The Unique HCV Genotype Distribution and the Discovery of a Novel Subtype 6u Among IDUs Co-Infected with HIV-1 in Yunnan, China

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The Yunnan province is the epicenter of HIV-1 epidemics in China and a center for drug trafficking to the other parts of the world. In six prefectures of this province, a total of 132 IDUs were recruited to determine the sero-prevalence of HCV and HIV-1 and the positive rates were 93.94% and 68.18%, respectively (P < 0.001). Co-infection with HCV and HIV-1 was found among 89 IDUs, of whom several HCV fragments were amplified and sequenced. Sequences of the HCV 5’NCR-C and NS5B region were determined from 82 IDUs. Phylogenetic analyses showed consistent genotyping among 80 IDUs. Among them HCV genotypes 1a, 1b, 3a, 3b, 6a, 6n, and a tentatively assigned novel 6u subtype were found in 1 (1.25%), 16 (20%), 19 (23.75%), 24 (30%), 4 (5%), 9 (11.25%) and 7 (8.75%) individuals, respectively. In two IDUs, genotyping results were discordant, suggesting mixed HCV infections or recombination. The proportion of patients with HCV 1b tended to decrease from the north to south and from the east to west in this province. Genotype 3 and 6 strains were more frequent in the southern prefectures. The novel subtype 6u strains were only detected in Dehong which borders Myanmar. Our findings showed a unique pattern of HCV genotype distribution, which is similar to that in the southeastern Asian countries but distinct from that among the general population in China. Routes of drug trafficking and the resulting high prevalence of HIV-1 infection may have contributed to this pattern of HCV genotype distribution.

KEY WORDS: HCV; HIV-1; co-infection IDUs; genotypes

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease. It infects an estimated 170 million people worldwide [Sy and Jamal, 2006]. With an overall sero-prevalence of 2–3% among the general population [Lei et al., 1999], HCV infection has become one of the most common causes for chronic hepatitis, cirrhosis, and hepatocellular carcinoma in China [Chen et al., 2002]. The worldwide prevalence of anti-HCV antibodies among injection drug users (IDUs) varies from 50% to 90% [Bobkov et al., 2001]. Although there is a shortage of such data in China, a few limited studies have indicated a similar range [Ruan et al., 2004; Garten et al., 2005; Zhang et al., 2002].

Xueshan Xia and Ling Lu contributed equally to this study. All sequences determined in this study have been submitted into Genbank accession nos. are EU081299-508 and EU119968-83.

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Due to shared transmission routes, co-infection with HCV and HIV-1 has become common among individuals who had high risks of blood exposures. According to the data from a large European cohort, the co-infection accounted for 85% of the hemophiliacs cases [Khalili and Behm, 2002]. Among IDUs who were positive for anti-HIV-1, the anti-HCV prevalence can be as high as 90% [Thomas et al., 1996; CDC, 1999]. HCV can accelerate the progression of HCV-related liver disease [Beld et al., 1998; Martinez-Sierra et al., 2003; Lichterfeld et al., 2005]. Although the effect of HCV on HIV-1 infection is controversial, most studies showed an increase in mortality due to liver disease [Khalili and Behm, 2002]. HCV may act as a direct cofactor to hasten the progression of AIDS [Beld et al., 1998; Carlos et al., 2004].

