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Molecular Epidemiology of Hepatitis C Infection in Cyprus: Evidence of Polyphyletic Infection

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The genetic diversity of the hepatitis C virus (HCV) in Cyprus is investigated for the first time in this study. Nucleotide sequence analysis of the CORE-E1 and NS5B regions of the HCV genome was performed on blood plasma samples obtained from 77 HCV patients in Cyprus, collected during 2005–2008. The amplified products were sequenced and compared to reference HCV strains of known genotype and subtype in order to classify the isolates found in this study. Genotype could be determined for all strains, and subtype for all but four isolates. Phylogenetic analysis revealed that 51 patients were genotype 1, of which 38 were subtype 1b, 9 were 1a, and 1 was unclassified, one patient was genotype 2c, 13 were genotype 3a, nine were genotype 4, of which six were subtype 4a, and three were of unclassified subtype, one was genotype 5a, two patients seem to carry a possible 2k/1b recombinant strain, and no genotype 6 strains were found. This study demonstrated a genetic heterogeneity of HCV infection in Cyprus, with five of the six known HCV genotypes on the island, including unclassified isolates in genotypes 1 and 4, and also the apparent introduction of the 2k/1b recombinant strain in intravenous drug users. ***J. Med. Virol. 81:238–248, 2009.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: HCV genotypes; NS5B region; CORE-E1 region; phylogenetic analysis

INTRODUCTION

Since its identification in 1989 [Choo et al., 1989], hepatitis C has been recognized as a major public health problem infecting nearly 170 million people around the world [WHO, 1999]. It is a life-shortening disease associated with complex and expensive morbidity and decreased quality of life, being a major contributor to liver cirrhosis and hepatocellular carcinoma. In 15–20% of acute HCV infections the patient recovers spontaneously, but in the large majority of cases the disease runs a chronic course and can even cause hepatocellular carcinoma [Seeff, 2002]. The most important route of HCV transmission is through exposure to infected blood and until the introduction of diagnostic screening in 1991, the virus was transmitted mainly through blood, blood products, hemodialysis, and organ transplantation [Memon and Memon, 2002]. HCV infection in the Western world now occurs primarily by parenteral exposure, the most common mode of transmission being intravenous drug use through sharing of needles or other injecting equipment. However, in developing countries unsafe therapeutic injection practices, inadequate disinfection practices, non-sterile medical and dental procedures, and unscreened blood transfusions may still account for significant HCV transmission and serve as a bridge to the general population. Although certain recent reports also link HCV transmission with sexual behavior [van de Laar et al., 2007; Richardson et al., 2008], this has been a controversial association for many years.

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The hepatitis C virus (HCV) is a small, enveloped, single-stranded, positive-sense RNA virus belonging to the genus *Hepacivirus* of the family Flaviviridae, consisting of a genome of approximately 9,500 nucleotides. Studies of the nucleotide sequences of HCV variants from different individuals and different geographical regions have revealed a high degree of genetic heterogeneity of the HCV genome, and this has led to a proposed consensus of six genotypes and numerous closely related subtypes based on sequence variation in the 5′ noncoding region (5′NCR), CORE, E1 and NS5B regions [Simmonds et al., 1993, 2005; Robertson et al., 1998; Simmonds, 1999]. At the level of full genomes, HCV exhibits around 30% variation between genotypes and 20–25% variation in subtypes of the same genotype. The genotype is presently a major factor in both the choice of treatment and prognosis [Zeuzem, 2004].

Genotype distribution differs by geographic region and by year and mode of transmission [Zein, 2000; Schroter et al., 2004]. Globalization, however, is changing the face of HCV epidemiology radically. Knowledge of genotype distribution in different parts of the world may help clarify the epidemiology and evolution of HCV, and has proven a useful tool for identifying risk groups and distinguishing different routes of transmission [Nakano et al., 2004]. Certain modes of transmission are associated with HCV subtype infections, suggesting separate HCV epidemics, but spill-over between different risk groups underlines the value of molecular epidemiological studies to gain insight into the origin and dynamics of HCV infections on a population level. In western Europe, HCV subtypes 1a and 3a predominate among intravenous drug users [Cochrane et al., 2002; van Asten et al., 2004] whereas subtypes 1b and genotype 2 are associated mainly with contaminated blood transfusions and other types of nosocomial transmission, especially in older patients. Geographically, genotypes 1, 2, and 3 are found globally, while 4–6 have a more restricted pattern. Genotype 4 is found mainly in North Africa and especially Egypt, but has recently been spreading to Europe largely through intravenous drug users with a high incidence in Greece [Savvas et al., 2005; Katsoulidou et al., 2006; Kamal and Nasser, 2008]. Genotype 5 is restricted primarily to South Africa [Chamberlain et al., 1997], but has also recently been found in West Flanders, Belgium [Verbeeck et al., 2006], central France [Henquell et al., 2004], and Syria [Antaki et al., 2008], albeit in much smaller numbers. HCV genotype 6 is found in Southeast Asia [Huy and Abe, 2004].

In the geographical area close to Cyprus, which is the eastern Mediterranean region, HCV genotypes are not distributed uniformly. In western Turkey, genotype 1b is the most prevalent [Altuglu et al., 2007]. Also, a study done in northern Cyprus with civilians, Turkish soldiers, and Northern Cyprus soldiers revealed genotype 1b as the most prevalent (92.4%) [Altindis et al., 2006]. In Egypt, the incidence of HCV infection is significantly higher than other countries worldwide and most cases are infected with subtype 4a [Abdel-Hamid et al., 2007]. Studies carried out with patients in the Middle East revealed a predominance of HCV genotypes 4 and 1 [Watson et al., 1999; Ramia and Eid-Fares, 2006]. In Greece, genotype 1 is the most prevalent (46.9%), followed by genotype 3 (28.1%), 4 (13.2%), 2 (6.9%), and 5 (0.4%) [Katsoulidou et al., 2006]. This pattern of diversity is much more similar to the results presented in this study.

