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Hepatitis C Infection Among Intravenous Drug Users Attending Therapy Programs in Cyprus

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The most high-risk population for HCV transmission worldwide today are intravenous drug users. HCV genotypes in the general population in Cyprus demonstrate a polyphyletic infection and include subtypes associated with intravenous drug users. The prevalence of HCV, HBV, and HIV infection, HCV genotypes and risk factors among intravenous drug users in Cyprus were investigated here for the first time. Blood samples and interviews were obtained from 40 consenting users in treatment centers, and were tested for HCV, HBV, and HIV antibodies. On the HCV-positive samples, viral RNA extraction, RT-PCR and sequencing were performed. Phylogenetic analysis determined subtype and any relationships with database sequences and statistical analysis determined any correlation of risk factors with HCV infection. The prevalence of HCV infection was 50%, but no HBV or HIV infections were found. Of the PCR-positive samples, eight (57%) were genotype 3a, and six (43%) were 1b. No other subtypes, recombinant strains or mixed infections were observed. The phylogenetic analysis of the injecting drug users' strains against database sequences observed no clustering, which does not allow determination of transmission route, possibly due to a limitation of sequences in the database. However, three clusters were discovered among the drug users' sequences, revealing small groups who possibly share injecting equipment. Statistical analysis showed the risk factor associated with HCV infection is drug use duration. Overall, the polyphyletic nature of HCV infection in Cyprus is confirmed, but the transmission route remains unknown. These findings highlight the need for harm-reduction strategies to reduce HCV transmission. ***J. Med. Virol. 82:263–270, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: molecular epidemiology; HCV genotypes; NS5B region; Core-E1 region; phylogenetic analysis

INTRODUCTION

Hepatitis C is a life-shortening disease recognized as a major public health problem worldwide, infecting more than 170 million people globally [Sy and Jamal, 2006]. It is caused by a small, enveloped single-stranded, positive-sense RNA virus of the *Flaviviridae* family. The HCV genome reveals high genetic heterogeneity, leading to a proposed consensus of six genotypes and numerous closely related subtypes [Simmonds et al., 2005]. Genotype distribution differs by geographic region but also by year and mode of transmission, but the face of HCV epidemiology is being changed radically by globalization [Esteban et al., 2008].

The most important route of HCV transmission is through exposure to infected blood and until diagnostic blood screening was introduced in the early 1990s the virus was transmitted mainly through blood, blood products, hemodialysis and organ transplantations. In the western world currently, HCV infection occurs mainly through parenteral exposure, the most common mode of transmission being intravenous drug use through sharing of needles or other injecting equipment [Shepard et al., 2005; Sy and Jamal, 2006; Esteban et al., 2008]. Long-term users display an extremely high prevalence of HCV infection at over 60%, whereas users who have been practicing intravenous drug abuse

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exhibit prevalence rates of 20–46%, which is still a significantly high rate [Shepard et al., 2005; Sweeting et al., 2009]. In general, large heterogeneity has been observed in the prevalence of HCV across different populations of injecting drug users [Hagan et al., 2007; Sutton et al., 2008].

In developed countries, the HCV subtypes that predominate among intravenous drug users are 1a and 3a, both showing exponential growth during the 20th century [Pybus et al., 2005; Esteban et al., 2008]. Subtype 3a has been associated significantly with transmission through intravenous drug use in industrialized countries. It is prevalent mainly in North and South America, Europe, and Australia, where intravenous drug use is common, and seems to have a common origin among injecting drug users [Pybus et al., 2005; Morice et al., 2006]. Lately genotype 4 strains, which have been restricted to north Africa, are also becoming increasingly prevalent among intravenous drug users, especially in southern Europe, and its introduction into this population seems to be more recent than that of 1a and 3a [van Asten et al., 2004; Esteban et al., 2008]. There is also one recombinant, the 2k/1b strain, which is associated with intravenous drug use, and has been found in Russia, Estonia, Uzbekistan, Ireland, and Cyprus [Kalinina et al., 2002; Moreau et al., 2006; Tallo et al., 2007; Kurbanov et al., 2008; Demetriou et al., 2009].