HCV has been classified into six genotypes. Variants within genotypes 1–4, and 6 are further divided into subtypes [Simmonds et al., 2005]. Geographically, genotypes 1–5 are associated with worldwide epidemic, while genotypes 4–6 are endemic [Simmonds, 2001]. Generally, HCV genotype 1b is the most prevalent in China that is followed by 2a. This distribution is similar to that was observed in Japan, Korea, and Taiwan [Lee et al., 1996; Yu et al., 2001a; Lu et al., 2005], but distinct from that in the Southeast Asian countries. Among the latter, genotype 3 is the most predominant followed by 1b and group 6 variants [Mellor et al., 1996; Kanistanon et al., 1997]. The Yunnan province is located in the far southern part of China bordering Laos, Vietnam, and Myanmar. Previously, the coexistence of multiple HCV genotypes (1b, 2a, 3a, 3b, 6k, and 6n) has been described among patients from Kunming, the capital city of Yunnan province [Lu et al., 2005]. Recently, HCV subtypes 1a, 1b, 3a, and 3b have also been isolated from IDUs who were co-infected with HIV-1 in Kunming [Zhang et al., 2004]. These two studies indicated that the Yunnan province is an area where the HCV genotype distribution is distinct from that in the other parts of the country. This may be due to the variety of the ethnic groups in this province or popular drug using. Since only a small number of cases were examined in the single Kunming city, further epidemiology studies of HCV are required to be performed in the other parts of this province, particularly in the rural areas and the areas bordering Southeast Asian countries.

In the present study, HCV was genotyped in IDUs who were co-infected with HCV and HIV-1. These individuals were sampled in six prefectures, Kunming, Dehong, Honghe, Wenshan, Qujing and Dali of the Yunnan province. A unique pattern of multiple HCV genotypes was identified. Moreover, a novel subtype of HCV genotype 6 variant with seven distinct strains was discovered. According to the updated HCV nomenclature [Simmonds et al., 2005], these variants were assigned subtype 6u.

**MATERIALS AND METHODS**

**Subjects and Specimens**

A total of 132 IDUs were recruited in this study. They were hospitalized in drug detoxification centers in six prefectures of the Yunnan province between 2000 and 2003. Among them, 26 were from Kunming, 29 from Dehong, 31 from Honghe, 22 from Wenshan, 18 from Qujing, and 6 from Dali. Guidelines set by the local ethical review committees were strictly followed. After recruitment, they were interviewed to determine ages, drug use histories, occupations, ethnic background and marital status. From these individuals whole blood samples were collected using K$_3$EDTA (ethylene-bis-iminoacetic acid tripotassium salt) as the anticoagulant. Plasma was separated by centrifugation. Both anti-HCV and anti-HIV-1 were determined by enzyme immunoassay (ELISA; Kehua Company, Shanghai, China). Anti-HIV-1 was further confirmed by Western blot assay (BioRad, Singapore). IDUs with specimens positive for both anti-HIV-1 and anti-HCV were considered to have HIV-1 and HCV co-infection.

**RT-PCR**

Plasma samples positive for both HCV and HIV-1 were subjected to RNA extraction and reverse transcription-PCR (RT-PCR). Virus RNA was extracted from 200 μl of plasma using High Pure Viral RNA Kit (Roche Applied Science, Mannheim, Germany) and following the manufacturer’s protocol. After extraction, RNA was used as templates for conducting one step RT-PCR with AMV RNA PCR Kit (TaKaRa, Ver. 3.0). Primers were designed or adopted from previous reports [Lu et al., 2005] based upon conserved sequences of common HCV genotypes and subtypes. The primer nucleotide sequences were listed in Table I. Each 25 μl of reaction volume was used, containing 4 pmol of each of the two outer primers. The reaction was incubated for 30 min at 50°C for reverse transcription. This was followed by 94°C incubation for 2 min, 35 PCR cycles each consisting of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 3 min. The final step was heating at 72°C for 10 min to terminate the reaction. The second round PCR was performed with Premix Taq Kit (TaKaRa, EX Taq™ Ver) each with 50 μl of volume. The reaction contained 5 μl of the first-round PCR product, 4 pmol of each of the two inner primers. The thermal cycling procedure was as the same as the first round PCR except for the reverse transcription. The final PCR products were resolved on 2% agarose gel stained with ethidium bromide and visualized under UV light.

Initially, the 5’ NCR through the Core region (5’NCR-C) was amplified for all the selected specimens. Of the 5’NCR-C positive specimens, the amplification of the NS5B region was then conducted. For specimens which amplicons were cloned into vector, the E1–E2 regions were further amplified.

