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Molecular Epidemiology and Genetic History of Hepatitis C Virus Subtype 3a Infection in Thailand

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Key Words
Hepatitis C virus subtype 3a · Epidemic · Genetic history · Evolution · Molecular clock

Abstract
Objective: Among all hepatitis C virus (HCV) infections, subtype 3a is the most common genotype in Thailand. This study investigates the molecular epidemiology and epidemic history of HCV subtype 3a in Thailand. ***Methods:*** Three hundred and fifty-six serum samples were collected from HCV-infected Thai patients. The virus was isolated, after which the core and NS5B regions were sequenced. Subsequently, the HCV genotype was classified by phylogenetic analysis based on the core and NS5B regions. Molecular evolution analysis of HCV subtype 3a was estimated using BEAST (Bayesian Evolutionary Analysis by Sampling Trees) v.1.5.4. ***Results:*** Based on our phylogenetic analyses, subtype 3a (38.5%) was the most prevalent, followed by 1a (21%), 1b (13.8%), genotype 6 (19.9%) [comprised of subtypes 6e (0.3%), 6f (11%), 6i (1.9%), 6j (1.9%) and 6n (4.8%)] and 3b (5.6%). Our phylogenetic tree indicates the existence of a specific group of HCV subtype 3a strains in the Thai population. Molecular evolutionary analysis dated the most recent common ancestor of the Thai HCV subtype 3a strains as existing approximately 200 ago, and a Bayesian skyline plot showed that this particular strain spread to Thailand during the mid-1970s and early 1980s. This period overlaps with the Vietnam War (1955–1975) and the widespread use of injection stimulants introduced by the US Army during this time. ***Conclusion:*** The estimated history of HCV subtype 3a infection in Thailand may help to predict the future burden of HCV-related diseases and facilitate better public health control and surveillance.

Introduction

Hepatitis C virus (HCV) is a major public health problem affecting approximately 170 million people worldwide [1], and persistent HCV infection may eventually lead to cirrhosis and culminate in hepatocellular carcinoma (HCC). In addition, serious HCV complications often occur without clinical symptoms until after two or three decades of viral incubation [2, 3]. More than 20

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years of epidemiological surveillance have proven that HCV-related HCC is increasing in developed countries [4]. Although in Europe and the United States an average of 3.7% of cirrhosis cases per year contribute to HCC incidence, the growing incidence in Japan is expected to reach a plateau in the near future [5, 6].

HCV is a member of Flaviviridae which can be classified into six major genotypes and many subtypes [7, 8]. Genotype distribution differs according to geographic regions, mode of transmission and treatment responses [2, 9, 10]. Genotypes 1, 2 and 3 are distributed worldwide and contribute to the majority of HCV infection. Subtypes 1a and 1b are most common in Europe, the USA and Japan, and subtypes 2a and 2b are predominant in Japan, North America and Europe, while subtype 3a is most prevalent in the Indian subcontinent and Thailand [11–13]. On the other hand, the less common genotypes 4, 5 and 6 are restricted to certain geographic areas. Genotype 4 is prevalent in the Middle East, genotype 5 in South Africa and genotype 6 in South China and Southeast Asia [13–16].

The mode of HCV transmission is associated with genotype distribution; for example, evolutionary analysis has suggested that HCV subtype 1a may be spread via blood transfusion and unsafe medical practices [17]. In addition, subtypes 1a and 3a are prevalent in young individuals and particularly in injecting drug users (IDUs) [18] in the USA, UK, Uzbekistan, Russia and Thailand [10, 19–21]. Past and present knowledge of HCV prevalence is important in order to be able to predict the future burden of HCV-related liver diseases. However, due to its asymptomatic nature and long-term complications, it is difficult to estimate the past prevalence of HCV infection from medical records. Thus, other methods are required. Coalescence theory is based on the hypothesis that all individuals originated from one common ancestor, and that populations which have evolved independently tend to genetically diverge over the time. Applying the coalescence theory and a molecular clock to phylogenetic reconstruction of present day HCV sequences allows HCV population dynamics to be estimated prior to the virus's discovery and investigation [9, 12], and can be used to estimate the modification of viral population size and viral epidemic behavior [22, 23]. A fine example of this method's efficacy comes from the elucidation that the high prevalence of HCV subtype 4a in Egypt was due to unsterile needle injection during mass treatment campaigns against schistosomiasis from the 1930s to the 1950s [9].

The distribution of HCV genotypes in Thailand has been investigated in this study and previously, and it has been found that subtype 3a is the dominant strain (39–51%) in all regions and populations. In addition, subtype 3a has a very high prevalence in IDUs (75–83%) and our phylogenetic analysis shows a specific clustering of Thai HCV, which indicates a specific transmission route. Other subtypes are also present in Thailand and the Thai population such as the 1a, 1b, 2a and 6 variants, but at a lower prevalence in comparison to subtype 3a [12, 24]. In Myanmar, Laos and Cambodia, Thailand's neighbors, HCV genotype 6 variants are predominant rather than 3a [14, 25, 26]. Thus, due to its high prevalence in the Thai population and geographic specificity, this study focused on the analysis of subtype 3a from Thai isolates along with reference strains from other countries.

Population-based surveys have shown that the HCV seroprevalence is 2.2% in Thailand [12]; however, HCC incidence caused by HCV infection is low in this country. Previous studies have demonstrated that the initial spread time of HCV infection is associated with the prevailing socioeconomic conditions, reflecting the present incidence as well as predicting the future development of HCC [11, 27–30]. Because HCC is associated directly with the incubation period of HCV infection and HCV subtype 3a is the predominant genotype [11, 30], the initial spread of HCV subtype 3a among the Thai population may occur later than in other countries.

Due to the epidemic history of HCV transmission, which reflects the present viral population and disease burden, the objective of the study is to estimate the time of HCV subtype 3a introduction and spread in the Thai population. The suspected mode of transmission history of HCV infection has also been described. This estimation may be useful for predicting the incidence of HCV-related diseases in the country and also used as data for HCV prevention and public health control.

Materials and Methods

Sample and Clinical Data Collection

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University before using the specimens. Serum samples were collected from HCV-infected patients at Chulalongkorn Hospital (Bangkok), the hospitals in Phetchabun Province (Phetchabun General Hospital, Lomsak General Hospital and Lomkao Crown Prince Hospital), as well as the National Blood Center, Thailand. Patients who were tested for anti-HCV and had positive results were sent to the Center of Excellence in Clinical Virology in order to investigate the viral RNA and genotype for treatment. Only samples with HCV RNA positive by RT-PCR were recruited for this study. Coded anonymous samples were collected from 2003 to 2009. All specimens

were stored at –70° until tested. Demographic data of patients such as age, sex, nationality, habitat and risk factors were also collected.