Among injected drug users, the HCV subtypes most prevalent are 3a and 1a, with both subtypes showing an exponential population growth during the 20th century [Pybus et al., 2005]. Genotype 3a, which originates in Asia, has been associated significantly with transmission through intravenous drug use in industrialized countries [Pawlotsky et al., 1995; McCaw et al., 1997; Bourliere et al., 2002]. It is prevalent mainly in North and South America, Europe, and Australia where practicing intravenous drug abuse is common, and seems to have been transmitted from a common origin through a unique worldwide epidemic that spread rapidly among drug users [Pybus et al., 2005; Morice et al., 2006]. Genotype 4 (mainly 4d) is also becoming increasingly prevalent in populations of intravenous drug users, especially in southern Europe, and its introduction into the European intravenous drug user population seems to be more recent than that of 1a and 3a [van Asten et al., 2004; Chlabicz et al., 2008; Kamal and Nasser, 2008].

Until 2001, HCV was thought to evolve in a clonal manner, with diversity generated through the accumulation of mutations. However, homologous recombination has been demonstrated between different genotypes or different subtypes of a genotype. A 2k/1b recombinant was found in St. Petersburg, Russia [Kalinina et al., 2002], and seems to be spreading among intravenous drug users in Russia. It has also been found in Ireland [Moreau et al., 2006], in Estonia [Tallo et al., 2007] and among intravenous drug users in Uzbekistan [Kurbanov et al., 2007]. Other natural recombinants of the virus found in certain parts of the world are a 2i/6p recombinant in Vietnam [Nopporpanth et al., 2006], a 2b/1b recombinant in the Philippines [Kageyama et al., 2006], a 1b/1a recombinant in Peru [Colina et al., 2004] and one between genotypes 2 and 5 in southwest France [Legrand-Abravanel et al., 2007]. HCV recombination break points have been located mainly in the non-structural proteins, but an intratypic recombinant with a break point in the structural region has also been identified [Cristina and Colina, 2006].

Cyprus is a small island with a population of approximately 800,000 and a large annual influx of foreigners mainly from tourism but also as political refugees. The molecular epidemiology of hepatitis C infection in Cyprus has never before been studied. The HCV genotype distribution on the island is presented here for the first time, revealing high genetic heterogeneity, multiple points of introduction, and the existence of possible recombinant strains.

MATERIALS AND METHODS

Patients and Samples

From 2005 to 2008 blood samples were obtained from 107 consenting chronically infected HCV patients aged 18–84 attending private clinics and public hospitals in the Nicosia, Larnaca, Limassol, and Paphos districts. All patients were tested positive for HCV antibodies by a second-generation immunoassay (INNO-LiPA), and for HCV RNA by diagnostic reverse transcription-polymerase chain reaction (RT-PCR; COBAS Amplicor, Roche Diagnostics, Branchburg, NJ). All samples were investigated by sequencing the CORE-E1 region and the NS5B region of the HCV genome.

RNA Extraction and RT-PCR

Blood was collected from the patients in BD Vacutainer® PPT™ (Becton Dickinson and Co., Franklin Lakes, NJ) tubes and the plasma was isolated after centrifugation at 1,100 RCF (relative centrifugal force) for 10 min in an Eppendorf Centrifuge 5810 R (Eppendorf). Viral RNA was extracted from 200 μl plasma using the QIAmp® UltraSens® Virus kit (Qiagen, Venlo, The Netherlands) and 15 μl of the RNA was used in a one-step RT-PCR using Superscript™ III One-Step RT-PCR Platinum Taq HiFi (Invitrogen, Carlsbad, CA), following a heat-shock step at 70°C for 20 sec to denature the RNA secondary structure. The RT-PCR was performed in a 50 μl reaction with 20 pmol each of the outer sense and antisense degenerate primers derived from the CORE-E1 and NS5B regions of the HCV genome, designed to amplify all HCV genotypes (see Table I). A nested PCR was performed using 3 μl of the RT-PCR product with 40 pmol each of the inner PCR primers (Table I), using Platinum® PCR SuperMix (Invitrogen) in a 50 μl reaction. PCR amplification was confirmed by visualization with ethidium bromide staining of a 2% agarose gel.

CORE-E1 and NS5B Sequencing and Phylogenetic Analysis

Cycle sequencing PCR was performed on the amplicons in both directions using the inner forward and reverse amplification primers for each region (Table I) by means of the BigDye® Terminator system v3.1 (Applied Biosystems, Foster City, CA). The products were purified using the DyeEx spin kits (Qiagen) and sequenced directly on the ABI 3300 Genetic Analyser (Applied Biosystems). The resulting readings were analyzed with the Sequencing Analysis Software v5.2 (Applied Biosystems). The obtained nucleotide sequences of both the CORE-E1 region (77 sequences, 417 bp, positions 867–1283) and NS5B region (70 sequences, 405 bp, positions 8277–8681) were aligned with the reference sequence of the H77 strain using the CLUSTALX 1.83 alignment software [Thompson et al., 1997]. Subtyping the sequence of each region was performed using Oxford HCV Subtyping Tool v1.0 [de Oliveira et al., 2005], after which the aligned sequences were compared to reference strains of known subtypes derived from the Los Alamos database [Kuiken et al., 2005] using the neighbor-joining method [Saitou and Nei, 1987] in MEGA version 4 [Tamura et al., 2007]. Pair-wise distance matrices were generated using the Kimura [1980] two-parameter distance estimation approach. 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.