**Sequence Analysis**

Amplicons of expected sizes (5’NCR-C: 1252 bp; E1–E2: 1346 bp; NS5B: 1037 bp) were purified with QIAquick PCR purification kit (QIAgen, Valencia, CA) according to the manual. The purified DNA was sequenced in both directions using ABI Prism dRhodamine...
terminators in an ABI Prism 3100 genetic analyzer (PE Applied Biosystems, Foster City, CA) with standard procedures. Selected sequencing primers (shown in Table I) were employed. The clear sequence information for 642 nt of the 5'0 NCR-C region, 884 nt of the partial NS5B region, and 581 nt of the E1 region were generated and selected for genotyping.

The original sequencing data was viewed and edited with the Chromas Version 14.5 (Conor McCarthy, School of Health Science, Griffith University, Queensland, Australia). The software Clustal X (Ver. 1.8) was used to align the nucleotide sequences with reference HCV strains. The resulted multiple alignments were then corrected manually to avoid the false gaps and substitutions. Using the maximum likelihood method under the HKY + I + G substitution model (gamma distribution approximated by using six rate categories), phylogenetic trees were reconstructed. The transition/transversion ratios, proportion of invariable sites, and gamma distribution shape parameters were estimated from the data. The base frequencies were adjusted to maximize the likelihood. Bootstrap resampling was performed using 1,000 neighbor-joining replicates.

**RESULTS**

**HCV and HIV-1 Infection Rates**

Of the 132 IDUs, 105 were males and 27 females. Their ages ranged from 18 to 50 years old, with an average of 29 years old (SD: 4.72). The earliest dates when they began injection drug use were between 1989 and 2001. Prior to the collection of serum samples, the duration of injection drug use ranged from 2 to 11 years, with a mean duration of 5 years (SD: 2.57).

Antibodies to HIV-1 were detected among 90 IDUs (68.2%) as determined by ELISA. Antibodies to HCV were detected in 124 IDUs (93.1%). Among the 90 IDUs who were positive for anti-HIV-1, 89 were positive for anti-HCV antibodies (98.9%).

Chi-square testing showed that among the anti-HIV-1 positive IDUs, the anti-HCV positive rate was significantly higher ($P < 0.001$) than among the anti-HIV-1 negative IDUs.

**Phylogenetic Analysis of the HCV Sequences**

Initially, a fragment of the 5'0 NCR-C region was targeted. The expected HCV amplicons were obtained from 82 (92.1%) of the 89 patients co-infected with HCV and HIV-1. Seven IDUs were negative for HCV PCR amplification and were most likely due to low viral titers. Samples from the 82 IDUs who were positive for the 5'0 NCR-C fragment were analyzed for the NS5B sequences. Samples from all the 82 IDUs were positive for this PCR amplification. The 20 patients whose 5'0 NCR-C and NS5B fragments were classified as genotype 6 had the E1–E2 genomic regions also amplified from serum samples. This gave a positive result among 16 IDUs (80%).

According to their phylogenetic presentation, we assigned these isolates as subtype 6u after the recently described subtypes 6r, 6s, and 6t [Murphy et al., 2007; Lu et al., 2008].

