ภาคผนวก

ตารางแสดงอัตราการติดเชื้อตับอักเสบ เอ ค่าความคลาดเคลื่อนและจำนวนตัวอย่างจำแนกตามกลุ่มอายุ

กลุ่มอายุ (ปี)	อัตราการติดเชื้อตับอักเสบ เอ (P:%)	ค่าความคลาดเคลื่อน (d :%)	จำนวนตัวอย่าง
15-20	40	5	369
21-30	60	5	369
31-40	80	5	246
41-50	90	5	138
51-60	90	5	138
รวม	-	-	1,260

ตารางแสดงอัตราการเป็นพาหะตับอักเสบ บี ค่าความคลาดเคลื่อนและจำนวนตัวอย่างจำแนกตามกลุ่มอายุ

กลุ่มอายุ (ปี)	อัตราการเป็นพาหะตับอักเสบ บี (p:%)	ค่าความคลาดเคลื่อน (d:%)	จำนวนตัวอย่าง
15-20	8	3.1	294
21-30	8	3.1	294
31-40	8	3.1	294
41-50	8	3.1	294
51-60	8	3.1	294
รวม	-	-	1,470

ตารางแสดงอัตราการติดเชื้อตับอักเสบ ซี ค่าความคลาดเคลื่อนและจำนวนตัวอย่างจำแนกตามกลุ่มอายุ

กลุ่มอายุ (ปี)	อัตราการติดเชื้อตับอักเสบ ซี (p:%)	ค่าความคลาดเคลื่อน (d:%)	จำนวนตัวอย่าง
15-20	4	2.2	305
21-30	4	2.2	305
31-40	4	2.2	305
41-50	4	2.2	305
51-60	4	2.2	305
รวม	-	-	1,525

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Annals of Tropical Medicine & Parasitology, Vol. 103, No. 4, 361–363 (2009)

SHORT COMMUNICATION

High seroprevalence of hepatitis A virus among migrant workers from Myanmar, Cambodia and Laos who are living in Thailand

In Thailand, the socio–economic development that has occurred over the last few decades has brought general improvements in living standards, sanitation and hygiene. In consequence, there has been a gradual decline in the general seroprevalence of infection with hepatitis A virus (HAV), from a hyper-endemic situation to the current meso-endemicity (Poovorawan *et al.*, 1997; Chatchatee *et al.*, 2002; Chatproedprai *et al.*, 2007). There is, however, some concern that areas with large populations of migrants from the neighbouring countries of Myanmar, Cambodia and Laos may still be hyper-endemic for HAV, although little is known about the HAV situation among these migrants or in their countries of origin. In 2004, the Thai Department of Employment estimated that there were >1,200,000 people from Myanmar, Cambodia and Laos working in Thailand (lib.doe.go.th/doeinfor/pagedata/ebookdoc/020400006369_1.pdf), and this estimate took no account of unregistered and illegal migrants. The annual influx of migrants is predicted to increase over the next few decades, and it is clear that the health problems of these workers could well have an impact on the non-migrant population.

In a cross-sectional study in 2008, the sero–epidemiology of HAV among migrants from Myanmar, Cambodia and Laos working in Thailand was explored. It was hoped that the data collected would help define the sero–epidemiology of HAV within the migrants' countries of origin and thus be useful in planning preventive strategies in those areas.

DOI: 10.1179/136485909X435085

All legal immigrant workers are registered with the Thai government and are required to have annual health check-ups. The subjects of the present study were legal workers from Myanmar, Cambodia and Laos who were aged between 16 and 60 years and had health insurance at the Bangkok 9 International Hospital, Bangkok. All had immigrated to Thailand within the previous 5 years and worked either in Bangkok or in provinces near Bangkok, such as Samutsakhon and Samutsongkhram. Potential subjects who had chronic illness, were receiving immunosuppressive therapy, and/or had the clinical signs or symptoms associated with HIV/AIDS or any other immunodeficiency-related disease were excluded. The protocol was approved by the ethics committee of Chulalongkorn University's Faculty of Medicine and by the director of the Bangkok 9 International Hospital.

The recruitment of subjects was sequential — as migrant workers presented at the Bangkok 9 International Hospital for their yearly check-ups, a blood sample was collected from each of them (a routine part of such check-ups). The blood (of each eligible subject) that was left after the routine haematology was used in the present study, with each subject given a code number to preserve his or her anonymity. Serum was separated off and stored at −20°C until it could be tested for anti-HAV IgG, in a commercial ELISA (Murex Biotech, Dartford, U.K.), at the Center of Excellence in Clinical Virology in Chulalongkorn University's Faculty of Medicine.

Overall, 1183 subjects (394, 394 and 395 from Myanmar, Cambodia and Laos, respectively) were investigated. The 594 males and 589 females investigated had a mean (S.D.) age of 28.1 (9.0) years. The recorded seroprevalences of anti-HAV (see Table) varied from 85.6% among the workers from Laos to almost 100% in those from Cambodia and Myanmar.

Immigrants from Thailand's neighbouring countries bring tuberculosis, syphilis, malaria, polio and filariasis into the country, and have contributed to the revival of infectious diseases that had been eradicated from Thailand, such as leprosy. In 2004, the seroprevalence of anti-HAV in the non-migrant Thai population was found to increase from 17.3% among subjects aged 16–20 years to approximately 75% among those aged >40 years (Chatproedprai *et al.*, 2007; see Table). The subjects of the present study showed no age-specific trends in HAV seroprevalence but even the youngest subjects were very likely to have anti-HAV antibodies (Table).

Although this study was confined to legal migrant workers, illegal immigrants only represent a small percentage of all immigrant workers in Thailand. It therefore seems likely that the present results give fairly accurate estimates of the seroprevalences of HAV among the adult immigrants from Myanmar, Cambodia and Laos who are working in Thailand (although it remains unclear if they also reflect the seroprevalences of HAV in the peoples who live in the countries that surround Thailand). Almost all of the present subjects had already been infected with HAV (presumably in their countries of origin) by the time they had reached the age of 16–20 years. Although information on anti-HAV seroprevalence in Myanmar, Cambodia and Laos is limited, those intending to travel to these countries, from areas where these is a low risk of HAV infection, should receive HAV immunization. Hepatitis A epidemics can occur as a consequence of non-immune travellers returning from HAV-endemic countries (Jong, 2005). Although the current recommendation is to immunize travellers against HAV at least 2 weeks before their trip, hepatitis A vaccine has proven effective in controlling outbreaks (Poovorawan *et al.*, 1994) and might be administered at any time before departure because, even if given immediately before the journey, it will still provide travellers with protection (Connor, 2005).

In Thailand, as in many other countries, access to immunization schedules — outside of the government-sponsored programmes of childhood vaccination — may be particularly challenging for migrant populations because of language, cultural and financial barriers. Programmes of hepatitis A immunization targeted at the migrant workers' children who are born in Thailand might be considered.

In conclusion, anti-HAV prevalence among the adult migrants from Myanmar, Cambodia and Laos who work in Thailand

TABLE. *The seroprevalences of IgG against hepatitis A virus recorded among immigrant workers, from Myanmar, Cambodia and Laos, in Thailand in 2008*

Age (years)	No. of subjects investigated and (% found seropositive)			
	Migrants from Myanmar	Migrants from Cambodia	Migrants from Laos	Non-migrant Thais*
16–20	127 (100)	134 (100)	120 (76.7)	369 (17.3)
21–30	135 (99.3)	83 (100)	173 (86.1)	380 (35.8)
31–40	97 (100)	82 (98.8)	86 (94.2)	446 (59.4)
41–60	35 (100)	95 (100)	16 (100)	659 (72.5)
16–60	394 (99.7)	394 (99.7)	395 (85.6)	1854 (50.9)

*Data collected in 2004 by Chatproedprai *et al.* (2007).

is very high, possibly reflecting high prevalences of hepatitis A in the migrants' countries of origin. Hepatitis A immunization prior to travelling to Myanmar, Cambodia and Laos should be considered.

ACKNOWLEDGEMENTS. This study received financial support from the Thai government's research fund, the National Research Council of Thailand, the Higher Commission of Education, the Ministry of Education, Chulalongkorn Hospital, the Center of Excellence in Clinical Virology, the CU Centenary Academic Development Project and The Center of Excellence Research Fund, Chulalongkorn University. The authors would like to thank all the staff in Bangkok 9 International Hospital for their assistance in the collection of blood samples, and the staff of the Center of Excellence in Clinical Virology, at Chulalongkorn University, for their help. The authors also thank P. Hirsch for reviewing the manuscript.