NS5B Phylogenetic Analysis of 3a Strains

Further phylogenetic analysis was performed on the isolated genotype 3a strains found in this study by constructing a tree from the NS5B sequences of these strains, and 50 intravenous drug use-related NS5B sequences from published work about strains from intravenous drug users [Kalinina et al., 2001; Cochrane et al., 2002; Morice et al., 2006] and other subtype 3a sequences with intravenous drug use as the stated source of infection retrieved from the HCV sequence database. The analysis was done using the neighbor-joining method [Saitou and Nei, 1987] in MEGA version 4 [Tamura et al., 2007]. Pair-wise distance matrices were generated using the Kimura [1980] two-parameter distance estimation approach. The reliability of the phylogenetic clustering was evaluated using bootstrap analysis with 1,000 replicates [Felsenstein, 1985].

Reference Sequences

The GenBank accession numbers for reference sequences used in phylogenetic analysis of the CORE-E1 region are: AB031663, AF064490, AF165045, AF169004, AF238486, AF271822, AF271876, AF271878, AF271886, AF290978, AJ000009, AY051292, AY434107, AY434119, AY434122, AY434128, AY434131, AY434134, AY434146, AY434149, AY434158, AY587845, AY706996, AY706999, AY754623, AY767506, AY767956, AY894540, AY894555, D10988, D14853, D28917, D43678, D50409, D63821, D90208, DQ418786, DQ418787, DQ418789, E10839, EF115767, EF115770, EF115798, EF115882, EF115883, EF115898, EF115900, EF115902, EF115906, EF115908, EF115915, EF115916, EF115923, EF589160, EF589161, L29589, L29609, L29610, L29620, L38350, L39282, L39310, NC_004102, NC_009823, NC_009824, X76414, Y11604, Y12083, Y13184. The GenBank accession numbers of the sequences used as references for phylogenetic analysis of the NS5B region are: AB031663, AF037235, AF037237, AF064490, AF165045, AF169004, AF238486, AF271799, AF290978, AJ000009, AY051292, AY265429, AY265435, AY434106, AY434108, AY434120, AY434123, AY434126, AY434132, AY434147, AY434157, AY548714, AY548717, AY548731, AY548736, AY587845, AY632098, AY632126, AY632144, AY632237, AY685046, AY743124, AY743160, AY743171, AY743182, AY743204, AY743208, AY743212, AY743213, AY754624, AY894553, D10988, D14853, D28917, D50409, D63821, D90208, DQ418786, DQ418787, DQ418789, DQ911240, E10839, EF115983,

TABLE I. RT-PCR Primers for CORE-E1 and NS5B Amplification and Sequencing

Name	Primer set	Polarity	Sequence[a]	Position[b]
CORE-E1				
735	Outer	Sense	5′-GACCTCATGGGGTACATYCCBSTCGTHGG-3′	735–763
1324	Outer	Antisense	5′-GGBGACCARTTYAKCATCATRTCCCAWGCC-3′	1,295–1,324
834	Inner	Sense	5′-GCAACAGGGAATYTDCCYGGTTGCTCYTTYTC-3′	834–865
1318	Inner	Antisense	5′-CAGTTCATCATCATRTCCCAWGCCATNCGRTGDCC-3′	1,284–1,318
NS5B				
8172	Outer	Sense	5′-TAYGGRTTCCARTACTCNCCHGVRCAGCGGGT-3′	8,172–8,203
8821	Outer	Antisense	5′-GARTTGACWGGRGWGTGTCKDRCTGTYTCCCA-3′	8,790–8,821
8244	Inner	Sense	5′-ATGGGBTTYKCRTATGAYACCCGHTGYTTTGA-3′	8,244–8,275
8713	Inner	Antisense	5′-GABACRTTKGAGGARCADGATGTTATNARCTC-3′	8,682–8,713

[a]Degenerate positions are shown with their IUB Base Codes (R: A or G; W: A or T; S: G or C; K: G or T; Y: C or T; B: C, G or T; D: A, G or T; H: A, C or T; V: A, C or G; N: A, C, G or T).
[b]Position numbering according to strain H77 (GenBank Acc. No NC_004102), genotype 1a.

EF115994, EF116013, EF116021, EF116118, EF116121, EF116125, EF116137, EF116138, EF116141, EF589160, EF589161, L29611, L29618, L38371, L48496, NC_004102, NC_009823, NC_009824, Y11604, Y12083, Y13184. The GenBank accession numbers of the reference sequences used in the subtype 3a tree for intravenous drug user strains are: AB327108, AB327110, AB327111, AB327112, AB327113, AB327114, AB327115, AF388439, AF388443, AF388447, AF388450, AF388451, AF388452, AF388455, AF388464, AF388466, AF388467, AF388469, AF388475, AF388476, AF388509, AF516369, AJ867081, AJ867088, AJ867093, AJ867098, AJ867101, AJ867105, AJ867106, AJ867162, AY100024, AY100031, AY100037, AY100045, AY100047, AY100051, AY100052, AY100055, AY100074, AY100077, AY100079, AY100081, AY100083, AY100084, AY100090, AY100093, AY100095, AY100107, AY100109, AY100111 and the tree was rooted with non-a genotype 3 strains E10839 and D63821.

Statistical Analysis

To test for a statistically significant correlation between the PCR results and demographic and clinical variables from the samples, the χ^2-test for categorical variables and the *t*-test for continuous variables were used.

Nucleotide Sequence Accession Numbers

GenBank accession numbers for the sequences obtained in this study are EU684591–EU684660 for the NS5B sequences and EU684661–EU684737 for the CORE-E1 sequences.

