The Myanmar Health Sciences Research Journal, Vol. 20, No. 1, 2008

Analysis of hepatitis C virus isolates from Tamu Township and identification of a new subtype of genotype 6

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Genotyping of hepatitis C virus (HCV) is important for designing therapeutic strategies and there is little information on the distribution of HCV genotypes in Myanmar. To determine the distribution of genotype/ subtype of HCV isolates from Tamu Township, a total of 501 blood samples were collected and tested for anti-HCV antibody by using the Ortho HCV Ab-PA test II kit. Genotyping was carried out by using reverse-transcriptase polymerase chain reaction (RT-PCR), direct DNA-sequencing and phylogenetic analysis of the partial core sequences. Sixty-four (12.8%) samples were anti-HCV positive and 59 (92.1%) HCV isolates could be genotyped. Genetic characterization of viral sequences indicated that there are three major genotypes (6, 3 &1) and six minor subtypes as 1a (1.7%), 1b (15.3%), 3b (25.4%), 6m (3.4%), 6n (52.5%) and a new 6 subtype (1.7%). This study showed that HCV genotype distribution in Tamu Township has a distinct geographic variation from other South-East Asia countries in terms of the existence of the new genotype 6 subtype.

INTRODUCTION

Hepatitis C virus is a unique, enveloped, positive, single stranded RNA hepacivirus of the Flaviviridae family. The RNA genome is approximately 9.6kb in length and encodes a single, large polyprotein that is post-translationally cleaved into multiple structural (core, E1 and E2) and non-structural (NS2–NS5) peptides [1]. Currently, all known HCV isolates have been divided into six phylogenetically distinct groups, referred to as clades, and more than 70 subtypes based upon nucleotide sequence and phylogenetic analysis [2]. HCV infection is a major health problem throughout the world. Collective estimates indicate over 175 million people are infected with HCV worldwide with up to 4 million new infections each year [3]. Infection with HCV is the most common cause of progressive liver disease, including cirrhosis and hepatocellular carcinoma (HCC) [4].

Three general approaches have been employed to characterize viruses and have led to the grouping of viral isolates [1]. The first approach, serotyping, examines the pattern of viral neutralization by antibodies. Another approach, phenotyping, assesses differences in disease patterns, cytopathology or response to antiviral agents when suitable model systems are available. Finally, investigators may look for differences in genetic sequences of viral genes or complete genomes to determine homology and detect variability that may alter viral replication and response to antiviral therapies. This last approach is referred to as genotyping. For some viruses, including HCV, there are no available cell culture systems or appropriate small animal models, making the characterization of these

viruses by serotype or phenotype very difficult. Therefore, genotypic classification and description of viruses has come to prominence in virology and the treatment of infectious diseases.

In addition to the identification and classification of a viral isolate, the genotype may be used to detect mutations in the genome that may have consequences with regards to viral infectivity, pathogenesis or response to different antiviral agents. The HCV genotype is an important predictor of response to interferon (IFN)-α based antiviral therapy [5, 6] and genotypes 1 and 4 are more resistant than genotypes 2 and 3 to the current standard of care. Patients with HCV genotype 6 gain a better response to combined-treatment with IFN-α and ribavirin than those with HCV genotype 1 [6]. No virological similarity between genotype 6 and 2 or 3 is reported and genotype 6 is not similar to genotype 2 or 3 by phylogenetic analysis [7].

The patterns of HCV genotypes can be identified throughout the genome by several methods [8]. However, it has been reported that mistyping between genotype 1 and genotype 6 could occur with the 5'UTR–Restriction Fragment Length Polymorphism (RFLP) [9] and some genotype 6 variants found in SEA have 5'UTR sequences identical to those of subtype 1a or 1b [10, 11]. Therefore, we analyzed HCV genotypes on sequencing of part of the core region of the genome followed by phylogenetic analysis in this study.

A higher prevalence of HCV infection ranging from 1.5% to 9% has been reported in SEA and the Indian subcontinent, with the highest rates of HCV (2-14%) present in northern and central Africa, the eastern Mediterranean, and Ukraine [12]. One study reported that among the HCC cases, 35% were positive for anti-HCV detected by using Anti-C100-3 EIA test kits [13]. The survey on HCV in Myanmar showed a high prevalence of hepatitis C infection in patients with thalassemia and in those with liver diseases [14]. HCV infection is highly prevalent in Myanmar both in the apparently healthy population as well as in patients with end-stage liver diseases [15].