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Received 17 November 2008,
Revised 24 March 2009,
Accepted 25 March 2009

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

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Key Words Hepatitis C virus • Seroprevalence • Genotype • Immigrant Workers • Cambodia • Myanmar

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Running title: HCV among workers from Cambodia and Myanmar

Abstract: 212 words,

Text: 2483 words,

Reference: 28 references,

Table: 2 tables,

Figure: 2 figures

Abstract

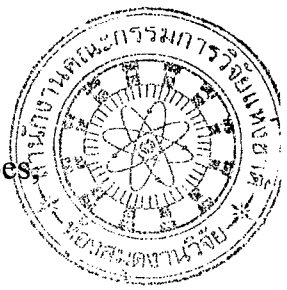
Objective: There are a large number of immigrant workers from Cambodia and Myanmar to Thailand. Our study was aimed at determining seroprevalence and genotypes of HCV in this group. ***Methods:*** Immigrants aged between 15 and 60 years (1431 Cambodians and 1594 Myanmarese) were recruited into this study. 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 Myanmarese which may reflect the prevalence in each country and closely relate to those prevalence in the guest country.

Introduction

A very high degree of genetic diversity of hepatitis C virus has lead to persistent infections. This agent currently infects approximately 170 million people around the world [1]. Chronic HCV carriers are at a significantly increased risk of liver cirrhosis and progression to hepatocellular carcinoma [2].

A high prevalence of hepatitis C virus has been found in Southeast Asia. HCV epidemiology is well documented in Vietnam and Thailand. The prevalence of HCV infection was 1% to 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 level 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.

Hepatitis C virus (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 to 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 relate 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 Bangkok 9 international hospital for their annual health check up were recruited. Sera collected from Cambodia and Myanmar workers amounted to 1431 and 1594 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 five 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 for 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°C until further tested.

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, IL). 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, Medison, WI). Viral RNA was detected by cDNA amplification of the 5' noncoding region as previously described [6]. Amplification of the non-coding region was performed with 2.5 μl 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 μM dNTP, 1.5 mM Mg^{2+}, 1.25 units of *Taq* DNA polymerase adjusted to a final volume of 25 μl with distilled water. The amplification conditions consisted of a pre-incubation step at 95°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 1 min and extension at 72°C for 1.30 min, and concluded by a final extension step at 72°C for 7 min. For nested PCR, 1.0 μl of PCR

product was amplified under the same conditions using primers IC3 (GGAACTACTGTCTTCACGCAG, position: 51-71) and IC4 (TCGCAAGCACCCTATCAGGCA, 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 (v.2.01), BioEdit (v.5.0.9) (Ibis Therapeutics, Carlsbad, CA) and SeqMan (DNASTAR, Medison, WI). All sequence results and reference strains of the core coding region were aligned using CLUSTALW version 1.83. Neighbor-joining trees were generated using the Gojobori-Ishi-Nei-six parameter method. Confidence values were calculated based on bootstrap resampling tests multiplied by 1000 (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, 1431 and 1594 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 to 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 21-35 years showed a high rate of HCV infection (table 2). Samples obtained from Myanmar workers, the most numerous immigrants to Thailand, showed 27 (1.69%) positive for HCV antibody. The 21-35-year age group showed high infection rate, whereas, none of the 36-40-year age group displayed anti-HCV positive (table 2). Fifteen samples proved positive for viral RNA. All RNA positive samples were subjected to further 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 was predominant in Cambodian workers

(56%), followed by 1b (24%), 3a (16%) and 3b (4%). This group showed at least 4 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 CBD3751 strain clustered most closely with subtype 6u (61% of 1000 bootstrap resampling tests, data not shown). The respective strain occupied a distinct branch of both core and NS5B phylogenetic trees.

Phylogenetic analysis 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%) (table 2). 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 hepatitis C virus infection in some South East Asian countries is quite limited, especially Cambodia and Myanmar. This study was carried out to determine the epidemiology of hepatitis C virus 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. They knew about HIV transmission associated with dirty needles but only half of the population 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 sero-positive samples from Cambodia and Myanmar, respectively. In agreement with various reports, the percentage of anti-HCV positive samples was ranging from 50-90% [17, 21]. Some individuals who have naturally cleared the virus may remain sero-positive 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 was likely to transmit 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 498091 and 26096 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, 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 from which may all contribute to similar patterns of virus transmission and genotype distribution.

HCV-6 is known as the genotype exclusive to South East 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 group 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.

Acknowledgements

This research was supported by the National Research Fund, the Center of Excellence in Clinical Virology Fund, Faculty of Medicine, Chulalongkorn University and, Thai Red Cross Society. Commission on Higher Education, Ministry of Education, Chulalongkorn University, CU Centernary Academic Development Project and the RGJ program of the Thailand Research Fund. Also, we would like to express our gratitude to the Bangpakok9 International Hospital for collecting the specimens. We also would like to thank Ms Petra Hirsch for reviewing the manuscript.

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Legend

Fig. 1. Phylogenetic tree constructed on partial core coding sequences. Sequences determined in this study are label as bold letters and black circle. Hepatitis C virus genotypes are indicated on the branch of the individual cluster. Reference sequences were obtained from GenBank database. Bootstrap values which more than 80 percent were indicated at each node.

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

Table 1. Prevalence of hepatitis C virus infection with age and sex among immigrant workers in Thailand (ND: no data).

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

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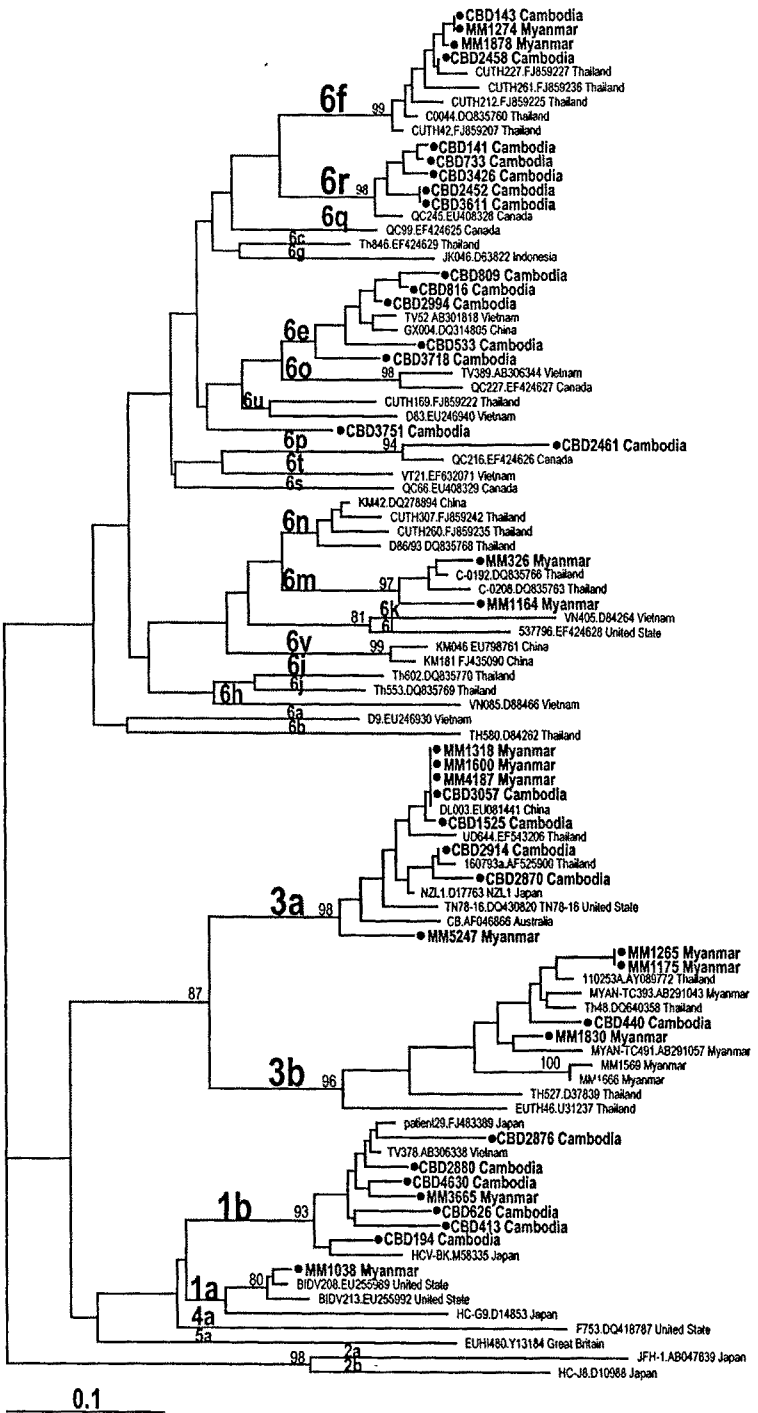

