

Original Article

Analysis of HCV Genotypes from Blood Donors Shows Three New HCV Type 6 Subgroups Exist in Myanmar

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The prevalence of hepatitis C virus (HCV) genotypes in Myanmar in comparison with the rest of Southeast Asia is not well known. Serum samples were obtained from 201 HCV antibody-positive volunteer blood donors in and around the Myanmar city of Yangon. Of these, the antibody titers of 101 samples were checked by serial dilution using HCV antibody PA test II and Terasaki microplate as a low-cost method. To compare antibody titers by this method and RNA identification, we also checked HCV-RNA using the Amplicor 2.0 test. Most high-titer groups were positive for HCV-RNA. Of the 201 samples, 110 were successfully polymerase chain reaction (PCR) amplified. Among them, 35 (31.8%) were of genotype 1, 52 (47.3%) were of genotype 3, and 23 (20.9%) were of type 6 variants, and phylogenetic analysis of these type 6 variants revealed that 3 new type 6 subgroups exist in Myanmar. We named the subgroups M6-1, M6-2, and M6-3. M6-1 and M6-2 were relatively close to types 8 and 9, respectively. M6-3, though only found in one sample, was a brand-new subgroup. These subtypes were not seen in Vietnam, where type 6 group variants are widely spread. These findings may be useful for analyzing how and when these subgroups were formed.

Key words: hepatitis C virus (HCV) genotype, type 6 variant, Myanmar, Southeast Asia, phylogenetic analysis

Hepatitis C virus (HCV) infection is a slowly progressive disease affecting about 175 million people worldwide [1], and more than one million new cases of infection are reported annually [2]. Twenty to thirty percent of chronic hepatitis patients progress to

cirrhosis over a 10- to 20-year period [3], and each year 2 to 5 percent of cirrhotic patients develop hepatocellular carcinoma. HCV infection is believed to be more prevalent than hepatitis B infection [2]. A higher prevalence of HCV has been found in South and Southeast Asian countries such as Thailand, Malaysia, and India than in other geographic locations. The first survey on HCV in Myanmar (Burma), published in 2000, showed a high prevalence of hepatitis C in patients with thalassemia and

in patients with liver disease [4]. HCV antibody screening of blood donors in Myanmar was started at blood centers in May 2000 with the support of JICA (Japan International Cooperation Agency) under Japan's Technical Cooperation Program. That screening found a 2.9% HCV-antibody rate among blood donors (unpublished result).

To predict treatment outcomes, it is important to distinguish between HCV genotypes. Usually genotype 2 or 3 is a high responder to interferon therapy, and genotype 1 is a low responder. HCV genotype surveys are performed throughout the world. In Southeast Asian countries, genotype 3a is highly prevalent. Genotype 6 is predominantly found in Hong Kong and Vietnam [5, 6].

Mizokami *et al.* re-evaluated the phylogenetic relationship of types 7, 8, and 9 and classified them as type 6 subtypes [7]. Therefore, in this paper we call them "types", not "genotypes". Types 7a, 7b, 8a, 8b, and 9a are common in Vietnam [6]; types 7c, 7d, 9b, and 9c are found in Thailand [8]; and types 10a and 11a are found in Indonesia [9]. Southeast Asian types 7, 8, and 9 showed higher sustained virologic responses than genotype 1b [10]. Myanmar is adjacent to India and Bangladesh, among other South Asian countries, so information on HCV genotypes in Myanmar is epidemiologically significant, yet such information is scarce there. In fact, only 2 reports about Myanmar HCV genotypes have been conducted, one testing 24 samples [11] and the other testing one serum of a type 6 variant [12].

In this study, we developed a cost-effective HCV-antibody titration method suitable for a developing country such as Myanmar. We also analyzed the genotypic classification of HCV-positive blood donors in and around the city of Yangon in Myanmar. Using phylogenetic analysis, we compared type 6-related subtypes (type 6 variants) with other reported sequences.

Materials and Methods

Serum samples. HCV antibody-positive serum samples were obtained from 201 healthy blood donors residing in Yangon or its suburbs. The donors' ages ranged from 18 to 51 years, and the ratio of males to females was 12:1. The serum samples were collected in May 2000, at the beginning of the hepatitis C control project, and were stored at -80°C until testing. HCV antibodies were detected by the Ortho HCV Ab PA

(Particle-Agglutination) test II kit (Ortho-Clinical Diagnostics K.K., Tokyo, Japan).

