity to each other. When a phylogenetic tree was constructed with G1 rotaviruses, these strains were located in lineage 6, sublineage S4, including rotaviruses reported mostly in India and Bangladesh recently (Fig 1A). Strains MMA08-8 and MMA08-10 exhibited >99%

sequence identity to rotaviruses in the sublineage S4 of VP7 gene, with slightly lower identities (96.5-97.9%) to rotaviruses in sublineages S1, S2, and S3, which comprise strains from China, Japan, Vietnam, and Thailand.

G2 strains, MMA08-4 and MMA08-5, having 98.8% VP7 gene sequence identity to each other, clustered with rotaviruses from India and Bangladesh in sublineage S3 of lineage 4, in the dendrogram of G2 rotavirus VP7 genes (Fig 1B). These G2 strains in Myanmar showed extremely high VP7 gene sequence identities (>99%) to most of Bangladeshi strains in the sublineage S3, but lower identities (<98%) to other rotaviruses, including those from China, Vietnam, Thailand, Australia, Uruguay, and some African countries.

When a phylogenetic tree of G3 rotavirus VP7 genes was constructed, the strain MMA08-11 clustered with many rotavirus strains reported recently in China, Thailand, Vietnam, Malaysia, Japan, and Russia in the sublineage S4 of the lineage 5 with extremely high sequence identities (>98%) (Fig 1C). Highest sequence identity (99.5%) was found with Chinese strains (E885 and Z235) and a Vietnamese strain (VN-374). On the other hand, MMA08-11 showed lower identities to Japanese strains in subclusters S1 and S2, and USA strains in sublineage S3 (95-97%).

VP8* genes of the P[8] strains, MMA08-10 (G1) and MMA08-11(G3), clustered with strains in the P[8]a lineage, sublineage S2 (Fig 2A). Among the strains in the P[8]a-S2 sublineage, an Indian strain ISO115 showed 99.9% identity to the G1 strain MMA08-10. Whereas VP8* of G1 strain, MMA08-8, was located in the P[8]b-lineage (OP354-like VP4-lineage) represented by Malawian strain OP354, and clustered with strains from