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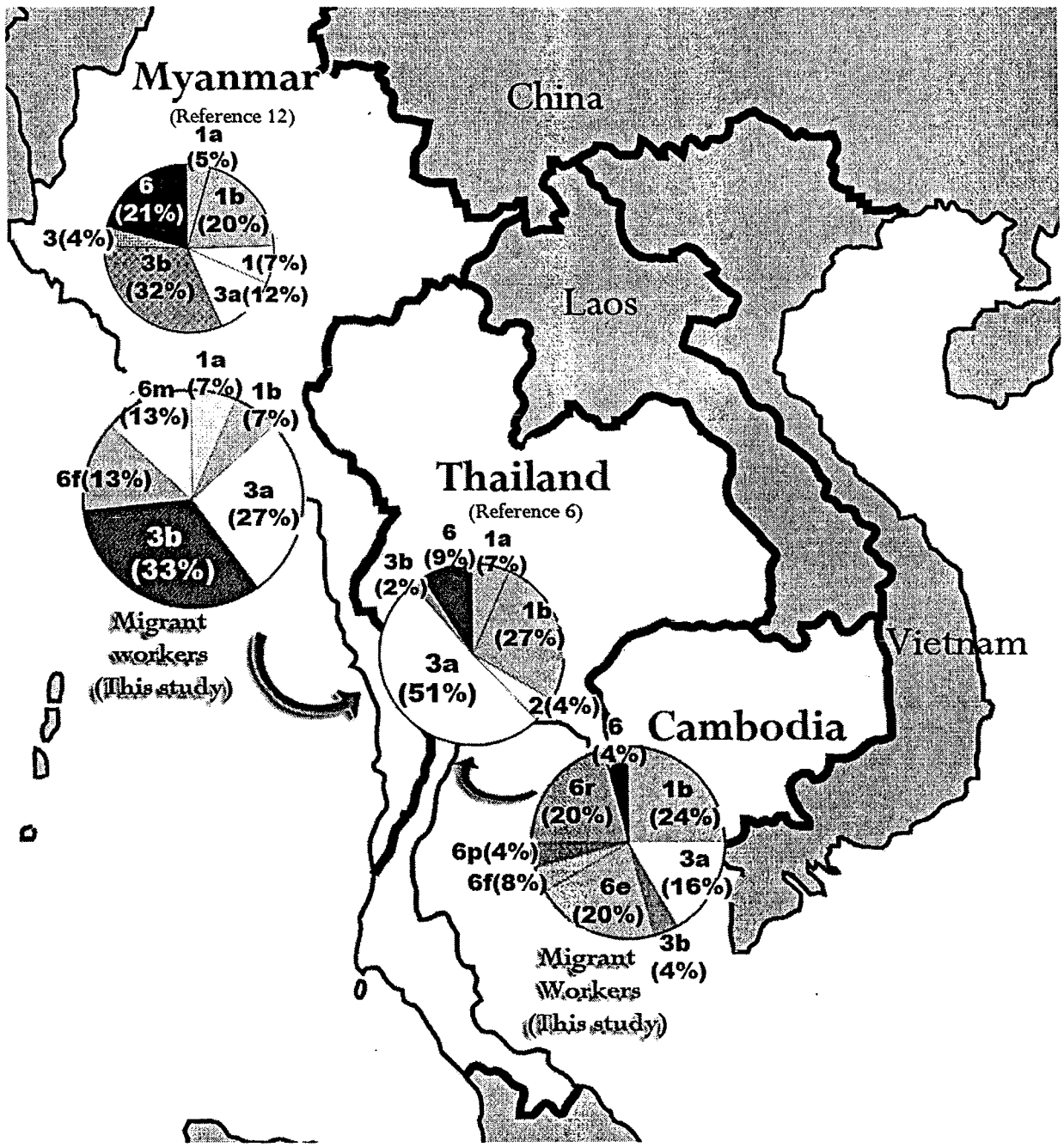

Table 1. Prevalence of hepatitis C virus infection with age and sex among immigrant workers in Thailand (ND: no data).

	Cambodia	Myanmar
	(n =1431)	(n = 1594)
Sex: Male	959 (67.02%)	631 (39.59%)
Female	469 (32.77%)	865 (54.27%)
ND	3 (0.21%)	98 (6.15%)
Mean age (SD)	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%)

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

Age group	Anti-HCV			Genotype										
(Years)	Male	Female	Total(%[≠])	1a	1b	3a	3b	6e	6f	6m	6p	6r	6	Total
Cambodia (n=1431)														
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[*])	11(2.3[*])	33(2.3[*])	0 (0[±])	6(24[±])	4(16[±])	1(4[±])	5(20[±])	2(8[±])	0(0[±])	1(4[±])	5(20[±])	1(4[±])	25(75.8[≠])
Myanmar (n=1594)														
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*)	18(66.7*)	27(1.7*)	1(6.7[±])	1(6.7[±])	4(26.7[±])	5(33.3[±])	0(0[±])	2(13.3[±])	2(13.3[±])	0(0[±])	0(0[±])	0(0[±])	15(55.6%[≠])

≠ Percent calculated with respect to total anti-HCV positive samples

* Percent calculated with respect to all samples of each country

± Percent calculated with respect to total RNA positive samples

Molecular Epidemiological Study of Hepatitis B Virus among Migrant Workers from Cambodia, Laos and Myanmar to Thailand

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Running head: Hepatitis B virus in migrant workers in Thailand

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ABSTRACT

Although hepatitis B virus (HBV) infection is endemic in Southeast Asia, molecular epidemiological data on HBV circulating in some countries are currently limited. The aims of this study were to evaluate HBV seroprevalence and its genetic variability present among migrant workers from Cambodia, Laos and Myanmar in Thailand.

Sera collected from 1,119 Cambodian, 787 Laotian and 1,103 Myanmarese workers were tested for HBsAg. HBV DNA was amplified and the *preS/S* region was sequenced for genotyping and genetic mutation analysis. HBsAg was detected in 282 (9.4%). The prevalence of HBsAg among migrant workers from Cambodia, Laos and Myanmar was 10.8%, 6.9% and 9.7%, respectively. Of 224 subjects positive for HBV DNA, 86% were classified as genotype C (99% were sub-genotype C1) and 11.6% were genotype B (30.8%, 34.6% and 30.8% were sub-genotypes B2, B3 and B4, respectively). Various point mutations in the 'a' determinant region were detected in approximately 18% of these samples, of which Ile126Ser/Asn was the most frequent variant. Sequencing analysis showed that 19.1% of samples had *pre-S* mutations, with *pre-S2* deletion as the most common mutant (7.7%) followed by *pre-S2* start codon mutation (3.8%) and both *pre-S2* deletion and start codon mutation (3.3%). High prevalence of HBV infection (approximately 7-11%) was found among migrant workers from Cambodia, Laos and Myanmar, which may reflect the current seroprevalence in their respective countries. Our data also demonstrated that HBV sub-genotype C1 was the predominant strain and various naturally occurring mutations of HBV were not uncommon among these populations.

Key words: Hepatitis B virus, seroprevalence, genotype, mutation, Southeast Asia

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major causes of chronic liver diseases ranging from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC) [Ganem and Prince, 2004]. HBV, a member of the family *Hepadnaviridae*, is a relaxed-circular double stranded DNA virus of approximately 3,200 base pairs in length, with four overlapping open reading frames encoding the polymerase (P), precore (PC)/core (C), envelope (pre-S1/pre-S2/S), and X proteins [Ganem and Prince, 2004]. HBV shows remarkable genetic variability and is currently classified into at least eight genotypes, designated A to H and four major serotypes, including *ayw*, *ayr*, *adw* and *adr* [Kramvis et al., 2005; Norder et al., 1992]. Each genotype can be further divided into sub-genotypes based on 4-8 % divergence of the viral genome. HBV genotype and sub-genotype distribution appears to show varying geographic patterns [Allain, 2006; McMahon, 2009]. For instance, genotypes A and D are predominant in Western countries and India, whereas genotypes B and C are common in Southeast Asia, China and Japan. Genotype E is restricted to Africa, while genotypes F and H are found in indigenous populations in Alaska and Central and South America. In Asia, sub-genotype B1 is predominant in Japan, while sub-genotypes B2–5 prevail in other countries. Sub-genotype C1 is prevalent mainly in Southeast Asia, whereas sub-genotype C2 is commonly found throughout the Far East as for example, in Japan, China and Korea [Allain, 2006; McMahon, 2009].