### Table I. List of the PCR Primers Used in this Study

<table>
<thead>
<tr>
<th>Region</th>
<th>Name</th>
<th>Sequences (5'-3')</th>
<th>Position</th>
<th>Sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'0 NCR-core</td>
<td>HCV1</td>
<td>GGCGACACTCCACCATGAAATCACT</td>
<td>18–41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1300</td>
<td>CCARTTCATCATCATTACRTCCASGCAT</td>
<td>1319–1293R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCV2</td>
<td>AATCTACCTGGTGGAGAAACTGCT</td>
<td>31–61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A1260</td>
<td>GGRTGDCCGKRTARATNGARCTRCA</td>
<td>1282–1253R</td>
<td>1252</td>
</tr>
<tr>
<td></td>
<td>S727</td>
<td>GCGAATCTCGGAGGTAATGTCA</td>
<td>733–751</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>A2232</td>
<td>ARTTBTDYDGTRCANGGRTARTGCA</td>
<td>2252–2272R</td>
</tr>
<tr>
<td>E1-E2</td>
<td>S854</td>
<td>CCYGTTGCTCTTCTCTATCTT</td>
<td>849–871</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A2175</td>
<td>GTNADCCARGHCHCNNGMNCCRCA</td>
<td>2194–2170R</td>
<td>1322–1346</td>
</tr>
<tr>
<td>NS5B</td>
<td>YF1</td>
<td>GGSTTYTGTTGATGTGAYACACAGCTGTTTGA</td>
<td>8247–8275</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A9521</td>
<td>CTACCCCTACRGSAYAGGAACTAAGGC</td>
<td>9351–9325R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YF2</td>
<td>GTNADCCARGHCHCNNGMNCCRCA</td>
<td>2174–2170R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A9479</td>
<td>GRGMYGRACACGCTGTGATASATGTC</td>
<td>9302–9274R</td>
<td>1037</td>
</tr>
</tbody>
</table>

**a**Genomic regions amplified.

**b**Primers used for sequencing were underlined.

**c**Nucleotide positions with reference to the numbering of HCV H77 strains (Genbank accession no. NC_004102).

**d**Expected sizes of the amplicons in bp.

Fig. 1. Phylogenetic trees based on sequences (A) from the 5′NCR-C region and (B) from the NS5B region. The 5′NCR and NS5B sequences correspond to nucleotides 41–681 and 8,343–9,226 in the numbering of H77 genome (NC_004102), respectively. HCV genotypes are indicated with 1–6 and subtypes are indicated with 1a–6u. Bootstrap values (%) are shown under trunks close to the related clusters. Reference sequences are displayed in bold with Genbank accession numbers. The sequences determined in this study are shaded and indicated with vertical lines and HCV subtypes on the right. Sequences from two IDUs (HH075 and DH102) are indicated by stars. They were suspected to have mixed HCV infection or virus recombination. The bar at the base of each figure shows the scale for nucleotide substitution per site.
China followed by 2a [Lu et al., 2005]. However, only 16 isolates (20%) in this study were 1b, and no 2a was identified. In addition, there was 1 isolate (1.3%) belonging to subtype 1a. Isolates HH075 and DH102 had inconsistent genotyping results. Their 5’NCR-C region sequences were classified as 6a and 6u, respectively (Fig. 1A). Both their NS5B region sequences were classified as 3a (Fig. 1B). These two patients were

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Fig. 2. Phylogenetic trees based on sequences (A) from a short segment of the E1 region and (B) from a short segment of the NS5B region. The E1 and NS5B segments correspond to nucleotides 915–1,289 and 8,343–8,615 in the H77 genome (NC_004102), respectively. Only genotype 6 sequences were analyzed and the D10988/2b was used as the outgroup. New genotype 6 variants were designated with 6? to indicate that their subtypes are not yet assigned. All the other designations were described in Figure 1.
suspected to have mixed HCV infections or virus recombination.

Isolates of HCV subtypes 1b, 3a, and 3b formed three firm clusters in both phylogenetic trees, each with bootstrap values greater than 75% (Fig. 1A,B). Most of their branches appeared shorter than those of the reference strains. The mean genetic distances were 0.0098 (5'NCR-C) and 0.0167 (NSSB) for 1b, 0.0131
(5'NCR-C) and 0.0215 (NS5B) for 3a, and 0.0100 (5'NCR-C) and 0.0328 (NS5B) for 3b. These results may suggest common sources of infection and network transmission. In contrast, the 6n group appeared rather divergent, containing three major branches. One branch included six isolates from three prefectures: HH079, HH082, KM012, KM030, WS132, and WS133. The second branch included two isolates from two prefectures: HH079 and DH091. The third branch led to a single isolate WS142 from the prefecture of Wenshan. The reference isolate km42 (DQ278894) was previously determined in Kunming; it clustered with the six isolates led by the first branch. The bootstrap values were 89% (Fig. 1A) and 99% (Fig. 1B) for the first branch, 97% (Fig. 1A) and 99% (Fig. 1B) for the second branch. The divergence of the subtype 6n group indicates either multiple origins or independent virus evolution.

