TABLE I. Clinical Features and Hepatitis Markers Among 124 Subjects With Chronic Liver Diseases in Tajikistan

Features	Total (n = 124)	Chronic hepatitis (n = 84)	Liver cirrhosis/HCC (n = 40)	P
Sex (M/F)	72/52	52/32	20/20	NS
Mean age (mean ± SD)	38.4 ± 14.9	36.67 ± 14.4	42.2 ± 15.5	NS
ALT median (range)	330.5 (171.9–2001.6)	297.5 (171.9–1946)	383.6 (177.9–2001.6)	NS
AST median (range)	251.35 (14–1189.6)	217.6 (14–1189.6)	282.2 (150.1–1000.8)	NS
ALP median (range)	173.55 (35.8–428.2)	166.8 (35.8–428.2)	180.7 (147.3–428.1)	<0.01
T. Bil. median (range)	21.9 (7.6–239.3)	21 (7.6–239.3)	24.6 (11.4–140.2)	NS
Chol. median (range)	3.6 (2.3–7.8)	3.8 (2.3–7.2)	3.25 (2.4–7.8)	<0.05
Anti-HCV/HCVcAg	57/124 (46)	40/84 (47.6)	17/40 (42.5)	NS
HCV RNA	52/57 (76.4)	40/40 (100)	12/17 (70.5)	NS
Anti-HBc	121/124 (97.5)	79/84 (94)	35/40 (87.5)	NS
Anti-HBs	15/124 (12.1)	14/84 (16.6)	1/40(2.5)	<0.05
HBsAg	51/124 (41.1)	34/84 (40.4)	17/40 (42.5)	NS
HBeAg	2/51 (3.9)	1/34 (2.9)	1/17 (5.8)	NS
HBV DNA	37/51 (72.5)	24/34 (70.5)	13/17 (76.4)	NS
Anti-HDV	12/51 (23.5)	6/34 (17.6)	6/17 (35.2)	NS
HDV RNA	10/12 (83.3)	6/6 (100)	4/6 (66.6)	NS
Patterns of mono or co-infection				
HCV only	50/124 (40.3)	33/84 (39.2)	17/40 (42.5)	NS
HBV only	27/124 (21.7)	20/84 (23.8)	7/40 (17.5)	NS
HCV + HBV	6/124 (4.8)	4/84 (4.7)	2/40 (5)	NS
HBV + HDV	12/124 (9.7)	6/84 (7.1)	6/40 (15)	NS
HCV + HBV + HDV	1/124 (0.8)	1/84 (1.2)	0/40 (0)	NS

Normal ranges: Alanine aminotransferase (ALT): 33.3–200.1 nM/L, asparate aminotrasferase (AST): 27.8–150.1 nM/L, alkaline phosphatase (ALP): 44.5–219.6 nM/L, total bilurubin (T. Bilu): 8.5–20.5 mM/L, cholestrol (Chol.): 3–6.2 nM/L.

nucleotide substitutions in the basal core promoter (BCP) of HBV are associated with HCC in infected patients, these include the 1762T/1764A double mutation [Kao et al., 2003], 1653T and 1753V [Takahashi et al., 1999; Ito et al., 2006]. However most of the studies were carried out on genotype C, and little is known about the other genotypes.

Hepatitis delta virus (HDV), a defective RNA virus that requires the "help" of HBV for packaging and transmission, is known to aggravate the chronic liver damage in patients infected chronically with HBV [Lusida et al., 2003]. HDV has been classified into three genotypes distributed geographically. Several studies suggested that HDV genotypes also influence the course of the disease [Casey et al., 1996; Wu et al., 1998; Hsu et al., 2002].

There has been no report on the prevalence of the hepatitis B and C viruses in Tajikistan. Therefore, the aim of the present study was to investigate the genotypic prevalence and clinical significance of HCV, HBV, and/or HDV among chronic hepatitis patients with and without liver cirrhosis and/or HCC in Tajikistan. Furthermore, the association between coinfection patterns or HBV specific mutations and disease progression were also analyzed in this study.

METHODS

Patients

Sera were obtained from 124 consecutive chronic liver diseases patients over a period of 6 months between February to August 2006. Patients in this study were classified into two clinical groups (i) Chronic hepatitis: patients with constantly high serum alanine aminotrasferase level (ALT), and (ii) Liver cirrhosis and HCC: patients, as diagnosed clinically based on ultrasonography, detection of serological tumor markers (AFP) and in a few cases histologically.

Serological Testing

Anti-HCV, HBsAg, HBeAg, anti-HBc and anti-HBs were determined by chemiluminescence with commercial assay kits (Fujirebio, Inc., Tokyo, Japan). HCV core antigen (HCVcAg), was measured [Aoyagi et al., 1999] using enzyme immunoassay (Fujirebio, Inc.).

Delta antibody (anti-HDV) was assessed only in HBsAg positive specimens using the recombinant HDVAg [Semiletov Iu et al., 2002] diagnostic kit ELISA

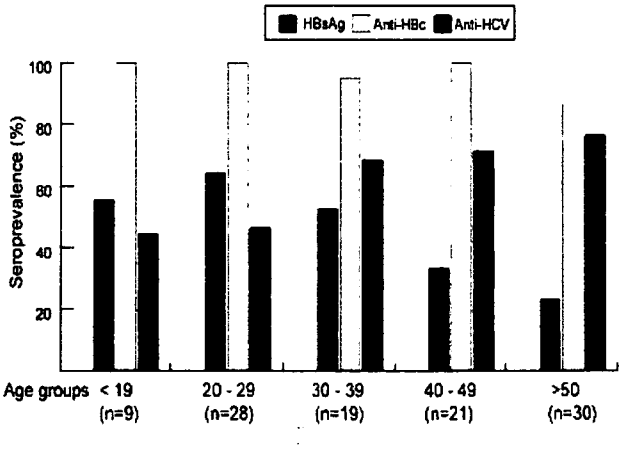


Fig. 1. Comparative prevalence of viral hepatitis markers in different age groups of studied chronic liver disease patients.

Anti-HDV (RPC Diagnostics Systems, Nizhnyi Novgorod, Russia).

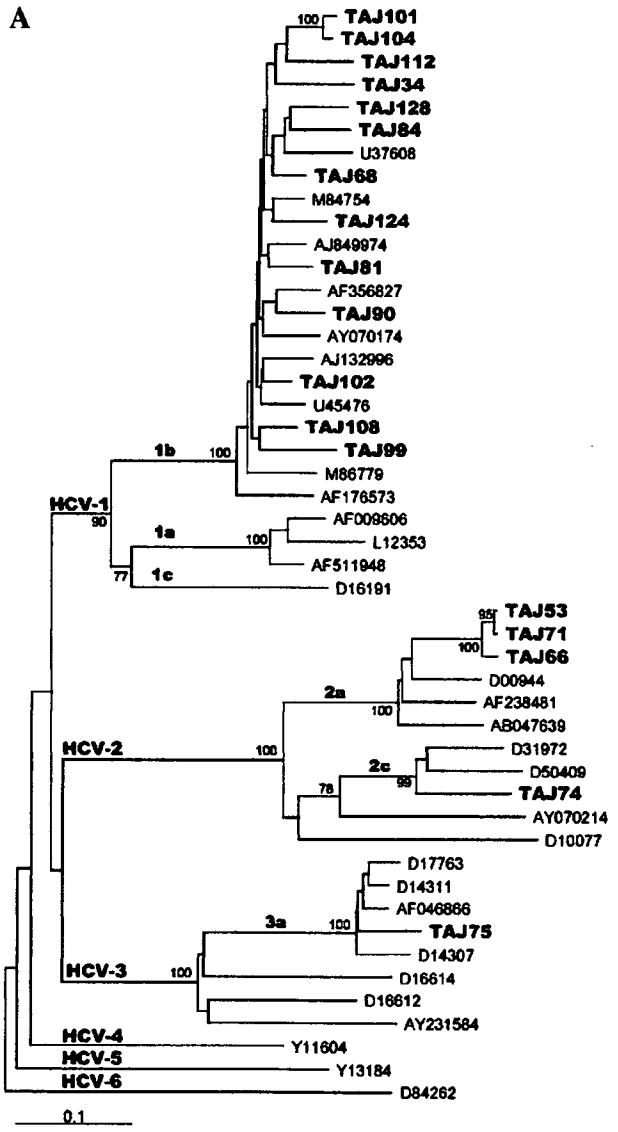
Biochemical markers, that is, ALT, AST, Alkaline Phosphatase, cholestrol, bilurubin were measured in all samples at the local hospital.

Genotyping of HCV

Total RNA was extracted from serum, reverse transcribed into cDNA using random hexamer primer as described previously [Ohno et al., 1997]. Confirmation of HCV RNA in the samples was carried by amplifying highly conserved 5'UTR region and HCV genotypes were determined for both; structural (E1/Core) and nonstructural (NS5B) viral genes using either or both genotyping PCR [Hashimoto et al., 1996; Ohno et al., 1997] and/or direct sequencing with genotype-universal primers [Tanaka et al., 2002].