Chronic HBV infection and its related hepatic complications are particularly important in Southeast Asian countries where the prevalence of the infection is relatively high, varying from 3-6% in Singapore, Malaysia and Brunei to approximately 6-12% in Indonesia, Philippines, Myanmar, Laos, Cambodia and

Vietnam [James, 2001; Merican et al., 2000; Sebastian et al., 1990; Alexander et al., 1990; Budihusodo et al., 1991; Amirudin et al., 1991; Utama et al., 2009; Lingao et al., 1989; Lansang, 1996; Nakai et al., 2001; Caruana et al., 2005; Jutavijittum et al., 2007; Thüring et al., 1993; Duong et al., 2009; Thuy et al., 2005]. In Thailand, the prevalence of HBV infection has declined upon implementation of the national HBV vaccination program, with present prevalence of approximately 4% [Luksamijarulkul et al., 2002; Suwannakarn et al., 2008; Theamboonlers et al., 1999]. The predominant HBV genotypes in this region are genoytypes C and B (Figure 1). Despite the high prevalence of HBV infection in Southeast Asia, data on its molecular epidemiology in this region are scarce, particularly in some countries such as Cambodia, Laos and Myanmar. At present, a large number of migrant workers, originating from these countries, are employed in various sectors of Thai industries located in Bangkok and neighboring provinces. In 2006, registered and non-registered foreign workers in Thailand were approximately 1,800,000 migrants [Martin, 2007]. In 2007, the distribution of working-age migrant workers of Cambodia, Laos and Myanmar was 111,391 (13.4%), 106,706 (12.9%) and 611,476 (73.7%), respectively [Pholphirul and Rukumnuyakit, 2007]. Growing influx of migrant populations may influence the prevalence of HBV infection and the resulting disease burden in Thailand. The present study has been aimed at evaluating the HBV seroprevalence and its genetic variability, including genotypes, antigenic subtypes and mutations present among these migrant workers. In addition, the phylogenetic relatedness of HBV strains isolated from these subjects was investigated.

MATERIALS AND METHODS

Study populations

The serum samples of migrant workers collected for a routine health check-up were stored at -70°C until further analysis. In this study, 3,009 serum samples collected from 1,119 Cambodians (353 females; 763 males and 3 unidentified), 787 Laotians (413 females, 364 males and 10 unidentified) and 1,103 Myanmarese (582 females, 423 males and 98 unidentified) were tested for Hepatitis B s antigen (HBsAg) by using commercially available automated ELISA assays (Murex, Biotech Limited, Dartford, Kent, England). Samples positive for HBsAg were subjected to further analysis aimed at molecular characterization of HBV. The project had been approved by the ethical committee of the Faculty of Medicine, Chulalongkorn University.

HBV DNA extraction, amplification and sequencing

HBV DNA was extracted from 100 microliters each of HBsAg-positive sera. The respective serum samples were incubated in lysis buffer (10 mM Tris-HCl ph 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS and 20 mg/ml proteinase K) at 50°C for 60 minutes followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The *Pre-S1/Pre-S2/S* region was amplified using primers Pre-S1F+ (5'-GGG TCA CCA TAT TCT TGG GAA C-3': position 2814-2835) and R5 (5'-AGC CCA AAA GAC CCA CAA TTC-3': position 1015-995) The total 25- µl reaction volume consisted of 10 µl of 2.5X 5 PRIME MasterMix solution (5 PRIME GmbH, Hamburg, Germany), 0.5 µl of 25 µM forward and reverse primers, 2 µl of DNA template and sterile distilled water. The thermocycler was programmed for HBV DNA

amplification as follows: initial denaturation at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, extension at 72°C for 1.30 minutes and a final extension step at 72°C for 7 minutes. The HBV DNA amplicons were isolated by 2% agarose gel electrophoresis at 100 volt for 60 minutes and stained with ethidium bromide. PCR product size was estimated in comparison with a 100-bp DNA ladder under UV light. The expected products were excised from the gel and purified using the Perfectprep® Gel Cleanup kit (Eppendorf, Hamburg, Germany). The purified samples were sent to a commercial DNA sequencing company (First BASE Laboratories Sdn Bhd, Selangor Darul Ehsan, Malaysia) for sequencing. Nucleotide sequences were edited by Chromas Lite program version 2.01 (Technelysium Pty Ltd., Queensland, Australia) and assembled by SeqMan (DNASTAR Lasergene software, Madison, WT).

Genotyping, subtyping and phylogenetic analysis

Each sample's sequences were aligned with each available human genotype stored at the GenBank database (National Center for Biotechnology Information, Bethesda, MD) by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Based on these alignments phylogenetic trees were constructed for genotyping using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ) for genotyping. The neighbor-joining method by Tamura-3 parameter was used for constructing phylogenetic trees. Some sequences were genotyped by the Viral Genotyping Tool (National Center for Biotechnology Information, Besthesda, MD). Genetic recombinants were further determined by SimPlot program and bootscanning analysis (Simplot version 3.5.1, Baltimore, MD). HBV nucleotides were translated into amino

acid sequences using the translation tool in ExPASy Proteomics Server (available on: http://www.expasy.ch/tools/dna.html). Subsequently, subtypes were identified based on the amino acids at positions 122 and 160 of the S protein.

HBV mutation analysis

HBV sequences were evaluated for mutations and deletions in the *pre-S1/pre-S2* regions. The amino acids at positions 120 and 160 of the S protein were indicative for 'a' determinant mutations.

Statistical analysis

Data were expressed as mean ± standard deviation (SD), and percentages as appropriate. Comparisons among groups were analyzed by the Pearson $\chi 2$ or Fisher's exact test for categorical variables and by One-Way ANOVA Bonferroni adjustment for quantitative variables. *P*-values below 0.05 were considered significant. All statistical analyses were performed using the SPSS software for Windows 17.0 (SPSS Inc., Chicago, IL).

RESULTS

HBsAg detection

HBsAg was detected in 282 of 3009 (9.4%) samples. This group comprised 121 Cambodians (10.8%), 54 Laotians (6.9%) and 107 Myanmarese (9.7%). Among these subjects, HBV DNA was detected in 102 Cambodians (84.3%), 42 Laotians (77.8%) and 80 Myanmarese (74.8%) (Table I).

Distribution of HBV genotypes and serotypes

All sequences obtained from this study were submitted to the GenBank database (accession nos. GQ855313-GQ85570 and GQ856585). Phylogenetic analysis was performed based on the *pre-S1/pre-S2/S* genes (Figure 2). Of those positive for HBV DNA, 194 of 224 (86.6%) cases were determined as genotype C (99% and 1% were sub-genotypes C1 and C5, respectively), 25 (11.2%) cases were identified as genotype B (32%, 36% and 32% were sub-genotypes B2, B3 and B4, respectively), 1 (0.44%) case as genotype A (sub-genotype A2) and 1 (0.44%) case as genotype D. As for antigenic subtype distribution, *adr* was the most common (68.3%), followed by *ayw* (8.9%), *adw* (6.7%) and *ayr* (0.9%). The prevalence of HBV genotype and subtype with respect to geographic location is shown in Table I. There were significant differences in genotype and serotype distribution among groups. Briefly, Cambodians and Laotians had significantly higher prevalence of genotype B but had significantly lower prevalence of genotype C than those of Myanmarese (p <0.05). In addition, Laotians had significantly higher prevalence of serotype *ayw* but had significantly lower prevalence of serotype *adr* than those of Cambodians and Myanmarese (p <0.05).

Although we did not sequence the entire genome in this study, three isolates with suspected inter-genotype recombinants were identified (isolate 31 with genotype B2/C1, accession no. GQ855407; isolate 612 with genotype B3/C1, accession no. GQ855454 and GQ855560; and isolate 3794 with genotype G/C1, accession no. GQ856585). Isolate 31 proved to be a recombinant of sub-genotypes B2 and C1, with its recombination breakpoint estimated at nucleotide 573 (Figure 3A). Isolate 3794 represented a recombinant of genotypes G/C1 with its recombination breakpoints between nucleotides 2006 and 157 (Figure 3B). Isolate 612 was classified as sub-

genotype B3 in the *pre-S/S* gene but showed sub-genotype C1 between nucleotides 1554 and 1974 (figure not shown).

Prevalence and characterization of the 'a' determinant mutations

In this study, various point mutations in the 'a' determinant region were detected in 35 out of 194 (18.0%) HBV isolates. Mutations were found in 19/94 (20.2%) of Cambodian samples, 6/38 (15.8%) of Laotian samples and 10/62 (16.1%) of Myanmarese samples. The most frequent mutation in Cambodian, Laotian and Myanmarese isolates was Ile126Ser/Asn. In addition, multiple point mutations in the 'a' determinant region were detected in 6 isolates (Table II). Amino acid sequence alignment of the partial S region of these 35 isolates is shown in Figure 4.

Prevalence and characterization of pre-S/S mutations

Upon direct sequencing, *pre-S* mutations were detected in 40 of 209 cases (19.1%). In this study, the prevalence of *pre-S* mutations/deletions among Cambodian, Laotian and Myanmarese migrant workers was 18.4%, 15.0% and 22.5%, respectively. As for the prevalence of site-specific *pre-S/S* mutations, *pre-S2* deletion was the most common (7.7%), followed by *pre-S2* start codon mutation (3.8%) and both *pre-S2* deletion and start codon mutation (3.3%) (Table III). Amino acid sequence alignment of the entire pre-S1/pre-S2 region of the 40 samples is shown in Figure 5.