Fig 1A

G1

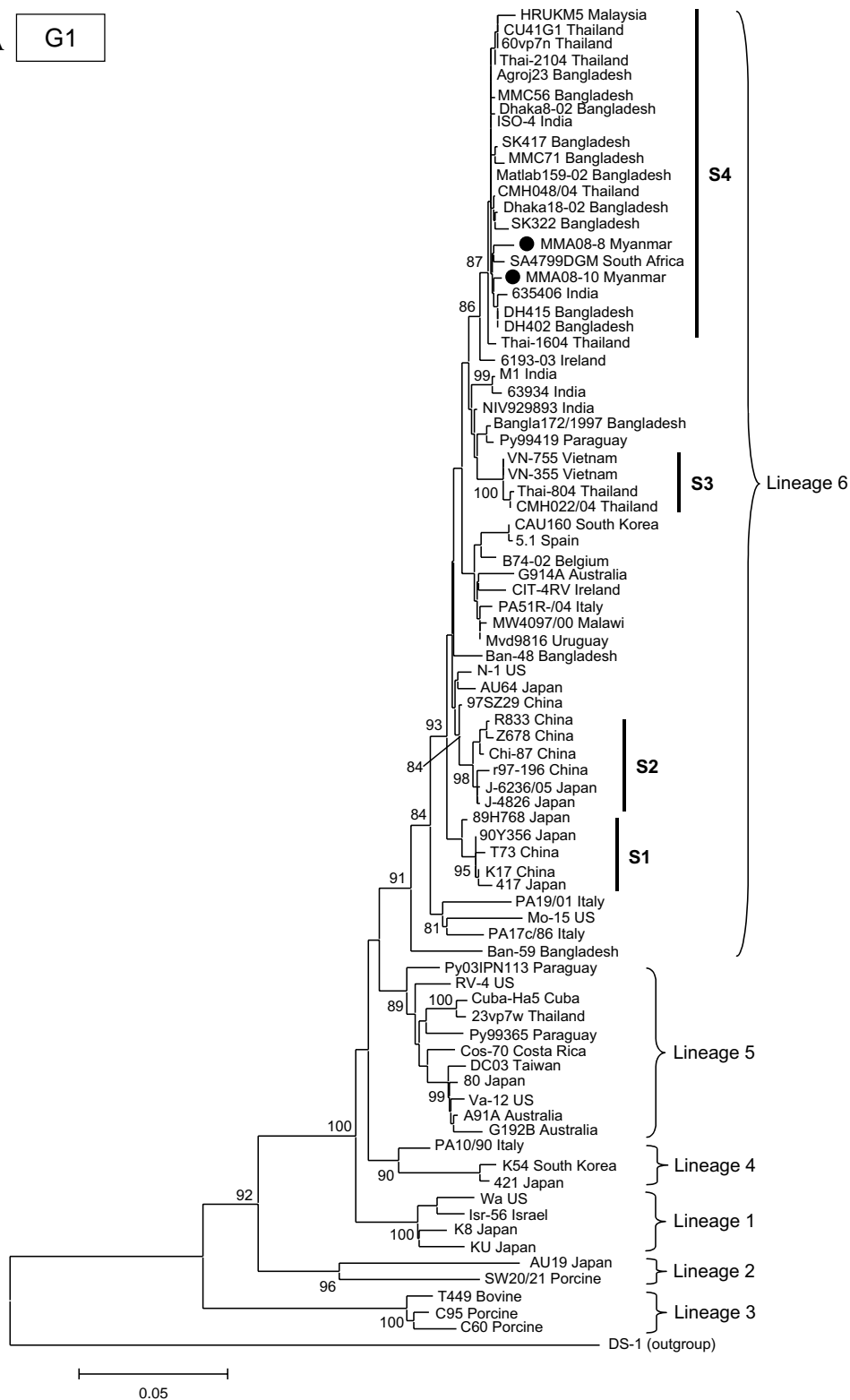
