Molecular epidemiology of Hepatitis B virus, Hepatitis C virus, and Hepatitis D virus in general population of Afghanistan

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ABSTRACT

Background/Aims: This study gives a clue about genotypes, subgenotypes, and subtypes of hepatitis B (HBV), Hepatitis C (HCV), and Hepatitis D (HDV) viruses in general population of Afghanistan.

Materials and Methods: A total of 234 Hepatitis B surface antigen (HBsAg), 44 anti-HCV, and 5 antidelta positive patients from 25–70-age group were studied through a rapid screening test among 5898 residents of Afghanistan. After quantifying viral load, genotyping of 61 HBV, 29 HCV, and 1 HDV samples were accomplished by sequencing of a segment of the HBV Pre S, HCV NS5B, and HDV Delta antigen regions, respectively. Clinically important variants of the HBV polymerase gene, the "a" determinant of HBsAg, HCV NS5B, and NS3 regions were assessed.

Results: All HBV isolates were dispersed throughout the genotype D branch and ayw2 was the only subtypes found. The anti-HDV prevalence among HBsAg-positive individuals was 2.2% and the single HDV sample, from HDV genotype I. Analysis of HCV isolates revealed subtype HCV genotype 1b (HCV-1b) in 75.86%, HCV-3a in 20.69%, and HCV-3b in 3.44% patients. The observed mutant variants in the major hydrophilic region (MHR) of HBsAg were Y100 15%, Q101 5%, G102 15%, T115 45%, P120 5%, and T131 5%. Likewise, S213T 10%, Q215P 5%, and N248H 100% mutations were detected in the HBV polymerase region. C316N mutation was prevalent in 72.7% of HCV-1b participants.

Conclusion: Genotypic variation in Afghan patients is in line with the ones existing in neighboring countries and regions. HBV genotypes D1, subtype ayw2, HDV RNA type I, and HCV RNA genotype 1b are likely to be dominant in Afghan patients.

Keywords: Hepatitis B virus, Hepatitis C virus, Hepatitis D virus, genotype, epidemiology, Afghanistan

INTRODUCTION

Infections caused by hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV) are one of the leading public health challenges in Afghanistan as it is worldwide mainly in low-income countries (1). There are very few studies on viral hepatitis in the country with inconsistent results. Most of the available data come from particular groups such as intravenous drug users, prisoners, sex workers, and women giving birth (2). Although limited studies estimate the prevalence of HBV, HCV among general population, and HDV among HBV asymptomatic carriers about 1.9%-5.6%, 0.2%-1%, and 2%, respectively, in Afghanistan, the molecular epidemiology of these viruses remains completely indefinite (3-8).

Viral hepatitis is a remarkable disease with serious consequences deriving from host and viral factors. The variation in the viral nucleotide sequences of the viruses is one of the main factors that affect the course and outcomes of infections (9, 10).

Viral nucleotide sequence variations in excess of 8% in HBV, 33%-35% in HCV, and 19%-38% in HDV on the whole nucleotide sequence, respectively, characterize the respective viruses to ten different HBV (A-J), at least six HCV (1-6), and eight HDV (I-VIII) genotypes with ethno-geographical distribution worldwide (11-18). HBV A-D and F genotypes are divided into various subgenotypes with 4%-8% nucleotide differences (11). In the same way, HCV genotypes are divided into several subtypes (designated a, b, and c) with at least 15% sequence differences (12-15).

The impact of genotypes on disease outcome was examined in many studies. Distinct HBV genotypes are associated with diverse clinical manifestations such as liver

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disease severity and chronicity, development of hepatocellular carcinoma, response to interferon treatment, and even transmission route and liver transplantation (19). The consequences of coinfection with HDV are usually more severe than HBV infection alone (20). The clinical impact of HDV-1 is very variable changing from mild to severe disease. HDV-2 is usually linked to a milder hepatitis, while HDV-3 can lead to fulminant hepatitis mostly. However, there is limited information on the clinical course of the other five HDV genotypes (21). Similarly, the impact of different genotypes of hepatitis C in the natural history, development of hepatocellular carcinoma, and response to treatment was confirmed in various studies (22-28).

Molecular epidemiological data are imperative for the development of health strategies. Due to lack of such information in Afghanistan, we aimed to assess the current molecular profile of circulating hepatitis viruses in general population of Afghanistan in this study.