DISCUSSION

Although chronic HBV infection prevails in Southeast Asia, the data on its molecular epidemiology in some countries in this part of the world are still limited. To our knowledge, this has been the first comparative study on molecular characterization of HBV circulating in Cambodia, Laos and Myanmar. Our study, which included identification of both viral genotypes and subtypes in a significant number of HBV carriers from these countries, demonstrated that the predominant HBV strains belong to categories C1/*adr*, which accounted for more than 85% of cases. These data were in agreement with previous reports that HBV genotype C was prevalent in Myanmar [Nakai et al., 2001], and sub-genotypes C1 and B4 were dominant strains in Cambodia [Huy et al., 2008]. These findings are not surprising but reflect the typical genotypes and subtypes circulating in Southeast Asia. The seroprevalence of HBsAg in these migrant workers was approximately 7-11%, similar to previous reports on seroprevalence in these countries but higher than a recent nationwide survey in Thailand (4%) [Luksamijarulkul et al., 2002; Theamboonlers et al., 1999]. This difference in seroprevalence among populations reflects a steady and remarkable decrease in chronic HBV carrier rate among Thai populations after the 1992 implementation of universal HBV vaccination.

HBV strains resulting from genomic recombination between different genotypes have been increasingly recognized in various parts of the world. In Asia, recombination of genotypes B/C has been reported in China, Hong Kong, Indonesia, Taiwan, Thailand and Vietnam [Sugauchi et al., 2002], whereas recombination of genotypes C/D has been detected in Tibet and China [Cui et al., 2002; Wang et al., 2005]. In addition, recombinants between genotypes A/C and genotypes A/D have

been documented in Vietnam [Hannoun et al., 2000] and India [Chauhan et al., 2008], respectively. Recently, a novel genotype I, with a complex recombination involving genotypes C, A and G has been reported in Vietnam and Laos [Huy et al., 2008; Olinger et al., 2008]. Although the entire genome sequence was not determined in this study, we identified three HBV isolates with suspected inter-genotype recombinants. It is of note that a hybrid of genotypes B3/C1 in this study displayed recombination breakpoints in the vicinity of the *preC/C* region, which is the most common site of inter-genotype recombination as previously described [Sugauchi et al., 2002]. Another recombinant of genotypes G/C with its recombination breakpoints between nucleotides 2006 and 157 was also demonstrated in this study. Interestingly, the site of breakpoints in this recombinant was different from that found in a hybrid of genotypes G/C previously identified by our group in a Thai patient with HCC [Suwannakarn et al., 2008].

Amino acid substitutions within the 'a' determinant domain could lead to conformational changes which may interfere with active and passive immunization against HBV infection [Carman et al., 1990]. The most common vaccine escape mutant results from the mutation at position 145 (Gly145Arg), which is located in the second loop of the 'a' determinant [Carman et al., 1990]. In this study, however, the most common amino-acid substitution found in Cambodian, Laotian and Myanmarese samples was located at position 126. In addition, the prevalence of 'a' determinant mutants among chronic carriers from these countries was approximately 15-20%, which was slightly higher than the prevalence among random chronic carriers recently reported (6-12%) [Echevarria and Avellon, 2006]. It has been proposed that vaccination might have increased a selection pressure on the emergence of surface mutants in relation to wild-type HBV, as has been observed in several regions of the

world [Carman et al., 1990; Coleman, 2006; Cooreman et al., 2001]. For example, a previous study in Taiwan demonstrated an increase in the prevalence of 'a' determinant mutants in children from 7.8% before to 23.1% 15 years after the introduction of universal vaccination against HBV [Hsu et al.,2004]. High prevalence of the variants among migrant workers in this study, however, might not be associated with previous vaccination because the coverage rates of HBV vaccine administration in their countries are generally low [Caruana et al., 2005; Soeung et al., 2009]. Thus, it is speculated that these mutants within the 'a' determinant region might have emerged in response to natural immunoselective pressure of the host. These infectious mutants have been circulating among individuals chronically infected with the virus.

Naturally occurring HBV *pre-S* mutations/deletions have been frequently reported in chronic HBV carriers. It has been shown that *pre-S* deletion mutants tend to accumulate during a later stage of persistent HBV infection, including cirrhosis and HCC [Chen et al., 2006]. In fact, the prevalence of these mutations/deletions is rather variable and considerably different, ranging from 0% to 36%, between diverse geographic areas [Huy et al., 2003]. In this study, the prevalence of *pre-S* mutations/deletions among Cambodian, Laotian and Myanmarese migrant workers amounted to 18.4%, 15.0% and 22.5%, respectively, which was higher than that determined by our previous study conducted on Thai populations (9.5%) [Suwannakarn et al., 2008]. As for the site of mutations, this study showed that *pre-S2* deletion was the most common mutation type, followed by *pre-S2* start codon mutation and the combined *pre-S2* deletion and start codon mutation. These results were in agreement with those recently reported from Japan, Korea and Thailand, according to which deletion in *pre-S2* regions and *pre-S2* start codon mutations were

among the most prevailing [Suwannakarn et al., 2008; Huy et al., 2003; Choi et al., 2007].

In conclusion, high seroprevalence of HBsAg (approximately 7-11%) was found among migrant workers from Cambodia, Laos and Myanmar, which may reflect the present prevalence of HBV infection in their respective countries. We also demonstrated that HBV sub-genotype/subtype C1/*adr* was the predominant strain circulating in these migrant workers. In addition, the 'a' determinant variants were frequently found in these populations, and might not be attributed to vaccine-induced mutation. Finally, *pre-S* mutations, especially *pre-S2* deletions and *pre-S2* start codon mutations were not uncommon among these populations.

ACKNOWLEDGEMENTS

This research was supported by the National Research Fund, the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Aniversary of His Majesty King Bhumibol Adulyadej, the Commission on Higer Education, the Thailand Research Fund, the King Chulalongkorn Memorial Hospital and the Center of Excellence in Clinical Virology, Chulalongkorn University, Bangkok, Thailand

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LEGENDS

Table I Prevalence of HBV genotypes and subtypes in migrant workers

Table II Prevalence of 'a' determinant mutations in migrant workers

Table III Prevalence of pre-S mutations in migrant workers

Figure 1 The prevalence and genotypes of HBV infection in Southeast Asia countries derived from previous reports. Charts in the left corner demonstrate the prevalence and subgenotypes among migrant workers from Cambodia, Myanmar and Laos in this study

Figure 2 Phylogenetic relationship of the sequence obtained in the present study and representative sequences of human HBV, orangutan and gibbon strains from GenBank. Region included in the comparison was the large S gene including *preS1*, *preS2* and HBsAg gene. Percentage bootstrap values (>75%) are shown at the respective nodes. The scale bar at the bottom indicates the genetic distance. The country of origin of migrant workers is indicated by a symbol (● - Cambodia, ▲- Laos and ■ - Myanmar)

Figure 3 Bootscanning analysis of suspected recombinant isolates. (A) complete *S* gene of isolate 31 was compared with HBV-B2 (AF121249) and HBV-C1 (AB112348); (B) Isolate 3794, nucleotide positions 2006 – 157, was compared with HBV-C1 (AB112348) and HBV-G (AB064310). Dashed line(s) indicate(s) the breaking point (s) of recombination. The number above the dashed line indicates the nucleotide position of each isolate compared with the reference strain (NC_003977)

Figure 4 Amino acid sequence alignment of the 'a' determinant region of 35 samples.

Figure 5 Amino acid sequence alignment of the entire pre-S1/pre-S2 region of 40 samples.

