Seroprevalence and Genotype of Hepatitis C Virus among Immigrant Workers from Cambodia and Myanmar in Thailand

Srunthron Akkarathamrongsin^{a, b} Kesmanee Praianantathavorn^a
Nisachol Hacharoen^a Apiradee Theamboonlers^a Pisit Tangkijvanich^c
Yong Poovorawan^a

^aCenter of Excellence in Clinical Virology, Faculty of Medicine, ^bInter-Department of Biomedical Sciences, Faculty of Graduate School, and ^cDepartment of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Key Words

Hepatitis C virus • Seroprevalence • Genotype • Immigrant workers • Cambodia • Myanmar

Abstract

Objective: There is a large number of immigrant workers from Cambodia and Myanmar in Thailand. The aim of our study was to determine seroprevalence and genotypes of hepatitis C virus (HCV) in this group. **Methods:** Immigrants aged between 15 and 60 years (1,431 Cambodians and 1,594 Myanmar) were recruited into this study. Each sample was screened for anti-HCV by ELISA. RNA was extracted from seropositive samples and RT-PCR was performed in order to amplify the HCV core region. Each sample was subsequently sequenced, and the genotype was determined by phylogenetic analysis. **Results:** The prevalence of HCV infection in immigrant workers from Cambodia and Myanmar was 33 (2.3%) and 27 (1.69%) samples, respectively. Of the anti-HCV-positive individuals, 25 (75.8%) from Cambodia and 15 (55.6%) from Myanmar harbored viral RNA. Phylogenetic analysis showed that the predominant HCV genotypes in this group were 1a, 1b, 3a, 3b and 6 (6e, 6f, 6m, 6p and 6r). Most HCV isolates can be found in Thailand, though some

subtypes of HCV-6 are uncommon. **Conclusions:** This study shows the HCV seroprevalence and genotypes among immigrant Cambodians and Myanmar which may reflect the prevalence in each country and closely relate to the prevalence in the guest country.

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Introduction

A very high degree of genetic diversity of hepatitis C virus (HCV) has led to persistent infections. Currently, approximately 170 million people around the world are infected with this agent [1]. Chronic HCV carriers are at a significantly increased risk of liver cirrhosis and progression to hepatocellular carcinoma [2].

A high prevalence of HCV has been found in Southeast Asia. HCV epidemiology is well documented in Vietnam and Thailand. The prevalence of HCV infection was 1–2% in Vietnam [3–5]. Seroprevalence of HCV in Thailand is approximately 2.2% in the whole population [6]. However, HCV prevalence in Cambodia and Myanmar has not been well studied. Cambodia has a high prevalence of HCV infection. In 1991, a community-based study reported that

6.5% of the population had developed antibodies to HCV [7]. Another report indicated that 10.4% of jaundice patients had antibodies to HCV [8]. In Myanmar, the first study conducted showed a high prevalence of HCV in thalassemia and liver disease patients [9]. Prevalence varies from approximately 2 to 11.6%, though most studies were performed based on a small sample size [10–12]. This variation may result from differences in geographical sampling area and target population.

HCV is a single-stranded RNA virus of positive polarity and the only member of the genus *Hepacivirus* in the Flaviviridae family. This virus shows an extremely high degree of genetic variation, and has been classified into six genotypes (1–6), which comprise various subtypes, assigned letters in alphabetical order [13]. A newly discovered seventh genotype has been documented [14]. Novel subtypes of HCV genotype 6 have been continuously identified in Southeast Asia [10, 12, 15, 16]. Thus, as yet unknown genotypes and subtypes remain to be elucidated in this part of the world.

Immigrant workers, especially from Myanmar and Cambodia have concentrated in Thailand. These groups may harbor some infectious diseases. New agents may be introduced into the indigenous population and impact public health. Therefore, it is essential to investigate and monitor some infectious agents, especially viral hepatitis C. This project has determined seroprevalence and genotypes of HCV among these groups and demonstrated that HCV prevalence of the migrant workers was closely related to the native population.