The previous study of HCV distribution in Yangon city was reported that three genotype 6 subtypes exist in Myanmar and according to the newly proposed nomenclature of hepatitis C, two subtypes (M6-1 and M6-2) belong to subtypes 6m and 6n respectively but one subtype has been unassigned yet [16]. Further to the reported study, we carried out the present study to investigate into the diverse distribution of HCV genotype 6. We assume that in border areas more diversity could exist as a result of human interlacing due to border trade and cross country migration. Thus, we aimed to analyze the HCV isolates in Tamu Township and to determine the genetic diversity of HCV isolates between Myanmar and neighboring country.

MATERIALS AND METHODS

Samples

A descriptive cross-sectional survey was conducted in Tamu Township, northwestern border area of Myanmar. A total of 501 peripheral blood samples were collected under informed consent. The sera were tested for anti-HCV antibodies by the Ortho HCV Ab-PA (Particle-Agglutination) Test II kit (Ortho-Clinical Diagnostic K.K., Tokyo, Japan) and stored in -80 ˚C.

RNA extraction

HCV RNA was extracted from 125 µl of positive serum by single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction [17] using Isogen-LS reagent (Nippon Gene Co., Toyama, Japan) according to the manufacture's instructions, and precipitated with isopropanolol and washed with 70% ethanol. The resulting pellet was resuspended in 20 µl of RNase free water.

RT-PCR and nested PCR

Reverse transcriptase polymerase chain reaction and nested PCR were performed according to the method of Ohno T *et al.* [18] with minor modification. Briefly, extracted RNA was first amplified with outer primers Sc2 and Ac2 at a portion of core region in a 20 µl reaction volume. The second round PCR was carried out using 1 U AmpliGold *Taq*DNA polymerase (Roche Applied Science) and 1 µl each of inner primers S7 and A5. The final PCR products were resolved by 3% agarose gels and stained with ethidium bromide and evaluated under UV light. The genotype 6 new subtype was further analyzed by reverse transcription and nested PCR designated to an amplified portion of NS5B region of the viral genome by using outer (1S, 6AS) primers [16] and inner (1203, 1204) primers [19]. To avoid the risks of false positive results, the PCR assays were done with strict precautions against cross contamination.

Directs sequencing of PCR products

The second round PCR amplicons were purified with MonoFas DNA Kit I and proceeded to sequencing reaction with Big Dye Terminator 1.1 cycle sequencing reaction kit. Sequencing was performed using an ABI PRISM 310Genetic Analyzer (Applied Biosystems, Foster city, CA).

Sequence alignment and phylogenetic analysis

Multiple alignments of our sequences with the reference sequences on core/NS5B region of different genotypes retrieved from DDBJ/GenBank database were carried out. Alignment of the sequences and phylogenetic tree were constructed by using the neighbor-joining method based on genetic distances calculated using the Kimura two parameter substitution model, as implemented in MEGA version 3.1 [20]. To access the reliability of the phylogenies, bootstrap re-sampling and re-estimation were performed in 1,000 replications for neighbor-joining trees. The sequences obtained in this study have been submitted to GenBank/DDBJ and have been assigned accession numbers from *AB269327* to *AB269352* (Core sequences), *AB269353* (NS5B sequence).

RESULTS

Anti-HCV screening of all 501 subjects revealed 64 (12.8%) positive cases in Tamu Township. The mean age was 30.7±15.4 years and 44.7% of the subjects were male. The prevalence of HCV infection by age, gender, and racial group is shown in Table 1. The prevalence rate by the 40-59 years age group was the highest (21.3%) and the lowest (2.9%) for the <20 years age group in this study. The youngest anti-HCV positive subject was 15 years old while the oldest was 67 years. Anti-HCV prevalence rate in male subjects was higher than female subjects. The prevalence rate in Chin race was highest (14.4%) among racial groups although Bamar population is the highest in the study. The higher rate (14.3%) in Narga race may be due to small number of subjects.

Table 1. Anti-HCV positivity with age, gender and racial group

Category	Number tested	Anti-HCV positive	Prevalence (%)
Age group (yr)			
<20	138	4	2.9
20-39	223	33	14.8
40-59	122	26	21.3
≥60	18	1	5.6
Gender			
Male	224	37	16.5
Female	277	27	9.7
Racial group			
Bamar	303	40	13.2
Shan	118	17	14.4
Chin	64	6	9.4
Narga	7	1	14.3
Others	9	0	0
Total	501	64	12.8

Among 64 samples, 59 samples (92.1%) could be amplified on the partial core genome and analyzed as three major genotypes and six minor subtypes named as 1a, 1b, 3b, 6m, 6n and new subtype of genotype 6. Genotype 6 (57.6%) accounted for the

majority of the samples, followed by genotype 3 (25.4%), and genotype 1 (17%). Neither genotype 2, 4 nor 5 was found. Subtype 6n was the most predominant subtype (52.5%), second most is subtype 3b (25.4%), followed by 1b (15.3%), 6m (3.4%), 1a (1.7%), and new 6 subtype (1.7%).