Table I Prevalence of HBV genotypes and subtypes in migrant workers

	Cambodia (n = 1,119)	Laos (n = 787)	Myanmar (n = 1,103)	Total (n = 3,009)	*P*-value
No. HBsAg positive	121 (10.8)	54 (6.9)	107 (9.7)	282 (9.4)	0.013*
No. HBV DNA positive	102 (84.3)	42 (77.8)	80 (74.8)	224 (79.4)	0.008*
Gender (M : F: ND[a])	81:20:1	31:11:0	46:28:6	158:59:7	0.030*
Age (yr; mean ± SD)	29.2±8.6	26.2±7.4	28.3±6.1	28.3±7.6	NS
Genotype					
A2[b]	1 (1.0)	0 (0)	0 (0)	1 (0.44)	NS
B	13 (12.7)	11 (26.2)	1 (1.25)	25 (11.2)	0.000*
B2	7 (6.9)	1 (2.4)	0 (0)	8 (3.6)	
B3	1 (1.0)	7 (16.7)	1 (1.3)	9 (4.0)	
B4	5 (4.9)	3 (7.1)	0 (0)	8 (3.6)	
C	86 (84.3)	30 (71.4)	78 (97.5)	194 (86.6)	0.000*
C1	86 (84.3)	29 (69.0)	77 (96.3)	192 (85.7)	
C5	0 (0)	1 (2.4)	1 (1.25)	2 (0.9)	
D[b]	0 (0)	0 (0)	1 (1.25)	1 (0.44)	NS
Suspected recombination					NS
B2/C1	1(1.0)	0 (0)	0 (0)	1 (0.44)	
B3/C1	0 (0)	1(2.4)	0 (0)	1 (0.44)	
G/C1	1 (1.0)	0 (0)	0 (0)	1 (0.44)	
Subtype					
adr	76 (74.5)	20 (47.6)	57 (71.25)	153 (68.3)	0.000*
adw	9 (8.8)	5 (11.9)	1 (1.25)	15 (6.7)	NS
ayr	1 (1.0)	1 (2.4)	0 (0)	2 (0.9)	NS
ayw	6 (5.9)	12 (28.6)	2 (2.5)	20 (8.9)	0.000*
Could not be identified	10 (9.8)	4 (9.5)	20 (25.0)	34 (15.2)	

Data were expressed as mean ± SD, no (%)

[a] Data not available; [b] *PreC* gene could not be amplified

* *P*-values □ 0.05; NS = no statistic significance

Table II Prevalence of 'a' determinant mutations in migrant workers

HBV DNA positive	Cambodia (n = 102)	Laos (n = 42)	Myanmar (n = 80)	Total (n = 224)
Amino acid substitution				
No. HBV sequencing available	94 (92.2)	38 (90.5)	62 (77.5)	194 (86.6)
Ile126Ser/Asn	6 (6.4)	2 (5.3)	4 (6.5)	12 (6.2)
Pro127Arg	1 (1.1)	0	0	1(0.5)
Gly130Arg	0	1 (2.6)	0	1(0.5)
Thr131Asn/Pro	0	1 (2.6)	2 (3.2)	3 (1.5)
Met133Thr	2 (2.1)	0	0	2 (1.0)
Phe134Leu	1 (1.1)	0	0	1(0.5)
Thr140Ile	0	0	1(1.6)	1(0.5)
Pro142Leu	1 (1.1)	0	0	1(0.5)
Gly145Arg/Ala	3 (3.2)	1 (2.6)	0	4 (2.1)
Trp156Leu	0	0	1(1.6)	1(0.5)
Ala157Gly	0	0	1(1.6)	1(0.5)
Ala159Val	1 (1.1)	0	0	1(0.5)
Pro120Thr + Ala128Asp + Cys138Tyr + Phe158Leu	0	1 (2.6)	0	1(0.5)
Lys122Gln + Thr131Asn + Met133Thr	1 (1.1)	0	0	1(0.5)
Gly130Arg + Met133Thr	1 (1.1)	0	0	1(0.5)
Thr131Asn + Phe134Tyr	1 (1.1)	0	0	1(0.5)
Thr131Asn + Phe134Tyr + Asp144Glu	1 (1.1)	0	0	1(0.5)
Ala128Val + Phe134Tyr + Phe158Leu + Ala159Gly	0	0	1(1.6)	1(0.5)
Total no. of 'a'determinant mutations	19/94 (20.21)	6/38 (15.79)	10/62 (16.13)	35/194 (18.04)

Data were expressed as no (%)

Table III Prevalence of pre-S mutations in migrant workers

Mutation/deletions	Cambodia (n = 102)	Laos (n = 42)	Myanmar (n = 80)	Total (n = 224)
No. Sequencing available	98 (96.1)	40 (95.2)	71 (88.8)	209 (93.3)
Pre-S1 start codon mutaion + pre-S1 deletion	1 (1.0)	0	0	1 (0.5)
Pre-S1 start codon deletion + pre-S2 deletion	0	0	1 (1.4)	1 (0.5)
Pre-S1 deletion	2 (2.0)	0	1 (1.4)	3 (1.4)
Pre-S1 deletion + pre-S2 deletion	1 (1.0)	0	0	1 (0.5)
Pre-S2 start codon mutaion	3 (3.1)	3 (7.5)	2 (2.8)	8 (3.8)
Pre-S2 start codon mutation + pre-S2 deletion	2 (2.0)	0	5 (7.0)	7 (3.3)
Pre-S2 start codon deletion + pre-S2 deletion	1 (1.0)	0	1 (1.4)	2 (1.0)
Pre-S2 start codon mutation + pre-S1 deletion	1 (1.0)	0	0	1 (0.5)
Pre-S2 deletion	7 (7.1)	3 (7.5)	6 (8.5)	16 (7.7)
Total no. of pre-S mutations	18 (18.4)	6 (15.0)	16 (22.5)	40 (19.1)

Data were expressed as no (%)

Figure 1 The prevalence and genotypes of HBV infection in Southeast Asia countries derived from previous reports. Charts in the left corner demonstrate the prevalence and subgenotypes among migrant workers from Cambodia, Myanmar and Laos in this study

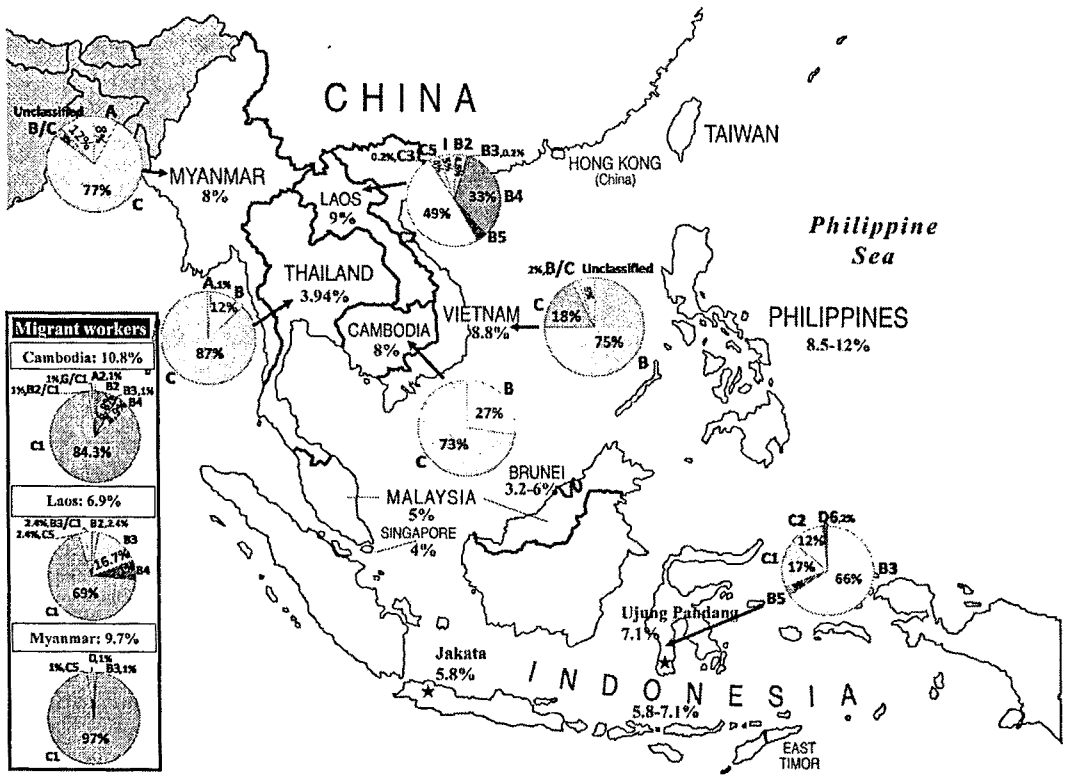
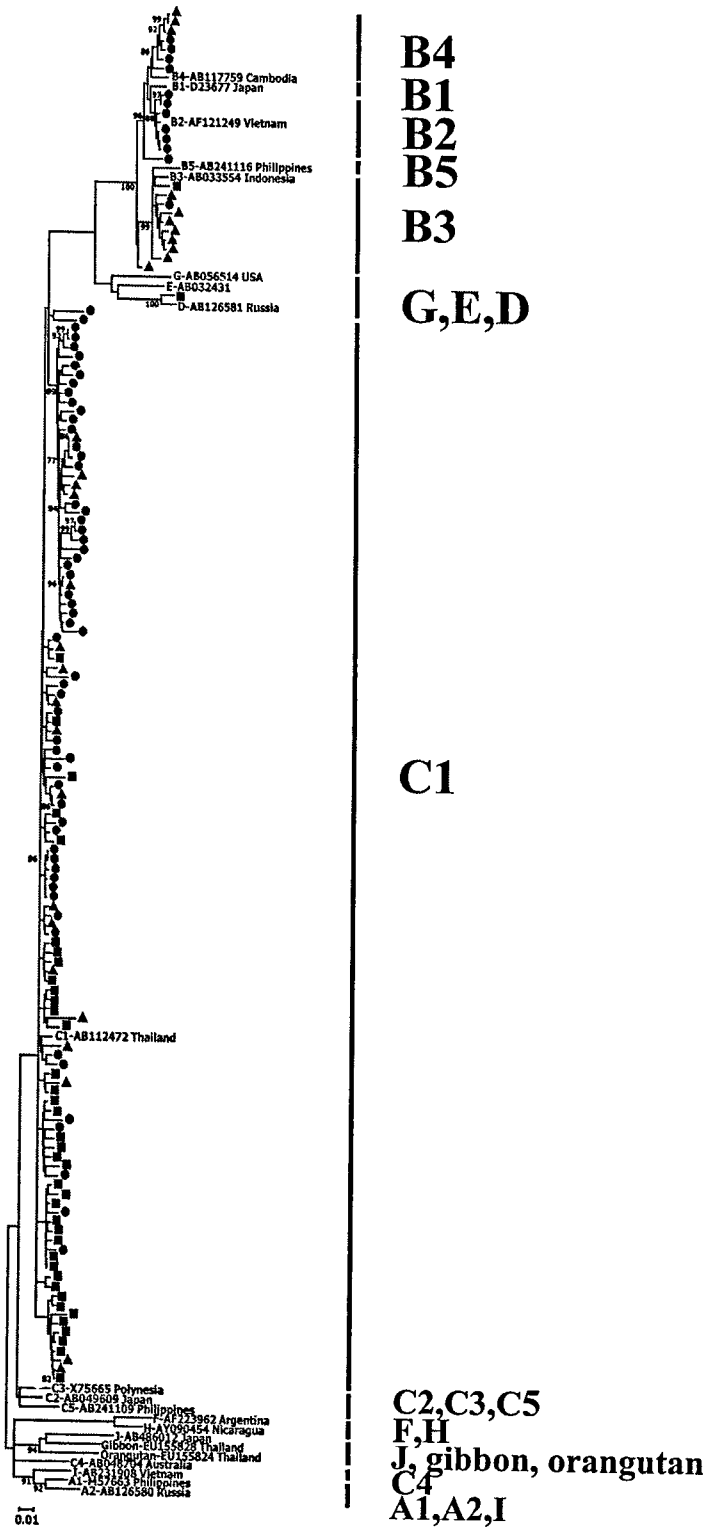