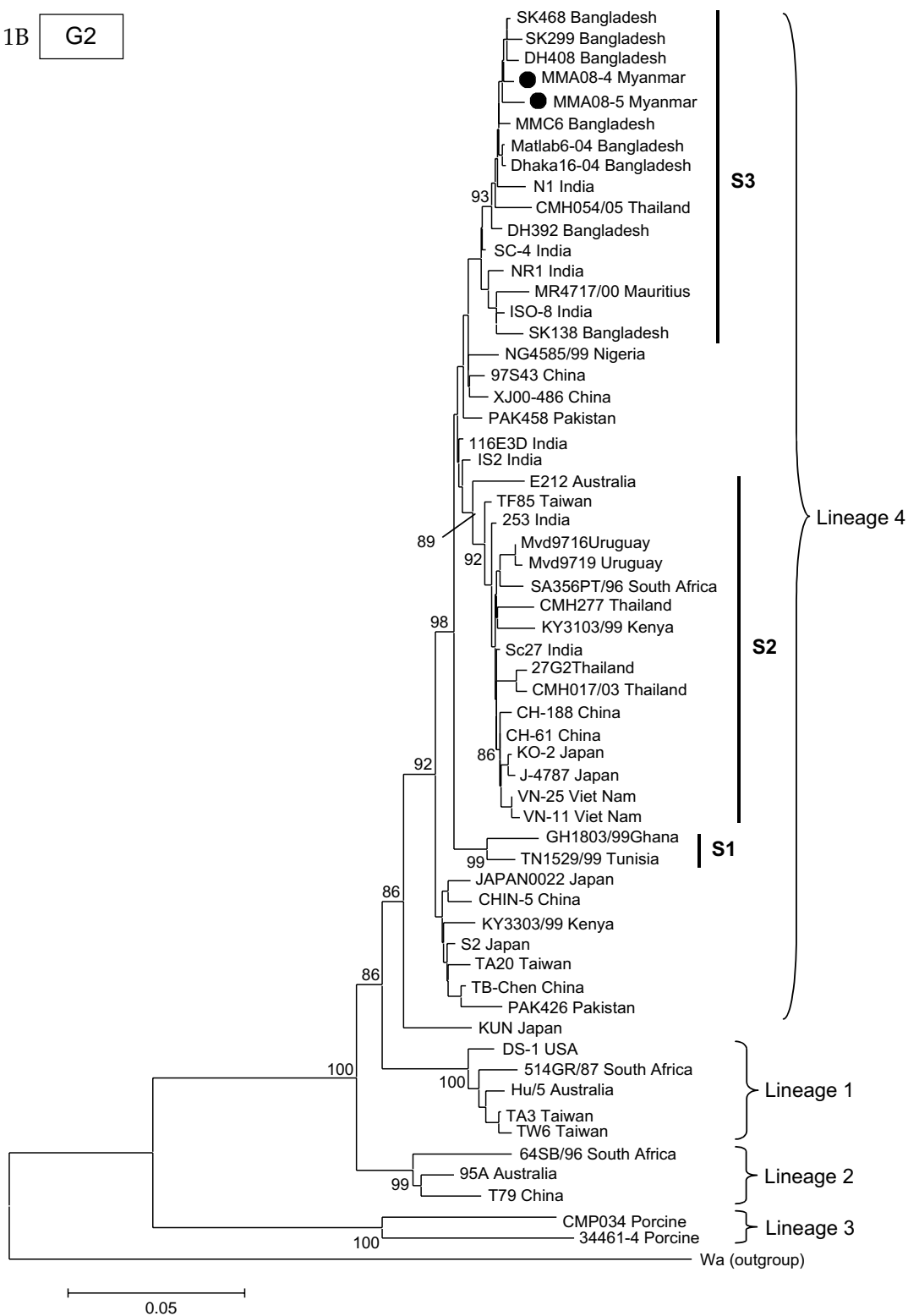