The molecular epidemiology of HCV infection in the general population of Cyprus was studied recently, revealing polyphyletic infection and a high genetic heterogeneity among strains on the island [Demetriou et al., 2009]. The presence of strains belonging to five of the six genotypes and putative isolates of the 2k/1b recombinant suggest multiple routes of introduction into the island from various geographical regions and by various routes of transmission. Cyprus is a small country, but unique with respect to its geographical position, as it lies between Europe, Africa, and Asia. It is very close to North Africa, where the prevalence of HCV is the highest in the world and Cyprus has a high rate of influx of immigrants from Eastern Europe and countries of the former Soviet Union. Also, as it is now a member state of the European Union, entry into Cyprus is easy, thus facilitating the introduction of new infectious diseases.

The molecular epidemiology of HCV, and also HBV and HIV infection specifically among a population of intravenous drug users in Cyprus was investigated in this study, following the recent discovery of subtypes associated with injecting drug use (1a, 3a, and 2k/1b) within the general population [Demetriou et al., 2009]. The aim of the study was to determine the prevalence of HCV, HBV, and HIV infection within this high-risk population, to determine the genotype distribution and investigate any phylogenetic relationships of strains found between themselves and with the strains found previously in the general population of Cyprus but also with the strains available in the Los Alamos HCV database (http://hcv.lanl.gov). The molecular epidemiology of hepatitis C infection among intravenous drug users in Cyprus has never been studied before and is presented for the first time, revealing genetic heterogeneity and multiple points of introduction of the virus within this high-risk population.

MATERIALS AND METHODS

Study Subjects and Sample Collection

The study subjects were consenting intravenous drug users seeking therapy in detoxification centers around the island at the time of sample collection. Two rounds of sampling were carried out in July and November 2008, covering detoxification centers treating intravenous drug users. The centers where the sampling took place were as follows: Aghia Skepi, a long-term inpatient therapeutic community which aims for withdrawal from substance abuse and social rehabilitation; Ghephyra, an outpatient substitution treatment program, for users who have failed with other therapeutic programs and buprenorphine is administered in parallel to psychosocial intervention and psychological and medical aid; Anosis, a long-term inpatient detoxification program, for abstinence from drug use and safe management of withdrawal symptoms through administration of analgesics and psychosocial support; Ploeghos, an outpatient psychosocial treatment program, for users who have failed with other therapeutic programs and buprenorphine is administered in parallel to psychosocial intervention and psychological and medical aid; Veresie Clinic, a clinic offering detoxification services through psychological and medical services, and perform naltrexone implant procedures.

An informed consent form was signed by each subject, a questionnaire was filled in with an interviewer, and blood samples were taken by qualified personnel as described previously [Demetriou et al., 2009]. All samples and questionnaires were coded at random with a laboratory identifier number so as not to reveal the personal identity of the study subjects. Forty individuals in total were included in this study, representing 87% of intravenous drug users requesting detoxification support at the time of sampling.

HCV, HBV, and HIV Tests

Immunoassays were performed on all samples for detection of HCV, HBV, HIV-1 and HIV-2 antibodies, using the AxSYM anti-HCV, HIV and HBsAg systems (Abbott Diagnostics, Chicago, IL).

RNA Extraction, RT-PCR and Sequencing in the Core-E1 and NS5B Regions

On all samples tested positive for HCV antibodies, RNA extraction, reverse transcription-PCR and sequencing were carried out in the regions Core-E1 and NS5B as described previously [Demetriou et al., 2009].

Subtyping and Phylogenetic Analysis

Subtyping of the strains found was done using the Oxford HCV Subtyping Tool v1.0 [de Oliveira et al., 2005]. Phylogenetic analysis was performed as described previously [Demetriou et al., 2009] to confirm the subtype of the strains and to investigate any possible phylogenetic relationship between the strains identified in this study from the intravenous drug users cohort and those identified in the general population of Cyprus [Demetriou et al., 2009]. Briefly, the sequences of both the Core-E1 region (14 sequences, 417 bp, positions 867–1283) and NS5B region (12 sequences, 405 bp, positions 8277–8681) were aligned and compared to reference strains from the Los Alamos database (http://hcv.lanl.gov) using the neighbor-joining method [Saitou and Nei, 1987] and the Kimura two-parameter distance estimation approach [Kimura, 1980] in MEGA v4 [Tamura et al., 2007]. The reliability of the phylogenetic clustering was evaluated using bootstrap analysis with 1,000 replicates [Felsenstein, 1985]. Bootstrap values above 70 were considered sufficient for subtype assignment. Clusters were determined by an arbitrary threshold of genetic distance (<0.05) [Lewis et al., 2008].