Screening for HCV Type RF1_2k/1b

Screening for natural recombinant type RF1_2k/1b strain was performed by the newly developed sensitive and specific method allowing detection up to 10 copies/



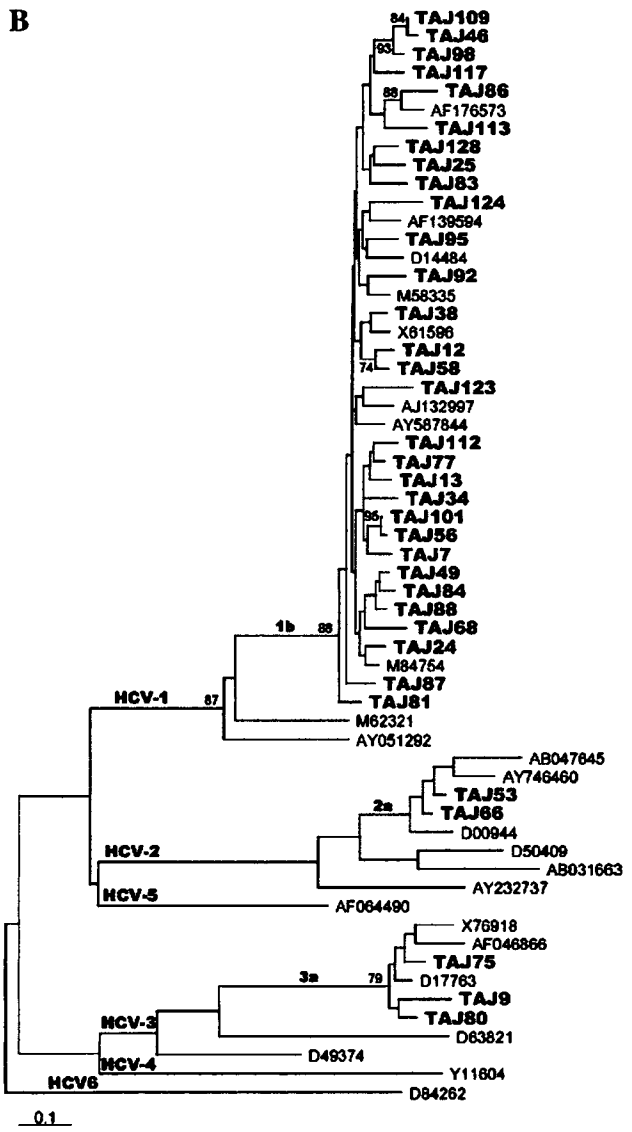


Fig. 2. A: Phylogenetic analysis of the hepatitis C virus (HCV) partial E1 region, sequences from Tajikistan and other countries. Tajikistan isolates (e.g., TAJ74, TAJ75, etc.) were subject to bootstrap resampling with all available sequences in E1 region retrieved from the EMBL/DDBJ/Gene Bank database. Closest neighbors used for phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/Gene Bank. B: Phylogenetic analysis of the HCV partial NS5B region, sequences from Tajikistan and other countries. Tajikistan isolates (e.g., TAJ108, TAJ75, etc.) were subject to bootstrap resampling with all available sequences in NS5B region retrieved from the EMBL/DDBJ/Gene Bank database. Closest neighbors used for phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/Gene Bank.

TABLE II. Comparison of Genotyping Results as Determined by Core, E1, and NS5B

Classification based on core and/or E1	Classification based on NS5B				
	1b	2a	3a	ND	n
1b	41			1	42
2a		3			3
3a			4		4
2c				1	1
ND	2				2
n	43	3	4	2	52

assay (100 copies/ml) of the target sequence even if it is present in minority in mixed heterogeneous template (Kurbanov et al., in submission). Briefly, the primers were designed to amplify a part of NS2 coding region between nt. 2986 and 3270 spanning the intergenotypic breakpoint described for RF1_2k/1b [Kalinina et al., 2002]. Target product's size was 347 base pair (bp) in the first round of PCR and 300 bp in the second round of the "hemi-nested" PCR.

Genotyping of HBV and HDV

All of the HBsAg-positive samples were subjected to genotyping by commercial EIA kit (Institute of Immunology Co., Ltd, Tokyo, Japan). The method allows discrimination among the seven major HBV genotypes (A–G) by monoclonal antibodies targeted to the preS2 epitopes [Usuda et al., 1999]. HBV DNA was extracted by QIAamp DNA Blood Mini Kit (Qiagen, Inc., Hilden, Germany) from 100 ml of all HBsAg-positive sera. Complete genome was successfully sequenced for 7 strains and a part of the HBV core and short S region was sequenced for other 30 strains using previously reported primers [Sugauchi et al., 2001].

HDV RNA was extracted from anti-HDV positive samples and transcribed reversely into a cDNA using random primer as previously described for HCV [Ohno et al., 1997]. A part of HDVAg coding region of the HDV was amplified using specific primers described previously [Nakano et al., 2001] and sequenced directly.

Phylogenetic Analysis

PCR products were sequenced directly with the Prism Big Dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer. All sequences were analyzed in both forward and reverse directions. Complete and partial HBV genomes were assembled using GENETYX version 11.0 (GENETYX, Tokyo, Japan). The sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GeneBank corresponding to the accession numbers mentioned in the trees. Alignment was accomplished using CLUSTAL W and NJ tree were constructed with Tamura-Nei distance correction model using online tools of the HCV database [Kuiken et al., 2006].

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number(s) AB330313 to AB330383.

Stastical Analysis

Statistical differences were evaluated by Fisher's exact probability test and Chi-square test with Yates' correction, where appropriate, using the STATA software version 8.0 (Stata Corp. LP, College Station, TX). Differences were considered significant for P values less than 0.05.

RESULTS

The HCV, HBV, and HDV Prevalence and the Co-Infection Patterns

Among 124 hepatitis patients, 84 (67.7%) were assigned into chronic hepatitis (group 1) and 40 (32.2%) into liver cirrhosis/HCC (group 2). The overall male to female ratio was 1.4, and the mean age (standard deviation) was 38.4 (± 14.9). These estimations are summarized in Table I. There was no significant difference in age between both groups. The ALT, AST, and total bilirubin, were high without significant difference between the both groups. Overall alkaline phosphatases (ALP) levels were within normal range in both groups. HCV-infection was defined upon detection of HCV RNA

TABLE III. Hepatitis C Genotypes, Stratified With Clinical Groups

Genotypes	HCV genotypes determination		
	Chronic hepatitis (n = 40)	Liver cirrhosis/HCC (n = 12)	n = 52
1b	32 (80.0)	12 (100)	44 (84.6)
2a	3 (7.5)	0 (0)	3 (5.7)
3a	4 (10.0)	0 (0)	4 (7.6)
2c	1 (2.5)	0 (0)	1 (1.9)

Numbers in brackets represent percentage (%).

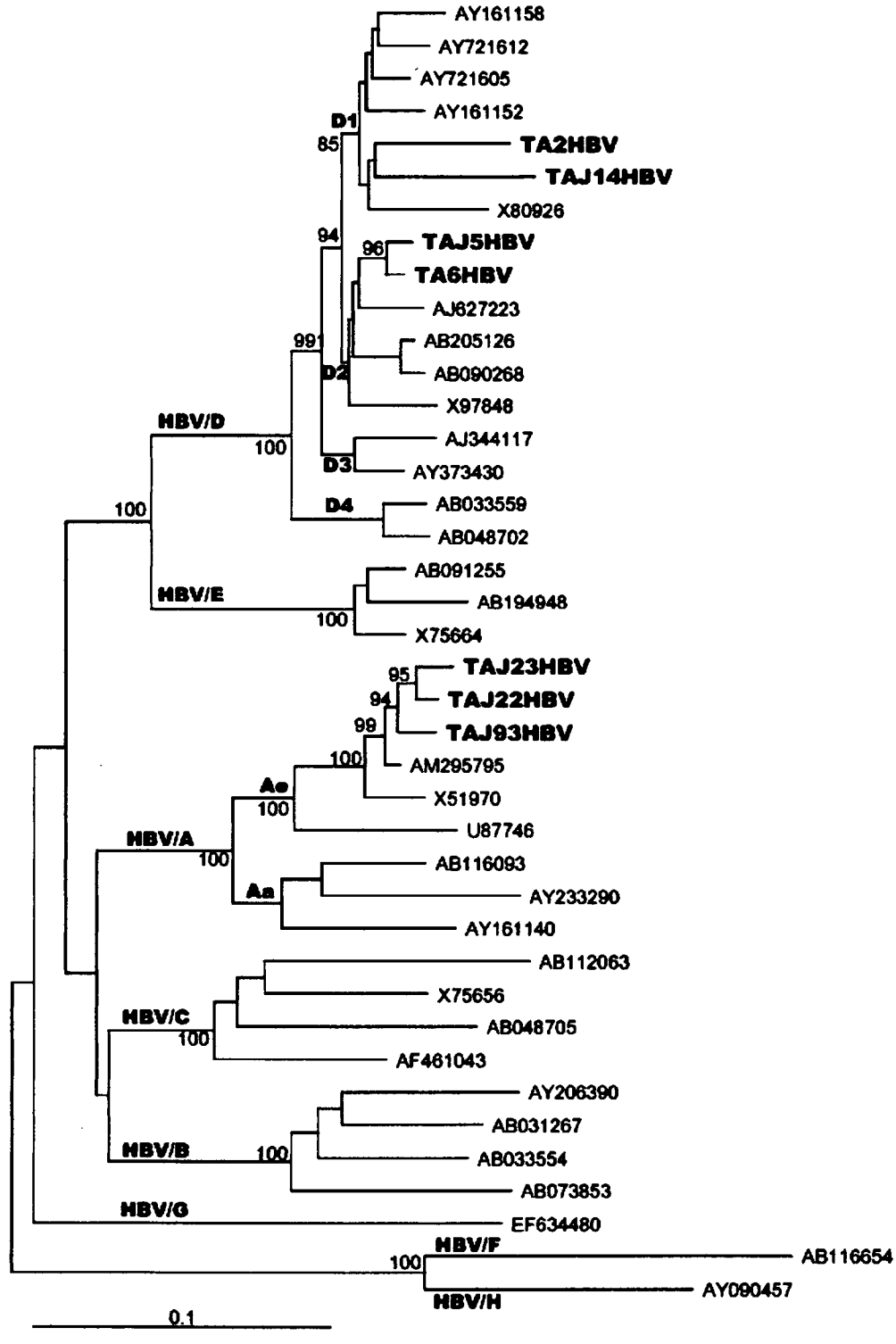


Fig. 3. Phylogenetic analysis of the hepatitis B virus (HBV) eight full genomes, sequences from Tajikistan and other countries. Tajikistan isolates (e.g., TAJ5, TAJ6, etc.) were subject to bootstrap resampling with all available full genome sequences retrieved from the EMBL/DDBJ/Gene Bank database. Closest neighbors used for phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/Gene Bank.