Fig 1B G2



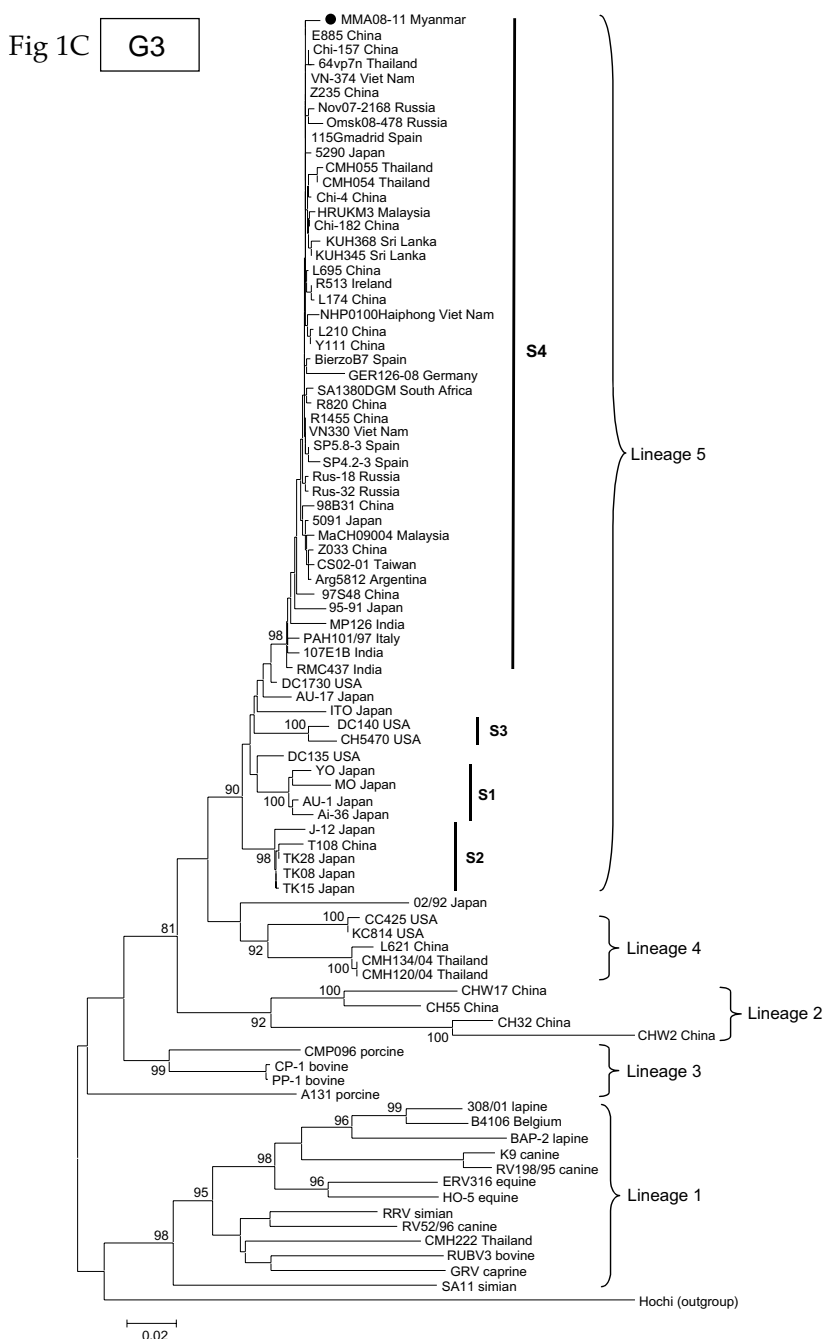