Additionally, for the purpose of a deeper investigation into possible phylogenetic relationships between the dataset obtained in this study and other HCV strains, each sequence was uploaded individually into the HCVBLAST tool on the Los Alamos HCV database website to discover the 50 most closely related sequences available in the database. Phylogenies for these sequences were constructed with MrBayes [Huelsenbeck and Ronquist, 2001] using a general time-reversible (GTR) model of nucleotide substitution with a proportion of invariant sites (ι) and gamma distribution of rates (Γ). The Monte–Carlo Markov Chain search was run for 5×10^6 generations, with trees sampled every 100th generation (with a burn-in of 50%) and a posterior consensus tree generated (from 25,000 trees). From this consensus tree, the posterior probability of nodes was used as phylogenetic support for each transmission cluster group. Cluster group size was determined using nodes with a posterior probability of 1.

Reference Sequences

The GenBank accession numbers for the reference sequences used in the phylogenetic analyses of the Core-E1 and NS5B regions are: AB031663, AF064490, AF169004, AF207752, AF238486, AF290978, AJ000009, AY051292, AY587845, D10988, D14853, D17763, D28917, D49374, D50409, D63821, D85516, D90208, DQ418786, DQ418787, DQ418789, EF108306, EF589160, EF589161, M62321, M67463, NC_004102, NC_009823, NC_009825, NC_009826, NC_009827, X76918, Y12083. The GenBank accession numbers for the sequences of the Cypriot strains of the general population used in the phylogenetic analysis for Core-E1 are EU684661–EU684737 and for NS5B are EU684591–EU684660.

Statistical Analysis

Variables were compared between hepatitis C positive and negative individuals using Mann–Whitney test for continuous variables and Chi-square test or Fisher-exact test where appropriate.

Nucleotide Sequence Accession Numbers

GenBank accession numbers for the sequences obtained in this study are GQ332540–GQ332553 for the Core-E1 sequences and GQ332554–GQ332565 for the NS5B sequences.

RESULTS

Demographic and Epidemiological Characteristics of the Study Subjects

The characteristics of the study subjects are listed in Table I, showing a comparison of those tested positive and those tested negative for HCV antibodies. Forty individuals in total were included in the study, of which the majority were male (85%) with a median age of 27 years (interquartile range 25–31) and of Cypriot nationality (64%). Other nationalities were either Greek or Eastern European. There was no statistical significance between the distribution of age or nationality between the HCV positive and negative individuals ($P = 0.61$ and 0.30 respectively). The results of the antibody testing showed that none of the intravenous drug users who took part in the study were infected with HIV or HBV at the time of sampling. However, 20 individuals of the 40 tested were positive for HCV antibodies, giving a prevalence of 50%.

The main risk factor which was statistically significant for HCV infection was the duration of injection drug use which, with a median duration of 10 years (IQR 5–14) was significantly higher in HCV-positive individuals as compared to a value of 6 years (IQR 1.5–8.5) in the HCV-negative group ($P = 0.02$). The association of the duration of intravenous drug use with the prevalence of HCV infection is seen in Figure 1. Another risk factor significantly different between the HCV positive and negative groups was a prison sentence. HCV-positive users reported a prison sentence more frequently than those who were negative ($P = 0.01$). All subjects reported to have practiced unprotected sex. Further analysis of known risk factors for HCV infection revealed no significant differences between the HCV positive and negative groups.

RT-PCR Sensitivity

Of the 20 samples found to be HCV positive from the AxSYM system, only 14 were PCR positive in the Core-E1 region and 12 were PCR positive in the NS5B region. PCR success rate was determined by comparing PCR results (positive or negative) with the results from the AxSYM anti-HCV system, which gives a value for the sample/cutoff rate ratio (S/CO) for each sample, where a value above 1 is considered positive. The S/CO values for the positive samples in this study were 4–124.