or HCVcAg positive and HCV recovered as anti-HCV positive but negative for HCVcAg or HCV RNA. HCV core antigen (HCVcAg) was measured in serum, where the cutoff value was set tentatively at 50 fmol/L. In this study 15 out of 68 anti-HCV positive samples were negative for HCV RNA. The HCVcAg was positive in only 4 of the 15 cases, suggesting that HCV RNA was degraded in these samples, while remaining 11 cases had resolved acute HCV infection. HCV infection was found to be high in both groups (group 1 = 47.6% and group 2 = 42.5%). HCV RNA was detected in 91% of cases (100% in group 1 and 70.5% in group 2). Anti-HBc was found to be high in both groups (94% and 87.5%, respectively). Although seroprevalence of anti-HBs was very low (12.1%) in the studied population but significantly high in group 1 (16.6%) than group 2 ($P = 0.0356$) while a few cases were positive for anti-HBeAg (2.9% and 5.8%, respectively). Positivity for HBsAg was regarded as indicating HBV infection. The seroprevalence of HBsAg was equally high in both groups, that is, group 1 = 40.4% and group 2 = 42.5%, whereas DNA positivity was 70.5% and 76.4%, respectively. Overall anti-HDV seroprevalence was 23.5%, 12 out of 51 HBsAg positive cases, relatively higher in group 2 (35.2%) compared to group 1 (17.6%) $P = 0.1990$. HDV viremia was detected in 83.3% of cases (100% in group 1 and 66.6% in group 2) (Table I). Based on the serological findings for hepatitis viruses, the patients were categorized into five groups, HCV only (40.3%), HBV only (21.7%), HCV/HBV (4.8%), HBV/HDV (9.7%), and HCV/HBV/HDV (0.8%) in Tajikistan (Table I).

When the seropositivity of hepatitis markers was analyzed in relation to age (Fig. 1), it was found that the prevalence of HBsAg was relatively high in younger group (<29 years old) and tended to decline with age. The opposite trend was observed in anti-HCV prevalence, which was increasing with the age of the patients. The seroprevalence of anti-HBc was high in all age groups, indicating that most patients had past HBV infection during childhood.

HBV, HCV, and HDV Genotypes

HCV genotyping was carried by two independent methods, that is, by type specific primers for core, NS5B region and phylogenetic analyses based on nucleotide sequences in E1 or NS5B region. No discrepancy was observed among the results between either method (Fig. 2A,B), however, one case (TAJ74) was determined as HCV genotype 2c (HCV/2c) only by the phylogenetic analysis in E1 region (Fig. 2A). A total of 7.6% of cases were untypeable by one of either method (Table II). Overall, HCV/1b was a predominant genotype (84.6%) in Tajikistan, followed by HCV/3a (7.6%), 2a (5.7%), and 2c (1.9%). None of the HCV-infected in this study carried recombinant 2k/1b strain. Other potential forms of recombination were also excluded by matching result of genotyping based on both structural and nonstructural genomic parts (Table II). Interestingly, all 12 LC/HCC patients had HCV/1b, not 3a or 2a (Table III).

HBV genotype D (HBV/D) was the predominant genotype (94.1%) in both groups, that is, group 1 = 97% and group 2 = 88.2%, followed by genotype A (2.9% and 11.7%, respectively). HBV genotypes were determined in 45 (88%) of 51 HBsAg-positive cases by EIA. The entire HBV genome was sequenced successfully for seven cases (Fig. 3), and the precore/core promoter region was sequenced in the other 25 cases. The remaining five cases were only positive by PCR for a short S region. The full genome analysis revealed that of the four HBV/D strains, two belonged to subgenotype D1, and the remaining two to subgenotype D2. All three HBV/A strains in this study were belonged to subgenotype Ae. The results of the phylogenetic genotyping were all concordant with EIA-genotyping results. For HDV strains, the phylogenetic analysis of the HDVAg region showed that all of the PCR-positive cases (10/12) belonged to the genotype 1 (Fig. 4).

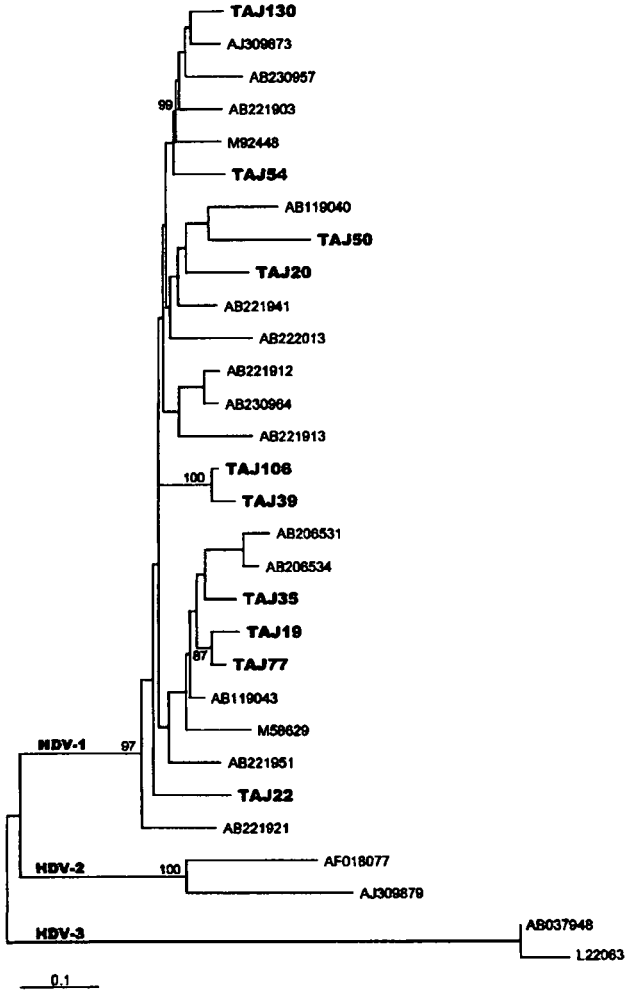


Fig. 4. Phylogenetic analysis of the hepatitis D virus (HDV) partial HDVAg coding region, sequences from Tajikistan and other countries. Tajikistan isolates (e.g., TAJ20-HDV, TAJ50-HDV, etc.) were subject to bootstrap resampling with all available sequences in partial HDVAg region retrieved from the EMBL/DDBJ/Gene Bank database. Closest neighbors used for phylogenetic tree are indicated under the corresponding accession numbers from DDBJ/EMBl/Gene Bank.

Variability of HBV in Enhance II, Basal Core Promoter (BCP), Pre-C/C Region

The prevalence of the nucleotide substitutions along the 33 sequence bearing the enhancer II/core promoter/precore/core regions of the HBV genome is summarized for both clinical groups (22 group 1 vs. 11 group 2) in the Table IV. Due to the small numbers of patients with liver cirrhosis (n = 8) and HCC (n = 3), these were combined in the "Group 2" to enable the comparative analysis with chronic hepatitis patients without the complications; "Group 1." The frequency of A1757 was high in both of the groups without the difference significant statistically. The T1762/A1764 double mutation was more frequent in the Group 2 and this was significant; $P = 0.0004$. The difference in prevalence of the A1896, A1899, and T1912 mutations did not reach statistical significance between the groups.

DISCUSSION

In the present study, the seroprevalence and molecular-genetic characteristics of the hepatitis viruses were investigated among chronic hepatitis (group 1) and liver cirrhosis/HCC (group 2) patients in Tajikistan. The HCV seroprevalence was equally high in both of the groups (47.6% and 42.5%, respectively) followed by HBsAg (40.4% and 42.5%, respectively). These results are slightly higher than the previous studies carried in neighboring Uzbekistan, where anti-HCV was detected in 26.8–41.9% and HBsAg in 25.6% of chronic hepatitis patients [Ruzibakiev et al., 2001; Kurbanov et al., 2003], such a difference may be explained by the different ages of studied patients different collecting time and also regional characteristics. A rather old study carried on the healthy population in Tajikistan and Azerbaijan showed that HBsAg detection was significantly higher in Tajikistan (7.2%) than in Azerbaijan (2.8%) suggesting that HBV is endemic in this country [Vorozhbieva et al., 1985]. Tajikistan introduced universal immunization against HBV for newborn children since 2002. Although, new anti-viral drugs have the potential to alter the natural progression of disease but their high cost prevents the mass use of therapy in Tajikistan (Dustov, personal communication).

One of the interesting observations in this study was the trend of seroprevalence of anti-HCV to increase and HBsAg do decrease with the age of the patients. Interestingly, the prevalence of anti-HBc was very high regardless the age of the patients, indicating that most of the patients had present or past infection of HBV. The age-related decline of the HBsAg prevalence observed in this study may be explained by either; seroclearance of HBsAg due to natural seroconversion [Lee, 1997] or by death of the HBV-infected patients at age younger than those with HCV, due to earlier development of the end stage liver disease [Oyunsuren et al., 2006; Kurbanov et al., 2007]. Alternatively, suppressive effect of the concurrent HCV infection might had caused the loss of the HBV antigenia [Sheen et al., 1994; Chu et al., 1998].