DNA Extraction and Sequencing

Viral RNA was extracted from 356 anti-HCV-positive serum specimens by using guanidine thiocyanate extraction [31]. Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Medison, Wisc., USA). Partial core and NS5B regions were amplified by RT-PCR using specific primer pairs (core: primers 410/951 and 953/951, NS5B: primers NS5BF1/R1 and NS5BF2/R2) as previously described [32]. The resulting cDNA fragments were purified (HiYield Gel/PCR DNA Fragment Extraction Kit, RBC Bioscience, Taipei, Taiwan) and subjected to direct sequencing (First BASE Laboratories, Selangor, Malaysia). Nucleotide sequences were edited and assembled using Chromas LITE (v.2.0.1), BioEdit (v.5.09; Ibis Therapeutics, Carlsbad, Calif., USA) and SeqMan (DNASTAR, Madison, Wisc., USA).

HCV Genotyping

The genotype of each sample was determined by phylogenetic analysis of both the core and NS5B sequences. All sequences were aligned with reference genotypes retrieved from the Los Alamos HCV database [33] using CLUSTALW v1.83. Neighbor-joining trees of partial core and NS5B sequences were constructed using the Gojobori-Ishi-Nei 6-parameter method with bootstrap resampling tests using 1,000 replicates (http://clustalw.ddbj.nig.ac.jp). Sequences clustering on the same branch as the reference strain were assigned the reference genotype. Some of the samples with HCV genotype 6 had been previously published elsewhere [32]. The core region represents nucleotide positions 377–669 and the NS5B region represents nucleotide positions 8282–8591 relative to the sequence M62321.There were discrepant subtypes between the core and NS5B trees; therefore, we reanalyzed all sequences by using another method. All sequences were subjected to FindModel (http://hcv.lanl.gov/content/sequence/findmodel/findmodel.html) to find the best substitution model. Then, the HCV genotype classification was confirmed by constructing maximum likelihood trees with the GTR+Γ model and bootstrapped 1,000 times using MEGA version 5.05.

Phylogenetic Analysis of HCV Subtype 3a

In total, 136 core sequences and 132 NS5B sequences clustering in subtype 3a were obtained. To analyze subtype 3a of Thailand, the available subtype 3a sequences of the core and NS5B genes were retrieved from the Los Alamos HCV database and aligned with the respective subtype identified in this study [33]. Redundant sequences originating from the same individual or from an individual with unknown nationality were excluded as were those of insufficient length in nucleotide sequence. Multiple alignments were calculated using CLUSTALW v.1.83 and the phylogenic relationships of the core and NS5B were constructed using the neighbor-joining method based on the 6-parameter model with a bootstrap test performed on 1,000 replicates (http://clustalw.ddbj.nig.ac.jp). Particular clusters originating from Thai subtype 3a strains were investigated.

Evolutionary Analysis of HCV Subtype 3a

The HCV subtype 3a sequences of the partial core and NS5B regions originating from the same sample, which grouped in the Thai cluster, were selected for further analysis. This left 74 sequences of the NS5B dataset which was 325 nucleotides long (representing nucleotide positions 8286–8610 relative to the sequence M62321) and subjected to evolutionary analysis. The most appropriate nucleotide substitution model for the NS5B sequences dataset was determined using the jModeTest version 0.1. The best-fitting model of these data sets was GTR+Γ. HCV subtype 3a population dynamics were estimated using the strict molecular clock and the uncorrelated lognormal model under the substitution model mentioned above. Since the samples were not systemically collected and contained insufficient temporal data to directly estimate the evolutionary rate, the external rate was used in both the strict and relaxed clock model. The previously estimated substitution rate of 5.8×10^{-4} substitutions per site per year obtained from the NS5B gene was applied as a prior to generate an evolutionary timescale of years [34]. The most appropriate clock model was selected based on the Bayes factors computed by Tracer v.1.5. The bayesian skyline plot approach implemented in BEAST (Bayesian Evolutionary Analysis by Sampling Trees) v.1.5.4 (http://evolve.zoo.ox.ac.uk/beast/) was used to calculate the epidemic history. Each Markov chain Monte Carlo analysis was run for 50,000,000 states and sampling every 5,000 states. Markov chain Monte Carlo convergence, effective sample size, the date of the most common ancestor of the cluster and Bayes factors were computed and investigated using Tracer v1.5, and annotated phylogenetic trees were displayed by FigTree v. 1.2.2 (http://evolve.zoo.ox.ac.uk/beast/).

Nucleotide Accession Number

All nucleotide sequences were submitted to the GenBank database. The accession numbers of partial core and NS5B sequences were HQ229038 and HQ229604, respectively. HCV genotypes in this study were classified according to the reference strains as follows: 1a: NC004102, EF407419,AB301742, AF511950, EU234064, DQ155558, EU256041, EU781772, D10749;1b: HM041987, HM041997, EF032894, AB016785, EU155228, U16362, D11355, FJ217354, EF032894, AF145454, EU155305, AB429050, AY587016, D90208, AF176573; 1c: AY051292; 2a: AB047639; 2b: D10988; 3a: D17763, AF046866, X76918, AF525902, AB472164, D14308, D10079, EF543248, AM423015, EF543249, X76918, AY003973, AF506583, D10078, AB327107, AB444431, AB444489; 3b: D37840, HM042021, D37854, D49374, D37853; 3k: D63821; 4a: NC009825, DQ418788; 4d: DQ418786; 5a: Y13184, NC009826; 6a: DQ480513, AY859526; 6b: D84262; 6c: EF424629; 6d: D84263; 6e: DQ314805; 6f: DQ835760, DQ835764; 6g: D63822, DQ314806; 6h: D84265; 6i: DQ835770, DQ835762; 6j: DQ835761, DQ835769; 6k: DQ278893, D84264; 6l: EF424628; 6m: DQ835765, DQ835763; 6n: AY878652, DQ835768; 6o: EF424627; 6p: EF424626; 6q: EF424625; 6r: EU408328; 6s: EU408329; 6t: EF632071; 6u: EU246940, and 6w: DQ278892.