MATERIALS AND METHODS

Patients

Afghanistan National Public Health Institute in collaboration with World Health Organization (WHO) randomly collected 5898 samples among adults between 25 and 70 years old from Nangarhar (eastern zone), Herat (western zone), Mazar-e-Sharif (northern zone), Kandahar (southern zone), and Kabul (central zone) using WHO stepwise approach. On the basis of the designed protocol, all the participants were given consent forms by the local authorities who were collecting samples. Temporary residents (<6 months) living in migrant camps or insecure areas were excluded. The main objective of the study was to determine the level of risk factor for noncommunicable diseases in main regional provinces of Afghanistan, but this opportunity was also used for rapid screening of HBsAg and anti-HCV through card tests (Standard Diagnostics, Korea/US) (4-7). The samples of 234 HBsAg-positive patients (mean age: 40.4±14.5 years; male/female: 143/91) and 44 anti-HCV–positive patients (mean age: 45.5±14.2 years; male/female: 22/22) were transferred to Ankara University Hepatology Institute of Turkey for further serological and molecular analysis.

MAIN POINTS

- *All HBV isolates were dispersed throughout the genotype D branch and ayw2 was the only subtypes found.*
- *The anti-HDV prevalence among HBsAg-positive individuals was 2.2% and the single HDV sample, from HDV genotype I.*
- *Analysis of HCV isolates revealed subtype HCV genotype 1b (HCV-1b) in 75.86%, HCV-3a in 20.69%, and HCV-3b in 3.44% patients.*
- *HBV genotypes D1, subtype ayw2, HDV RNA type I, and HCV RNA genotype 1b are likely to be dominant in Afghan patients.*

Serological Analysis and Viral Load Determination

HBsAg and anti-HCV–positive samples were retested by chemiluminescent microparticle immunoassay (Abbott Laboratories, Illinois, USA). The same method was used to detect anti-HDV positivity in the samples of HBsAg-positive patients. HBV DNA and HCV RNA levels were measured using the COBAS AmpliPrep/COBAS TaqMan HCV test, version 2.0 (Roche, CA, USA). HDV-RNA was quantitated by a previously published in-house real-time polymerase chain reaction (PCR) based method (29).

HBV DNA, HCV RNA, and HDV RNA Genotyping

Direct sequencing of amplified segment of the HBV pre-S region, HCV NS5B region, and HDV Delta antigen region were used for HBV, HCV, and HDV genotyping, respectively (11, 30-32). To confirm the HBV genotyping results of the short segments, we also amplified whole genome of HBV in parallel in 20 samples. For the amplification of HBV pre-S segments, we used just the sera of the 88 patients having viral load more than 2000 IU/mL. In addition to HBV subtyping, HBV vaccine escape, and antiviral resistance mutations analysis, a segment of related S gene (coding for aa100-167) and HBV polymerase region (coding for amino acid 160-257) was amplified and sequenced, respectively. The sera of all 29 patients with anti-HCV positivity were used for PCR amplification. The sequences obtained from 61 HBV, 29 HCV, and 1 HDV Afghan isolates were used in genotyping and phylogenetic analysis and stored at GenBank with accession numbers MH048707 to MH048767, MH048808 to MH048836 and MH048837, respectively.

PCR Amplification and DNA Sequencing of HBV Pol Gene and HCV NS5B and NS3 Regions

PCR-DNA sequencing of NS3 and NS5B region of HCV genome was performed according to a previously published paper in order to detect existing mutation patterns related to antiviral resistance (33, 34). In addition, PCR-DNA sequencing of HBV Pol gene was achieved in 20 samples to identify existing common antiviral–resistant patterns (30). HBsAg protein gene and HBV polymerase gene were also sequenced and subsequently used for variant analyses are available at GenBank with accession

numbers MH048768 to MH048787 and MH048788 to MH048807, respectively.

Phylogenetic Analysis

All sequence isolates obtained from HBV, HCV, and HDV positive sera were manually edited and aligned with corresponding regions of reference sequences retrieved from the GenBank database. Phylogenetic comparison was done by distance matrix/UPGMA analysis using Kimura 2-parameter by MEGA7 software package program (Kumar et al. Institute for Genomics and Evolutionary Medicine, Temple University) (35). In addition to phylogenetic analysis, available database was also used to confirm the handled results (36, 37).