HCV/1b has been found as the dominant genotype (84.6%) in Tajikistan, followed by 3a (7.6%), 2a (5.7%), and 2c (1.9%). The similar epidemiological situation was also described in the former Soviet Union Republics (Russia, Belarus, Moldova, and Uzbekistan) [Viazov et al., 1997; Kurbanov et al., 2003; Shustov et al., 2005] as well as in most of Asian countries, namely Mongolia [Lvov et al., 1996], Taiwan [Yu et al., 2001], China

TABLE IV. BCP and PC Mutations and Clinical Disease

	Clinical diagnosis		
	Chronic hepatitis (n = 22)	Liver cirrhosis/HCC (n = 11)	*P*
Age (mean ± SD)	39.0 ± 15.6	41.6 ± 17.3	NS
Gender (M/F)	15/7	8/3	NS
Genotype D	21 (95.4)	9 (81.8)	NS
Genotype A	1 (4.5)	2 (18.1)	NS
Nucleotide substitutions			
T1653	3 (13.6)	4 (36.3)	NS
T1678	15 (68.2)	3 (27.2)	0.0613
G1737	13 (59)	5 (45.4)	NS
C1752	5 (22.7)	2 (18.2)	NS
V1753 (not T)	6 (27.2)	4 (36.3)	NS
A1757	17 (77.2)	7 (63.6)	NS
T1762/A1764	2 (9)	8 (72.7)	0.0004
T1764/G1766	2 (9)	0	ND
A1768 alone	0	2 (18.2)	ND
Deletions 1766–1773	4 (18.2)	0	ND
C1773 alone	3 (13.6)	3 (27.2)	NS
C1772/C1773	2 (9)	1 (9)	NS
G1775	3 (13.6)	1 (9)	NS
A1896	7 (31.8)	6 (54.5)	NS
A1899	4 (18.2)	5 (45.4)	NS
T1912	8 (36.3)	5 (45.4)	NS
G1915	1 (4.5)	2 (18.2)	NS

Numbers in brackets represent percentage (%).

[Suzuki et al., 1997], and Japan [Noguchi et al., 1997] where genotype 1b was predominant.

The HCV core antigen (HCVcAg) has significant correlation with HCV RNA [Tanaka et al., 2000]. In this study 15 out of the 68 anti-HCV positive simples, had the HCV RNA undetectable by PCR method, however only 4 of the 15 cases had the HCVcAg detectable, suggesting that the viral RNA has degraded in these samples prior study [Tanaka et al., 2003], while the remaining 11 cases may have had an acute HCV infection resolved in the past.

HBV genotypes have a characterstic geographical distribution [Miyakawa and Mizokami, 2003; Norder et al., 2004]. Particularly, genotype D is widespread, predominating in the Mediterranean area and in the Middle East region. Genotype A is present in Europe, India, Africa, and North America. The present results have shown that 94% of HBV isolates in Tajikistan belonged to genotype D and 5.8% to genotype A. Similar epidemiological situation was observed in a previous study carried in Uzbekistan, where HBV/D found in 87% and HBV/A in 13% of HBV infected patients [Kato et al., 2002]. Complete genome analysis of the four HBV/D strains determined in present study allowed distinction into two subgenotypes; D1 and D2. Previous reports indicate that HBV/D subgenotypes have no specific geographical distribution; however strains from the Middle East mainly belong to subgenotype D1 and those from India, Russia and Europe to D2 [Norder et al., 2004]. No specific clustering was observed in Tajikistan strains, which is in agreement with previous observations regarding HBV/D molecular epidemiology. HBV genotype A has three major geographical clusters, the European-North American (Ae), the Afro-Asian (Aa) [Sugauchi et al., 2004], and the Central African (Ac) [Kurbanov et al., 2005]. The HBV genotype A strains isolated in this study were all belonged to the subgenotype Ae.

Increasing evidence suggests that specific HBV genomic mutations may increase the risk of HCC in the patients infected chronically. Particularly, a high incidence of the T1762/A1764 double mutation was found among HBV/B or C infected Asian population [Kao et al., 2003] and with HBV/E infected African patients [Baptista et al., 1999]. Similarly, in the present study, the frequency of the double mutation was significantly high in group 2 (6HBV/D and 2 HBV/A) as compared to group 1 (1HBV/D and 1HBV/A). In vitro studies [Buckwold et al., 1996], indicated that the T1762/A1764 mutants show enhanced replication and might be associated with high incidence of HCC.

The prevalence of coinfection with HBV and HDV found to be relatively higher in group 2 compared to group 1 (15% vs. 7.1%), however the difference was not statistically significant possibly due to the small size of cases ($P = 0.1990$). Previous studies have demonstrated that the coinfection with these viruses was also contributing in high HCC incidence in Mongolia [Oyunsuren et al., 2006].

It is concluded that HBV and HCV are the major cause of viral hepatitis in Tajikistan. Among HBV-infected patients, the T1762/A1764 mutations were associated with liver cirrhosis/HCC in this population.

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Original Article

Detection of hepatitis C virus natural recombinant RF1_2k/1b strain among intravenous drug users in Uzbekistan

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Aim: A series of recent studies have indicated the presence of natural intergenotypic recombinant hepatitis C virus (HCV) strains in distinct parts of the world. The majority of the current genotyping methods are based on analysis of either 5'UTR, structural (Core/E1/E2) or non-structural (NS5B) genomic regions of the virus.

Methods: In the present study, based on both structural and non-structural regions, we determined the genotype of 55 anti-HCV-positive intravenous drug users (IDUs) in Uzbekistan.

Results: HCV-3a (67.3%) was the most prevalent genotype in this cohort, followed by HCV-1b (27.3%). A discrepancy in results was observed between structural and non-structural regions in one case (1.8%). Phylogenetically this strain was related to the previously reported RF1_2k/1b variant. Based on accumulated sequences, specific primers were designed for polymerase chain reaction (PCR) spanning the tentative inter-genotypic crossover point of RF1_2k/1b. The sensitivity and specificity of the method were assessed using generated template clones of HCV-1b, 2a, 2k and RF1_2k/1b. The method was applied to 55 cases in the present study and only one case showed a positive result, indicating that in these individuals, the variant is not present as a minor quasispecies clone.

Conclusion: In conclusion, the finding of RF1_2k/1b in Central Asia indicates that the variant has wide geographic distribution. The PCR-based screening method developed in this study should be useful in further epidemiological and clinical studies on the recombination phenomenon in HCV.

Key words: 2k/ 1b, HCV genotyping, HCV, natural recombination

INTRODUCTION

BASED ON NUCLEOTIDE sequence heterogeneity in the genome and phylogenetic relationship, hepatitis C virus (HCV) has been classified into six genotypes (1 to 6) and numerous subtypes (1a, 1b, etc).[1,2] The genotypes are strongly associated with responses to antiviral therapy, thus making genotyping important in the determination of dose and duration of the therapy (reviewed in[3]). Characterization of genotypes and subtypes is also important for vaccine development and epidemiological investigations.[1] A series of recent studies in distinct parts of the world have indicated the presence of natural intergenotypic hybrid strains, having genotype 2 sequence in the structural, and genotype 1,[4–6] 5,[7] or 6[8] in the non-structural half of the genome. Since the majority of the genotyping studies performed to date have been based on analysis of either the structural or the non-structural parts of the HCV genome, there is a substantial possibility that the prevalence of natural recombinant HCV variants is underestimated. Moreover, a recent study on chimpanzees with different genotypes/subtypes of HCV indicated that the intersubtypic recombinant clones were present in two animals as a minor clone of quasispecies population,[9] which may

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also result in underestimation of the natural recombinant strains if they present as a minor clone population in infected carriers.

Frequent HCV superinfection has been previously reported in intravenous drug users (IDUs), both intra- and inter-genotype,[10,11] which increases the possibility of the detection of recombinant strains in this category of the population.

In the present study we aimed to investigate the possibility of circulation of the intergenotypic recombinant strains among IDUs in Uzbekistan. Furthermore, we developed and evaluated an easy, sensitive and specific method allowing the detection of the recombinant RF1_2k/1b strain even when it is present in a minority among populations of quasispecies in a specimen.

METHODS

Studied population

THE ANTI-HCV-POSITIVE SERUM samples were collected in 2006, in the State Reference Laboratory of the Ministry of Public Health, Tashkent City, Uzbekistan. The sera were obtained from IDUs who were undergoing hospitalization at the National Narcological Clinic of Public Health, Tashkent City, Uzbekistan, during 2005 and 2006. The material collection was approved by the Institutional Ethics Committee and was conducted according to the Declaration of Helsinki. Risk factors for HCV infection were assessed during interview with each participant. Serum specimens were obtained only from patients who had no history of antiviral treatment. Finally, a total of 55 HCV-RNA-positive IDUs were enrolled in this study. The mean age in this cohort was 31.8 years (standard deviation ± 6.7, range 18–49); 48 subjects were male; the male/female ratio was 6.9.

Serological and molecular confirmation of the HCV infection

The presence of anti-HCV was determined on LUMIPULSE automated Chemiluminescence Enzyme Immunoassay system (Fujirebio, Tokyo, Japan) using the kit produced by Ortho Clinical Diagnostics, Tokyo, Japan. Total RNA was extracted from 200 μL of anti-HCV-positive sera using the SepaGene RV-R kit (Sanko Junyaku, Tokyo, Japan) and reversely transcribed into complementary DNA (cDNA) using SuperScript II RNase H⁻ transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamer primer (Takara Shuzo, Shiga, Japan) in total reaction volume of 20 μL. Two microliters of the obtained cDNA were used as a template in 50 μL reaction volume for polymerase chain reaction (PCR)-amplification of the HCV 5'UTR by previously described primers KK30 (nucleotides [nt.] 58–77) and KM3 (nt. 313–294).[12] The threshold of the method was as low as 100 copies per milliliter; therefore the primers were used to define HCV-RNA-positive cases.

HCV genotyping

The HCV genotype in each case was determined in both structural (Core/E1) and non-structural (NS5B) viral genes using the corresponding genotyping PCR, as described previously.[13,14] Cases showing an unexpected size of PCR bands were further subjected to PCR and direct sequencing using the genotype-universal primers targeting Core/E1 and NS5B regions.[15]

Phylogenetic analyses

The PCR products were directly sequenced using Prism Big Dye ready reaction kit (Applied Biosystems, Foster City, CA, USA) on the ABI 3100 DNA automated sequencer.

The phylogenetic relationship of the strains sequenced in this study, as well as those reported previously, were investigated by the neighbor-joining method using tools available online from HCV databases (http://s2as02.genes.nig.ac.jp/).[16] Genetic distances were corrected by the Tamura–Nei method, and the bootstrap test was performed on 1000 resamplings.