Comparison of HCV antibody titers by a low-cost method and HCV-RNA detection.

HCV antibody screening systems are not popular in Myanmar because of their high cost. Therefore, we used a serial dilution method with a Terasaki microplate (Nalge Nunc International, Rochester, NY, USA) and the Ortho HCV Ab PA test II for antibody titration as a cost-effective alternative. Using this method, we were able to save 75% of PA test II reagents. HCV titers were graded as follows: 2^{12} or more: high titer, 2^6 to 2^{11} : middle, 2^5 or less: low. HCV-RNA detections were performed by the Amplicor HCV 2.0 test (Roche Diagnostic Systems, Inc., Branchburg, NJ, USA). A total of 101 samples were tested for HCV titers and HCV-RNA detection. We compared HCV titers and the HCV-RNA-positive rate to determine whether this HCV titer screening is useful as a cost-effective method for testing the Myanmar population.

Determination of HCV antibody using the RIBA HCV 3.0 SIA test kit.

Among the HCV titers tested, 5 samples of HCV antibody titer 2^{13} and all samples of HCV antibody titer 2^4 to 2^{12} were tested by the Chiron RIBA HCV 3.0 SIA test kit (Chiron Co., Emeryville, CA, USA) according to the manufacturer's instructions. The RIBA HCV 3.0 SIA is an *in vitro* qualitative enzyme immunoblot assay for the detection of antibodies to individual proteins encoded by the hepatitis C virus (anti-HCV) in human serum or plasma. A negative, indeterminate, or positive interpretation is based on the reaction pattern present on the strip according to the manufacturer's criteria.

RNA extraction. Amplicor HCV 2.0-positive sera were HCV-RNA extracted for further testing. RNA was extracted from 0.125 ml of serum by the single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction [13] using Isogen-LS reagent (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. Extracted samples were precipitated with isopropanol and washed with 70% ethanol. The resulting pellet was resuspended in 30 μl of RNase-free water.

RT-PCR for HCV-RNA. Extracted RNA was amplified by reverse transcriptase (RT)-polymerase chain reaction (PCR) using sense primer KY80S and antisense primer KY78AS at a portion of the 5' untranslated region (UTR) (Table 1). RT-PCR was performed by

Table 1 Oligonucleotide primers used for PCR sequencing of the 5'UTR or NS5b region

Primer	Nucleotide Sequence	Nucleotide Position
KY80S	5'-GCAGAAACGTCTAGCCATGGCGT-3'	nt.56-79
KY80-4S	5'-GCGTCTAGCCATGGCGTTA-3'	nt.63-82
KY78AS	5'-CTCGCAAGCACCCCTATCAGGCAGT-3'	nt.276-299
1S	5'-AARBCVGCYCGNCTNATYGT-3'	nt.8059-8078
6AS	5'-GTTBCCYARCCADGARTT-3'	nt.8813-8830
2S	5'-GVGTBMGBGTNTGYGAAAA-3'	nt.8094-8112
5AS	5'-ACRTTKGABGARCADGATGT-3'	nt.8687-8706
3S	5'-CHTAYGGNTTYCARTACTC-3'	nt.8166-8184
4AS	5'-CTRGTCATRGCTCCGTGAA-3'	nt.8612-8631

R, A.G; B, C.G.T; V, A.C.G; Y, C.T; N, A.C.G.T; D, A.G.T; M, A.C; K, G.T; H, A.C.T; S represents the sense primer; AS represents the antisense primer.

the following protocol. We first prepared 20 μ l of reaction mixture, containing 4 μ l of 5 x reaction buffer, 0.8 μ l of RNase inhibitor, 0.8 μ l of rTTH DNA polymerase (Toyobo, Osaka, Japan), 0.25 μ M of each primer, and 0.3 mM of deoxynucleotide. The thermal cycler was programmed at 60 °C for 30 min and 95 °C for 1 min for the RT step; 95 °C for 15 sec and 60 °C for 20 sec for 2 cycles; and 90 °C for 15 sec and 60 °C for 20 sec for 38 cycles, followed by extension at 60 °C for 4 min.