Statistical Analysis

Analysis was performed using VassarStats online Statistical Computation program (Richard Lowry, Vassar College ~ Poughkeepsie, NY USA) (38). To determine prevalence of each genotype and also the frequency of clinically important mutations, results were summarized using descriptive data.

RESULTS

Of the 234 HBsAg-positive persons (143 [61.1%] male and 91 [38.8%] female,40.4±14.5) HBV DNA was detected in 225 (96%) serum samples (Table 1). The viral load of HBV DNA measured by the real-time PCR assay ranged from 6.1×10^{1} to 1.2×10^{9} IU/mL in the samples. Out of 88 patients having viral load higher than 2000 IU/mL (Table 2), we were able to amplify the sequences of pre-S segment in 61 patients. NS5B region of HCV could be amplified in 29 of 44 anti-HCV–positive patients. All anti-HDV–positive samples confirmed by real-time PCR were subjected to whole genome PCR amplification. However, we failed to amplify whole genome in all five samples because of insufficient sensitivity of whole genome PCR settings. However, we succeeded to amplify a part of genome, namely Hepatitis delta antigen (HDAg), only in one patient. Therefore, just one sample could be used for genotyping of HDV RNA by partial sequencing of HDAg (Tables 1 and 2).

Comparison of the sequences with HBV reference strains previously published in GenBank, and using the HBV database genotyping tool (36), all samples of this study were classified as genotype D. Phylogenetic analysis confirmed that 60 samples were dispersed throughout subgenotype D1 and one sample in subgenotype D4 branch with a 99% bootstrap value (Figure 1). Phylogenetic analysis done by 20 whole genomes of HBV was also in line with this result (Data not shown).

In addition, the small surface antigen region and reverse transcriptase domain of the HBV polymerase gene were amplified and subsequently sequenced in 20 participants. Subtype-related amino acid residues in the HBsAg region were 122 Arg, 127 Pro, 140 Thr, 159 Gly, and 160 Lys in all Afghan individuals. Therefore, the dominant subtypes were determined as *ayw2*. The observed mutant variants were Y100 (15%; 3/20), Q101 (5%; 1/20), G102 (15%; 3/20), T115 (45%; 9/20), P120 (5%; 1/20), and T131 (5%; 1/20; Table 3). Amino acid alterations in the reverse transcriptase domain of HBV polymerase gene linked to antiviral resistance, such as those in residues 169 Ile, 173 Val, 180 leu, 181 Ala, 184 Thr, 202 Ser, 204 Met, 207 Val, 208 val, 213 Ser, 215 Gln, 217 Leu, 219 Ser, 221 Phe, 233 Ile,

Table 1. Population characteristics and distribution of HBV, HCV, and HDV genotypes.

	HBV	HCVHDV	
Seropositive samples	HBsAg-positive (N=234)	Anti-HCV positive (N=44)	Antidelta positive (N=5)
Age (mean±SD)	40.4±14.5	45.5±14.2	49.2±5
Male, n	143	22 2	
Female, n	91	22 3	
Real-time PCR positive, n	225	29 5	
PCR-DNA sequencing, n	61	29 1	
Genotype, (%)	D 61 (100)	1 22 (75.9)	1 1 (100)
		3 7 (24.1%)	
Subgenotype	D1 60 (98.4%)	1b 22 (75,9 %)	—
	D4 1 (1.6%)	3a 6 (20,7 %)	
		3b 1 (3,4 %)	

Table 2. Distribution of HBV, HCV and HDV Viral load among samples.