Table 2 shows the distribution of HCV genotypes stratified by age, gender and race. Most HCV genotypes were distributed in 20-39 years and 40-59 years age groups. Subtype 6n and 3b were predominant in 20-39 years age group and 40-59 years age group respectively. Subtypes 6n, 3b, and 1b were prevalent in male gender. All six HCV subtypes were distributed in Bamar, three subtypes in Chin and Gorahkar and only one in Narga. The most predominant genotype was subtype 6n and second most predominant was subtype 3b. The new genotype 6 subtype was found only in the Bamar race.

Table 2. HCV genotype distribution with age, gender and racial group

HCV genotypes (No)	Age group (yrs)				Gender		Race			
	<20	20 l 39	40 l 59	≥60	M	F	Bamar	Chin	Gorakhar	Narga
1a(1)		1				1	1			
1b(9)	1	4	4		6	3	3	5	1	
3b(15)	1	4	9	1	9	6	6	6	3	
6m(2)	1	1			1	1	2			
6n(31)		19	12		18	13	23	5	2	1
6 §(1)			1	0	1		1			
Total (59)	3	29	26	1	35	24	36	16	6	1

§ Referred to as new genotype 6 subtype
M=Male, F=Female

Fig. 1 shows that phylogenetic tree constructed on partial core sequences (nucleotides 380-659) along with sequences of different genotypes retrieved from GenBank /DDBJ database. HCV genotypes are indicated on the roots of cluster or individual branch. The bar at the base of the figure shows the scale for nucleotide substitution per site. The six HCV genotypes were indicated with numbers 1-6 and subtypes were designated 1a-6n.

DISCUSSION

In this study, we could characterize the three major HCV genotypes and six subtypes in Tamu Township with predominance of genotype 6. This finding was different with the genotype predominance of other reports [11, 16, 21]. That might be due to either the regional difference or transmission pattern of HCV infection in Myanmar. It may be probable that (i) the frequency of infection with genotype 6 group subtypes differs between geographical regions within Myanmar or (ii) there are differences in its distribution in different risk groups for infection or age of subjects or (iii) different genotyping methods or regions of genome were chosen to analyze. One sample of HCV isolate in this study could be provisionally assigned as a new subtype of genotype 6 by analysis on core and NS5B region, according to the consensus proposals for a unified system of nomenclature of HCV genotypes by Simmond P *et al*.

In fact, the routes of transmission of HCV infection or the factors involved were not identified in the present study. There was only a little evidence of history of tattooing or history of hepatitis in family. We could propose that HCV may be transmitted by the probable routes such as (a) blood transfusion in remote areas where HCV testing is not easily available (b) tattooing or ear piercing as religious practice, and (c) intravenous drug users with sharing needles. We are conducting further investigations to clarify the transmission route of this infection.

Comparing the genotypic pattern of HCV infection with neighboring country, HCV genotype distribution in Tamu Township was different from those in India. It has been reported that genotype 3 is the most prevalent genotype in India [22]. Some genotypes/subtypes may predominate within the regions and different HCV genotypes have unique patterns of geographic distribution [23]. The subtypes 1a, 1b, 2a, 2b and 3a are distributed globally and rarer,