**The Further Confirmation of Novel Subtype 6u**

The five subtype 6u isolates were further confirmed by E1–E2 region sequencing (Genbank accession no: EU119975-79) (Fig. 2A). Recently, many new genotype 6 variants have been described [Murphy et al., 2007]. In order to place the novel subtype 6u isolates phylogenetically with these new HCV variants, short segments of the E1 and NS5B regions were analyzed. These segments correspond to the nucleotides 915–1,289 and 8,343–8,615 in the numbering of H77 genome (Genbank accession no. NC_004102). Apart from the 21 assigned 6a–6u subtypes, there appeared additional 19 branches (marked with ?) in the phylogeny reconstructed with the E1 segment sequences (Fig. 2A). These branches may represent novel subtypes within genotype 6 that remain unassigned. Five isolates from the IDUs in the Dehong prefecture were tightly grouped into subtype 6u with a bootstrap value of 100%. This group was independent of all the other 20 assigned subtypes and the 19 unassigned branches (Fig. 2A). It represents a new subtype that was confirmed with seven isolates and sequences from three genomic regions. Similarly, subtypes 6a–6u were also demonstrated in the phylogeny reconstructed with the short NS5B segment sequences (Fig. 2B). In addition, there were seven branches leading to variants that may represent novel subtypes. Within the newly assigned subtype 6u seven isolates were grouped. These isolates were from the IDUs in the Dehong prefecture. They grouped with two other unassigned isolates MYAN-3E-3 (AB103143) and MYAN-TC148 (AB269353) that were retrieved from Genbank. The latter two sequences were recently determined from Myanmar [Shinji et al., 2004; Lwin et al., 2007].

**Geographic Distribution of the HCV Isolates**

The viral isolates were obtained from plasma samples of IDUs in six prefectures of the Yunnan province. HCV genotype distribution was characterized based on their geographic origins as shown in Figure 3. Two subtypes
(1b and 3b) accounted for the majority of the isolates in Kunming and Qujing, located in the north-middle and eastern parts of the province. Subtype 1b (Kunming: 6/14; Qujing: 4/9) was the major subtype because the two prefectures are neighbors and are more interconnected with the other provinces of China where 1b is the most predominant genotype. Subtype 3b (Kunming: 5/14; Qujing: 4/9) was the second most prevalent, while only two isolates of 3a were found in these two prefectures. In addition, two isolates from Kunming were classified as 6n, whose existence was only recently described [Lu et al., 2005]. Four and five HCV subtypes were identified in Wenshan and Honghe, respectively. These two prefectures are located in the southeastern parts of the province bordering Vietnam and Laos. In these two prefectures 3a or 3b was the most predominant subtype, followed by genotype 6 (6a and 6n) and 1b isolates. In detail, three 1b, six 3a, one 6a, and three 6n isolates were identified among 13 IDUs from Wenshan. Three 1b, six 5a, seven 3b, three 6a, and two 6n isolates were identified among 21 IDUs from Honghe. A suspected HCV recombination or mixed infection (HH075: 6a/3a) was also from Honghe (Table II). Dehong prefecture has been known as a hotspot of great genetic diversity of HIV-1 where multiple genotypes and unique recombinants have been identified [Takebe et al., 2003]. In this study, a distinct pattern of HCV genotype distribution was also observed. Genotype 3 was the most prevalent among the 22 IDUs from Dehong. These included four 3a, seven 3b and seven novel 6u isolates. In addition, a single 1a, two 6n, and a suspected 3a/6u recombination or mixed infection was found among IDUs from Dehong. An obvious trend was noted that the 1b proportion decreased from north to south, from east to west in the province. Subtype 1b represented nearly a half of the genotyped isolates in Qujing, the north-eastern prefecture. However, no 1b isolates were found in Dehong, the most south-western prefecture of the province. On the other hand, more genotype 3 isolates were present in the southern parts of this province. The co-existence of multiple HCV genotypes among IDUs in the Yunnan province may have provided environments for HCV mixed infection and recombination.