Regions Viral load	Herat No of Pts (M:F)	Kabul No of Pts (M:F)	Kandahar No of Pts (M:F)	Mazar No of Pts (M:F)	Nangarhar No of Pts (M:F)	Total No of Pts (M:F)	General Age mean±SD
HBV							
<2000 IU/mL	35 (15:20)	21 (14:7)	15 (9:6)	24 (18:6)	42 (25:17)	137 (81:56)	38.8±13.2
2000-10,000 IU/mL	3 (1:2)	6 (5:1)	2 (2:0)	33 (12:11)	6 (4:2)	40 (24:16)	41.7±12.5
>10,000 IU/mL	5 (2:3)	7 (4:3)	5 (3:2)	22 (12:10)	9 (6:3)	48 (27:21)	37.5±12.7
Total	43 (18:25)	34 (23:11)	22 (14:8)	69 (42:27)	57 (35:22)	225 (132:93)	39.1±13
HCV							
<2000 IU/mL	2 (0:2)	2 (1:1)	1 (0:1)	2 (1:1)	1 (0:1)	8 (2:6)	43.0±15.6
2000-10,000 IU/mL	4 (1:3)	3 (1:2)	2 (2:0)	3 (2:1)	4 (3:1)	16 (9:7)	44.8±14.1
>10,000 IU/mL	1 (1:0)	1 (1:0)	0 (0:0)	2 (1:1)	1 (0:1)	5 (3:2)	51.8±13.1
Total	7 (2:5)	6 (3:3)	3 (2:1)	7 (4:3)	6 (3:3)	29 (14:15)	45.5±14.2
HDV							
<10,000 cps/mL	1 (0:1)	1 (0:1)	2 (1:1)	1 (1:0)	0 (0:0)	5 (2:3)	47.4±6.5
>10,000 cps/mL	0 (0:0)	0 (0:0)	0 (0:0)	0 (0:0)	0 (0:0)	0 (0:0)	00.0±0.0
Total	1 (0:1)	1 (0:1)	2 (1:1)	1 (1:0)	0 (0:0)	5 (2:3)	47.4±6.5

Table 3. Frequency of clinically important mutations in HBV S gene, HBV Pol gene, and HCV NS5B and NS3 region among Afghan isolates.

Related gene	Mutation	Frequency (%)
HBV S gene (coding for aa100-167) (MHR domain)		
	Y100	3/20 (15)
	Q101	1/20 (5)
	G102	3/20 (15)
	T115	9/20 (45)
	P120	1/20 (5)
	T131	1/20 (5)
HBV pol gene (coding for aa160-257)		
	S213T	2/20 (10)
	Q215P	1/20 (5)
HCV NS5B (coding for 242-340)	C316N	17/29 (59)
Genotype 1b	—	16/22 (72.7)
HCV NS3 (coding for 242-340)	31V	2/2*
Genotype 1b	93H	1/2 **

HCV NS5B region C316N pattern is related with Tegobuvir-Sofosbuvir-Dasabuvir resistance.
HCV NS3 region 31V and 93H patterns are related with reduced susceptibility to Daclatasvir-Ombitasvir and Daclatasvir-Elbasvir-Ledipasvir-Ombitasvir-Velpatasvir resistance, respectively.
* HCV NS3 region could be amplified in 2 of 29 samples.
** HCV NS3 region 31V+93H pattern was found in one sample.

236 Asn, 248 Asn, and 250 Met were analyzed and S213T (10%; 2/20) and Q215P (5%; 1/20) mutations were identified (Table 3).

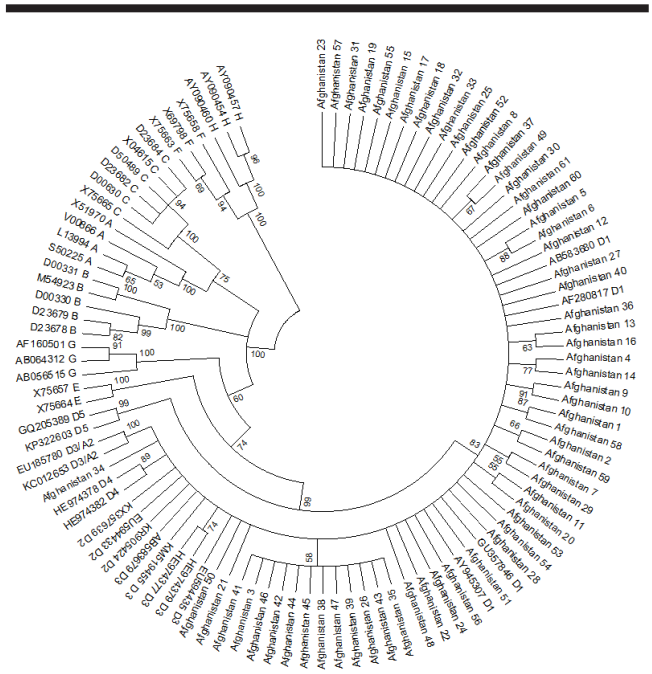

Figure 1. Phylogenetic tree obtained by distance matrix/UPGMA comparison (with Kimura-2 correction) after bootstrapping 1000 replicates of sequence segment from the Pre-S region of HBV

Among 225 HBsAg-positive patients with detectable HBV DNA, 5 patients with mean age 49.2±5 years had coinfection with HDV with viral loads ranging from 5.3×10^3 to 2.2×10^4 IU/ml. We were able to amplify and

sequence only one of the samples partially which resided in branch genotype I in phylogenetic analysis (Figure 2).