TABLE I. Characteristics of the Study Subjects

Characteristics[a]	Subjects (N = 40)	HCV +ve	HCV −ve
Age (years) (%)			
Median (IQR)	27 (25–31)	27 (24–34)	27 (25–29)
Gender (%)			
Male	35 (85)	16	19
Female	6 (15)	3	3
Nationality (%)			
Cypriot	25 (64)	10	15
Greek	8 (21)	4	4
Russian	2 (5)	2	0
Bulgarian	1 (3)	1	0
Georgian	1 (3)	1	0
Romanian	1 (3)	0	1
Not stated	1 (3)	0	1
Risk factors (%)			
Age at first injection (years) (IQR)	20 (17–23)	19.5 (16–21)	21 (18–25)
Duration of injecting drug use (years) (IQR)		10 (5–14)	6 (2–9)
Served a prison sentence	11 (28)	9	2
Shared injecting equipment	27 (68)	13	14
Can find sterile equipment easily	26 (65)	12	14
History of blood transfusion	3 (8)	3	0
History of surgical procedure	24 (60)	12	12
Tattoos	25 (63)	12	13
Have used syringes/needles abroad	10 (25)	7	3
Sexual practices (%)			
Heterosexual	35 (92)	17	18
Homosexual	1 (3)	1	0
Bisexual	2 (5)	0	2
Unprotected sexual practices	40 (100)	19	21
RT-PCR[b]	*Core-E1*	*NS5B*	
Positive	14	12	
Negative	6	8	
AxSYM anti-HCV S/CO values (IQR)[c]	*Core-E1*	*NS5B*	
PCR positive	86.8 (47.8–95.7)	88.1 (77.8–95.9)	
PCR negative	8.8 (4.6–25.7)	14.6 (5.5–45.6)	
Genotype[d]	*Core-E1*	*NS5B*	
1b	6	4	
3a	8	8	

[a]IQR, interquartile range.
[b]Only performed on samples which were HCV seropositive.
[c]AxSYM anti-HCV system, the HCV antibody test used in this study; S/CO, signal/cutoff rate ratio is the result obtained from the HCV antibody test.
[d]Only obtained for samples which were PCR positive.

A Mann–Whitney test showed that the PCR positive samples were those with significantly higher S/CO values than the PCR negative samples for both regions tested, as seen in Table I ($P = 0.001$).

Core-E1 and NS5B Phylogenetic Analyses

Phylogenetic trees of the Core-E1 and NS5B sequences obtained in this study with reference sequences and the sequences obtained from the general population infected with HCV in Cyprus are seen in Figures 2 and 3, respectively. The results of the subtyping showed that the strains of this study group fell into only two subtypes. Eight samples (57%) belong to subtype 3a, and six samples (43%) belong to subtype 1b, with bootstrap values higher than 98. Two of the 3a strains are not seen in the NS5B tree, as they were PCR negative in this region. There was concurrence for all other samples between the two regions.

No clusters were observed between the sequences from the intravenous dug users and those of the general population, however three small clusters were seen within the injecting drug users group with small genetic distances (<0.05) and high bootstrap values. These clusters are between samples CYIDU06, CYIDU07, and CYIDU33 within genotype 1b, CYIDU24 and CYIDU37 also within 1b, and CYIDU20, CYIDU26, and CYIDU27 within genotype 3a. The two subjects clustering together in the 1b subtype (CYIDU24 and CYIDU37) interestingly reported the use of speedball. Only one other individual reported this, CYIDU02, but they tested negative for HCV antibodies.