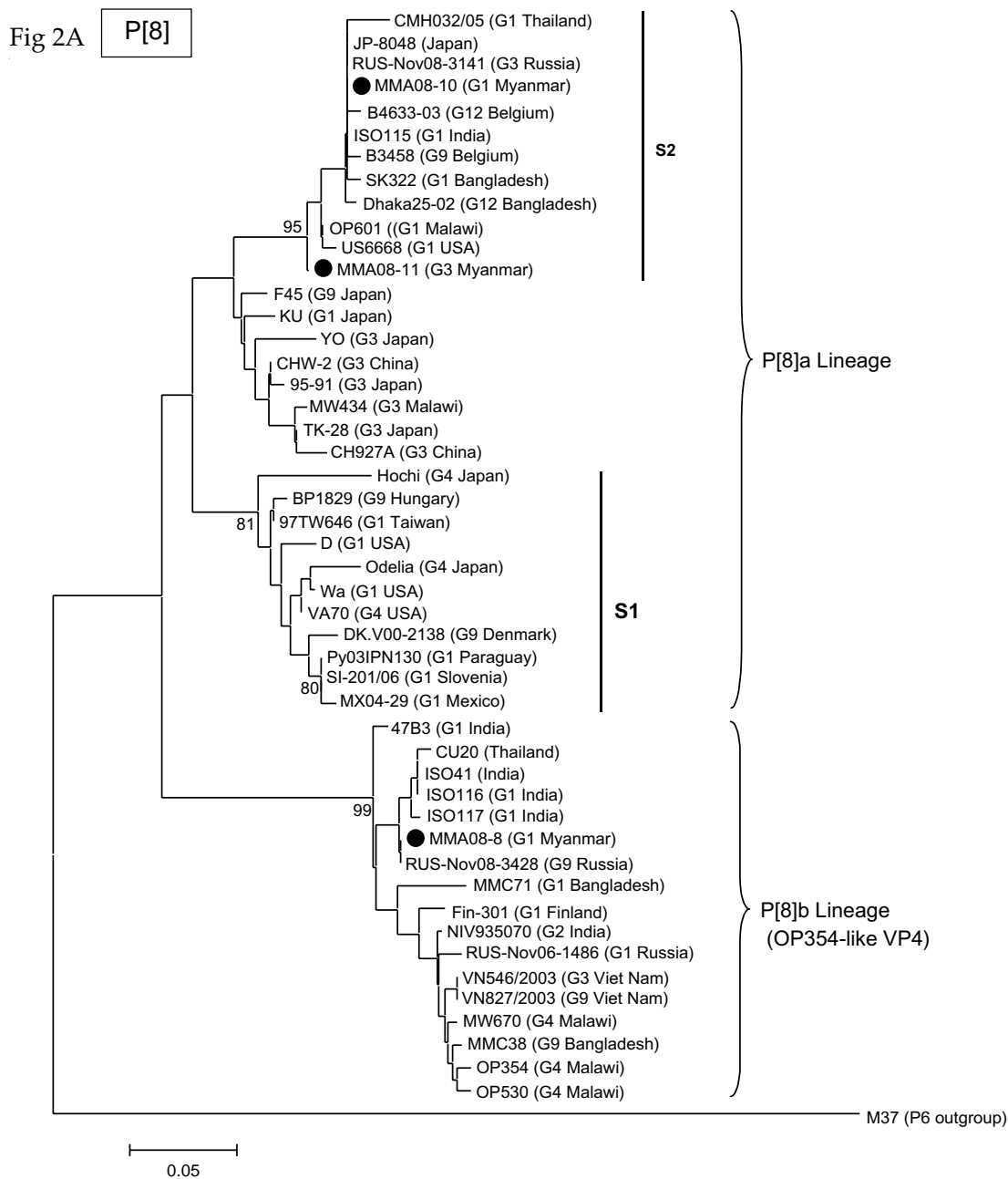