RESULTS

Clinical and Epidemiological Features of Study Subjects

The study group consisted of 107 HCV seropositive patients between the ages of 18 and 84, from two private gastroenterology clinics and public hospitals in Cyprus. The epidemiological features of the study subjects varied, as 53 (51.5%) patients were Cypriots and the rest were various other nationalities, 36 (35%) being from countries of the former Soviet Union (Russia, Georgia, Moldova, and Ukraine). Thirty-nine patients (36.4%) had a history of transfusion with blood products, 4 (3.7%) stated they were intravenous drug users, 10 (9.3%) traced infection to dental or surgical procedures, and 51 (47.7%) did not know the source of infection (see Table II for the demographic details).

Viral RNA Extraction From Plasma and RT-PCR

Seventy-seven samples were PCR-positive for CORE-E1 and 70 for NS5B, presenting 73.8% and 71.0% PCR success rates, respectively. Statistical analysis to determine the association between PCR result and whether the patients were on therapy revealed a more frequent negative PCR result for patients on therapy with *P*-values <0.016. Fifty-six patients (52.3%) were on interferon therapy when blood was taken, and 51 patients (47.7%) were taking ribavirin. Of all patients on treatment, 49 were on interferon–ribavirin combination therapy. Forty-nine patients (45.8%) were not on therapy at the time blood was taken. Considering the two drugs separately, from the patients on interferon therapy, 35 exhibited PCR-positive results and 21 showed negative results, compared to 44 positive results and 7 negative results from the patients not on interferon ($P = 0.008$). Of the patients taking ribavirin, 32 had positive PCR results and 19 had negative results compared to 47 positive and 9 negative samples from patients not taking ribavirin ($P = 0.016$). Furthermore, the correlation between PCR result and viral load showed that samples from patients with a detectable viral load (more than 135 copies/ml) exhibited more frequently a positive PCR result than those with undetectable viral load with a *P*-value of 0.002. From the patients with undetectable viral load at the time blood was taken, 6 had positive PCR results and 13 had negative results, compared to patients with detectable viral load, of which 58 were positive and 21 were negative for PCR ($P = 0.002$; patients with unknown viral load were excluded from this analysis).

TABLE II. Characteristics of the Study Subjects

Characteristics	Patients (N = 107)	
Gender (%)[a]		
Male	53 (50)	
Female	51 (48)	
Age (years) (%)[a]		
Median (IQR)	40 (32–57)	
18–29	21 (20)	
30–39	31 (30)	
40–49	11 (11)	
50–59	22 (21)	
60 and older	19 (18)	
Region of origin (%)[b]		
Cyprus	53 (52)	
Russia	15 (15)	
Georgia	14 (14)	
Greece	5 (5)	
Moldova	4 (4)	
Ukraine	3 (3)	
Egypt	3 (3)	
Other—Europe	4 (4)	
Other—Asia	2 (2)	
Route of transmission (%)		
Blood transfusion	39 (36)	
Surgical procedure	6 (6)	
Dental procedure	4 (4)	
IVDU	4 (4)	
Tattoo	2 (2)	
Sexual transmission	1 (1)	
Other/unknown	51 (48)	
Use of medication (%)		
Interferon/ribavirin	58 (54)	
No medication	49 (46)	
Plasma HCV-RNA (log copies/ml)		
Median (IQR)	5.5 (2.5-6.2)	
	CORE-E1	NS5B
RT-PCR (%)		
Positive	79 (74)	76 (71)
Negative	28 (26)	31 (29)
Genotype (%)		
1	51 (48)	48 (45)
2	3 (3)	1 (1)
3	13 (12)	13 (12)
4	9 (8)	7 (7)
5	1 (1)	1 (1)
Unknown	30 (28)	37 (35)

IQR, interquartile range.
[a]Information available for 104 patients.
[b]Information available for 103 patients.

CORE-E1 and NS5B Sequencing and Phylogenetic Analyses

The neighbor-joining trees for the CORE-E1 and NS5B regions are seen in Figure 1. HCV genotype 1 was the most frequent (47.7% in the CORE-E1 region), followed by genotypes 3, 4, 2, and 5 (12.1%, 8.4%, 2.8%, and 0.9%, respectively in the CORE-E1 region). There is concordance between the trees for all samples that were positive for both regions, except for two strains from Georgian patients (designated with an asterisk in Fig. 1). These strains were identified as subtype 2k in the CORE-E1 region and as 1b in the NS5B region. From these results, the strains appear to be 2k/1b recombinants, as they cluster together with the St. Petersburg 2k/1b recombinant strain in both trees. However, further clonal analysis is required to confirm this.

For genotype 1, 36 sequences recovered in this study classify with subtype 1b. Two additional strains also classify as subtype 1b in the CORE-E1 region, but were PCR-negative in the NS5B region and could not be analyzed. Twenty of these strains are from patients originating from Cyprus, six from Georgia, five from Russia, three from Moldova, and one each from Greece, Ukraine, and Romania. One strain in particular, from a Greek patient, has a 3-nucleotide insertion in the CORE-E1 sequence between positions 1044 and 1045 (numbers according to strain H77).

Nine strains classify within the 1a subtype in both regions with bootstrap values higher than 90. Three further isolates also classify as subtype 1a in the CORE-E1 region but were PCR-negative in the NS5B region. Of the nine strains, six are from Cypriot patients, two from Georgian patients and one each from a British, an Iranian, an Italian, and a Greek patient. One strain from a Cypriot patient does not cluster with any specific genotype 1 subtype, having used reference sequences from all available assigned genotype 1 subtypes in this genomic region in the phylogenetic trees. This is also the case for the NS5B tree, where all available assigned subtypes in this region were also included in the phylogenetic analysis. To investigate this strain further, BLAST was performed using the HCV BLAST tool on the HCV Sequence database website, recovering the 100 closest matches. These were downloaded into a FASTA file along with the Cypriot strain and a phylogenetic tree was constructed to explore the relationship with the closest sequences available in the database (data not shown). The strain in question did not cluster with any of the sequences from the database. This sequence is thus labeled unclassified, and due to lack of data, could not be assigned as a new subtype.