Primers for HCV type RF1_2k/1b-specific detection

New primers were designed to amplify a part of the HCV genome between nt. 2986 and 3270, spanning the tentative intergenotypic crossover point in the RF1_2k/1b strains.[4] The selection of appropriate primer sites was accomplished manually using alignments of the sequences previously published in the DDBJ/GenBank, including five strains of the recombinant RF1_2k/1b type (accession numbers AY070170–AY070172, AY070214 and AY070215),[4,17] and selected reference sequences of other HCV genotypes.[16]

The sequences of the primers used in this study are shown in Table 1. Amplification using the primers was optimal for our samples under the following PCR conditions: total reaction volume 25 μL, including 2.5 μL of 10 × PCR buffer (Roche, Indianapolis, IN, USA) containing 15 mM $MgCl_2$, 2.0 μL of dNTPs (2 mM each), 1.0 μL of forward and 1.0 μL of reverse primer, 2 units of Taq polymerase (Ampli-Taq Gold; Roche) and 1.0 μL of cDNA. Thermal cycle conditions were as follows: hot start at 96°C with 7 min hold followed by 40 2-step

Table 1 Oligo-nucleotide primers for PCR

Primer ID	Direction	5'-3' sequence and nucleotide position†		Target genotype	
NS2_uni_2415f	Forward	2415	CTC CAC CAA AAC ATC GTG GA	2434	1 & 2
NS2_2ak_2948f‡	Forward	2948	CCG YGA YGG CAT CAT ATG GG	2968	2 (2a & 2k)
NS2_2ak_2994f‡	Forward	2994	GTG TTT GAC ATA ACC AAG TGG	3014	2 (2a & 2k)
NS2_1b_3100f	Forward	3100	GTG CAT GCA TGT TGG TGC GGA	3120	1 (1b)
NS2_1b_3295r‡	Reverse	3295	GTG ATG ATC TTG GTC TCC ATG TCR GA	3270	1 (1b)
NS2_1b_3377r	Reverse	3377	CAG AAG TAT CTC CCT CCC CCT	3357	1 (1b)
NS2_2ak_3417r	Reverse	3417	AGT TTC CAC CCC TTG GAA GT	3398	2 (2a & 2k)

†Relative to the reference strain H77 (NC_004102).
‡The primers used in hemi-nested screening polymerase chain reaction (PCR) are emboldened.

cycles at 95°C for 1 min and 60°C for 1 min, with ramp speed at approximately 1°C per sec. The second PCR products were visualized by electrophoresis in 3% agarose gel with ethidium bromide staining. The approximate size of the target product is 347 bp after the first PCR round (with outer sense primer) and 300 bp after the second "hemi-nested" (with inner sense primer) (Fig. 3a).

Preparation of the "competitive" PCR templates

To evaluate the specificity and sensitivity of the screening primers, the following three clones were generated from the stored environmental serum samples collected at the Department of Molecular Informative Medicine of Nagoya City University: Cln_1b (Japan), Cln_2a (Japan), Cln_2k (Altai, Russia). Obtained cDNA was amplified using the relevant set of primers: ***NS2_uni_2415s*** along with ***NS2_1b_3377as*** or ***NS2_2ak_3417as*** (Table 1), then directly inserted into a plasmid vector (pCR2.1-TOPO; Invitrogen) and cloned in *E. coli* (DH 5 alpha; Toyobo, Osaka, Japan). The resulting colonies were further transferred from LB agar type medium into LB medium and cultivated for 16 h. The obtained clones were purified using QIAprep kits (Qiagen, Valencia, CA, USA) and quantified by measuring optical density and molecular purity on Beckman spectrophotometer (Beckman Instruments, Fullerton, CA, USA). Based on the obtained values, aliquots were prepared using serial dilution.

Statistical analyses

Statistical differences were evaluated by Fisher's exact probability test and χ^2-test with Yates' correction, where appropriate, using the STATA software version 8.0 (StataCorp LP, College Station, TX, USA). Differences were considered significant for *P*-values less than 0.05.

RESULTS

HCV genotypes

THE PREVALENCE OF HCV genotypes in the studied cohort was determined in both Core and NS5B regions. The results are summarized in Table 2. The most prevalent genotype was HCV-3a, determined in 37/55 (67.3%) cases. All of these had concurring genotyping result in both Core and NS5B regions. The second most common genotype was HCV-1b, determined in 15/55 (27.3%) cases in the Core and 16/55 (29.1%) cases in the NS5B, revealing a 1/55 (1.8%) untypable case by Core-genotyping-PCR. The third genotype found in this cohort was HCV-2a, detected in 2/55 (3.6%) cases showing mutual agreement of results between the Core and NS5B PCR. One case (identified in this study as *"UZ-IDU19"*) showing an undetermined result with Core-PCR was subjected for E1 region sequencing. Additionally, 38 cases were also subjected

Table 2 HCV genotypes determined in Core/E1 and NS5B coding region

Core[13]	NS5B[14]			Total
	1b	2a	3a	
1b	15			15 (27.3)
2a		2		2 (3.6)
3a			37	37 (67.3)
ND	1			1 (1.8)
Total	16 (29.1)	2 (3.6)	37 (67.3)	55 (100)

HCV, hepatitis C virus; ND, not determined.

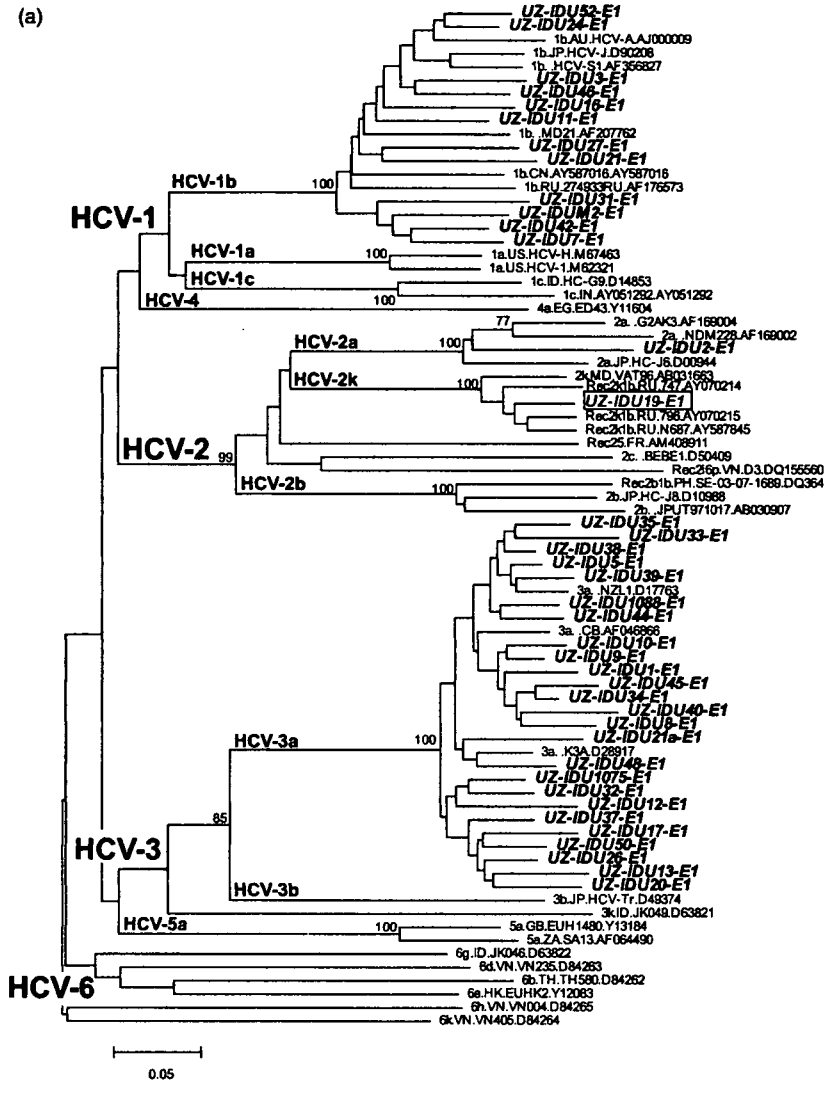

Figure 1 Phylogenetic relation of the hepatitis C virus strains. NJ tree constructed on the basis of 438 nucleic acids sequence of the Core/E1 coding region (nt. 861–1298) (a) and 286 nt. of the NS5B coding region (nt. 8283–8568) (b). Distances were estimated on the basis of synonymous substitutions under a modified Nei–Gojobori model. Bootstrap re-sampling indices exceeding 70% are indicated near to the roots of the corresponding cluster. Strains isolated in this study are indicated in bold italic font. Framed is the strain (*UZ-IDU19*) which was untypable by core genotyping.

for E1 amplification and direct sequencing with the aim of confirming genotyping results. The phylogenetic relationship of the strains sequenced in this study and those reported previously is depicted in Figure 1a. Interestingly, specimen *UZ-IDU19* was clustered phylogenetically together with the HCV-2k[18] and RF1_2k/1b strains from Russia.[4] Of the remaining 38 strains, 25 were clustered with HCV-3a references, 12 with HCV-1b and one with HCV-2a, thus indicating agreement with the results that had been obtained by Core and NS5B PCR-genotyping (Table 2). Furthermore, 14 specimens including the *UZ-IDU19* and 13 other cases in which genotypes 3a ($n = 9$) or 1b ($n = 4$) were determined by all Core-PCR, NS5B-PCR (Table 2) and E1 phylogenetic analysis (Fig. 1a), were subjected to sequencing and phylogenetic analysis in the NS5B region. The resultant tree (Fig. 1b) indicates clustering of the *UZ-IDU19* strain with 1b reference strains along with the four strains obtained in this cohort. The remaining nine strains were clustered with HCV-3a reference sequences, confirming the results obtained by the genotyping methods outlined above.