Figure 2 Phylogenetic relationship of the sequence obtained in the present study and representative sequences of human HBV, orangutan and gibbon strains from GenBank. Region included in the comparison was the large S gene including *preS1*, *preS2* and HBsAg gene. Percentage bootstrap values (>75%) are shown at the respective nodes. The scale bar at the bottom indicates the genetic distance. The country of origin of migrant workers is indicated by a symbol (● - Cambodia, ▲- Laos and ■ - Myanmar)


B4-AB117759 Cambodia
B1-D23677 Japan
B2-AF121249 Vietnam
B5-AB241116 Philippines
B3-AB033554 Indonesia
G-AB056514 USA
E-AB032431
D-AB126581 Russia
C1-AB112472 Thailand
C3-X75665 Polynesia
C2-AB049609 Japan
C5-AB241109 Philippines
F-AF223962 Argentina
H-AY090454 Nicaragua
J-AB480414 Japan
Gibbon-EU155828 Thailand
Orangutan-EU155824 Thailand
C4-AB048704 Australia
I-AB241108 Vietnam
A1-AB457953 Philippines
A2-AB126580 Russia
0.01
B4
B1
B2
B5
B3
G,E,D
C1
C2,C3,C5
F,H
J, gibbon, orangutan
C4
A1,A2,I

1

2

3

Figure 3 Bootscanning analysis of suspected recombinant isolates. (A) complete *S* gene of isolate 31 was compared with HBV-B2 (AF121249) and HBV-C1 (AB112348); (B) Isolate 3794, nucleotide positions 2006 – 157, was compared with HBV-C1 (AB112348) and HBV-G (AB064310). Dashed line(s) indicate(s) the breaking point (s) of recombination. The number above the dashed line indicates the nucleotide position of each isolate compared with the reference strain (NC_003977)

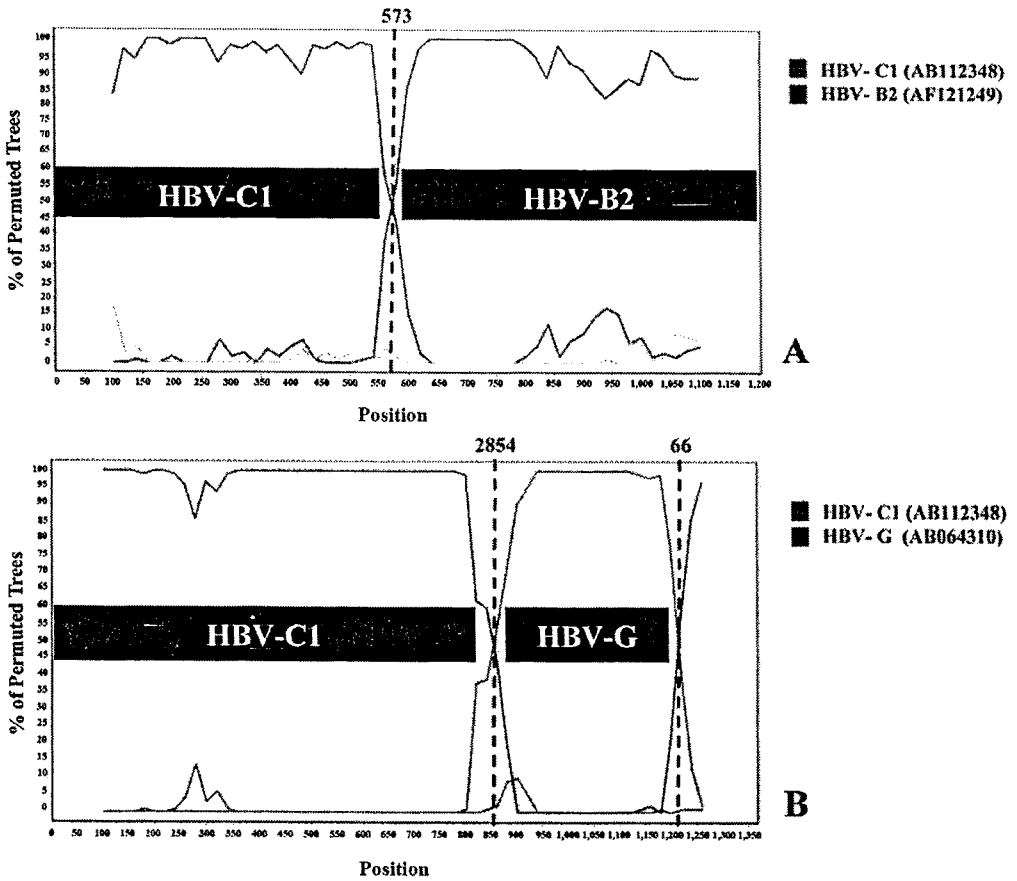
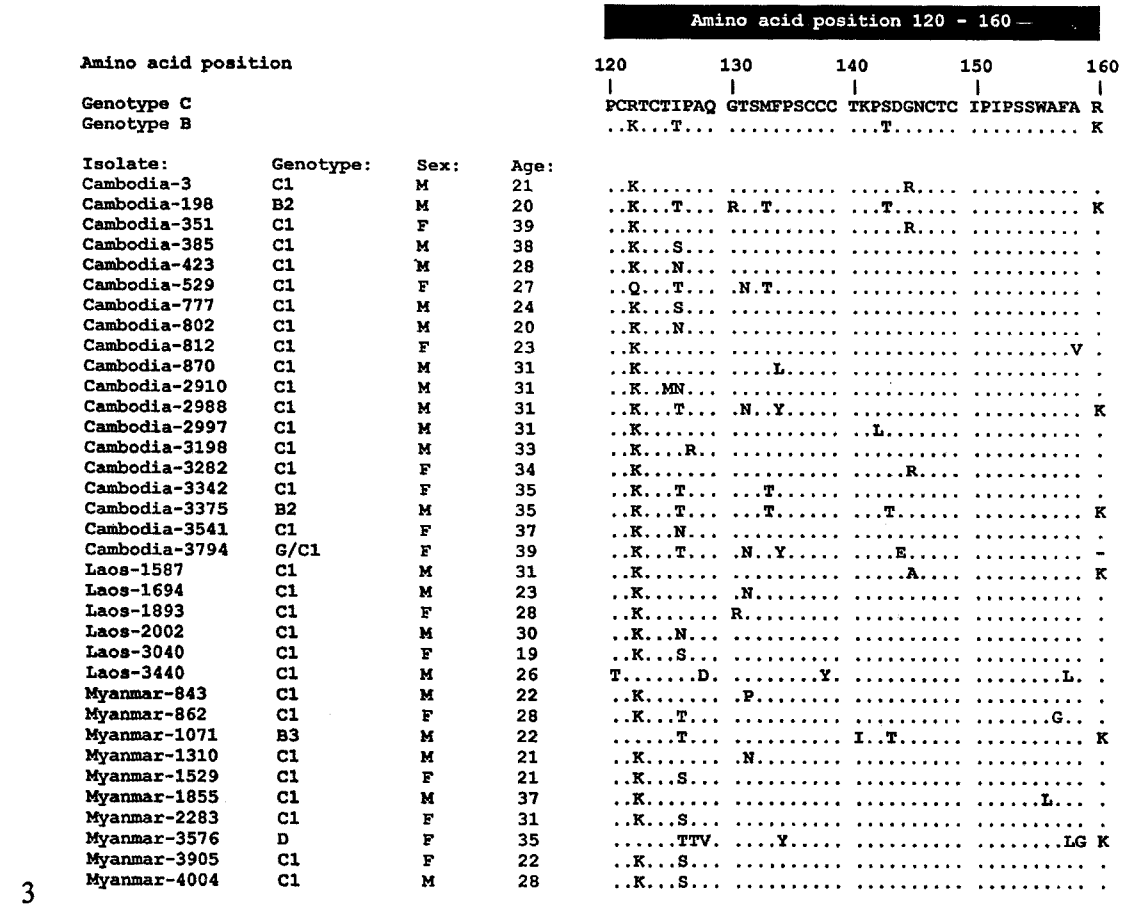