Materials and Methods

All study protocols were approved by the Ethics Committee of the Hospital and Faculty of Medicine, Chulalongkorn University. The anti-HCV positive blood samples were chosen from the specimens obtained during the routine annual check-up compulsory for immigrant workers. All the studied specimens were anonymous with a coding number for analysis, and permission was granted by the director of the hospital. In addition, all specimens were used exclusively for academic research and the patients were not remunerated.

Sample Collection

Serum samples were collected from immigrant workers in Thailand. Immigrants from Cambodia and Myanmar aged between 15 and 60 years who attended Bangpakok 9 International Hospital for their annual health check up were recruited. Sera collected from Cambodia and Myanmar workers amounted to 1,431 and 1,594 samples, respectively. Serum samples were collected from August 2007 to January 2009. Individuals of general good health were included. Immigrants resident in Thailand for more

than 5 years were excluded as prolonged residence in the guest country might increase the potential for de novo HCV infection, and thus HCV prevalence detected would not be indicative of the country of origin. Also, individuals receiving immunosuppressive drugs, infected with HIV or displaying signs of immunodeficiency were excluded. This protocol was approved by the Ethics Committee, Ministry of Public Health and Faculty of Medicine, Chulalongkorn University, Bangkok. The specimens were labeled as anonymous with a coding number. Sera were collected and kept at -70° until further analysis.

Serological Tests and RT-PCR Amplification

All samples were subjected to enzyme-linked immunosorbent assay for anti-HCV detection using a commercially available kit (Murex anti-HCV v.4.0; Abbott Laboratory, North Chicago, Ill., USA). RNA was extracted from anti-HCV-positive serum samples applying the guanidine thiocyanate method [17]. Reverse transcription was performed using random primers and M-MLV reverse transcriptase (Promega, Madison, Wisc., USA). Viral RNA was detected by cDNA amplification of the 5' noncoding region as previously described [6]. Amplification of the noncoding region was performed with 2.5 μ l cDNA and the outer primer pair OC1 (GCCGACACTCCACCATGAAT, position: 18–37) and OC2 (CATGGTGCACGGTCTACGAG, position: 325–344). The PCR reaction mixture contained 5 pmol of each primer, 200 μ M dNTP, 1.5 mM Mg^{2+} , 1.25 units of Taq DNA polymerase adjusted to a final volume of 25 μ l with distilled water. The amplification conditions consisted of a preincubation step at 95° for 3 min followed by 35 cycles of denaturation at 94° for 1 min, annealing at 49° for 1 min and extension at 72° for 1.30 min, and concluded by a final extension step at 72° for 7 min. For nested PCR, 1.0 μ l of PCR product was amplified under the same conditions using primers IC3 (GGAAGTACTGTCTTCACGCAG, position: 51–71) and IC4 (TCGCAAGCACCTATCAGGCA, position: 290–310). Nucleotide positions in this study refer to GenBank accession number M62321. The DNA fragment of the core region was amplified by nested PCR using specific primers (954 and 410 for the first round of amplification, and 953 and 951 for nested PCR) as described elsewhere [6, 18]. Some samples which showed ambiguous genotypes were subjected to further amplification of the NS5B region using specific primer pairs [19].

Sequencing and Phylogenetic Analysis

After gel electrophoresis, the PCR product of the core region was purified (HiYield Gel/PCR DNA Fragments Extraction Kit; RBC Bioscience, Taiwan) and subjected to sequencing. The sequences were edited manually using Chromas LITE (v2.01), BioEdit (v5.0.9; Ibis Therapeutics, Carlsbad, Calif., USA) and SeqMan (DNASTAR, Madison, Wisc., USA). All sequence results and reference strains of the core coding region were aligned using CLUSTALW v1.83. Neighbor-joining trees were generated using the Gojobori-Ishi-Nei six-parameter method. Confidence values were calculated based on bootstrap resampling tests multiplied by 1,000 (<http://clustalw.ddbj.nig.ac.jp>). The reference sequences were retrieved from GenBank, DDBJ and EMBL DNA database.