The second PCR was performed using sense primer KY80-4S (Table 1) and antisense primer KY78AS at 95 °C for 10 min and at 95 °C for 15 sec; and 60 °C for 20 sec for 30 cycles followed by 60 °C for 4 min.

The resultant PCR product was electrophoresed on 2 % agarose gel containing ethidium bromide in 0.5 \times TBE (Tris-borate/EDTA electrophoresis buffer) and evaluated under UV light. To avoid the risk of false-positive results, the PCR assays were done with strict precautions against cross-contamination.

The HCV-RNA-positive samples proceeded to the next steps. Direct sequences were performed for 5'UTR PCR products and analyzed for genotyping. Type-specific nested PCR for core regions was also performed to confirm genotyping [14]. Genotype 6 subgroups are difficult to distinguish from genotype 1 by 5'UTR sequences [10]. Therefore, samples that were classified into genotype 1 by 5'UTR sequences but not confirmed by core-region type-specific PCR proceeded to NS5b region RT-PCR and were direct sequenced and subjected to phylogenetic analysis.

Direct sequencing. Direct sequencing was performed as follows. Five microliters of crude PCR product was digested for 15 min at 37 °C by using 2 μ l of

exosap-IT followed by heat inactivation at 80 °C for 15 min. Cycle-sequencing reactions were completed with the addition of 2 μ l (3.2 pmol) internal sense or antisense sequencing primer using KY80-4S or KY78AS, plus 2 μ l of RNase-free water and 8 μ l of the dye terminator included in the Big Dye Terminator 3.0 (Applied Biosystems, Foster City, CA, USA) cycle sequencing reaction kit. Thermal cycling was performed on a Gene Amp 9600 thermal cycler at 96 °C for 10 sec, at 50 °C for 5 sec, and at 60 °C 4 min, for a total of 25 cycles. Sequencings were done using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Genotype distribution was based on the comparison of sequence information from 7 distinct informative regions found within the 5'UTR with information obtained from previously published databases [15, 16].

Hepatitis C genotyping by PCR and sequencing at the NS5b region. HCV-RNA-positive sera were further analyzed for HCV-RNA by reverse transcription and nested PCR designated to an amplified portion of the NS5b region of the viral genome. We prepared three pairs of primers and selected 2 of these pairs for nested PCR. When the NS5b region was successfully amplified, sequencing of 329 bp spanning nucleotides 8279-8607 was performed [6]. HCV subtypes were determined by phylogenetic analysis.

Phylogenetic analysis. ODN (version 1.1.1) [17] using the six-parameter method [18] was used to determine the number of nucleotide substitutions per site (genetic distance) between the isolates. Based on these values, a phylogenetic tree was constructed by the neighbor-joining (N-J) method [19]. The tree was plotted by a program from DNA Data Bank of Japan

(DDBJ) with the mid-point rooting option. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1,000 times [20].

Nucleotide sequence accession numbers. The sequences obtained in this study have been submitted to the DDBJ, GeneBank, and European Molecular Biology Laboratory (EMBL) nucleotide sequence databases and have been assigned accession numbers AB103135 to AB103157.

Results

Prevalence of HCV high-titer group in blood donors. We titer-checked 101 HCV sera samples. Among these, 58 were found to have high titration of HCV antibody (2^{12} or more) (Table 2).

Forty-five samples of HCV titer 2^{12} or under, plus 5 samples of HCV titer greater than 2^{12} , were subjected to the RIBA HCV 3.0 SIA test. All high-titer group serum samples were RIBA positive, and all the low-titer group samples (2^5 or under) were RIBA negative or indeterminate (Table 3).