In contrast, out of the 44 anti-HCV–positive (22 [50%] male and 22 [50%] female, mean age 45.5±14.2), HCV-RNA was detected in 29 patients with viral loads ranging from 9.5×10^{1} to 6.7×10^{4} IU/mL (Table 2). Nucleotide sequence analysis of NS5B region of HCV genome (codon 242-340) and subsequent phylogenetic analysis showed subtype HCV genotype 1b (HCV-1b) in 22 (75.86%), HCV-3a in 6 (20.69%), and HCV-3b in 1 (3.44%; Figure 3). In addition, geno2Pheno database was also used for analysis (41), which confirmed our results and demonstrated HCV NS5B C316N mutation in 16/22 HCV-1b patient. NS3 region of HCV could be amplified in just 2 out of 29 samples yielding one 31V and one 31V plus 93H patterns related to antiviral resistance (Table 3).

DISCUSSION

This is the first molecular epidemiological study of HBV, HCV, and HDV aiming the determination of genotypes, subgenotypes, and subtypes in the general population in Afghanistan. In addition, the nucleotide patterns in MHR of HBV S gene, HBV RT-Polymerase, HCV NS3, and HCV NS5B genes, determining HBsAg antigenicity and antivi-

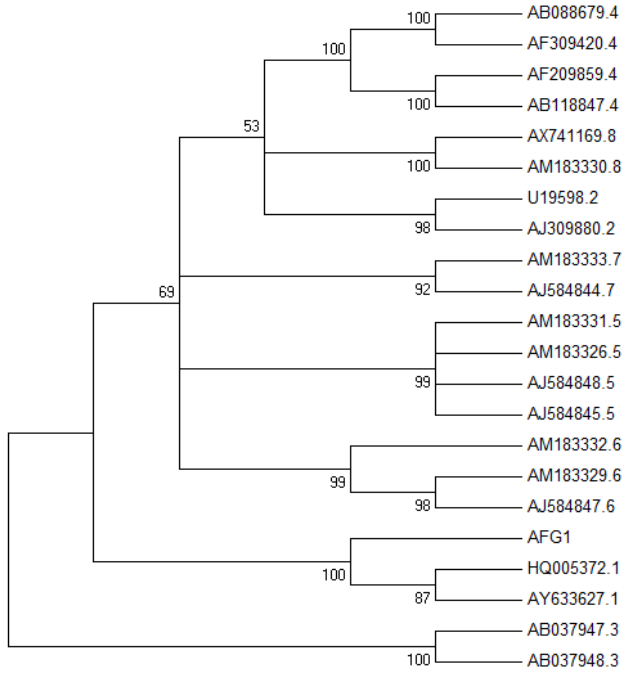

Figure 2. Phylogenetic tree obtained by distance matrix/UPGMA comparison (with Kimura-2correction) after bootstrapping 1000 replicates of sequence segment from HDAg.