Nucleotide Sequence Accession Numbers

The nucleotide sequences of HCV from Cambodia and Myanmar have been submitted to the GenBank database under accession numbers GU186925–GU186964.

Results

Seroprevalence of HCV among Immigrant Workers

In total, 1,431 and 1,594 serum samples were collected from Cambodia and Myanmar immigrant workers in Thailand, respectively. Male gender predominated among immigrants from Cambodia, in contrast to those from Myanmar (table 1). All subjects were between 15 and 57 years old, with a mean age of 27.13–27.77 years (table 1). The majority of the subjects in the present study were 24–26 years old. Samples retrieved from Cambodian workers showed 33 (2.3%) positive for HCV antibody by ELISA, as well as 25 (75.8%) samples positive for viral RNA upon RT-PCR of the 5'UTR. Participants aged between 21 and 35 years showed a high rate of HCV infection (table 2). Among the samples obtained from Myanmar workers, the most numerous immigrants to Thailand, 27 (1.69%) were positive for HCV antibody. The 21–35 years age group showed high infection rate, whereas none from the 36–40 years age group was anti-HCV positive (table 2). Fifteen samples proved positive for viral RNA. All RNA-positive samples were subjected to fur-

ther analysis of the core region and subsequently to direct sequencing.

Phylogenetic Analysis of HCV Genotypes

HCV genotype of all sequences was determined by phylogenetic analysis based on the core region. HCV-6

Table 1. Prevalence of HCV infection with age and sex among immigrant workers in Thailand

	Cambodia (n = 1,431)	Myanmar (n = 1,594)
Sex		
Male	959 (67.02%)	631 (39.59%)
Female	469 (32.77%)	865 (54.27%)
No data	3 (0.21%)	98 (6.15%)
Age, years	27.77 ± 8.14	27.13 ± 6.19
Anti-HCV positive	33 (2.31%)	27 (1.69%)
RT-PCR positive	25 (75.76%)	15 (55.56%)

Values for age are expressed as mean ± SD.

Table 2. Distribution of anti-HCV-positive samples and genotypes among different age groups of Cambodian and Myanmar immigrant workers

Age group years	Anti-HCV			Genotype										
	male	female	total ³	1a	1b	3a	3b	6e	6f	6m	6p	6r	6	Total
Cambodia (n = 1,431)														
21–25	5	0	5 (15.2)	0	2	0	0	0	1	0	0	0	0	3
26–30	3	3	6 (18.2)	0	0	1	0	1	0	0	0	1	1	4
31–35	5	2	7 (21.2)	0	2	1	1	0	1	0	0	0	0	5
36–40	2	2	4 (12.1)	0	1	0	0	0	0	0	0	2	0	3
41–45	1	3	4 (12.1)	0	1	0	0	3	0	0	0	0	0	4
46–50	3	1	4 (12.1)	0	0	0	2	0	0	0	1	1	0	3
>50	3	0	3 (9.1)	0	0	0	0	2	0	0	0	2	0	4
Total	22 (2.3) ¹	11 (2.3) ¹	33 (2.3) ¹	0 (0) ²	6 (24) ²	4 (16) ²	1 (4) ²	5 (20) ²	2 (8) ²	0 (0) ²	1 (4) ²	5 (20) ²	1 (4) ²	25 (75.8) ³
Myanmar (n = 1,594)														
15–20	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
21–25	4	4	8 (29.6)	0	0	1	4	0	0	1	0	0	0	6
26–30	2	3	5 (18.5)	1	0	1	0	0	1	0	0	0	0	3
31–35	2	5	7 (25.9)	0	1	1	0	0	0	1	0	0	0	3
36–40	0	0	0 (0)	0	0	0	0	0	0	0	0	0	0	0
41–45	1	3	4 (14.8)	0	0	1	1	0	1	0	0	0	0	3
46–50	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
>50	0	1	1 (3.7)	0	0	0	0	0	0	0	0	0	0	0
Total	9 (33.3) ¹	18 (66.7) ¹	27 (1.7) ¹	1 (6.7) ²	1 (6.7) ²	4 (26.7) ²	5 (33.3) ²	0 (0) ²	2 (13.3) ²	2 (13.3) ²	0 (0) ²	0 (0) ²	0 (0) ²	15 (55.6) ³

Figures in parentheses indicate percentages.