For genotype 2, one strain from a Cypriot patient clusters in subtype 2c in the CORE-E1 tree and NS5B tree with bootstrap values of 98 and 83, respectively. Also the two putative recombinant strains mentioned

Fig. 1. Neighbor-joining phylogenetic trees for the 77 CORE-E1 sequences (400 nucleotides, corresponding to positions 884–1283; left) and the 70 NS5B sequences (328 nucleotides, corresponding to positions 8277–8604; right) of HCV strains obtained from patients in Cyprus, based on the Kimura two-parameter method for estimation of genetic distance. Trees were constructed using 35 representative reference sequences from 6 known subtypes (1 through 6) and 1 recombinant strain (2k/1b) taken from the HCV sequence database of Los Alamos National Laboratory [Kuiken et al., 2005]. The sequences determined in the study are color-coded, with colors corresponding to the patients' country of origin: Cyprus (yellow), Russia (pink), Georgia (blue), Moldova (light green), Greece (gray), Egypt (lilac), Bulgaria (beige), Iran (light blue), Italy (brown), Pakistan (purple), Romania (black), Ukraine (green), United Kingdom (red). The asterisks indicate putative 2k/1b recombinant strains. The divergence between any two sequences is obtained by summing the branch length, using the scale at the lower left of each tree. The numbers indicated at genotype and subtype-determining nodes are percentage bootstrap support for 1,000 replicates. The brackets on the right side of the trees indicate the determined subtypes as described in Results.

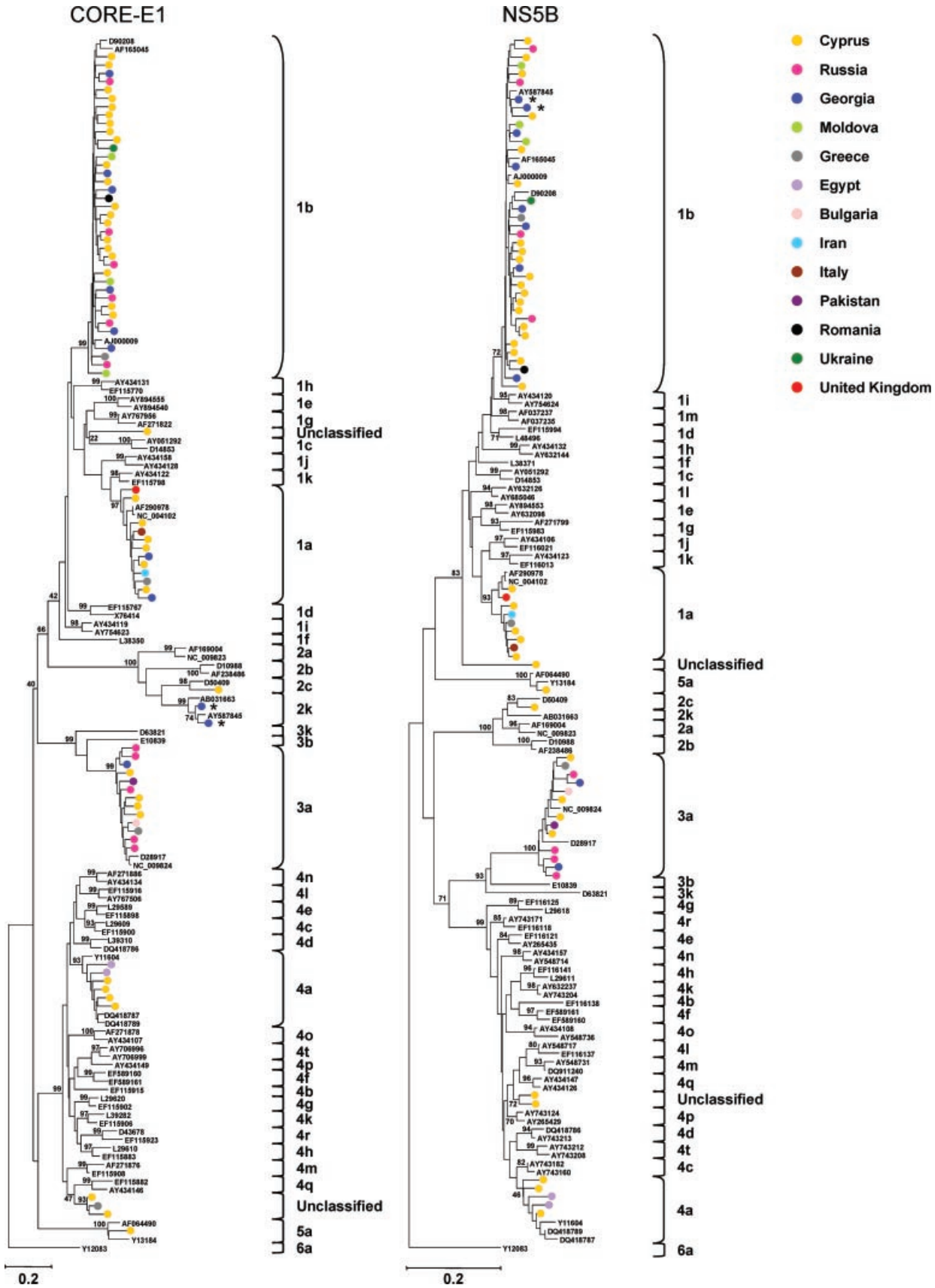
CORE-E1
NS5B
Cyprus
Russia
Georgia
Moldova
Greece
Egypt
Bulgaria
Iran
Italy
Pakistan
Romania
Ukraine
United Kingdom
1b
1h
1e
1g
Unclassified
1c
1j
1k
1a
1d
1i
1f
2a
2b
2c
2k
3k
3b
3a
4n
4l
4e
4c
4d
4a
4o
4t
4p
4f
4b
4g
4k
4r
4h
4m
4q
Unclassified
5a
6a
1i
1m
1d
1h
1f
1c
1l
1e
1g
1j
1k
1a
Unclassified
5a
2c
2k
2a
2b
3a
3b
3k
4g
4r
4e
4n
4h
4k
4b
4f
4o
4l
4m
4q
Unclassified
4p
4d
4t
4c
4a
6a
0.2
0.2

Fig. 1.

earlier cluster with the 2k subtype in the CORE-E1 tree with a bootstrap of 99, but with the 1b subtype in the NS5B region.