Results

Clinical Data and Patient Background

A total of 356 serum samples collected from patients with detectable HCV-RNA were subjected to RT-PCR amplification and sequencing of the core and NS5B regions. Subject age ranged between 17 and 73 years and the mean age was 43.01 ± 10.9. Out of 187 patients with

Table 1. Genotype of HCV determined in this study

Core genotype	NS5B genotype											
	1a	1b	2a	3a	3b	6e	6f	6i	6j	6n	ND	total
1a	75											75 (21.1)
1b	1	48										49 (13.8)
2a			2									2 (0.6)
3a				130	1						5	136 (38.2)
3b					17						1	18 (5.1)
6e						1						1 (0.3)
6f							37				2	39 (11)
6i								7				7 (2.0)
6j									7			7 (2.0)
6n										17		17(4.8)
ND		1		2	2							5 (1.4)
Total, n (%)	76 (21.3)	49 (13.3)	2 (0.6)	132 (37.1)	20 (5.6)	1 (0.3)	37 (10.4)	7 (2.0)	7 (2.0)	17 (4.8)	8 (2.2)	356 (100)

Values are given as n (%). Genotypes are classified based on core and NS5B sequences. ND = not detected.

clinical reports, 92% were diagnosed with chronic liver disease, 5.3% with cirrhosis and 2.7% with HCC. The mean age of the patients in the HCC group was higher than in the chronic hepatitis and cirrhosis group (57, 43.7 and 46.3 years, respectively). Among patients in the chronic hepatitis group, males were predominant with a 2:1 male-to-female ratio. Approximately 92% of the patients lived in the central area of Thailand. One hundred and thirty (36.5%) subjects had known risk factors such as blood transfusion (33.1%), tattooing (27.7%), intravenous drug use (26.9%), unsafe medical injection (23.8%), shaving by a barber (23.1%), prostitution (13%), needle stick injury (11%), HIV coinfection (1.5%) and a spouse with HIV/HCV (1.5%) (see online supplementary tables 1, 2; for all online suppl. material, see www.karger.com/doi/10.1159/000351621). Among all risk factors, intravenous drug use incurred the greatest diversity of HCV genotypes identified (genotypes 1, 2, 3 and 6). In cases where the patient's background showed that more than one risk factor could be associated with multiple routes of transmission, the exact route could not be specified in this study (suppl. table 3).

HCV Genotype

Phylogenetic analysis based on partial core and NS5B sequences (n = 356 sequences) showed that most Thai patients carried subtype 3a (n = 137, 38.5%), followed by subtype 1a (n = 75, 21.1%), 1b (n = 49, 13.8%), genotype 6 (n = 71, 19.9%) and subtype 3b (n = 20, 5.6%). Genotype 6 was classified as 6e (n = 1, 0.3%), 6f (n = 39, 11%), 6i (n = 7, 2.0 %), 6j (n = 7, 2.0%) and 6n (n = 17, 4.8%) (suppl. fig. 1). Genotype 2 was detected in only 2 cases. Two samples, CUTH45 and CUTH233, showed discordant subtypes based on the core and NS5B, in that one sample was 1b/1a and the other was 3a/3b, respectively (table 1). To test that the discordant core and NS5B genotypes were not an artifact of a selected substitution model, the genotype was reclassified by other phylogenetic and nucleotide substitution models. The results showed the same classification was given to both discordant samples by the maximum likelihood trees and the neighbor-joining trees (suppl. fig. 2, 3).

HCV genotype 3 could be detected in all regions of the country; genotype 2 was identified in 2 patients residing in the central area, while genotypes 1 and 6 were not found in the southern and western regions, (suppl. table 2). These results may be due to the fact that most of the patients in this study were recruited from Chulalongkorn Hospital, hence central Thailand. In addition, HCV genotypes 1 and 3 were found in all stages of liver disease, while genotype 2 was found only in chronic hepatitis and genotype 6 was not detected in any HCC patients (suppl. table 1).

Phylogenetic Analysis of HCV Subtype 3a

HCV subtype 3a isolated in this study was analyzed in comparison with reference sequences available from the Los Alamos HCV database. Figure 1 shows the neighbor-

joining tree estimated from the partial core and NS5B sequences. In total, 136 core and 132 NS5B sequences clustered together and were classified as subtype 3a (table 1). In this clade, subbranches exhibited a phylogeographic structure by grouping samples from the same origin together, e.g. HCV strains from the former Soviet Union, Pakistan and Thailand (fig. 1a, b).

The phylogenetic tree of partial core sequences isolated in this study had 92 isolates in the Thai cluster, while the tree constructed from NS5B sequences had 82 isolates (fig. 1a, b). To define these Thai clusters more clearly, 74 samples from both the core and NS5B sequences from these same clusters were selected for further evolutionary analysis.

Evolutionary Analysis of HCV Subtype 3a

The NS5B dataset was analyzed using BEAST under a strict and uncorrelated lognormal relaxed molecular clock with a coalescence bayesian skyline plot model. Model comparison was undertaken through comparison of estimated marginal likelihoods of each model calculated by the Tracer v. 1.5 program. The results show that the relaxed molecular clock had a higher marginal likelihood than the strict model, ln P (model|data) was –3,028.84 and –3,039.35, respectively (log10 Bayes factor of >4.5). Using the data from the relaxed clock model, the origin of the most recent common ancestor of the HCV subtype 3a strains in Thailand existed around 200 years ago (95% credible interval, 573–15 years ago). The mean rate under relaxed clock (ucld.mean) was 9.28×10^{-4} (95% credible region: 1.23×10^{-6} to 2.04×10^{-3}) and the standard deviation (ucld.stdev) was 0.29 (95% credible region: 6.51×10^{-2} to 0.49). If the ucld.stdev is zero, it means no variation in the rate among branches. Rate heterogeneity among the site (α) of NS5B was 0.267 while the coefficient of variation (COV) was 0.3 (95% credible region: 0.05–0.49), which showed heterogeneity among branches. COV measures the rate variation among lineages. A small value of COV represents less rate variation and close to clock-like evolution. So, the evolution will be the strict molecular clock if the COV is equal to zero. Our COV was close to the previous reported value for HCV genotype 6 and Dengue virus [35, 36]. Covariance parameter of the relaxed clock was –0.0079 (95% credible region: –0.1642 to 0.151).