The Bayesian analysis of the Cypriot intravenous drug users sequences with the most genetically related

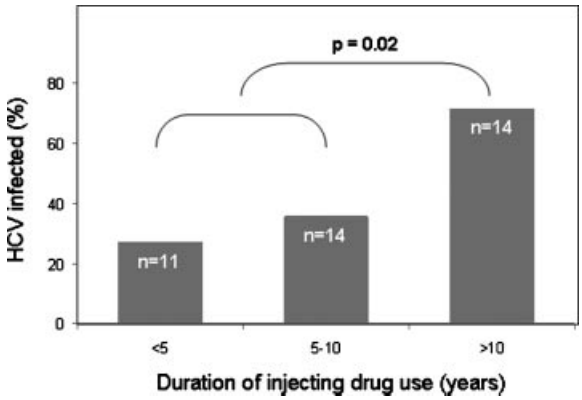

Fig. 1. Bar chart indicating the percentage prevalence of HCV seropositive users in three groups of different intravenous drug use duration. In the group of individuals who had been users for up to 5 years, 3 out of 11 were anti-HCV positive (prevalence is 27%); within the individuals who had practiced IDU for 5–10 years, 5 out of 14 were anti-HCV positive (prevalence is 36%); within the individuals who had been practicing IDU for over 10 years, 10 out of 14 were positive (prevalence is 71%). Intravenous drug users who had been practicing for more than 10 years were significantly at higher risk of being HCV seropositive than short-term users ($P = 0.02$).

database sequences, which resulted from the BLAST search, identified no significant clustering, as indicated by posterior probabilities that did not exceed 0.90 (data not shown).

DISCUSSION

In this study, the prevalence and molecular epidemiology among intravenous drug users seeking therapy in Cyprus is presented. Forty consenting individuals took part in this work, providing demographic and epidemiological characteristics as well as information on risk groups and drug use behavior. The study subjects were predominantly young men, and mostly Cypriots. The nationalities of the subjects who were not Cypriot demonstrate the influx of young people from Eastern Europe to the island and its strong association with Greece.

The results of the study showed that within the group tested, the prevalence of HCV infection was 50%, which is comparable to or lower than known prevalence rates in intravenous drug users [Shepard et al., 2005; van de