Analysis of both genomic regions revealed 13 strains for genotype 3, all of which belong to subtype 3a, clustering with the 3a reference strains with bootstrap values higher than 95 in both trees. These strains were isolated from four Cypriot patients, four Russian patients, two Georgian patients, and one patient each from Greece, Pakistan, and Bulgaria.

For genotype 4, five strains found in this study are assigned as subtype 4a in both regions, plus one additional strain for which only the CORE-E1 region could be sequenced. These strains were isolated from four Cypriot patients and two patients of Egyptian origin. A cluster of a further three strains was found to belong to genotype 4 in the CORE-E1 region but these sequences do not cluster with any particular assigned subtype of genotype 4 and were therefore labeled as unclassified. These strains were isolated from two Cypriot patients and one Greek patient. Two of these strains were seen to behave similarly in the NS5B region, the third being PCR-negative for NS5B, and therefore could not be sequenced in this region. For both trees, sequences from all available subtypes in the corresponding genomic regions were used and this cluster appears to be genetically closest to subtypes 4q, 4m, and 4l. These sequences were further investigated by uploading them into the HCV BLAST tool of the Los Alamos HCV sequence database and retrieving the 100 closest matches. These sequences were analyzed together and a phylogenetic tree was constructed to look for a possible relationship between the strains found in this study and any other similar sequences in the HCV database (data not shown). This was done for the sequences in both regions. In the CORE-E1 region, the unassigned Cypriot strains cluster with seven other sequences from the database, four from subjects of African origin from a Canadian submission (Acc. No. EF115885, EF115899, EF115905, and EF115910) [Murphy et al., 2007] and three from patients of unknown epidemiological and demographic details from a UK submission (Acc. No. AY766949, AY767036, and AY767953), which are also genotype 4 of unassigned subtype. The sequences do not cluster with sequences of assigned subtype and are genetically closest to the subtype 4q group. A similar pattern is revealed in the NS5B tree, where the two Cypriot sequences cluster with the same four Canadian sequences (Acc. No. EF116108, EF116122, EF116128, and EF116133), apart from assigned subtypes and genetically closest to subtype 4q. They cluster with no other isolates and the British sequences were not sequenced in the NS5B region.

Lastly, one genotype 5 strain was also identified in a Cypriot patient and is classified as subtype 5a on both trees with corresponding bootstrap values of 100 in both cases.

No genotype 6 strains were found in this population study.

NS5B Phylogenetic Analysis of Subtype 3a Strains

To further assess the relationship of the genotype 3a sequences discovered in this study with intravenous drug use, these sequences were examined in a new dataset context. A new tree was constructed using the NS5B region of only the 3a strains from this study and 50 3a NS5B sequences derived from publications that studied sequences from intravenous drug users from various countries [Kalinina et al., 2001; Cochrane et al., 2002; Morice et al., 2006] and also from a search for 3a strains from intravenous drug users in the HCV Sequence Database (Fig. 2). As found previously [Morice et al., 2006], the geographical areas of origin of the subjects do not seem to harbor HCV-3a populations distinct from each other. The tree shows no clearly defined subclade of subtype 3a strains isolated from intravenous drug users from different geographical areas; it can therefore not be verified that the HCV-3a strains found in this study are introduced by intravenous drug use in specific geographical regions.

DISCUSSION

In this study, viral RNA extraction from blood plasma, RT-PCR and nucleotide sequence analysis of the CORE-E1 region and NS5B region were used successfully to genotype HCV strains and determine the genetic heterogeneity in a sample study group of patients in Cyprus. The RT-PCR assay design is considered successful as over 70% of samples were PCR-positive in both regions and those that were not belonged mainly to patients receiving therapy and/or with low viral load. The patients investigated were epidemiologically diverse, showing a wide range of ages, countries of origin, and routes of transmission for HCV. Most patients were either from Cyprus or from countries of the former Soviet Union, but the group also included patients from other countries, including Greece, Britain, Pakistan, Italy, Egypt, Iran, Romania, and Bulgaria. The route of transmission for a large percentage of patients was unknown, mainly because this virus can be carried for many years without diagnosis and because safety measures for the prevention of HCV transmission

Fig. 2. Neighbor-joining phylogenetic trees for the 13 NS5B sequences (240 nucleotides, corresponding to positions 8316–8555) of HCV subtype 3a strains obtained from patients in Cyprus, based on the Kimura two-parameter method for the estimation of the genetic distance. Trees were constructed using 50 intravenous drug use-related subtype 3a sequences taken from published studies [Kalinina et al., 2001; Cochrane et al., 2002; Morice et al., 2006] and from searches in the HCV sequence database of Los Alamos National Laboratory [Kuiken et al., 2005]. The tree is rooted with two non-a genotype three reference sequences. The reference sequences are color-coded, with the colors corresponding to the sampling country: Australia (yellow), France (blue), Russia (pink), Uzbekistan (brown), Brazil (gray), United Kingdom (dark green) and USA (purple). The Cypriot strains are indicated with gray circles. The numbers indicated at the nodes are percentage bootstrap support for 1,000 replicates. The divergence between any two sequences is obtained by summing the branch length, using the scale at the lower left of the tree.