Figure 2 shows the bayesian skyline plot estimated from the NS5B dataset. The effective population size was relatively constant from the early 20th century before changing to exponential growth in the mid-1970s. From the early 1980s to the present the rate has been on a relatively slow decline and seems to be steady after the year 1990.

Discussion

In this study, we have described the genotypic and evolutionary analysis of HCV-infected patients in Thailand. While the severity of liver disease was associated with advanced age, the number of cirrhotic and HCC cases remained small. However, previous studies have found that the mean age of patients with HCC is significantly higher than that of chronic hepatitis patients, suggesting that protracted infection may increase the likelihood of severe complications [13, 15, 30].

In Thailand, HCV genotype 3 was the predominant genotype with subtype 3a being the most common subtype. Genotype 1 and 6 variants were also prevalent, followed by subtype 3b and subtypes 2a and 2c [12, 37]. However, although the genotypic distribution of HCV found in this study is similar to previous reports, these results likely do not accurately represent Thailand's overall genotype distribution because most of the examined samples were obtained from patients living in the central region of Thailand. Also, two samples, 1b/1a and 3a/3b, showed discordance between subtypes based on the core and NS5B regions (table 1, suppl. fig. 1, 2). Intragenotypic recombination of HCV has been reported before, thus additional analysis should be performed to identify the recombination breakpoint of these discrepant samples to confirm the recombination event [38].

Some of the HCV genotypes show phylogeographic distribution, e.g. genotype 2 is common in Africa and genotype 6 is common in East Asia [29, 35]. Phylogenetic analysis indicated that there was a HCV subtype 3a-specific cluster for Thai strains (fig. 1), which may be attributable to a specific route of transmission. In addition, these strains shared a most recent common ancestor that existed approximately 200 years ago. Based on our phylogenetic trees (fig. 1), the Thai subtype 3a strains did not form a monophyletic cluster, thus various 3a strain ancestors may have been introduced into the country in varying regions and at different time points. However, because most of the HCV subtype 3a stains were collected from central Thailand, we were unable to analyze the possible entry point of the original HCV strain into Thailand, while the emergence of subtype 3a in Thailand may be related to the introduction of Western medical practices such as vaccinations and operations [39].

Phylogenetic reconstruction (fig. 1) showed the Thai and Indian/Pakistani clusters in close proximity, which supports the argument of a close relationship between the HCV subtype 3a viruses circulating in those countries. Historically, the Indian culture has had a profound influ-

Color version available online

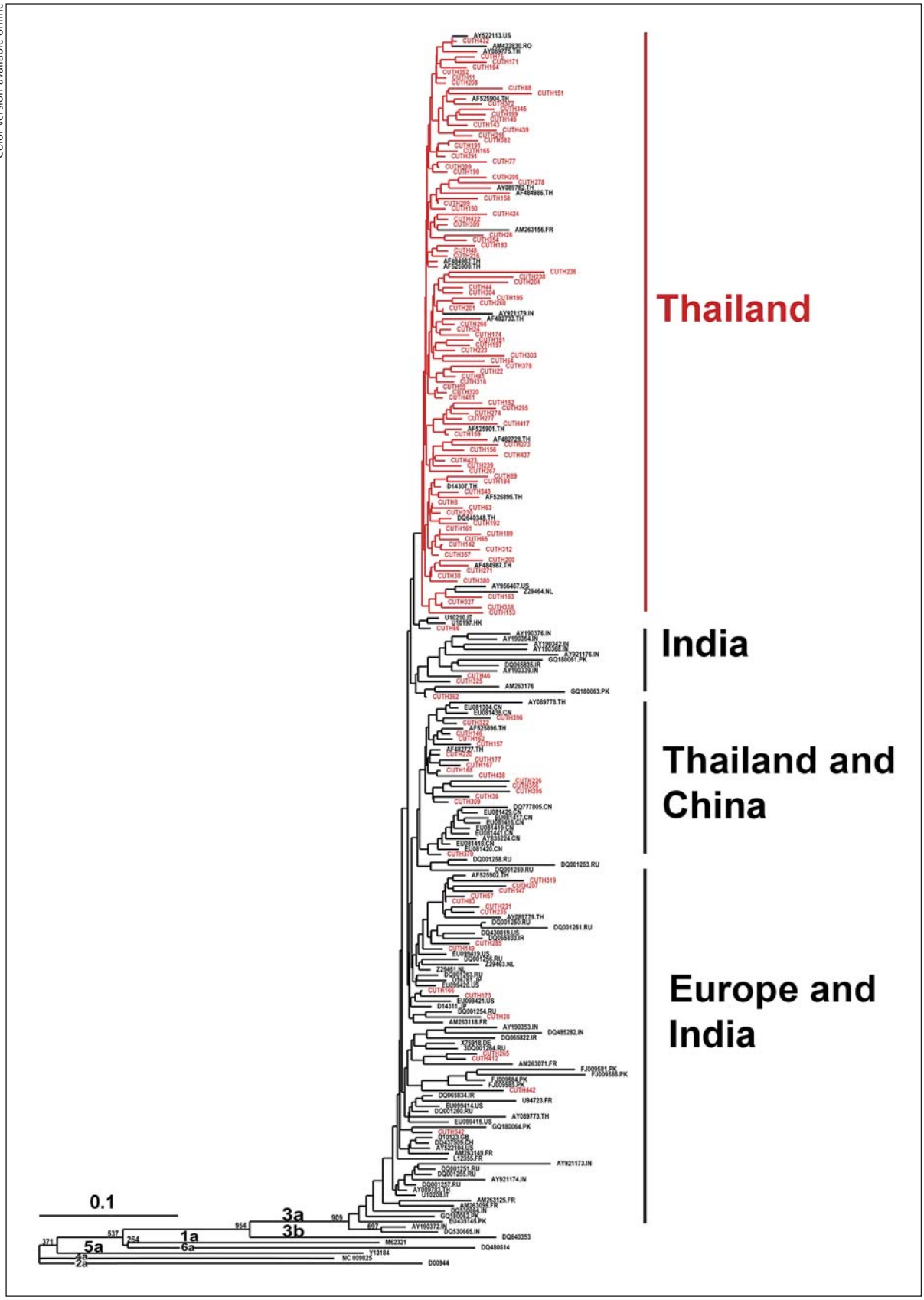
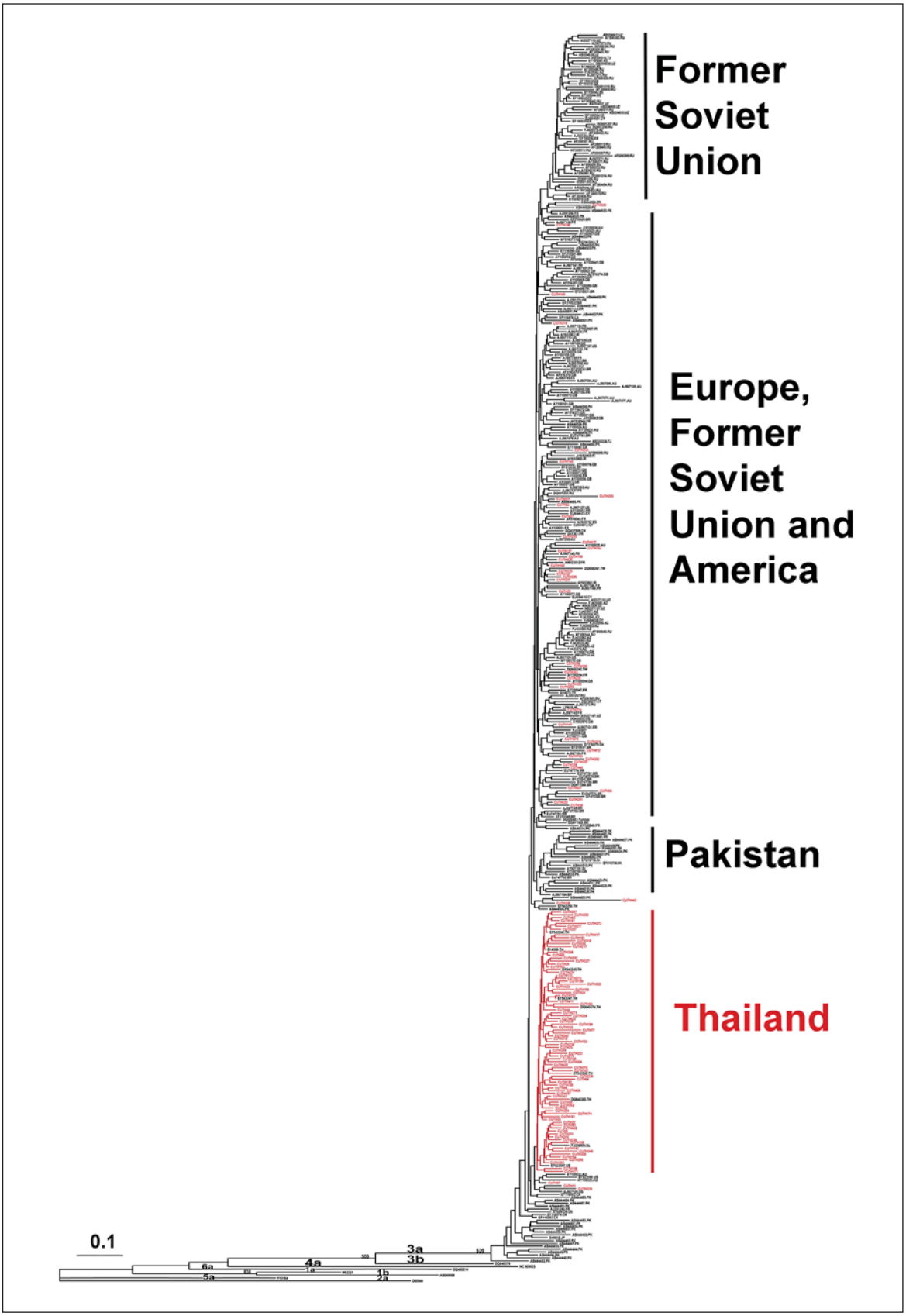