The collective result of the genotyping revealed that one case in this cohort (*UZ-IDU19*) had phylogenetic evidence of the intergenotypic recombination previously reported as RF1_2k/1b. To confirm this, the *UZ-IDU19* case was subjected to direct sequencing using primers spanning the intergenotypic crossover point previously

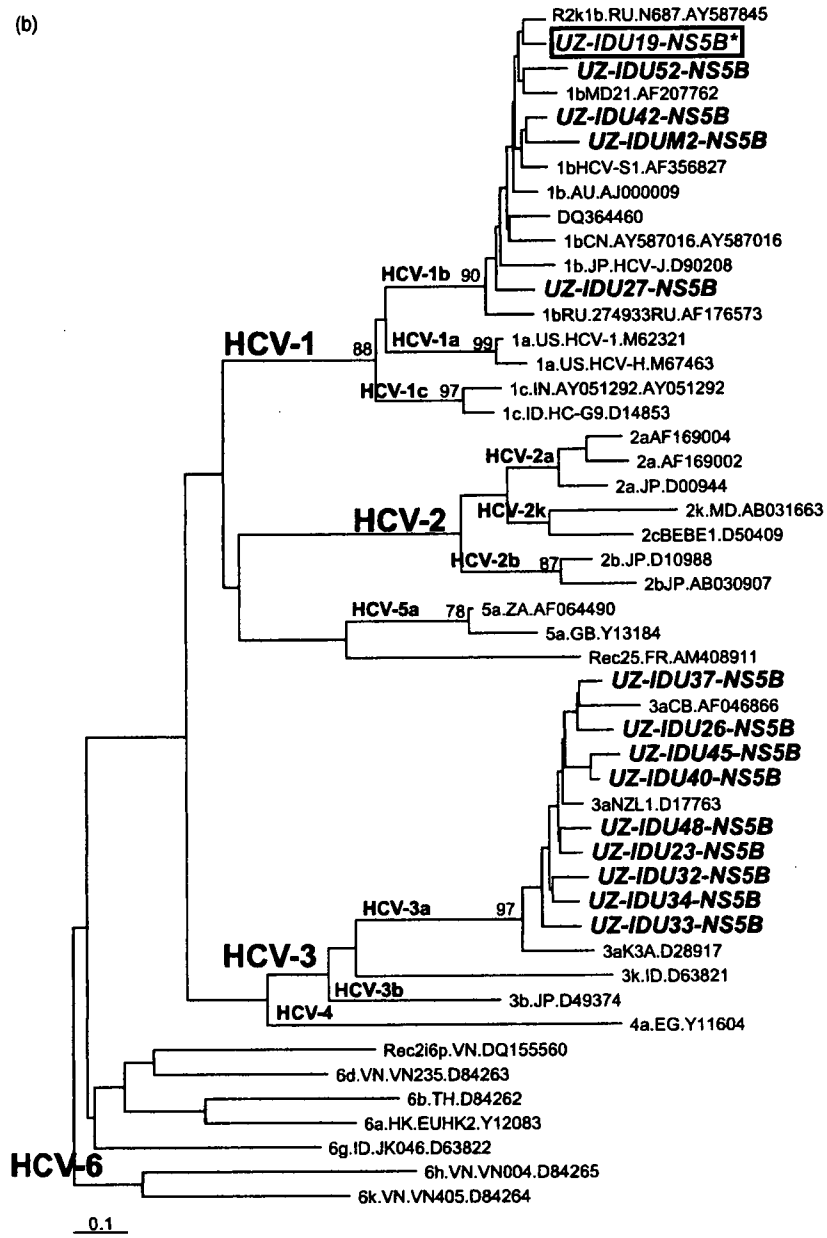

Figure 1 *Continued*

reported within the NS2 coding region (nt. ~3175). The resulting nucleotide sequence was aligned with those of RF1_2k/1b and references of HCV-2 k and HCV-1b previously reported, and the crossover point has been confirmed within the same nucleotide positions (Fig. 2).

Clinical and epidemiological characteristics of the RF1_2k/1b-infected carrier

As was defined by retrospective epidemiological investigation, the carrier of the recombinant HCV variant strain (specimen # *UZ-IDU19*) was a 32-year-old male Caucasian who had no clinical symptoms of active hepatitis (total bilirubin, alanine aminotransferase and aspartate aminotransferase were all within the normal range). The carrier reported neither a history of blood transfusion, nor travel to Russia or other countries (except Kazakhstan), nor any contact with subjects from foreign countries. The carrier has been registered at Republic Narcological Center since 2001, and had a reported history of intravenous drug use since 1994. No

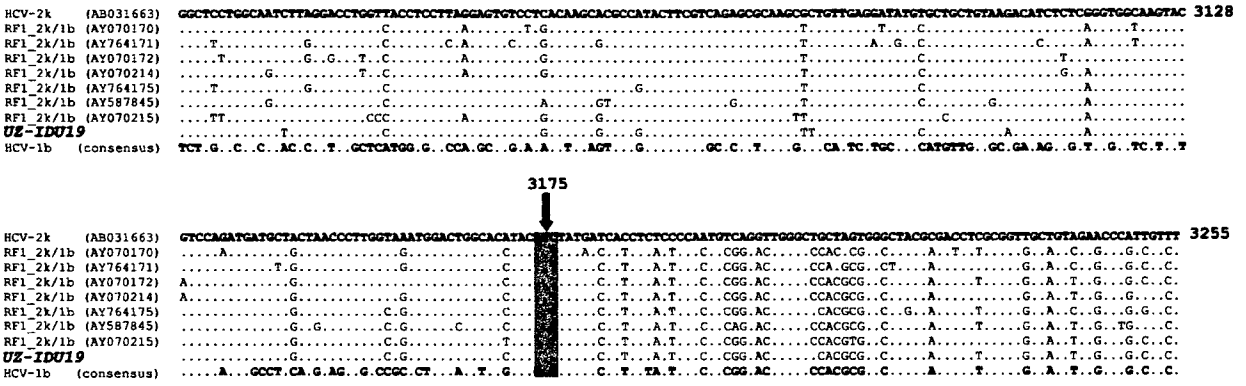

Figure 2 Alignment of 255 nucleic acids sequences of the NS2 coding region (nt. 3015–3269) of the hepatitis C virus (HCV) strains. The RF1_2k/1b strain determined in this study is indicated in bold italic font. Previously published HCV reference sequences identified by previously established genotype and accession number (in parentheses). The tentative crossover point is indicated by an arrow and the corresponding codon is shaded in gray.

records prior to this are available for serological investigation of the subject.

Sensitivity and specificity of the RF1_2k/1b-specific primers

To examine the hypothesis that the recombinant strain may be present in HCV-infected subjects carrying non-recombinant HCV genotypes (i.e. those with determined HCV-1b, 2a and 3a genotypes in this study), we designed a specific PCR amplification strategy, which is graphically depicted in Figure 3a and primers included in Table 1. To evaluate the specificity and sensitivity of the method we used different concentrations of the target PCR templates. The established detection limit of the method was 10 copies per assay. As shown in Figure 3b, specific amplification was successful even when the RF1_2k/1b clone was present in a tested sample along with the HCV-1b clone in a concentration ratio $10–10^5$ copies per assay, respectively. This was confirmed with clones of genotypes 3a, 2a and 2k used as a competitive control template (not shown).

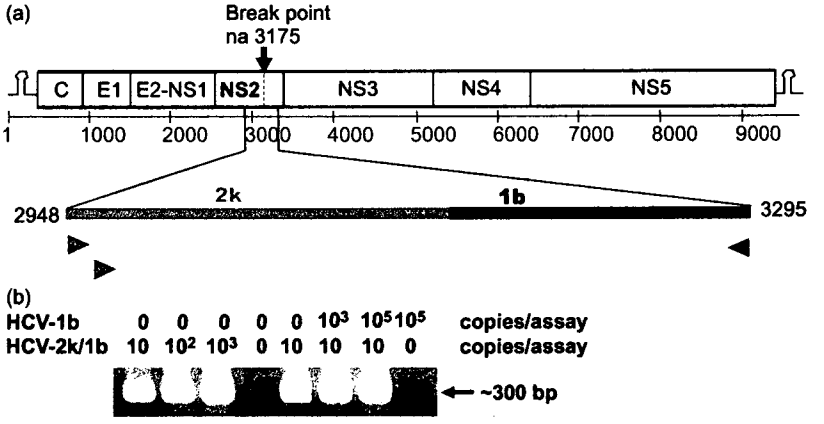

Figure 3 An outline of the amplification strategy of the polymerase chain reaction (PCR)-based detection method for the hepatitis C virus (HCV) type RF1_2k/1b strain (a), sensitivity and specificity of the method (b). (a) HCV genomic region corresponding to nt. 2948–3295 is targeted by the designed primers. A part of the amplicon corresponding to HCV-2 k sequence (in RF1_2k/1b strain) and 2k-specific primers (outer and inner sense) are depicted in gray; a part corresponding to the HCV-1b sequence and 1b-specific antisense are depicted in black. (b) Agarose gel picture showing specific bands obtained after the amplification of RF1_2k/1b template alone, or mixed with non-recombinant strain in different concentrations.

This amplification strategy was applied to 55 cases in the present study and only one case (*UZ-IDU19*) showed a positive result, indicating that the variant is not present as a minor clone of quasispecies population among these individuals.

DISCUSSION

THE RESULTS OF this study indicate the circulation of the RF1_2k/1b natural recombinant strain among IDUs in Uzbekistan (one of the former Soviet Union countries located in Central Asia). This genetic variant of HCV was previously reported only in European parts of Russia[4] and in Russian immigrants to Ireland[6] and Estonia.[19] The detection of the strain within the relatively small cohort of Uzbek IDUs in the present study may indicate a substantial possibility that its actual prevalence is underestimated. Because little is known about the epidemiological and clinical impact of the natural HCV recombinants, further studies are required on RF1_2k/1b. The PCR-based strategy designed in this study allows specific and sensitive detection of the RF1_2k/1b strain and could be used in further large-scale screenings of an HCV infected population.