Fig 1—Phylogenetic dendrograms of VP7 genes for G1 rotaviruses (A), G2 rotaviruses (B), and G3 rotaviruses (C), dendrograms were constructed by neighbor-joining method with MEGA.4 program. Dendrograms are rooted with the strains at the bottom. Variation scale (substitution per site) is indicated below the dendrogram. Percent bootstrap support is indicated by values at each node, and the values <80 are omitted. Strains analyzed in the present study are indicated by closed circles. Reference sequences used in the analysis were obtained from the GenBank database. Lineage numbers modified from those published previously (Paul *et al*, 2008; Wang *et al*, 2009) were employed. Strains included in individual sublineages (S1-S4) are indicated by vertical bars.



India, Thailand, Bangladesh, Vietnam, Russia, and Finland. Within the P[8]b lineage, the MMA08-8 strain had the highest VP8* sequence identity (98.6-99.5%) to strains from eastern India (eg, ISO116), Thailand and Russia, and showed 98.0%

identity to strain OP354, and 95.4-97.7% identities to other strains. However sequence identity between MMA08-8 and strains in the P[8]a lineage was 86-89%.

P[4] strains MMA08-4 and MMA08-5 had 97.8% identity of VP8* gene to each

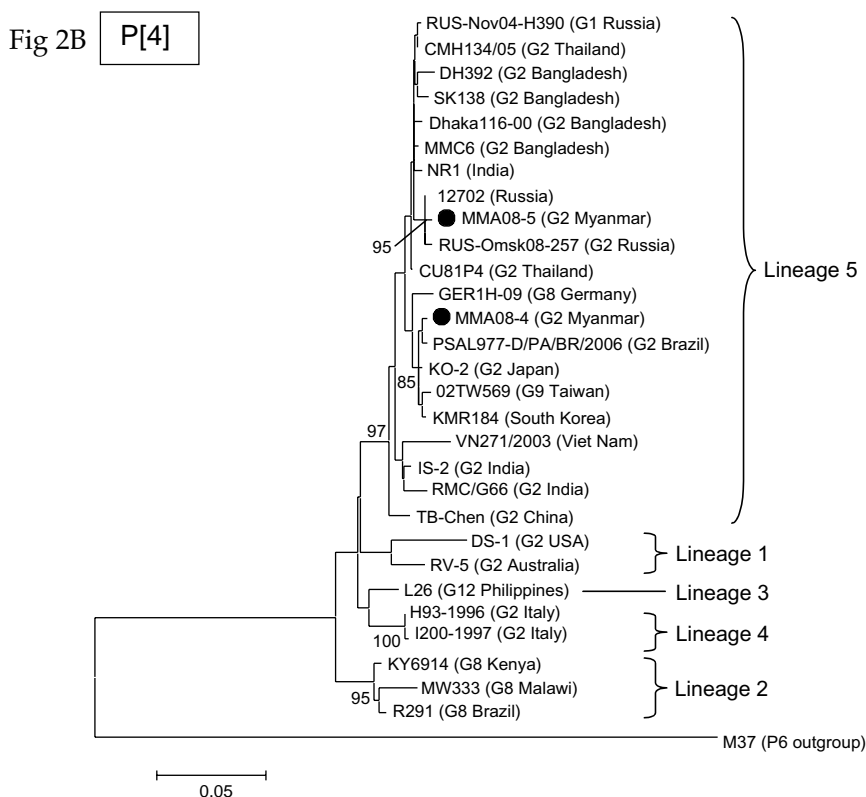


Fig 2—Phylogenetic dendrograms of VP8* genes for P[8] rotaviruses (A) and P[4] rotaviruses (B). Dendrograms were constructed by neighbor-joining method with MEGA.4 program. Dendrograms are rooted with the strains at the bottom. Variation scale (substitution per site) is indicated below the dendrogram. Percent bootstrap support is indicated by values at each node, and the values <80 are omitted. Strains analyzed in the present study are indicated by closed circles. Reference sequences used in the analysis were obtained from GenBank database.

other, and clustered with the majority of G2 rotavirus strains detected in Asian countries in lineage 5 (Fig 2B). VP8* of the two G2 strains in Myanmar exhibited 96.7-99.7% identity to strains in lineage 5, and lower identity (92.0-94.7%) to those in lineages 1-4.

DISCUSSION

Accumulation of extensive epidemiological data of rotavirus genotypes has shown that over 88% of the strains ana-

lyzed worldwide are represented by 4 common G types, G1-G4, with G1 being the most common in Asia, Europe, Americas and Australia (Bányai *et al*, 2004; Reidy *et al*, 2005; Santos and Hoshino, 2005; Lennon *et al*, 2008). However, the predominant genotypes differ depending on region or country, and the incidence of genotypes in the same region shows a yearly fluctuation. In Bangladesh, G2P[4] has been the most prevalent type in both rural and urban areas, followed by G1P[8] since 2004 or 2005, although previous predominant

types are G4 (1990-1996), G9 (1997-2001), and G1 (2001-2004) (Rahman *et al*, 2007a; Paul *et al*, 2008; Dey *et al*, 2009; Zaman *et al*, 2009). Similarly in India, highest prevalence of G2P[4] was reported from 2004 to 2007 (Kang *et al*, 2009; Tatte *et al*, 2010). However, recent detection of G3 strain was extremely rare in India and Bangladesh (Kang *et al*, 2009; Zaman *et al*, 2009). In contrast, G3P[8] has been a single most prevalent type (>80%) in China since 2000 (Fang *et al*, 2005; Wang *et al*, 2007, 2009; Duan *et al*, 2009). Similarly, an overwhelming predominance of G3 rotavirus from 2005 or 2006 was noted also in Mongolia and Vietnam (Ngo *et al*, 2009; Nyambat *et al*, 2009). Phylogenetic analysis revealed that G3 rotaviruses recently prevailing in China was distinct from G3 strains in China from the late 1980s to the 1990s, and have acquired some genetic variations compared with the older G3 viruses (Wen *et al*, 1997; Trinh *et al*, 2007; Wang *et al*, 2009). Therefore, the current G3 virus in China has been considered as a "new variant G3 rotavirus", and its VP7 gene sequence is mostly identical to those of recent strains from Japan, Malaysia, Thailand, and Vietnam (Phan *et al*, 2007; Ngo *et al*, 2009; Wang *et al*, 2009). In China, Mongolia and Vietnam where G3 has been recently dominant, the frequency of G2 is substantially low.