Fig. 1. Phylogenetic tree constructed based on the core (**a**) and NS5B (**b**) region of HCV subtype 3a. Thai isolates identified in this study are labeled in red. The red branches represent a particular cluster of Thai which subjected to evolutionary analysis. Isolates of the same origin tend to cluster together and their countries are shown as abbreviation names. AU = Australia; AZ = Azerbaijan; BR = Brazil; CA = Canada; CH = Switzerland; CN = China; CY = Cyprus; EE = Estonia; ES = Spain; FR = France; GB = United Kingdom; IN = India; IR = Iran; JP = Japan; LK = Sri Lanka; LT = Lithuania; NL = The Netherlands; PK = Pakistan; RU = Russia; TH = Thailand; TJ = Tajikistan; TN = Tunisia; TW = Taiwan; US = United States; UZ = Uzbekistan.



1b

(For legend see page 289.)

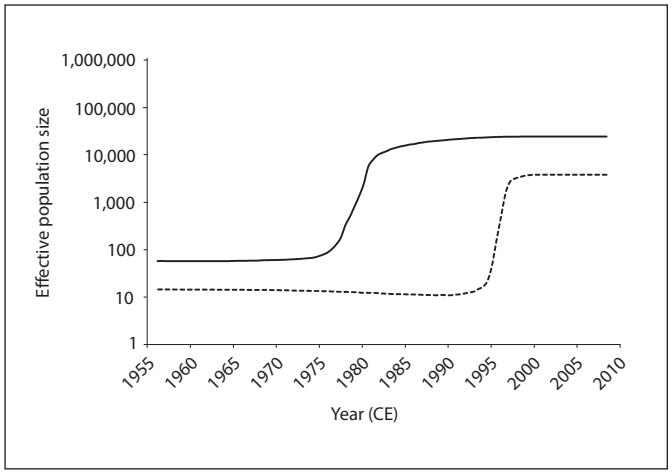


Fig. 2. Bayesian skyline plot for HCV subtype 3a of the Thai cluster. The black line indicates the estimated median of effective numbers of infection based on the NS5B gene and the dotted lines represent 95% highest posterior density confidence interval.

ence on Thai culture, language, ceremony and food. In addition, there has been a history of trade migration between both countries, which may have resulted in a long period of mutual HCV transmission. The widespread introduction of HCV subtype 3a in the Thai population occurred around the mid-1970s and was later introduced to Pakistan [11]. A prior study suggested that the subtype 3a population in Pakistan is older than it is in other countries, and helps contribute to the high number of HCC patients in that country. Khan et al. [11] estimated that the 3a subtype was introduced to the Pakistani population around the 1950s and thus prior to its emergence in the former Soviet Union (ca. 1960s) [30] and Thailand (ca. 1970s). So, it is possible that the most recent common ancestor may have existed in this area.