To date, only three other reports on the complete or nearly complete HCV genome analysis have described natural intergenotypic recombinant strains.[5,7,8] Interestingly, all of these reports concur with the initial report[4] in respect to the following observations: (i) the intergenotypic breakpoint is located between the structural and non-structural genomic parts of the HCV, within the NS2 coding region near to the NS2/NS3 junction (nt. 3175–3455); and (ii) all of the chimeric variants had genotype 2 sequences (subtype 2b, 2i and 2k) in the structural part of the genome.

The HCV genotype distribution in this study was similar to previous reports, suggesting that genotype 3a is frequent among IDUs in Uzbekistan[20] and Russia,[21] with genotype 1b being the second most common. Interestingly, no evidence of recombination between the prevalent HCV genotypes (i.e. 3a and 1b) was observed in this cohort. Furthermore, genotype 2 in this study was found to be in the minority, and none of the strains was phylogenetically related to HCV-2k. These observations may suggest that, unlike hepatitis B virus, recombination in HCV may not be associated with the cocirculation of genotypes in a population. This hypothesis concurs with previous reports that have demonstrated rare dual genotype coinfection among HCV infected individuals.[22,23] One of the possible explanations could be the superinfection exclusion phenomenon, depriving the viral strain of its translational and/or replication ability inside a cell that is primarily infected by another homologous virus. This was recently proven for HCV by delicate *in vitro* studies using genotypes 1a, 1b and 2a.[24,25] Collectively, these studies may indicate the possibility that minor HCV variants such as 2b, 2k, 2e and 2i may possess the unique ability to overcome the superinfection exclusion. Further experimental studies are required to confirm this hypothesis.

In conclusion, this study demonstrated that the first of the reported natural HCV recombinants, RF1_2k/1b, is widely scattered and not associated with cocirculation of the predominant genotypes. The specific PCR-based detection method developed in this study could prove useful in further investigations of the epidemiological and clinical impact of the recombination in HCV.

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Tracing hepatitis C and Delta viruses to estimate their contribution in HCC rates in Mongolia

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ABSTRACT. An estimated incidence of hepatocellular carcinoma (HCC) in Mongolia is currently one of the highest in the world. According to previous reports, the sero-prevalence of hepatitis B (HBV) and hepatitis C (HCV) viruses in general population of the country is very high (HBV, 10% and HCV, 15%, respectively). Moreover, the majority (75–100%) of the HBV-infected individuals have co-infection with hepatitis Delta virus (HDV). Despite reported observations that HBV + HDV/HCV co-infection have significantly stronger association with HCC when compared with HCV-monoinfection, the later is still frequently observed among Mongolian HCC patients (39%). In this study, an approach based on principles of population genetics and mathematical epidemiology was used to trace an epidemic history of HCV and HDV. In agreement with the sero-epidemiological and social-historical background of the country, the results have demonstrated that the viruses had different epidemic dynamics in Mongolia; HCV was characterized by earlier epidemic expansion, whereas HDV spread with approximately 50 years lag. This may explain the comparable contribution of the HCV-monoinfection and HBV + HDV co-infection in current HCC rate despite different levels of risk of carcinogenesis. Used approach is useful in evaluation of current and prospective disease burden.

Keywords: coalescence theory, hepatitis C virus, hepatitis Delta virus, hepatocellular carcinoma, molecular epidemiology, Mongolia.

INTRODUCTION

Age adjusted primary liver cancer mortality rate estimated per 100 000 men is geographically divergent, ranging from 2.1 in Central America up to 35.5 in Eastern Asia with the world's highest level recorded in Mongolia (from 56.0 to 98.93, according to different reports) [1,2]. Great majority of the primary liver cancer corresponds to hepatocellular carcinoma (HCC), which is a terminal consequence of the chronic viral hepatitis [1]. According to previous reports, Mongolia is distinguished for a very high sero-prevalence of the hepatitis B (HBV) and hepatitis C (HCV) viruses that constituted 10% and 15%, respectively in apparently healthy general population. Furthermore, over 75% of the HBV-infected individuals also had markers of the hepatitis Delta virus (HDV) infection [3–5]. Evidence of the viral aetiology of HCC was present in 92% of Mongolian chronic hepatitis patients [6]. It was also indicated that the chronic hepatitis patients with HBV + HDV double-infection or HBV + HDV + HCV triple infection despite their younger age had significantly higher frequency of HCC in comparison with those with HCV monoinfection [6,7]. As summarized in Fig. 1, according to previous studies, the co-infection (mainly HBV + HDV) was detected in 9% of healthy general population, in 30% of chronic hepatitis patients and in 50% of patients with HCC. However, HCV monoinfection is still detected in 39% of patients with HCC. Knowing the time when each of these prevalent hepatitis viruses spreads in Mongolia can be helpful in understanding the current epidemiological pattern and in explaining the contribution of these agents in the current and future disease burden.

Recently developed method, based on the coalescent theory, allowed inferring the past epidemic growth of HCV population [8,9]. This method was implicated to estimate (trace) the history of changes in viral population size using the phylogenetic trees reconstructed from sampled viral gene

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB295119–AB295193.

Abbreviations: HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDV, hepatitis Delta virus; PCR, polymerase chain reaction.

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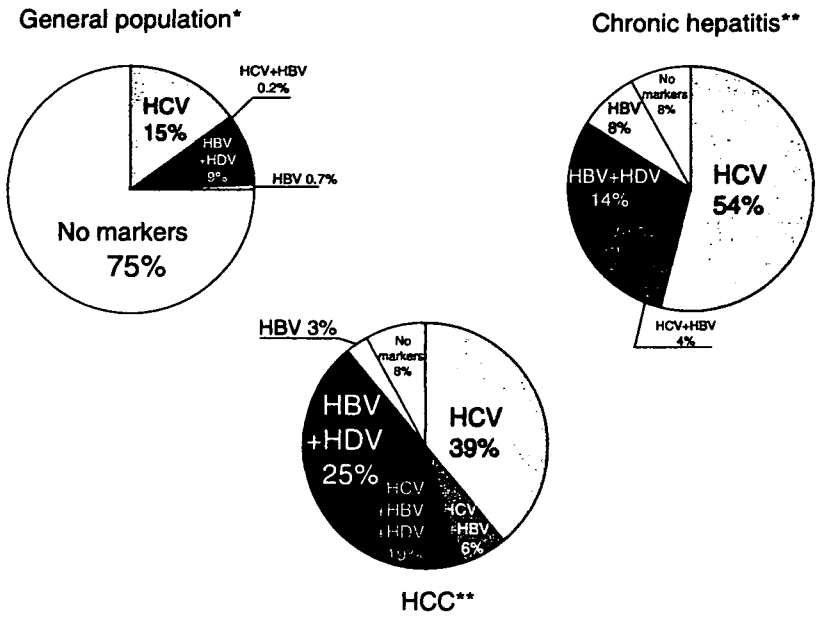

Fig. 1 Pie diagrams indicating rates of infection/coinfection patterns in General population (n = 538) *[4,5], chronic hepatitis patients (n = 184) and HCC patients (n = 108) in Mongolia **[6]. Infection was determined on the basis of detection of anti-HCV (for HCV), HBsAg (for HBV) and either or both anti-HDV/HDV RNA (for HDV).

sequences. In previous studies, the tracing of the epidemic history of HCV in different countries of the world has indicated its remarkable association with HCV-related HCC mortality in the corresponding geographical areas [10–13]. Allowing evaluation of the current and prediction of the further disease burden, this approach can be useful especially in regions where epidemiological data on HCC are unavailable. Furthermore, this method was successfully applied in investigations of the epidemic history of human immunodeficiency virus [14–16], dengue viruses [17] and HBV [18].

In present study we applied this approach to compare history and dynamics of changes in the viral population size of HCV and HDV and have analysed these results along with previously published epidemiological data and the socio-historical background of Mongolia.

MATERIALS AND METHODS

Patients

Patients were selected from previously reported cohort of 292 chronic hepatitis patients who attended for medical recourse at the Liver Clinics and the National Cancer Center, Ulaanbaatar, Mongolia between April and August, 2005 [6]. Aliquots of blood serum were stored in −70 °C until examination. Clinical Laboratory examination, HCC diagnosis, and serological examination including HBV surface antigen (HBsAg), hepatitis B e antigen (HBeAg), hepatitis Delta virus antibody (Anti-HDV) and hepatitis C virus antibody (Anti-HCV) were carried out as previously described [6]. HCV genotypes were determined using genotype specific primers for core region [19]. A part of HDVAg coding region of the HDV was amplified using previously reported specific primers [20] and directly sequenced in both forward and reverse direction.

HCV sequencing in part of NS5b gene

Sequences spanning 339 nucleotides (nt) in the NS5B region were amplified by polymerase chain reaction (PCR) with primers described previously [10]. PCR products were directly sequenced using Prism Big Dye (Applied Biosystems, Foster City, CA, USA) in an ABI 3100 DNA automated sequencer.

Analyses of HCV and HDV sequence

The sequences were analysed in respect to phylogenetic relation with previously reported strains by neighbour joining principle using tools implemented in online databases [21]; genetic distances were corrected by Gojobori 6-parametr method, and bootstrap test was performed on 1000 resamplings.

Demographic model of effective population size

To estimate changes in the viral population size, we used a previously described approach [8,9,13]. Maximum-likelihood (ML) trees were reconstructed using a heuristic search with the nearest neighbour-interchange algorithms. Tree likelihood scores were calculated using HKY85 + G method implemented in PAUP software (version 4.0b; Sinauer Associates Sunderland, MA, USA) with the molecular clock enforced to make the branch lengths of the output genealogies, proportional to time. Furthermore 'demographic' history was inferred from estimated phylogenies, using an appropriate model implemented in Genie v.3.5 software (2002 Pybus & Rambaut freeware) [9].