Figure 4 Amino acid sequence alignment of the 'a' determinant region of 35 samples.



Amino acid position				120	130	140	150	160
Genotype C				PCRTCTIPAQ	GTSMFPSCCC	TKPSDGNCTC	IPIPSSWAFA	R
Genotype B				..K...T...	....	...T......	....	K
Isolate:	Genotype:	Sex:	Age:					
Cambodia-3	C1	M	21	..K.....	....	.....R....	....	.
Cambodia-198	B2	M	20	..K...T...	R..T......	...T......	....	K
Cambodia-351	C1	F	39	..K.....	....	.....R....	....	.
Cambodia-385	C1	M	38	..K...S...	....	....	....	.
Cambodia-423	C1	M	28	..K...N...	....	....	....	.
Cambodia-529	C1	F	27	..Q...T...	.N.T......	....	....	.
Cambodia-777	C1	M	24	..K...S...	....	....	....	.
Cambodia-802	C1	M	20	..K...N...	....	....	....	.
Cambodia-812	C1	F	23	..K.....	....	....	V	.
Cambodia-870	C1	M	31	..K.....	....L.....	....	....	.
Cambodia-2910	C1	M	31	..K..MN...	....	....	....	.
Cambodia-2988	C1	M	31	..K...T...	.N..Y.....	....	....	K
Cambodia-2997	C1	M	31	..K.....	....	..L.......	....	.
Cambodia-3198	C1	M	33	..K....R..	....	....	....	.
Cambodia-3282	C1	F	34	..K.....	....	.....R....	....	.
Cambodia-3342	C1	F	35	..K...T...	...T......	....	....	.
Cambodia-3375	B2	M	35	..K...T...	...T......	...T......	....	K
Cambodia-3541	C1	F	37	..K...N...	....	....	....	.
Cambodia-3794	G/C1	F	39	..K...T...	.N..Y.....	....E.....	....	-
Laos-1587	C1	M	31	..K.....	....	.....A....	....	K
Laos-1694	C1	M	23	..K.....	.N........	....	....	.
Laos-1893	C1	F	28	..K.....	R.........	....	....	.
Laos-2002	C1	M	30	..K...N...	....	....	....	.
Laos-3040	C1	F	19	..K...S...	....	....	....	.
Laos-3440	C1	M	26	T.......D.	........Y.	....	........L.	.
Myanmar-843	C1	M	22	..K.....	.P........	....	....	.
Myanmar-862	C1	F	28	..K...T...	....	....	.......G..	.
Myanmar-1071	B3	M	22	......T...	....	I..T......	....	K
Myanmar-1310	C1	M	21	..K.....	.N........	....	....	.
Myanmar-1529	C1	F	21	..K...S...	....	....	....	.
Myanmar-1855	C1	M	37	..K.....	....	....	......L...	.
Myanmar-2283	C1	F	31	..K...S...	....	....	....	.
Myanmar-3576	D	F	35	......TTV.	....Y.....	....	........LG	K
Myanmar-3905	C1	F	22	..K...S...	....	....	....	.
Myanmar-4004	C1	M	28	..K...S...	....	....	....	.

Figure 5 Amino acid sequence alignment of the entire pre-S1/pre-S2 region of 40 samples.

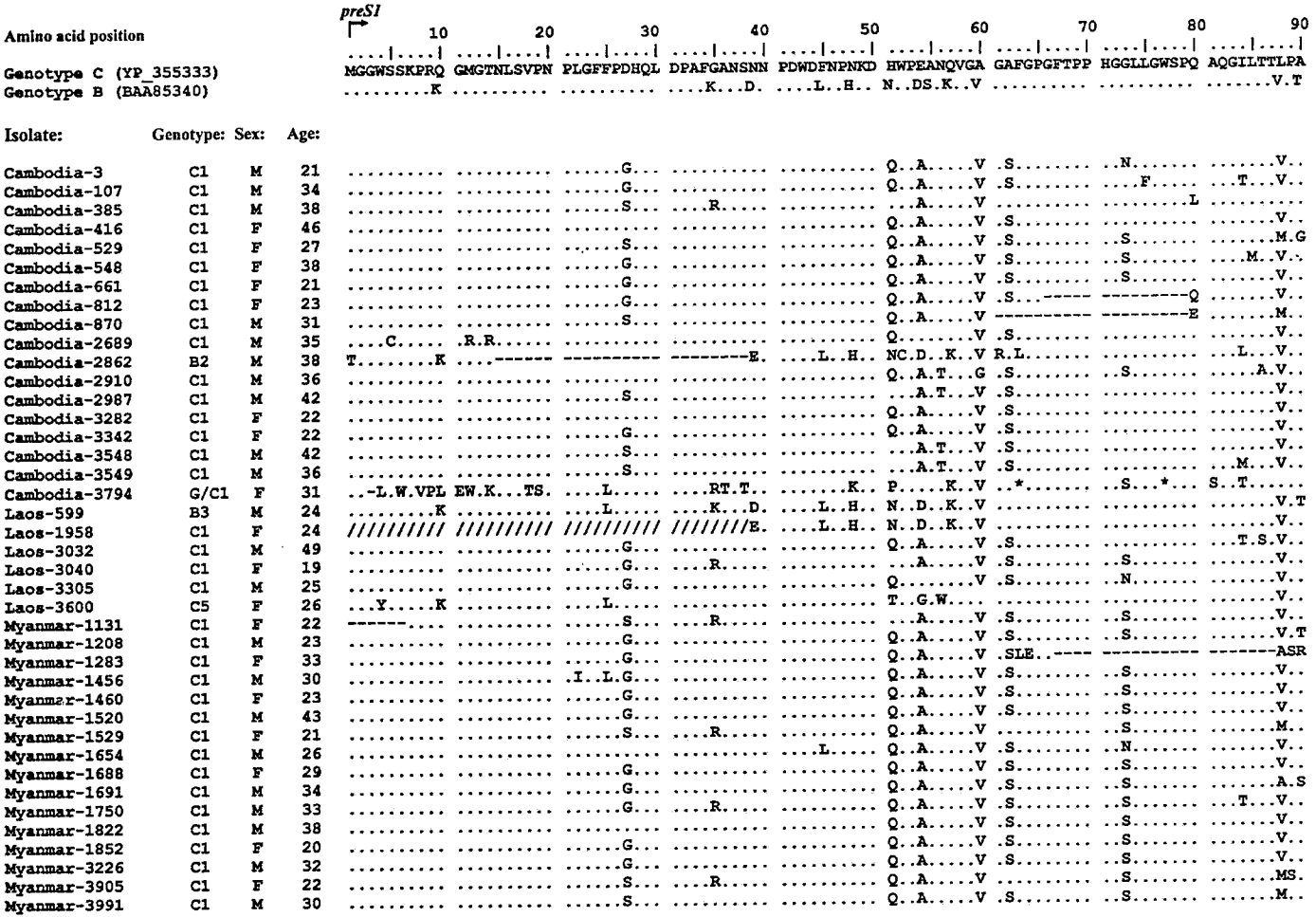


Figure 5 (Continued)