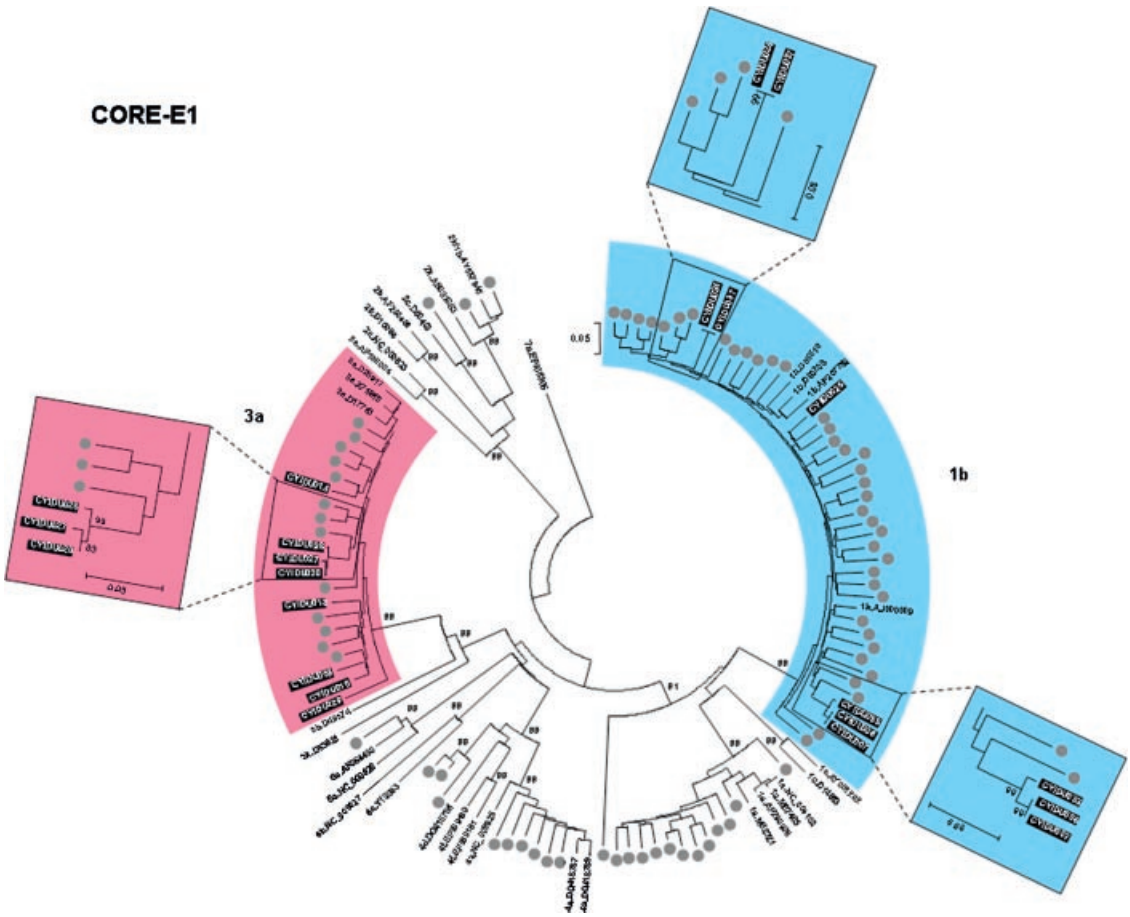

Fig. 2. Neighbor-joining tree of Core-E1 sequences, constructed as described in the "Materials and Methods" section. The intravenous drug users' strains are indicated with their laboratory codes and highlighted in black with white font. The reference strains are written in black font. The strains of the general population are indicated with gray circles. The subtypes in which the injecting drug users' strains fall are colored: 1b is blue and 3a is pink. The observed clusters of intravenous drug users' strains are indicated with a square and magnified for better viewing. The numbers indicated at the nodes are percentage bootstrap support values for 1,000 replicates. The divergence between any two sequences is obtained by summing the branch length using the scale at the top of the tree.