Table 2 Correlation between HCV titer and RNA positivity

HCV titer		Number of subjects	Number of RNA-positive subjects	RNA-positive ratio (%)
High	2^{13}	53	51	96
	2^{12}	5	5	100
Middle	2^{11}	6	3	50
	2^{10}	5	3	60
	2^9	2	0	0
	2^8	1	0	0
	2^7	5	0	0
	2^6	11	1	9.1
Low	2^5	8	0	0
	2^4	2	0	0
	$< 2^3$	3	0	0

Table 3 Correlation between HCV antibody titer, RIBA HCV 3.0 SIA test, and RNA positivity

HCV titer	Number of subjects	RIBA HCV 3.0 SIA test			HCV RNA	
		Positive	Negative	Indeterminate	Positive	Negative
High	2^{13}	5	5	–	5	–
	2^{12}	5	5	–	5	–
Middle	2^{11}	6	6	–	3	3
	2^{10}	5	5	–	3	2
	2^9	2	2	–	–	2
	2^8	1	1	–	–	1
	2^7	5	–	5	–	5
	2^6	11	3	6	2	10
Low	2^5	8	–	5	3	–
	2^4	2	–	2	–	2

Correlation between HCV antibody high titer and RNA positivity. Of the 101 HCV titer-checked samples, 63 were RNA positive (62.4%). Of the 58 high antibody titer samples (2^{12} titer or more), 56 samples were HCV-RNA positive (96.6%). Of the 30 middle antibody titer samples, 7 samples were HCV-RNA positive (23.3%). In the low-titer group, no sample was RNA positive (Table 2).

Genotype distribution of blood donors. All antibody-positive samples were tested for RNA positivity. Among the 201 HCV antibody-positive samples, 110 were positive for HCV-RNA, in the following distribution: 35 (31.8%) were genotype 1 (1a: 5 (4.5%), 1b: 22 (20.0%), subtype undetermined: 8 (7.3%)); 52 (47.3%) were genotype 3 (3a: 13 (11.8%); 3b: 35 (31.8%); subtype undetermined: 4 (3.6%)); 23 (20.9%) were type 6 variant (Fig. 1). Samples that failed to amplify at the Core region or NS5b region by RT-PCR, or spent all sera in stock, were classified "subtype undetermined".

Phylogenetic analysis revealed 3 distinct subgroups of type 6 variants in Myanmar (Fig. 2). We named them M6-1, M6-2, and M6-3. M6-1 was found only in Myanmar and northern Thailand [21, 22], whereas M6-2 was found commonly in Thailand [12]. M6-1 and M6-2 are relatively close to types 9 and 8. M6-3, though occurring in only one sample, was a new subgroup. These genotypes have not been seen in Vietnam, where type 6 group variants are widespread [6].

A comparison of sequences of the NS5b region

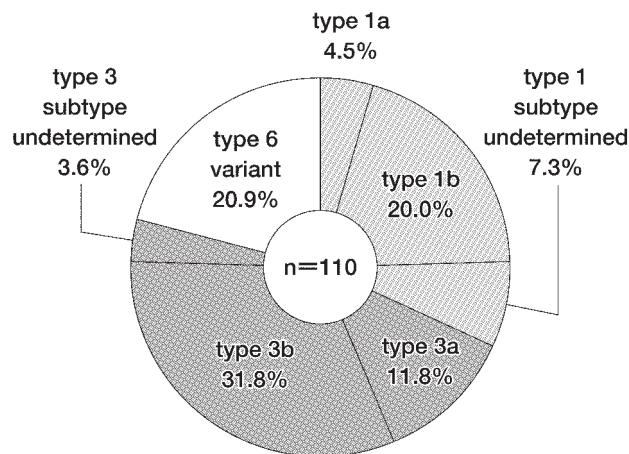


Fig. 1 Genotype distribution of HCV-RNA-positive blood donors in Myanmar. The pie chart indicates the proportions of various HCV genotypes.

(nucleotides 8279–8607) of type 6 subgroups including representatives of M6-1 to M6-3 is shown in Fig. 3.

Discussion

HCV antibody screening systems are not popular in Myanmar because of their high cost. Therefore, we used Terasaki microplates for HCV antibody titration as a cost-effective alternative. Cost effectiveness is a very important issue in the attempt to expand the HCV antibody screening system for blood donors in the coun-