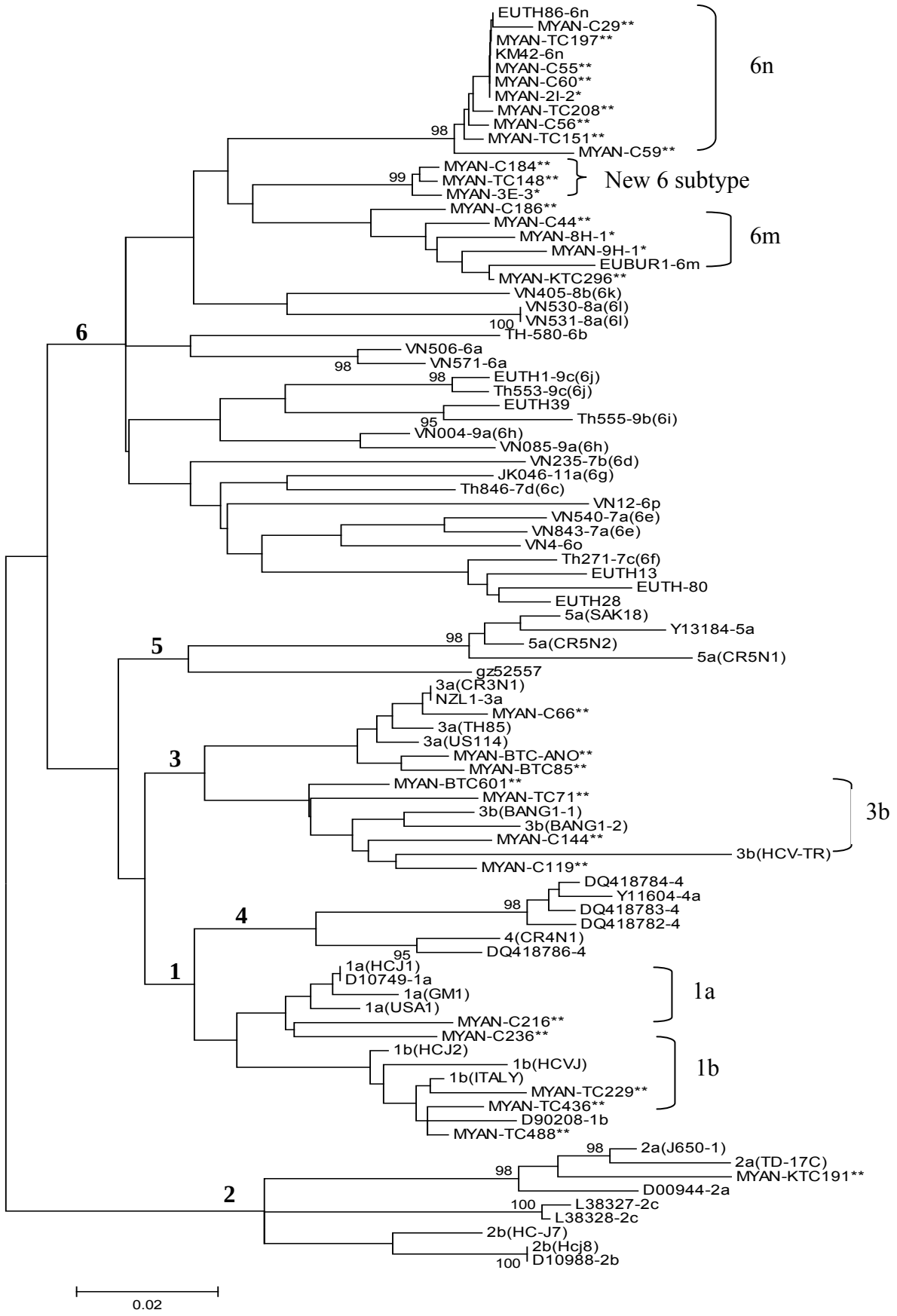

Fig.1. Phylogenetic tree constructed on partial core sequences (nucleotides 380-659) along with sequences of different genotypes

genotypes have also been localized in certain geographical regions. For example, subtype 3k has only been found in Indonesia [10], genotype 4 in the Middle East and Egypt [24], genotype 5 in South Africa [25], and genotype 6 and its variants in South-east Asia (SEA) [11, 26]. Racial heterogeneity exists firmly in Myanmar with eight major groups (Bamar, Shan, Kayin, Kachin, Mon, Yakhine, Chin and Kayar), 135 minor indigenous groups and those races of neighboring countries, predominantly China, India and Bangladesh. The Bamar indigenous people settled predominant in any region of Myanmar. Both indigenous group and non-indigenous group resided in border area. Bamar and Chin were two major indigenous groups in this study (Table 1). We have shown that HCV genotypes were more prevalent in the middle aged group and in Bamar and Chin indigenous group especially for subtype 6n and 3b. Although we could not postulate a definite relationship between HCV genotypes and age or racial group factors, we could provide a pattern of genotype distribution among different racial groups. Regarding the prevalence of HCV in this study, it was higher than those of the previous studies on the apparently healthy subjects [15] and the blood donors in Myanmar [16]. Although HCV prevalence survey could not be considered to be representative of the population at large, it could be concluded that significant higher rates of HCV infection exists among apparently healthy populations residing in border area. It is probable that the frequency of HCV infection will differ in age of subjects, different risk groups or transmission patterns of the infection but no risk factors could be explored in this study. Therefore, further large-scale in-depth studies will be needed to clarify the situation that will lead to the development of more effective prevention and control measures against HCV infection in this particular area.

Conclusion

This study highlighted that HCV infection was highly prevalent and various HCV genotypes/subtypes with great genetic diversity of HCV genotype 6 were found in Tamu Township. In particular, the new genotype 6 subtype found in this study could be used to describe the epidemiology of HCV transmission in Myanmar and to enhance the understanding of the HCV sequence variation in SEA countries.

ACKNOWLEDGEMENTS

We would like to thank Minister Professor Dr. Kyaw Myint and Deputy Minister Professor Dr. Paing Soe, Ministry of Health, Myanmar for kind permission to conduct this study and we wish to express our sincere thanks to all participants who contributed to the study.

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