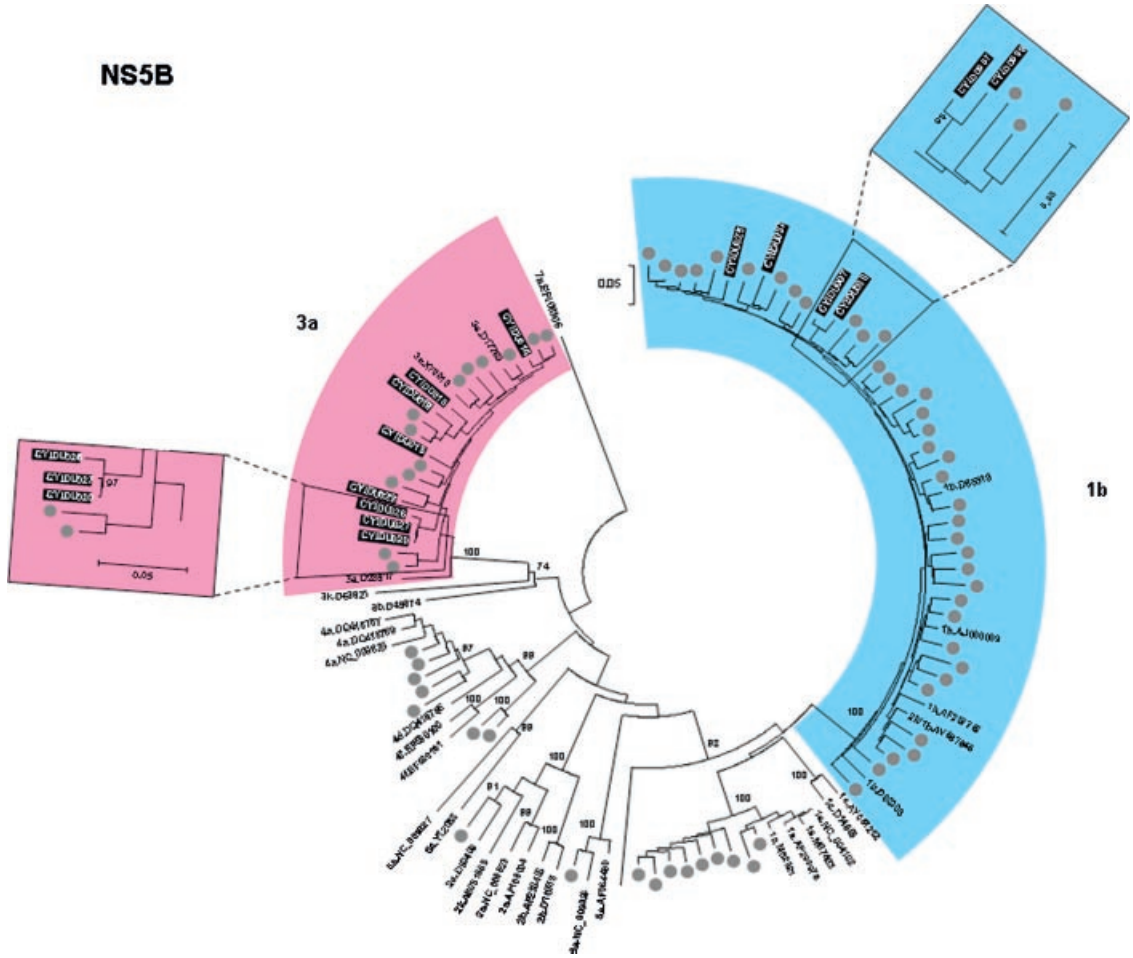

Fig. 3. Neighbor-joining tree of NS5B sequences, constructed as described in the "Materials and Methods" section. The intravenous drug users' strains are indicated with their laboratory codes and highlighted in black with white font. The reference strains are written in black font. The strains of the general population are indicated with gray circles. The subtypes in which the injecting drug users' strains fall are colored: 1b is blue and 3a is pink. The observed clusters of intravenous drug users' strains are indicated with a square and magnified for better viewing. The numbers indicated at the nodes are percentage bootstrap support values for 1,000 replicates. The divergence between any two sequences is obtained by summing the branch length using the scale at the top of the tree.

Laar et al., 2005; Shapatava et al., 2006; Sutton et al., 2008; Tan et al., 2008]. On the other hand, it was surprising to find that no subjects were infected with HIV or HBV. The prevalence of HIV and HBV is known to be lower than that of HCV, as fewer sharing partners are necessary to sustain HCV transmission than for other blood-borne viruses, and indirect drug sharing and preparation practices have also been associated with HCV transmission [Crofts et al., 1999; Alter, 2007], which it is considered applies to this population since the percentage of injecting drug users requesting therapy which was sampled in this study is high (87%).

From studies carried out on intravenous drug users, the main genotypes found to be associated with this transmission route are 1a and 3a [Esteban et al., 2008]. Subtyping of the sequences obtained in the Core-E1 and NS5B region of the HCV genome and, the frequent presence of 3a strains in this population have been confirmed, but subtype 1a, which appears in the general population infected with HCV in Cyprus [Demetriou et al., 2009], was not found in any individuals of this cohort. Interestingly, 43% of the strains were subtype 1b, showing that this subtype has a significant prevalence in intravenous drug users on the island, and possibly illustrates spill-over between different risk groups in this geographical region and the countries where the strains have possibly originated from, that is, eastern and southern Europe. This would have to be confirmed by phylogenetic analysis with HCV sequences from these countries. About 10% of the sequences submitted into the Los Alamos HCV database as ones which have been obtained from intravenous drug users are subtype 1b (http://hcv.lanl.gov), and it has been discovered in other such studies, mostly at a lower prevalence [Mathei et al., 2005; Zhou et al., 2006; Tallo et al., 2007; Peng et al., 2008; Oliveira Mde et al., 2009]. Finally, no 2k/1b strains were found in this group, even though such isolates have been identified on the island and they are known to circulate among intravenous drug users [Kalinina et al., 2002; Demetriou et al., 2009].

The phylogenetic analysis of the sequences resulted in no clustering between the injecting drug users' samples

and those of the general population. Even with such a permissive arbitrary threshold of genetic distance as 0.05, there were no other clusters observed, confirming the polyphyletic nature of HCV infection on the island and illustrating that the HCV epidemic in the selected population runs in parallel to the general population infected with HCV.