**DISCUSSION**

The worldwide sero-prevalence of HCV is estimated to be 3.1% [Sy and Jamal, 2006], and in China this rate has been estimated to be 2.5–4.9% [WHO, 2000]. The rate can be low in urban areas that have improved hygienic conditions, whereas in some rural areas the rate can be as high as 9.6% [Zhang et al., 2005]. Based on these estimates, the total number of HCV-infected people in China might exceed 50 million. Among IDUs the HCV prevalence varies between 70% and 95%, depending on the length of drug use and local factors [Lorvick et al., 2001]. Despite a recent increase in sexual transmission, sharing needles/syringes during injection drug use remains to be the major route of HIV-1 infection [Cohen, 2004]. In the cohort studied by Garten et al., the quick adoption of injection heroin use in the Guangxi province has introduced more HCV than HIV-1 infections [Garten et al., 2004]. According to one of our recent studies of 242 IDUs from three cohorts in Yunnan, an average prevalence of HIV-1 infection was 71.9% [Zhang et al., 2002]. In the current study, the positive rate of anti-HIV-1 among 132 IDUs was 68.2%, while the positive rate of anti-HCV was 93.9%. The HCV rate was significantly higher than the HIV-1 rate (P < 0.001).

These results suggest that the HCV transmission through injection drug use is more efficient than HIV-1. This was also indicated in the current study where 83.3% of the IDUs who were negative for anti-HIV-1 were positive for anti-HCV.

Young persons represent the major population of illicit drug users in China. They quickly switch from smoking to drug using [Lai et al., 2000]. Their rate of HCV infection could reach a peak level in excess of 70% after 1 year of injection drug use. The rate could continue to rise to over 90% within 4 years [Zein, 2000; Garten et al., 2004]. In the present study, most of the IDUs had an average age of 28.9 years. The duration of their injection drug use was between 2 and 11 years, with a mean duration of 5.1 years. This longer duration may help to explain why the rate of HCV infection in this cohort of individuals was as high as 93.9%.

Different methods have been used to determine HCV genotypes and subtypes. These include serological genotyping, RT-PCR with specific primers, restriction fragment length polymorphism, reverse hybridization, and DNA sequencing [Nolte, 2001]. Among them the DNA sequencing is the gold standard and preferred, but it requires phylogenetically informative regions to be targeted for amplification. The highly conserved 5'-NCR is often used for sensitive PCR-based detection of HCV. However, genotyping based on this region does not differentiate subtypes within genotype 1 [Chen and Weck, 2002] and between genotypes 1 and 6 [Mellor et al., 1996]. A multicenter study based on sequencing the NS5B region has provided sensitive and specific results. The results could meet the requirements for most clinical and epidemiological genotyping purposes [Laperche et al., 2006]. An updated proposal about HCV nomenclature has recommended that sequences from the core, E1, and NS5B regions be determined and extensively analyzed for genotyping [Simmonds et al., 2005]. Accordingly, the phylogenies reconstructed in the current study used sequences of 642 nt of the 5’NCR-C and 884 nt of the NS5B regions. Some genotype 6 isolates were further analyzed by sequencing 581 nt of the E1 region. Except for two individuals who possibly had mixed infection or recombination, HCV genotypes and subtypes among the 80 IDUs were determined.