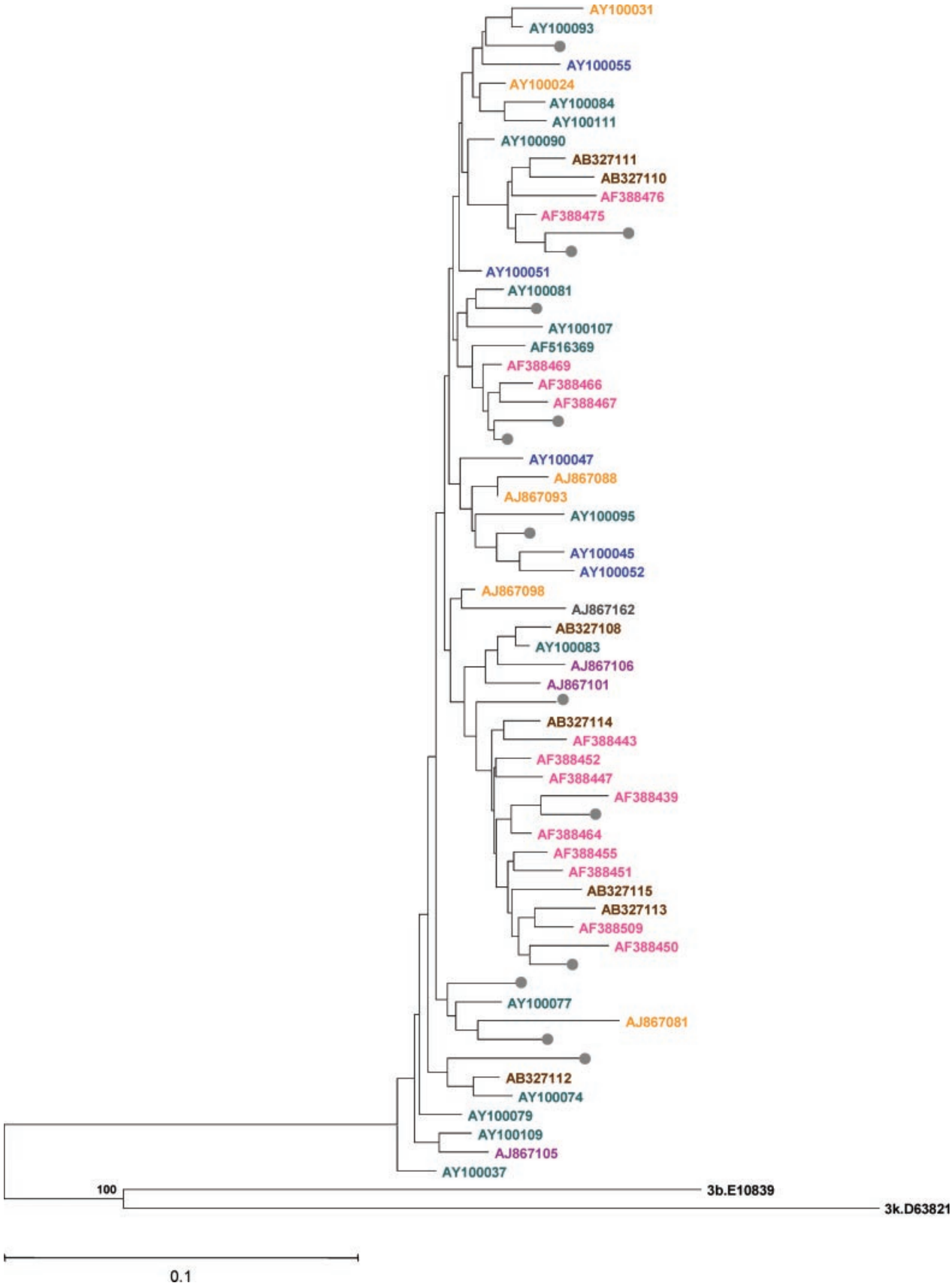
AY100031
AY100093
AY100055
AY100024
AY100084
AY100111
AY100090
AB327111
AB327110
AF388476
AF388475
AY100051
AY100081
AY100107
AF516369
AF388469
AF388466
AF388467
AY100047
AJ867088
AJ867093
AY100095
AY100045
AY100052
AJ867098
AJ867162
AB327108
AY100083
AJ867106
AJ867101
AB327114
AF388443
AF388452
AF388447
AF388439
AF388464
AF388455
AF388451
AB327115
AB327113
AF388509
AF388450
AY100077
AJ867081
AB327112
AY100074
AY100079
AY100109
AJ867105
AY100037
100
3b.E10839
3k.D63821
0.1

Fig. 2.

in hospitals and screening of donated blood and blood products were not carried out until the early 1990s. Also, considering the patients' diversity of nationality, it is worth noting that unsafe nosocomial practice still occurs in developing countries.

For genotype 1, 38 variants belonging to subtype 1b and 12 belonging to subtype 1a were identified. It is interesting that one strain, from a Greek patient, has a three nucleotide insertion in the CORE-E1 sequence between positions 1044 and 1045 (numbers according to strain H77), resulting in an addition of one amino acid in the E1 protein sequence. The epidemiological origin or significance of this insertion is not currently known. One genotype 1 variant has been labeled unclassified as it does not cluster with any of the groups of assigned subtypes for which sequences are available in the HCV sequence database. This strain could be a new subtype in genotype 1, but more epidemiologically distinct strains that cluster with this variant are needed to provisionally assign a new subtype; such sequences were neither found in this study nor in the HCV sequence database.