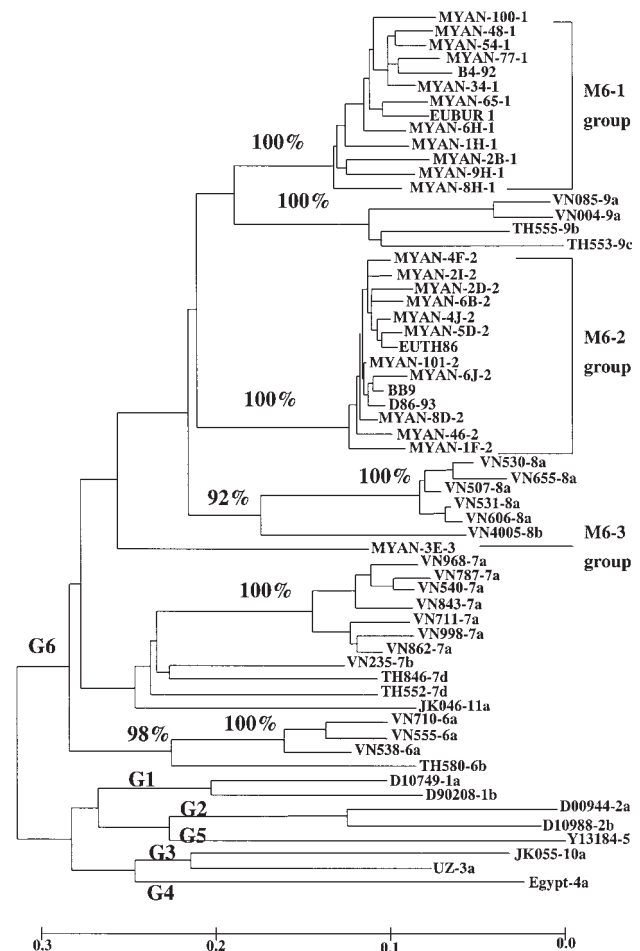


Fig. 2 Phylogenetic tree constructed on the NS5b region of 23 HCV type 6 variants in Myanmar and previously reported type 6 group variants, along with representatives of the other 5 genotypes. Bootstrap values are shown along each main branch. The length of the horizontal bar indicates the number of nucleotide substitutions per site. Isolates labeled "MYAN-" were sequenced in the present study.

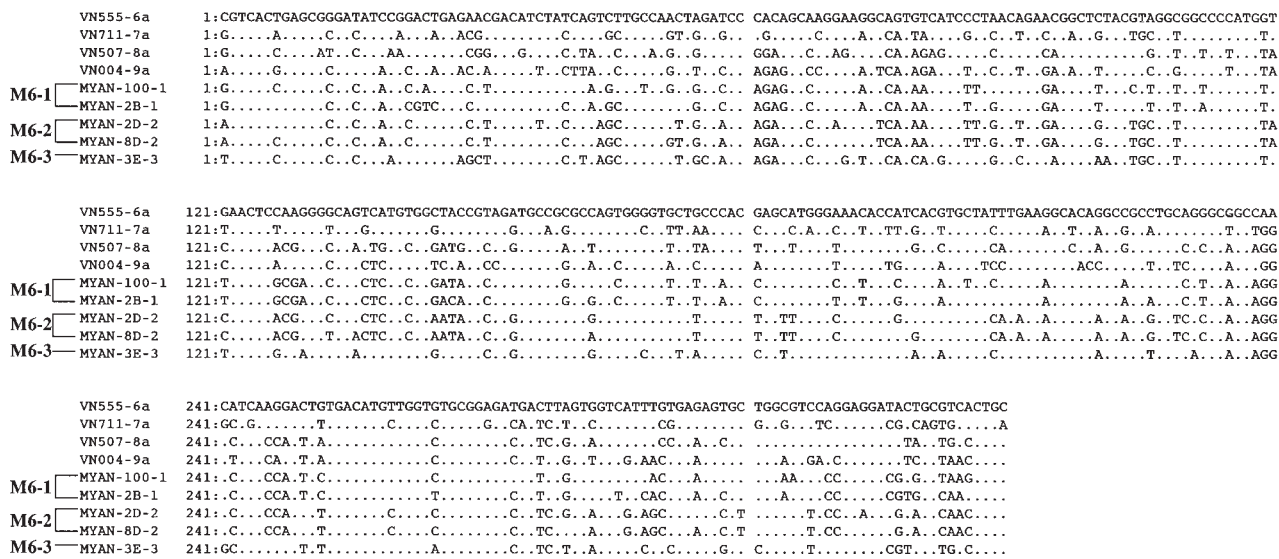


Fig. 3 Comparison of sequences of the NS5b region (nucleotides 8279-8607) of type 6 subgroups. VN555 represents type 6a. VN711, VN507, and VN004 represent type 7a, 8a, and 9a, respectively. MYAN-100-1 and MYAN-2B-1 represent M6-1 subgroups; MYAN-2D-2 and MYAN-8D-2 represent M6-2 subgroups. MYAN-3E-3 represents M6-3. Dots express identical nucleotides.