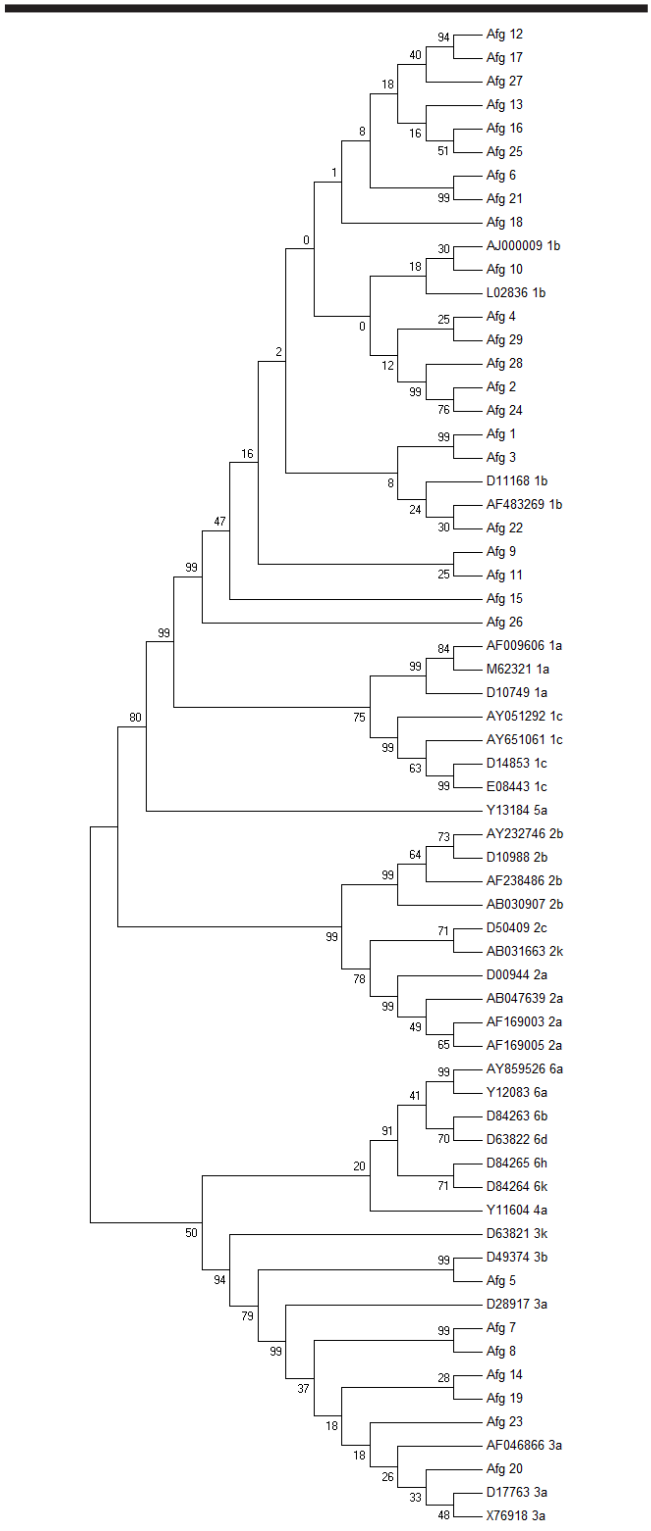

Figure 3. Phylogenetic tree obtained by distance matrix/UPGMA comparison (with Kimura-2 correction) after bootstrapping 1000 replicates of sequence segment from the NS5B region of HCV.

ral resistance were identified. HBV and HCV are endemic in Afghanistan (39). There is no updated data about HDV prevalence in this country (35). Earlier studies are limited and were mainly conducted on high risk groups such as intravenous drug users, prisoners, sex workers, and intrapartum women using mostly serological assays (2).

This study shows that genotype D is the only HBV genotype found in Afghan patients and D1 is the dominant subgenotype. Likewise, HBsAg subtypes were found to be *ayw2* in all tested patients. A previous study on 12 HBsAg-positive Afghan patients in Iran was in line with the results obtained in this study (40). However, in another study by Attaullah et al (41), a heterogeneous distribution of HBV genotypes was reported in Afghan refugees residing in Pakistan. This discrepancy may be due to the method, failure of type-specific PCR for HBV genotyping used in the study. HBV subgenotype D1, D2, and D3 are prevalent throughout the world. Subgenotype D3 is common in Asia (East India), South Africa, and Europe, while subgenotype D4 is found in Australia and subgenotype D5 is frequent in Eastern India and Japan (42). In Iran, the western neighbor of Afghanistan, several studies have shown that genotype D is dominant in most provinces, but genotype B has also been reported in some parts of the country (43, 44). Likewise, HBV genotype D is the most common genotype in all regions of Pakistan followed by the A+D combination and A genotype, respectively (45). Further evidence suggests that genotype D (up to 88%) is the dominant HBV infection in Central Asian countries as well (46). However, it seems that molecular characteristic of the HBV infections in Afghanistan are similar to those of neighboring countries such as Iran in the west, Pakistan in the southeast, and central Asian countries in the north. HBV genotype D results in more severe disease and higher rates of drug resistance in comparison with other HBV genotypes (47).

The last study on HDV in Afghanistan dates back to 1984 (8). In this study, HDV prevalence in chronic liver disease, inactive HBsAg carriers, and acute HBV was 18%, 2%, and 5%, respectively. The anti-HDV prevalence among HBsAg-positive patients in our study was 2.2%, which suggests no significant change over the years. HDV RNA genotype was identified as genotype 1 in a unique sample in which amplification was possible. Although analysis of one sample for genotyping is really inadequate for generalizing the result to the entire population, it nevertheless suggests that HDV genotype in Afghanistan is genotype 1 similar to the situation in neighbor countries.