While 81% of HCV patients in Pakistan carry HCV subtype 3a [11], the prevalence of subtype 3a in India varies upon the sampling population and location. For example, in the northeastern part of India, HCV subtype 3a constitutes around 8% of all genotypes [40]; however, in North and Central India the prevalence of genotype 3 is around 80% [41]. Also, in Bangladesh, an analysis of 168 samples showed that subtype 3b was most prevalent (45.5%), followed by subtype 3a (18.7%) [42]. The prevalence of HCV subtype 3a in the Indian subcontinent is in contrast to reports on Southeast Asian countries – except Thailand – in which the most prevalent genotypes are genotypes 1 and 6 [25, 32, 43–46].

Bayesian phylogeography has provided evidence that HCV subtype 3a was transmitted via heroin trafficking and IDU stemming from the Golden Triangle and Golden Crescent to the Chinese provinces of Yunnan in 1981 and Xinjiang in 1990, which are adjacent to the Golden Triangle and Golden Crescent, respectively. Those two provinces served as a hub for the drug trade and also played an important role in the viral transmission of viruses such as HIV and HCV to other regions of China and Asian [47]. In addition, subtypes 1a and 3a were proposed to have been introduced from an endemic area to the industrial nations by IDU [18]. According to all of these studies, HCV subtype 3a may circulate in an endemic area for a long period and then migrate to other parts of the world through trading, population migration and drug trafficking with subsequent IDU.

As evidenced by our molecular clock estimation, Thai HCV subtype 3a strains have experienced a rapid expansion of their effective population from the mid-1970s to the early 1980s (fig. 2). This time period of expansion is consistent with an influx of IDUs in Thailand as a result of US Army deployment in Southeast Asia during the course of the Vietnam War (1955–1975). At that time, the US Army set up camp at several locations throughout the North and Northeast of Thailand, and the use of stimulants via intravenous administration was widespread [48]. More recently, however, the trend of drug administration has changed from needle to noninjection (non-IDU) practices such as inhaling or oral uptake. Specifically, methamphetamine use has dramatically risen since the 1990s and has largely replaced intravenous drug use. HCV prevalence among IDUs (approx. 86%), however, is still proportionally much higher than non-IDUs (approx. 5%) [21, 49].

Thus, even though we propose that needle sharing may be implicated as the major route of transmission of subtype 3a in Thailand, other factors may have a part in maintaining the present day transmission rates. Risk factor profiles of our patients showed that blood transfusion represented the most profound risk factor in this study (suppl. table 3). Thus, we hypothesize that while intravenous drug use may have been the major cause of the viral expansion in the Thai population during the mid-1970s through the 1980s, contaminated blood products likely play a role in maintaining the current and new viral strains in the population.

In the past, anonymous blood donation was not a popular practice among Thai people, who traditionally preferred to receive blood from their friends or relatives (http://www.blooddonationthai.com/, in Thai). Howev-

er, by 1985 this behavior had changed and blood transfusions from anonymous donors gradually replaced the old tradition. In 1986, donors from convicts were excluded, and in 1990 the National Blood Bank, Thai Red Cross Society implemented universal screening for anti-HCV. At this time, it was found that 2% of first-time donors were positive for HCV antibodies, though this gradually declined to 0.5% by 2009 [50]. Presently, the blood screening policy of the National Blood Center consists of questionnaires to exclude high-risk donors, anti-HCV serology screening of all blood units using chemiluminescent immune assay with some negative samples being further screened by nucleic acid amplification technology [50]. Furthermore, coverage of nucleic acid amplification technology testing throughout the country has been increased from 34% in 2009 to 80% in 2012 (The 21st Academic Annual Meeting of National Blood Centre 2013, Bangkok, Thailand). However, even though the rate of HCV infection among current donors in Thailand is low due to the blood screening policy implemented, the HCV seroprevalence survey was still as high as 2.2% in 2004 [12].

In 2007, the Bureau of Epidemiology reported that HCV was endemic in Thailand's Phetchabun Province and proffered that the route of transmission was due to an unhygienic medical injection procedure [51] given by inadequately trained medical personnel [52]. The infection rate was 70% for intravenous drug users compared to a rate of 0.98% in new blood donors [21]. Thus, initially, needle sharing combined with receiving contaminated blood products significantly escalated the risks of HCV transmission; however, transfusion-related HCV infection has been under control since the implementation of the screening program in 1990. In addition, the first 2 cases of HIV were first reported in Thailand in 1984, and since then health and biosafety education concerning HIV includes information on HCV also [53].

In 2009, the Thailand National Cancer Institute reported that liver and bile duct cancer was the third most common cause of cancer in Thailand, ranking as the third most common cause in males (12.4%) and as the fifth most common cause in females (4%) [54]. The report also showed that most hepatitis cases were the result of infection with HBV (approx. 51.9%), followed by HCV (approx. 11.8%) and, to a lesser extent, HAV, HDV, HEV and others [55]. Therefore, although HCV is generally the major cause of new HCC cases in developed countries [13], HBV is the most predominant cause of HCC in Thailand [56]. Due to the course of disease progression, HCV-related liver disease may take 20–30 years before progressing to liver cirrhosis or HCC [2, 3, 27]. Thus, the spread of HCV in Thailand during the 1970s and 1980s may soon manifest itself phenotypically. Fortunately, however, since 2012 the National Health Security Office (NHSO) has launched a policy of free HCV treatment by registered specialists for chronic hepatitis patients with HCV genotype 2 or 3, which are the genotypes most likely to be cured by the standard viral therapy of pegylated interferon plus ribavirin. Accordingly, if most of the favorable HCV genotype patients can be accessed and receive treatment, the incidence of HCV-related diseases may not be as high as expected in the future.

Similar to previous reports, our study indicates that HCV subtype 3a is the most prevalent strain in Thailand followed by genotypes 1 and 6. Evolutionary analysis based on the coalescence theory estimated that common HCV subtype 3a was introduced and spread into the Thai population during the mid-1970s to the early 1980s. This event coincided with the Vietnam War period which is also when intravenous drug use and needle sharing was widespread. Sharing needles may have been a primary source of the HCV epidemic in the country, and receiving contaminated blood products may have been the primary factor for high HCV prevalence in the Thai population. However, HCV prevalence seems to have steadily declined since anti-HCV antibody screening was implemented in blood donation centers. Our results suggest that the cirrhosis and HCC incidence of this population may increase in the future unless effective treatment and public health policy is introduced to help prevent further disease progression.