¹ Percent calculated with respect to all samples of each country.

² Percent calculated with respect to total RNA-positive samples.

³ Percent calculated with respect to total anti-HCV-positive samples.

was predominant in Cambodian workers (56%), followed by 1b (24%), 3a (16%) and 3b (4%). This group showed at least four clusters of HCV-6, -6e, -6f, -6p and -6r (table 2). One sequence, CBD3571, did not cluster with any of the reference sequences but was grouped close to the clade of 6e and 6u (fig. 1). Subtype 6e from Vietnam and China was grouped with the Cambodian cluster (fig. 1). It seemed that subtype 6e was transmitted from Cambodia. Based on the cohort study and previous report, subtypes 6p and 6r were found mainly in Cambodia (fig. 1) [14].

To analyze the ambiguous isolates, the highly divergent strains, CBD3571 was further subjected to amplification and sequencing of the NS5B region using specific primer sets [19]. Phylogenetic analysis of the neighbor-joining tree generated by the 6-parameter model showed that the CBD3571 strain clustered most closely with subtype 6u (61% of 1,000 bootstrap resampling tests, data not shown). The respective strain occupied a distinct branch of both core and NS5B phylogenetic trees.

Phylogenetic analysis (table 2) showed that samples from Myanmar were mainly genotype 3b (33.2%), the most prevalent genotype in this study. The remaining strains were 3a (26.7%), 6 (26.7%), 1a (6.7%) and 1b (6.7%). Subtypes 6f and 6m were identified in this group. Subtype 6f was grouped with Cambodian and Thai strains (fig. 1). Subtype 6m is generally detected in Myanmar and Thailand. There was no specific cluster of subtype 1b, 3a, 3b and 6m isolates in this study. Subtype 6f from Cambodia and Myanmar has likely migrated from Thailand (fig. 1).

Discussion

Information on HCV infection in some Southeast Asian countries is quite limited, especially Cambodia and Myanmar. This study was carried out to determine the epidemiology of HCV among foreign immigrant workers from Cambodia and Myanmar. Anti-HCV seroprevalence of Cambodian workers was 2.3%, which was quite similar to the 2.2% determined for Thailand [6]. Myanmar immigrants showed low prevalence of anti-HCV at 1.69%. The subjects from the two countries recruited into the study were mainly young people with a mean age of 26–27 years, which may account for the low prevalence of anti-HCV in this survey, while older age groups tend to show a higher prevalence of HCV infection [6].

A high level of HCV infection (6.5%) has been detected mainly in adult males from Cambodia. Intravenous injection of various drugs, a popular habit in the Takeo

province, may constitute the major source of infection [7]. Another report from rural Cambodia has shown that even in young age groups, HCV prevalence was very high (10.4%) [8]. In 2002, a community-based survey suggested that intravenous drug abuse was common and administered at excess rate among the general population. The population knew about HIV transmission associated with dirty needles, but only half were concerned that hepatitis virus could be transmitted by the same route [20]. In contrast to previous studies, the present study demonstrated a lower level of HCV infection (2.3%) mainly representative for healthy male Cambodians. Place of residence in their home country could not be identified. In the meantime, the Cambodian government has made an effort to discourage intravenous drug injection and improve public health [20]. Hence, the decrease in HCV infection rate observed with the samples tested could imply that the health care infrastructure of Cambodia has improved.

Viral RNA was detected in 75.8 and 55.6% of the anti-HCV seropositive samples from Cambodia and Myanmar, respectively. In agreement with various reports, the percentage of anti-HCV positive samples ranged from 50 to 90% [17, 21]. Some individuals who have naturally cleared the virus may remain seropositive without exhibiting viremia. However, owing to low viral load, HCV RNA could not be detected in some infected individuals. This study has provided information on various HCV genotypes detected in immigrants from Cambodia and Myanmar. The primer sets in this study can be used to detect various genotypes of the virus, especially the divergent HCV genotype 6 [18, 19, 22]. HCV genotypes and subtypes can potentially be determined based on the nucleotide sequence of the core region [23].