According to the single report on the prevalence of G/P types of rotavirus in Myanmar, the most prevalent type is G1 (46%), followed by G3 (30%) and G2 (14%), but the number of strains analyzed was relatively small ($n = 37$) (Moe *et al*, 2009). Thus, the present epidemiologic feature in terms of G type prevalence in Myanmar seems to be different from those in India/Bangladesh or China. The present study indicated that VP7 genes of G1 and G2 rotaviruses in Myanmar belong to the same sublineages/lineages as those of cur-

rently prevailing viruses in Bangladesh and India, and a G3 rotavirus in Myanmar is virtually identical to the new variant G3 virus predominating in China and Vietnam. These findings suggest that G1/G2 rotaviruses and G3 rotavirus may have been transmitted recently into Myanmar from India/Bangladesh and China, respectively. Because Myanmar is bordered on the west by India and Bangladesh and on the northeast by China, it is conceivable that the spread of predominant rotaviruses from these regions may readily occur via movement of people. The close relatedness of the Myanmar rotaviruses to Indian-Bangladeshi viruses was supported also by the analysis of VP8* genes of P[8]a-lineage in the present study, and was described previously for group B rotavirus (Aung *et al*, 2009).

It is worth noting that the rare subtype of P[8], *ie*, P[8]b (OP354-like P[8]), was identified in a G1 rotavirus strain in the present study. The P[8]b-VP4 gene is genetically distinct from the common P[8] subtype of VP4 gene designated as P[8]a, with sequence identity being 86-89% between the two subtypes (Nagashima *et al*, 2009). The P[8]b subtype was first reported for a G4 rotavirus strain OP354 isolated in Malawi in 1998 (Cunliffe *et al*, 2001), thereafter detected in Vietnam (Nguyen *et al*, 2007), India (Samajdar *et al*, 2008) and Bangladesh (Nagashima *et al*, 2009). Rotaviruses with P[8]b subtype have been detected in only seven countries, including Myanmar, most of which are located in South and Southeast Asia. Despite the limited information, P[8]b was found to be associated with various G types, *viz*, G1, G4, and G9 (Cunliffe *et al*, 2001; Nagashima *et al*, 2009). In the present study, almost genetically identical G1-VP7 genes were shared by strains MMA08-10 with P[8]a and MMA08-8 with P[8]b. These findings

suggest that the P[8]b-VP4 gene may have been reassorted in the genetic background of G1 rotaviruses, as well as other G type rotaviruses. Furthermore, results of the phylogenetic analysis of MMA08-8 VP8* gene suggest the distribution of P[8]b rotavirus in a wide region, from eastern India to Myanmar and Thailand. Although the significance of P[8]b subtype has yet to be clarified, further investigations will be necessary on the spread of rotaviruses with P[8]b and the efficacy of current rotavirus vaccines against these virus strains.

Although only a limited number of rotaviruses were analyzed in the present study, a key to delineate the epidemiologic feature of rotaviruses in Myanmar was obtained. It suggests that Myanmar has been affected by the spread of rotaviruses from neighboring countries, or has been mediating the transmission of rotavirus as a geographical "crossing" point between the two rotavirus-epidemic regions. To elucidate these features more clearly in order to control rotavirus infections in Myanmar, accumulation of epidemiologic data of G/P genotypes and genetic information of more rotavirus strains is necessary.

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