Among the general population and IDU communities of south and southeastern Asian countries, patterns of HCV genotype distribution were distinct from that observed in China [Mellor et al., 1996]. For example, subtype 3a was the most prevalent, followed by subtype 1b, genotype 6 and subtype 1a in Thailand [Kanistamon et al., 1997; Agdamag et al., 2005]. A similar HCV
genotypes distribution was also observed in Myanmar where the genotype 6 variants included three possible new subgroups [Shinji et al., 2004]. Vietnamese patients were infected most frequently by genotype 6 viruses, secondly by subtype 1b and less by genotype 2 [Noppornpanth et al., 2006]. To a greater extent, genotype 3 was more prevalent in southern Asian countries, such as India and Pakistan [Akhtar et al., 2002; Lole et al., 2003]. In contrast, a pattern of subtype 1b followed by 2a was observed among the general population and paid blood donors in most parts of China [Zhang et al., 2004; Lu et al., 2005]. Some studies from China also showed that a higher frequency of genotype 3 and 6 variants occurred among IDUs [Garten et al., 2004, 2005]. In the present study, a distinct pattern of HCV genotype distribution was found among IDUs. More than a half of the IDUs were infected with HCV genotype 3, followed by genotype 6 variants and then subtype 1b. This pattern appears more similar to that observed in the southeastern Asian countries. Furthermore, a trend was observed that more subtype 1b isolates were identified in the inner prefectures, Kunming and Qujing, while more genotypes 3 and 6 strains were detected in the border prefectures, Wenshan, Honghe, and Dehong. These results suggest that HCV infection in the Yunnan province has been affected by human movements possibly from two directions. One travels from the inner provinces of the country through common routes. The other is border crossing transmission from southeastern Asian countries, most likely through drug trafficking. An overland drug trafficking route via Yunnan has been suggested to transport heroin from Southeast Asia to China and then worldwide [Beyrer et al., 2000; Yu et al., 2001b]. This route has played a critical role in the transmission of HIV-1 infections to the other parts of the country [Li et al., 2005]. Our study provided further information to support that this trafficking route has also influenced the transmission of HCV among IDUs in the Yunnan province.

Among the 80 IDUs, HCV genotype 3 was the most frequent, accounting for 53.8%. Subtype analysis showed 3a (23.8%) and 3b (30%) in slightly different percentages. In contrast, 1b accounted for 20% and no 2a viruses were identified, although they represent the first and second most prevalent subtypes among the general population of China [Lu et al., 2005]. Since none of these 1b isolates clustered into the two 1b groups we previously identified as being unique in China [Lu et al., 2005], different origins for these viruses were suggested. Isolates of genotype 6 were found to be the second most predominant genotype in this study. They accounted for 25% of isolates and consisted of four 6a, nine 6n, and seven isolates tentatively assigned the novel subtype 6u. The 6a subtype, attributed to IDU network transmissions, has been found to be widely spread in Hong Kong [Zhou et al., 2006], the Pearl River Delta [Lu et al., 2005], and Guangxi [Garten et al., 2004, 2005]. In contrast, other variants of this genotype were exclusively seen in southeastern Asian countries with an endemic pattern [Mellor et al., 1996]. Geographically, Yunnan province neighbors southeastern Asian countries. During recent years, drug use and trafficking have become more common in this region. Our data in conjunction with previous studies suggest that genotype 6 subtypes may possess a propensity for transmission by IDU [Lu et al., 2005].

In the present study, nine 6n variants formed three diverse clusters with origins from four different prefectures. In contrast, seven 6u isolates formed a close group that was derived from a single prefecture, Dehong. The 6n pattern suggests three independent historical introductions. The 6u pattern may reflect the recent entry from bordering Myanmar where 6u isolates were first identified [Shinji et al., 2004]. It is also possible that 6u variants are indigenous to the Dehong area and that previous studies have missed this subtype due to its great genetic variation or a limitation of the assays used. Alternatively, the prevalence of 6u may be extremely low, and only an insufficient number of infected individuals were sampled previously. Once seeded in the IDU network, 6u has become transmitted more efficiently. It now accounts for more infections, especially among patients with their immunity comprised by HIV-1 co-infection.

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