Molecular evolutionary rates of HCV and HDV

Mutation rate implemented in this study for HDV was estimated using direct linear regression in previous study based on sequence data recovered from serial samples taken in distant periods of time (more than 10 years): 1.1 (range, 0.9–2.3) $\times 10^{-3}$ nucleotide substitutions/site/year [22]. The evolution rate for HCV was previously estimated as 0.58 (range, 0.53–0.61) $\times 10^{-3}$ nucleotide substitutions/site/year [10].

Statistical analyses

Statistical differences were evaluated by Fisher exact probability test and chi-squared test with Yates' correction, where appropriate, using the STATA software version 8.0 (StataCorp. LP, College Station, TX, USA). Differences were considered significant for *P*-values <0.05.

RESULTS

Phylogenetic relation of indigenous HCV and HDV populations in Mongolia

A part of NS5b gene sequences was subjected to analysis of the phylogenetic relation among HCV strains isolated in this study and those previously published in EMBL/DDBJ/GeneBank. In the preliminary trees (not shown), great majority of the HCV strains grouped together according to their origin. After omission of the homologous sequences, and exceptional (not grouping according to their origin) strains, 187 strains were selected at random to represent phylogenetic relation of HCV clusters in geographical regions (Fig. 2a). In total, 95% (60/63) of HCV sequences obtained in this study were grouped together, forming Mongolian cluster with more than 75% confidence by interior branch test.

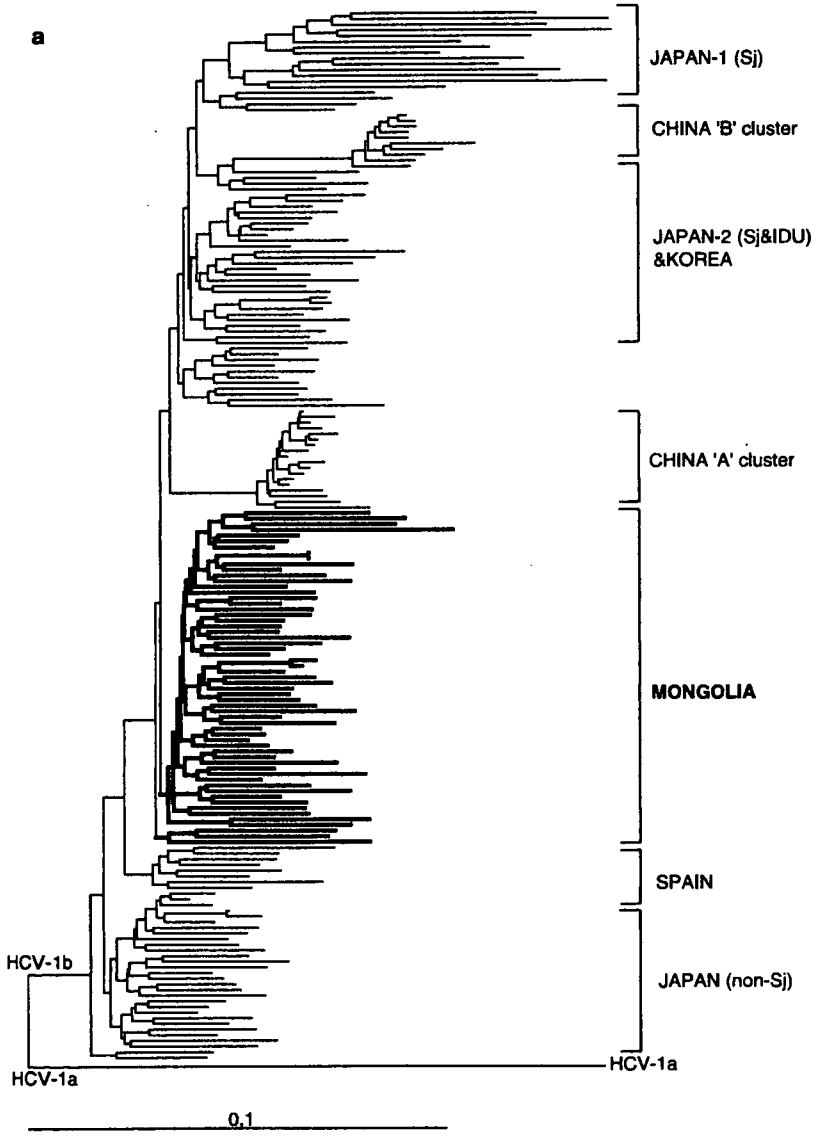

Fig. 2 (a) NJ tree constructed using 63 HCV strains' NS5b sequences obtained in this study (bold-line branches) and 124 corresponding reference sequences retrieved from EMBL/DDBJ/GeneBank. The indicated origins of the phylogenetic clusters determined if more than 70% of the strains within a particular cluster were reported from the same population. The following clusters were described previously Japan-1/2 (Sj) and Japan (non-Sj) [36], Spain [13], China 'A' and 'B' [37]. (b) NJ tree constructed using 24 HDV strains' Delta antigen coding region sequences obtained in this study and 264 reference sequences retrieved from EMBL/DDBJ/GeneBank. The indicated origins of the phylogenetic clusters determined if more than 70% of the strains within a particular cluster were reported from the respective geographical areas. Cluster with bold-line branches was used for further analyses to reconstruct the epidemic history of associated local epidemic.

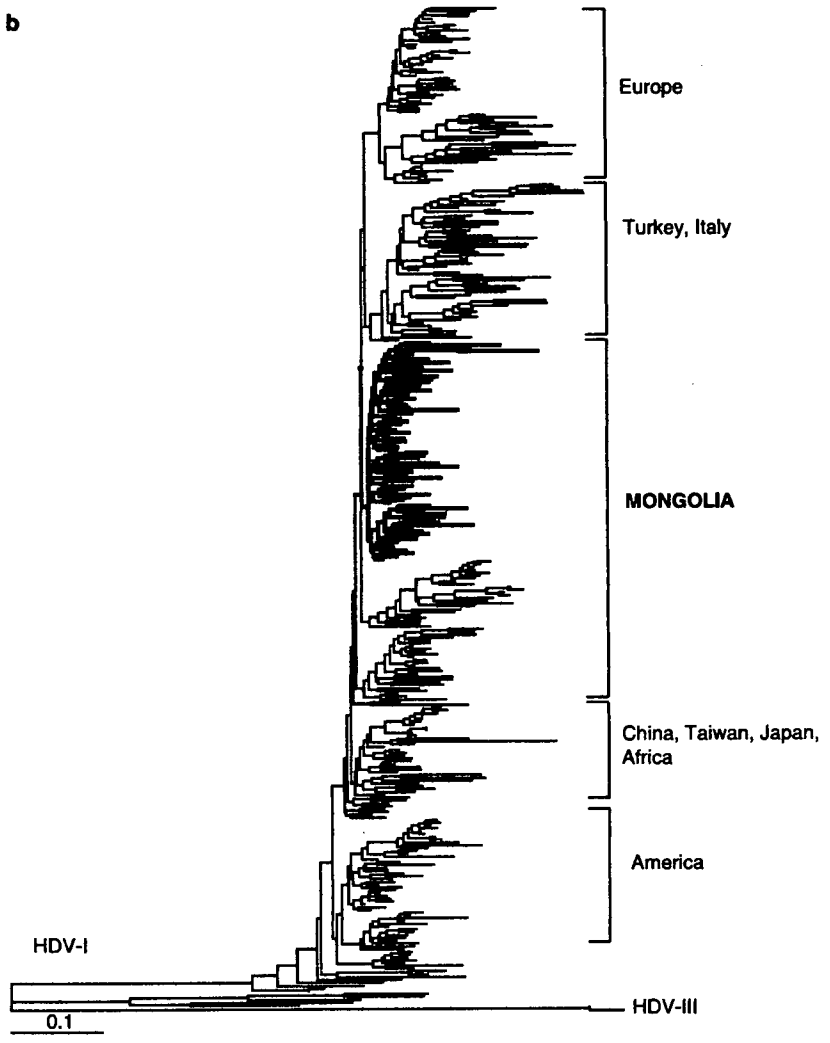

Fig. 2 (*Continued*)

A total of 24 HDV strains obtained in this study underwent preliminary analysis phylogenetically along with 578 strains from database (trees not shown). Similar to HCV phylogeny, there was an observed trend for the HDV strains to cluster respectively to their geographical origin. Mongolian strains sequenced by us and others were grouped into several phylogenetic clusters with bootstrap test indexes ranging from intermediate to low (not shown). There were several Mongolian strains clustering with European strains (Turkey, Italy); however, only strains that clustered separately from those of other countries were considered to represent local epidemic. After omission of the most homologous entries and exceptional sequences, three phylogenetic clusters that mainly consisted of Mongolian HDV strains (91.4%, 117/128) were found as shown in Fig. 2b; one of them contained 95% (74/78), second – 79% (19/24) and the third 92% (24/26) of Mongolian strains retrieved from international database. The larger cluster (indicated by bold branches on the Fig. 2b) was considered to play the most important role in the local epidemic process.

Modelling of the HCV and HDV epidemic evolution in Mongolia

A total of 52 HCV strains (isolated in this study) and 32 HDV strains (isolated in this and previous studies) were selected at random from those representing indigenous Mongolian epidemic cluster (as confirmed by bootstrap tests). As shown in Table 1, the groups of HCV and HDV carriers were matched according to their gender, age and clinical diagnosis. The ML estimates of N(t), corresponding to the effective number of infections throughout the time were performed for each HCV and HDV phylogenies (ML trees not shown). The ML analysis was carried out under 'piecewise expansion' model, which was approved by likelihood ratio testing implemented in GENIE v3.5 (2002 Pybus&Rambaut freeware). The results plotted into Microsoft (Redmond, WA, USA) Excel graphs are presented in Fig. 3 and ML approximations are summarized in Table 1. Estimated by the maximum likelihood analysis, the most recent time when currently circulating strains were diverged from their common ancestor was 1880s and 1950s