Of the genotype 2 variants isolated in this study, one strain was identified as subtype 2c. This was isolated from a Cypriot patient who was born in Argentina and was infected there by a blood transfusion in the late 1970s. According to Re et al. [2007], 2c is a subtype found in high prevalence in central Argentina, however it is unknown whether this was the case at the time this patient became infected, nor is it known where in Argentina the blood transfusion took place. Two other strains were classified as subtype 2k in the CORE-E1 region but not in the NS5B region and they are considered to be putative 2k/1b recombinants, discussed below.

For genotype 3, 13 variants belonging to subtype 3a were identified from the analysis of both regions. These strains were isolated from four Cypriot patients, four Russian patients, two Georgian patients, one patient from Greece, one from Pakistan, and one from Bulgaria. Because there is an established correlation of genotype 3a with intravenous drug use [Morice et al., 2006], these strains were analyzed further by comparing them to genotype 3a sequences from intravenous drug users available in the database. This analysis yielded no significant evidence for the Cypriot strains clustering with each other or with isolates from intravenous drug users from other countries. Also it has been shown previously that there is a phylogenetic mixing of HCV subtype 3a strains from drug users and non-drug users in various countries, supporting the existence of a unique origin for subtype 3a [Kalinina et al., 2001; Cochrane et al., 2002; Samimi-Rad et al., 2004; Morice et al., 2006]. The observation of no country-specific phylogenetic clustering for strains isolated from intravenous drug users has been made for all genotypes [van Asten et al., 2004].

For genotype 4, nine strains were identified in this study group, six of which were classified into subtype 4a, four from Cypriot patients, and two from patients originating from Egypt, which is the geographical area with a significantly high prevalence of HCV-4a. It is significant to note the origin of the patients with HCV-4a and the fact that this subtype was not isolated from patients of any other ethnic origin in this study group, which includes a high percentage of patients from countries of the former Soviet Union and other countries. This highlights the fact that this subtype is more restricted to a certain geographical radius than genotypes 1, 2, and 3. Of particular interest was the finding of three isolates that do not classify within any known subtype of genotype 4. Three strains in the CORE-E1 region do not cluster with any particular subtype of genotype 4, having used all available assigned genotype 4 subtypes in this region as reference strains in the phylogenetic analysis. Two of these strains are seen to behave similarly in the NS5B region. The third sample was PCR-negative for NS5B, and therefore could not be sequenced in this region. For both trees, sequences from all available subtypes in the corresponding genomic regions were used.

In genotype 5, just one strain was identified and this was classified as subtype 5a, which is a subtype found primarily in South Africa [Chamberlain et al., 1997], but also in west Flanders, Belgium [Verbeeck et al., 2006], central France [Henquell et al., 2004], and Syria [Antaki et al., 2008]. This strain is from a Cypriot patient who had a transfusion with 17 units (17×450 ml) of blood in 1975 in Johannesburg after a serious accident, but was only diagnosed with hepatitis C in 2006.

The strains indicated on the CORE-E1 and NS5B trees with asterisks (Fig. 1) appear to be 2k/1b recombinants and both were isolated from Georgian patients who stated routes of infection as intravenous drug use and sexual transmission. The first identified 2k/1b recombinant found was recovered in St. Petersburg in intravenous drug users [Kalinina et al., 2002] and has since been found only in Estonia, Ireland, and Uzbekistan in intravenous drug users [Moreau et al., 2006; Kurbanov et al., 2007; Tallo et al., 2007]. The putative recombinants found in this study cluster together and with the St. Petersburg strain in both the CORE-E1 and NS5B trees (Fig. 1). These strains are currently being investigated with clonal analysis along the full genome to identify the putative point of recombination (unpublished results).

Overall, in the Cypriot patients of this study group, all HCV genotypes and subtypes reported in this study were found, except the putative 2k/1b recombinant, revealing high genetic diversity. In the patients coming from countries of the former Soviet Union, the HCV strains identified belonged to subtypes 1b, 1a, 3a, and possibly the 2k/1b recombinant strain, corresponding to the HCV subtypes circulating in the eastern Europe [Naoumov, 1999; Kalinina et al., 2001; Kurbanov et al., 2003; Tallo et al., 2007]. In the Egyptian patients, only subtype 4a was found, reflecting the HCV situation in their country [Abdel-Hamid et al., 2007]. For patients from other European countries (Greece, UK, Italy, Romania, and Bulgaria) the genotypes identified were 1a, 1b, 3a, and 4, which are genotypes found commonly in western and southern Europe [Trepo and Pradat, 1999; Ansaldi et al.,

2005; Katsoulidou et al., 2006; Esteban et al., 2008]. Finally, among patients of Asian ethnicity (Iranian and Pakistani), the genotypes discovered were 1a and 3a, respectively, again corresponding to the most prevalent types of HCV in their countries [Samimi-Rad et al., 2004; Idrees, 2008]. It is, however, difficult to make any further epidemiological conclusions, as for many patients the mode of transmission is unknown and, equally important, so is the country of infection.

The genetic diversity of HCV in Cyprus, as shown in this study, is similar to the findings of HCV diversity in Greece [Katsoulidou et al., 2006], and unlike the findings in Turkey, where subtype 1b is predominant [Altindis et al., 2006; Altuglu et al., 2007], Egypt, which has mainly subtype 4a [Abdel-Hamid et al., 2007], or other countries in the Middle East, where genotypes 4 and 1 predominate [Watson et al., 1999; Ramia and Eid-Fares, 2006]. The heterogeneity in HCV genotypes found in Cyprus is probably due to imported strains from repatriated Cypriots, Cypriots traveling abroad and the large tourism industry. The ethnic background of the study group and the finding of the possible 2k/1b recombinant strains also emphasize the impact of immigrants from eastern Europe and increasing use of intravenous drugs in Cyprus on the multiple points of introduction and risk of widespread transmission of HCV strains on the island.

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