try. We compared the HCV antibody titers with HCV-RNA detection by Amplicor 2.0 and found that this antibody titration method could be useful for a developing nation such as Myanmar.

Only one serum of 2⁶ titer showed as HCV-RNA positive. By RIBA HCV 3.0 SIA test, this sample was c100, c22 positive and c33c, NS5 negative. The donor might be in an acute HCV infection state after a window period, but further information is not available.

In this study, we identified the HCV genotypes of 110 HCV-RNA-positive sera from blood donors in and around the city of Yangon in Myanmar. In this area, genotype 3 was dominant and other major genotypes included genotype 1 and variants of type 6. Genotype 2 was not identified. This result was similar to the genotype distributions in other Southeast Asian countries, such as Thailand and Vietnam [12], but different from those in South Asian countries, such as India [23, 24] and Nepal [25].

HCV type 6 variants are common in the Southeast Asian countries, and they also are restricted to this region. They are classified into many subgroups from 6a to 11a. We were interested in this variety and locality of the HCV type 6 variants. Thus, we further investigated the type 6 variants by phylogenetic analysis. For this analysis we used the six-parameter neighbor-joining (N-J)

method, because it offered more reliability to our study than did the unweighted pair-group method with the arithmetic mean (UPGMA) [7].

Interestingly, the type 6 variants of Myanmar blood donors were classified into 3 distinct subgroups, M6-1, M6-2, and M6-3 (Fig. 2). Among previously reported but not classified sequences, B4-92 and EUBUR1 belong to the M6-1 subgroup, and EUTH86, BB9, and D86-93 belong to the M6-2 subgroup. EUBUR1 is a sample from Myanmar [12], and B4-92, BB9, EUTH86, and D86-93 are from Thailand [12, 21, 22]. Among them, B4-92, BB9, and D86-93 are from northern Thailand [21, 22]. These subgroups seem to exist in restricted regions of Southeast Asia. M6-1 was restricted to Myanmar and northern Thailand, whereas M6-2 appears to be common in Myanmar and Thailand. These 2 groups were not seen in Vietnam, where type 6 variants exist widely [6]. This result is important for an epidemiological understanding of how HCV spread in Southeast Asian countries.

Recently, Tanaka *et al.* [26] examined the time origin of the HCV epidemic in Japan and the United States from serial samples. Using this method, we may be able to clarify when these subgroups of HCV began to spread in Myanmar and Southeast Asia

Furthermore, we found one subgroup, M6-3, which

may be classified as a brand-new subgroup, because its sequence has not yet been reported in the world. It does not appear to be common in the Yangon area. How extensively this type of subgroup is spread in Myanmar is unknown. Further study is needed.

Blood donors in this study were living in and around the city of Yangon, in southeastern Myanmar. Most of the people of Myanmar are Mongoloid but live within several ethnic groups. Burmese is the major ethnic group in the Yangon city area. It is important to study HCV genotypes in other areas, such as along the border with India or China, for epidemiological study. We are planning to survey these areas.

A previous study of HCV genotypes in Myanmar was performed with sera from diseased patients [11], and type 6 subgroups were not mentioned. Type 6 subgroups of hepatitis C patients might have been included with the 8% of "unclassified" patients, although this is lower than the prevalence among blood donors (8% *vs.* 20.9%). Type 6 subgroups may not be more progressive than other genotypes, but until now no detailed studies about type 6 variants have been reported in Myanmar. Dev *et al.* [10] reported that type 7, 8, and 9 showed higher sustained viral response (SVR) to interferon and rebavirin treatment than did type 1b. Therefore, we also plan to study HCV genotypes of patients' sera. Further, we want to compare HCV genotypes according to the stages of liver disease, and we want to characterize pathogenicity according to HCV genotypes.

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