Three small clusters were observed within the intravenous drug users group itself, with small genetic distances (<0.05) and high bootstrap values. These clusters are between samples CYIDU06, CYIDU07, and CYIDU33 within genotype 1b, CYIDU24 and CYIDU37 also within 1b, and CYIDU20, CYIDU26, and CYIDU27 within genotype 3a. In the first cluster (CYIDU06, CYIDU07, and CYIDU33), the sequences were obtained from three males between the ages of 24 and 47, all sampled from the same town, two of which from the same center. Two were Cypriot and the other was Greek. They all stated that they had practiced intravenous drug abuse within the past year, and interestingly all had been practicing intravenous drug use for over 10 years. In the second cluster (CYIDU24 and CYIDU37) the samples were taken from a 27-year-old Bulgarian male and a 31-year-old Cypriot male, sampled in different towns. They both mention the use of speedball as the drug used when first practicing injecting drug use, and have both been practicing intravenous drug use for over 13 years. Only one other individual reported this, CYIDU02, however this person tested negative in the HCV antibody testing. In the third cluster (CYIDU20, CYIDU26, and CYIDU27), are samples from three Cypriots, one female and two males aged between 19 and 24. Two of the samples were obtained from users in the same detoxification clinic and the other from another center in another town. The males and females from the same detoxification clinic stated a duration of intravenous drug abuse as less than five years and the other individual did not state how long he had been using. These small clusters indicate the probability of sharing injecting equipment between these users. Also Cyprus is a small island and easy to get around, with a relatively small injecting drug user community. Therefore it is not surprising that strains cluster from users who were sampled in different towns or rehabilitation centers.

The Bayesian analysis showed that there was no clustering between the Cypriot intravenous drug users' sequences and any sequence from the Los Alamos HCV database (http://hcv.lanl.gov). Lack of sequence data in the database impairs the ability to investigate molecular epidemiological and phylogenetic relationships between newly obtained sequences and HCV sequences from anywhere around the world. From these results it cannot be determined where the HCV infections were transmitted, for them to make their way into Cyprus through intravenous drug users. More sequence data from intravenous drug users, especially from eastern and southern European countries, would be required to obtain a better picture of the HCV epidemic and to gain insight on the origin and dynamics of HCV infection on a population level. This point is the most severe limitation to this study and to all work on phylogenetic analysis of HCV strains.

In this population of highly experienced intravenous drug users the only significant factor predicting HCV was the duration of injection drug use, a factor known to be a predictor of higher HCV prevalence [Shepard et al., 2005; Hagan et al., 2007; Sweeting et al., 2009]. Also, having served a prison sentence appears to be reported more frequently in HCV-positive individuals, highlighting the occurrence of unsafe injecting practices in correctional facilities. Interestingly, there were no significant differences between the HCV-positive and negative groups with regards to having shared injecting equipment or having easy access to clean syringes, or other stated risk behaviors. All subjects stated that they had practiced unprotected sex, but only two stated MSM behavior, given the recent reports regarding MSM being a route of transmission [van de Laar et al., 2007].

This study is the first description of the molecular epidemiology of HCV infection among intravenous drug users in Cyprus and provides confirmation of the polyphyletic nature of the epidemic, already seen in the general population on the island infected with HCV. This is seen by the limited clusters appearing within the sequences obtained from injecting drug users and none with those obtained from the general population. Also, there appears to be a circulation of only subtypes 1b and 3a among intravenous drug users seeking therapy in Cyprus, and no indication of subtype 1a. Furthermore, the results showed that the duration of intravenous drug abuse was a main factor to acquiring HCV infection compared to other risk factors in this cohort. The prevalence of HCV infection among the studied population was 50%, comparable to the rest of the world but also significant enough to raise an alarm for the requirement of harm reduction strategies to be applied for the protection of intravenous drug users, especially considering the high influx of tourists and political refugees on the island. Overall, this study reinforces the idea of multiple points of introduction of HCV into Cyprus and also the risk of widespread transmission of these strains. Further investigation would be necessary with a more extended sampling group and by sampling through snowballing instead of through detoxification services, to obtain a more complete picture of the epidemiology of HCV among intravenous drug users on the island and rule out the presence of more genotypes and subtypes in this population.

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