This study provides molecular profile of HCV genotypes circulating among general population of Afghanistan for the first time. Regarding HCV RNA genotyping in the general population, HCV-1b is the most common subtype of HCV in Afghanistan followed by HCV-3a. Only one study among intravenous drug users revealed information related to the distribution of HCV genotype in Afghanistan, referred by all subsequent reviews. On the basis of the aforementioned study, genotype 3a (62%) was the predominant genotype circulating in injecting drug users (IDU's); genotype 1a was observed in 35.2% of intravenous drug users (IVDUs) followed by genotype 1b (2.8%) (48). Various studies throughout the world confirmed that the distribution of HCV genotypes among IDU's is different from the general population (49). HCV subtypes 1a, 3a, and 1b are the most frequent ones in Iran, respectively (50-52). The HCV genotype 3 is also predominant in all regions of Pakistan followed by genotypes 1 and 2 in frequency (53). Contrary to HBV infections, the genotype distribution of HCV in Afghanistan is different from that of western and southeastern neighbors and similar to common genotypes in Central Asian countries, where the HCV genotype 1 (70%), subtype 1b, is the most frequent HCV genotype followed by genotype 3 (19.6%) and genotype 2 (8.6%), respectively (54,55). HCV genotype 1b was linked to more severe liver disease compared with infection with other HCV genotypes (56).

Several viral factors including genotype, viral load, and specific viral mutations have been associated with disease progression and response to treatment (57,58). The reverse transcriptase domain (amino acid 160-257) of the HBV polymerase gene and the "a" determinant (amino acid 124-147) in the major hydrophilic domain of HBsAg (amino acid 99-166), respectively, are the main targets of novel antiviral agents against HBV and epitopes for vaccine development or serological immunoassay (59). Although the G145R substitution is the most common immune escape mutation, the P120S, identified in one sample in this study is a known immune escape mutation as well (60,61). Another detected variant, T131I, was reported in patients with occult infection and maybe associated with failed HBsAg detection (62). However, it was seen that the major mutations affecting the antigenicity of HBsAg or leading to HBV antiviral resistance are not frequent in Afghan isolates. Mutations in the N248 and Q215 locus in the HBV polymerase gene are frequently found but its relation with drug resistance is controversial (63,64). However, it was suggested that patients with the rtQ215S mutation have a higher risk of progression of

liver disease (63), S213T is a candidate mutation associated with hepatocellular carcinoma (65), and N248H can reduce HBV susceptibility to antiviral drugs during treatment (64).

The C316N mutation pattern in the NS5B region of HCV found in the majority of genotype 1b HCV (16/22) chronically infected patients, seems to be related to reduced response rates to sofosbuvir, tegobuvir, and desabuvir (66). HCV NS3 region was amplified in two samples (2/29) and yielded 31V and 93H patterns, which is linked to reduced susceptibility to daclatasvir, ombitasvir, daclatasvir, elbasvir, ledipasvir, ombitasvir, and velpatasvir, respectively. The NS3, NS5A, and NS5B regions of HCV, which are the main target of HCV antiviral drugs, should be studied in more detail and thoroughly in the Afghan population.

Seroepidemiological and molecular characterization studies for blood-borne diseases are basic needs for National Health authorities to develop public health polices, and the results of this study may contribute to this goal (67,68).

Although this study may adequately reflect the epidemiological status of HBV and HCV in general population, the evaluation of HDV with one individual is the main limitation of study.

In conclusion HBV, HCV, and HDV genotypic variations in the Afghan population may not be heterogeneous. HBV subgenotype D1 and subtype ayw2 are observed in almost 100% of HBV infections. Likewise, the HCV-1b (75.86%) and HCV-3a (20.69%) are the most common subgenotype in Afghan patients. The mutation patterns related to antiviral resistance in chronic C hepatitis should be taken into consideration. Additionally, the mutant variations within HBsAg and HBV polymerase gene that are clinically important are not common in Afghan population. Vaccination policies against HBV should be considered accordingly. More comprehensive studies including serologic and molecular investigation of HDV should be carried out.

Ethics Committee Approval: Ethics committee approval was received for this study from the Afghanistan National Public Health Institute, Feb/10/2016, 361537.

Informed Consent: Written informed consent was obtained from the patients who participated in this study.

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