Various HCV genotypes were detected among Cambodian immigrants in this survey. Some genotypes are common in Thailand (1b, 3a, 3b, 6e and 6f), while some subtypes of HCV-6 are not found in the native population (6p, 6r and 6u; fig. 1) [19]. Subtype 6e is likely transmitted from Cambodia to other countries such as China and Vietnam (fig. 1). Subtype 6r seemed to originate from Cambodia in correlation with a previous study (fig. 2) [14].

There is a large influx of immigrants from Myanmar and Cambodia to Thailand. In 2007, the annual report from the Office of foreign worker administration Thailand showed that 498,091 and 26,096 people had immigrated from Myanmar and Cambodia, respectively (<http://115.31.137.7/workpermit/main/Stat/syear.asp>, reported in Thai). As a large sample size was available,

Fig. 1. Phylogenetic tree constructed on partial core coding sequences. Sequences determined in this study are in bold. HCV genotypes are indicated on the branch of the individual cluster. Reference sequences were obtained from GenBank database. Bootstrap values >80% were indicated at each node.

Fig. 2. Comparison of HCV genotypes in this study with those reported in previous studies in Thailand [6] and Myanmar [12].

healthy workers were included in this study and they may have migrated from different parts of the country. Based on the results of this study, the trend of HCV infection could be extrapolated to the general population. Even though the HCV infection rate was lower than expected [10, 11], the predominance of genotype 3 (3a; 26.7% and 3b; 33.5%) of Myanmar immigrant workers in this survey was similar to previous studies [11, 12]. HCV-3 is also the predominant genotype in Thailand followed by genotype 1b and genotype 6 (fig. 2) [6]. However, we have no data based on the previous study of HCV genotypes in Cambodia for comparison. Subtype 3a from Cambodia and Myanmar had mingled with subtypes from other countries (fig. 1). Genotype 3a is globally prevalent in injection drug users [24, 25], as well as common in some Asian countries [6, 12, 18, 26]. Therefore, unsafe needle sharing or drug abuse may introduce this genotype to the general population. Furthermore, these two countries are connected by trade, travel and migration all of which may contribute to similar patterns of virus transmission and genotype distribution.

HCV-6 is known as the genotype exclusive to Southeast Asia and as the most diverse genotype [19, 22, 27].

HCV-6 was predominant and subtype 6a was most prevalent in North Vietnam [28]. A previous report based on GenBank, EMBL and BBDJ database study suggested that subtype 6f was most prevalent and seemed to originate in Thailand [19]. The present study showed that this subtype is also circulating in Myanmar and Cambodia which may be due to the close connection and dynamic movement of migrating people among these countries. However, some subtypes are restricted to a specific geographical area. Thus, subtype 6r is specific for Cambodia, subtype 6p is found in Cambodia and Vietnam. Subtype 6m appeared to have migrated from Myanmar and mingled with the subtype prevalent in Thailand (fig. 1). It could be speculated that novel unassigned genotypes or subtypes may have accumulated in this area.

As immigrants can easily find employment in Thailand, their numbers are steadily increasing. Their respective original residence in their home countries could not be ascertained in this study. Despite the low incidence of HCV infection in these foreign workers, infectious diseases such as HIV, HAV and HBV may affect these groups. Hence, additional studies ought to be performed.

The prevalence of HCV infection in Cambodia and Myanmar immigrant workers determined in this study is similar to Thailand. Participants were mainly of a young age, which may provide an explanation for lower infection levels than previously reported. Various and as yet unclassified subtypes of HCV-6 may have accumulated in Southeast Asia. Further research should be focusing on HCV genotype distribution, novel subtypes of HCV-6, the evolution of the virus and incidence of HCV-related HCC in Southeast Asian countries.

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