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References

1 Lavanchy D: The global burden of hepatitis C. Liver Int 2009;29:74–81.
2 Hoofnagle JH: Course and outcome of hepatitis C. Hepatology 2002;36:S21–S29.
3 Seeff LB: Natural history of chronic hepatitis C. Hepatology 2002;36:S35–S46.
4 Trinchet JC, Ganne-Carrie N, Nahon P, N'Kontchou G, Beaugrand M: Hepatocellular carcinoma in patients with hepatitis C virus-related chronic liver disease. World J Gastroenterol 2007;13:2455–2460.
5 Fattovich G, Stroffolini T, Zagni I, Donato F: Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology 2004;127:S35–S50.
6 Yoshizawa H: Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 2002;62:8–17.
7 Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin IT, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A: Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 2005;42:962–973.
8 Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, Beall E, Yap PL, Kolberg J, Urdea MS: Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 1993;74:2391–2399.
9 Pybus OG, Drummond AJ, Nakano T, Robertson BH, Rambaut A: The epidemiology and iatrogenic transmission of hepatitis C virus in Egypt: a bayesian coalescent approach. Mol Biol Evol 2003;20:381–387.
10 Simmonds P: Genetic diversity and evolution of hepatitis C virus – 15 years on. J Gen Virol 2004;85:3173–3188.
11 Khan A, Tanaka Y, Azam Z, Abbas Z, Kurbanov F, Saleem U, Hamid S, Jafri W, Mizokami M: Epidemic spread of hepatitis C virus genotype 3a and relation to high incidence of hepatocellular carcinoma in Pakistan. J Med Virol 2009;81:1189–1197.
12 Sunanchaikarn S, Theamboonlers A, Chongsrisawat V, Yoocharoen P, Tharmaphornpilas P, Warinsathien P, Sinlaparatsamee S, Paupunwatana S, Chaiear K, Khwanjaipanich S, Poovorawan Y: Seroepidemiology and genotypes of hepatitis C virus in Thailand. Asian Pac J Aller Immunol 2007;25:175–182.
13 Zein NN: Clinical significance of hepatitis C virus genotypes. Clin Microbiol Rev 2000;13:223–235.
14 Akkarathamrongsin S, Praianantathavorn K, Hacharoen N, Theamboonlers A, Tangkijvanich P, Tanaka Y, Mizokami M, Poovorawan Y: Geographic distribution of hepatitis C virus genotype 6 subtypes in Thailand. J Med Virol 2010;82:257–262.
15 Elkady A, Tanaka Y, Kurbanov F, Sugauchi F, Sugiyama M, Khan A, Sayed D, Moustafa G, Abdel-Hameed AR, Mizokami M: Genetic variability of hepatitis C virus in South Egypt and its possible clinical implication. J Med Virol 2009;81:1015–1023.
16 Lu L, Nakano T, Li C, Fu Y, Miller S, Kuiken C, Robertson BH, Hagedorn CH: Hepatitis C virus complete genome sequences identified from China representing subtypes 6k and 6n and a novel, as yet unassigned subtype within genotype 6. J Gen Virol 2006;87:629–634.
17 Romano CM, de Carvalho-Mello IM, Jamal LF, de Melo FL, Iamarino A, Motoki M, Pinho JR, Holmes EC, de Andrade Zanotto PM: Social networks shape the transmission dynamics of hepatitis C virus. PLoS One 2010;5:e11170.
18 Pybus OG, Cochrane A, Holmes EC, Simmonds P: The hepatitis C virus epidemic among injecting drug users. Infect Genet Evol 2005;5:131–139.
19 Kurbanov F, Tanaka Y, Avazova D, Khan A, Sugauchi F, Kan N, Kurbanova-Khudayberganova D, Khikmatullaeva A, Musabaev E, Mizokami M: Detection of hepatitis C virus natural recombinant RF1_2k/1b strain among intravenous drug users in Uzbekistan. Hepatol Res 2008;38:457–464.
20 Shustov AV, Kochneva GV, Sivolobova GF, Grazhdantseva AA, Gavrilova IV, Akinfeeva LA, Rakova IG, Aleshina MV, Bukin VN, Orlovsky VG, Bespalov VS, Robertson BH, Netesov SV: Molecular epidemiology of the hepatitis C virus in western Siberia. J Med Virol 2005;77:382–389.
21 Verachai V, Phutiprawan T, Theamboonlers A, Chinchai T, Tanprasert S, Haagmans BL, Osterhaus AD, Poovorawan Y: Prevalence and genotypes of hepatitis C virus infection among drug addicts and blood donors in Thailand. Southeast Asian J Trop Med Public Health 2002;33:849–851.
22 Rosenberg NA, Nordborg M: Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. Nat Rev Genet 2002;3:380–390.
23 Pybus OG, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH: The epidemic behavior of the hepatitis C virus. Science 2001;292:2323–2325.
24 Jutavijittum P, Jiviriyawat Y, Yousukh A, Pantip C, Maneekarn N, Toriyama K: Genotypic distribution of hepatitis C virus in voluntary blood donors of northern Thailand. Southeast Asian J Trop Med Public Health 2009;40:471–479.
25 Lwin AA, Shinji T, Khin M, Win N, Obika M, Okada S, Koide N: Hepatitis C virus genotype distribution in Myanmar: predominance of genotype 6 and existence of new genotype 6 subtype. Hepatol Res 2007;37:337–345.
26 Hubschen JM, Jutavijittum P, Thammavong T, Samountry B, Yousukh A, Toriyama K, Sausy A, Muller CP: High genetic diversity including potential new subtypes of hepatitis C virus genotype 6 in Lao People's Democratic Republic. Clin Microbiol Infect 2011;17:E30–E34.
27 Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, Alter HJ: Inaugural article: a comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci USA 2002;99:15584–15589.
28 Nakano T, Lu L, He Y, Fu Y, Robertson BH, Pybus OG: Population genetic history of hepatitis C virus 1b infection in China. J Gen Virol 2006;87:73–82.
29 Markov PV, Pepin J, Frost E, Deslandes S, Labbe AC, Pybus OG: Phylogeography and molecular epidemiology of hepatitis C virus genotype 2 in Africa. J Gen Virol 2009;90:2086–2096.
30 Tanaka Y, Kurbanov F, Mano S, Orito E, Vargas V, Esteban JI, Yuen MF, Lai CL, Kramvis A, Kew MC, Smuts HE, Netesov SV, Alter HJ, Mizokami M: Molecular tracing of the global hepatitis C virus epidemic predicts regional patterns of hepatocellular carcinoma mortality. Gastroenterology 2006;130:703–714.
31 Theamboonlers A, Chinchai T, Bedi K, Jantarasamee P, Sripontong M, Poovorawan Y: Molecular characterization of hepatitis C virus (HCV) core region in HCV-infected Thai blood donors. Acta Virol 2002;46:169–173.
32 Akkarathamrongsin S, Praianantathavorn K, Hacharoen N, Theamboonlers A, Tangkijvanich P, Poovorawan Y: Seroprevalence and genotype of hepatitis C virus among immigrant workers from Cambodia and Myanmar in Thailand. Intervirology 2011;54:10–16.
33 Kuiken C, Yusim K, Boykin L, Richardson R: The Los Alamos hepatitis C sequence database. Bioinformatics 2005;21:379–384.
34 Mizokami M, Tanaka Y, Miyakawa Y: Spread times of hepatitis C virus estimated by the molecular clock differ among Japan, the United States and Egypt in reflection of their distinct socioeconomic backgrounds. Intervirology 2006;49:28–36.
35 Pybus OG, Barnes E, Taggart R, Lemey P, Markov PV, Rasachak B, Syhavong B, Phetsouvanah R, Sheridan I, Humphreys IS, Lu L, Newton PN, Klenerman P: Genetic history of hepatitis C virus in East Asia. J Virol 2009;83:1071–1082.
36 Drummond AJ, Ho SY, Phillips MJ, Rambaut A: Relaxed phylogenetics and dating with confidence. PLoS Biol 2006;4:e88.
37 Kanistanon D, Neelamek M, Dharakul T, Songsivilai S: Genotypic distribution of hepatitis C virus in different regions of Thailand. J Clin Microbiol 1997;35:1772–1776.
38 Cristina J, Colina R: Evidence of structural genomic region recombination in hepatitis C virus. Virol J 2006;3:53.

39 Charuluxananan SC, Chentanezb V: History and evolution of Western medicine in Thailand. Asian Biomed 2007;1:5.

40 Medhi S, Goswami B, Das AK, Singh TB, Husain SA, Sehgal A, Kar P: New insights into hepatitis C virus infection in the tribal-dominant part of northeast India. Arch Virol 2012; 157:2083–2093.

41 Hissar SS, Goyal A, Kumar M, Pandey C, Suneetha PV, Sood A, Midha V, Sakhuja P, Malhotra V, Sarin SK: Hepatitis C virus genotype 3 predominates in North and Central India and is associated with significant histopathologic liver disease. J Med Virol 2006;78: 452–458.

42 Islam JH, Jamiruddin A, Ahmed R, et al.: Prevalence of hepatitis C virus genotypes in Bangladesh. 13th Annual Scientific Conference (ASCON), Dhaka, 2011, SP132, p 197.

43 Pham DA, Leuangwutiwong P, Jittmittraphap A, Luplertlop N, Bach HK, Akkarathamrongsin S, Theamboonlers A, Poovorawan Y: High prevalence of hepatitis C virus genotype 6 in Vietnam. Asian Pac J Allergy Immunol 2009;27:153–160.

44 Pybus OG, Rambaut A: Evolutionary analysis of the dynamics of viral infectious disease. Nat Rev Genet 2009;10:540–550.

45 Utama A, Tania NP, Dhenni R, Gani RA, Hasan I, Sanityoso A, Lelosutan SA, Martamala R, Lesmana LA, Sulaiman A, Tai S: Genotype diversity of hepatitis C virus (HCV) in HCV-associated liver disease patients in Indonesia. Liver Int 2010;30:1152–1160.

46 Kageyama S, Agdamag DM, Alesna ET, Abellanosa-Tac-An IP, Corpuz AC, Telan EF, Que ER, Leano PS, Jereza LD, Emphasis YE, Prasetyo AA, Tanimoto T, Ichimura H: Tracking the entry routes of hepatitis C virus as a surrogate of HIV in an HIV-low prevalence country, the Philippines. J Med Virol 2009;81: 1157–1162.

47 Liu J, Zhang C: Phylogeographic analyses reveal a crucial role of Xinjiang in HIV-1 CRF07_BC and HCV 3a transmissions in Asia. PLoS One 2011;6:e23347.

48 Pattha P: The Impact of American Bases on Socio-Economic Condition of Udornthani, A.D. 1962–1977; thesis, Silpakorn University, Bangkok, 2007, pp 48, 61.

49 Jittiwutikarn J, Thongsawat S, Suriyanon V, Maneekarn N, Celentano D, Razak MH, Srirak N, Vongchak T, Kawichai S, Thomas D, Sripaipan T, Netski D, Ananthakrishnan A, Nelson KE: Hepatitis C infection among drug users in northern Thailand. Am J Trop Med Hyg 2006;74:1111–1116.

50 Chimparlee N, Oota S, Phikulsod S, Tangkijvanich P, Poovorawan Y: Hepatitis B and hepatitis C virus in Thai blood donors. Southeast Asian J Trop Med Public Health 2011;42: 609–615.

51 Buathong R: Hepatitis. Annu Epidemiol Surveill Rep 2007:88–92.

52 Tanwandee T, Piratvisuth T, Phornphutkul K, Mairiang P, Permpikul P, Poovorawan Y: Risk factors of hepatitis C virus infection in blood donors in Thailand: a multicenter case-control study. J Med Assoc Thai 2006; 89:S79–S83.

53 Limsuwan A, Kanapa S, Siristonapun Y: Acquired immune deficiency syndrome in Thailand. A report of two cases. J Med Assoc Thai 1986;69:164–169.

54 Attasara PB, et al: Hospital-Based Cancer Registry 2008. Bangkok, National Cancer Institute Thailand, 2009.

55 Santayakorn S: Hepatitis. Annu Epidemiol Surveill Rep 2009;100–104.

56 Tangkijvanich P, Mahachai V, Suwangool P, Poovorawan Y: Gender difference in clinicopathologic features and survival of patients with hepatocellular carcinoma. World J Gastroenterol 2004;10:1547–1550.