Distribution of Hepatitis B Virus Genotypes in Azerbaijani Patients With Chronic Hepatitis B Infection

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Background: Hepatitis B virus (HBV) has been classified into ten genotypes (A-J) based on genome sequence divergence, which is very important for etiological and clinical investigations. HBV genotypes have distinct geographical distributions worldwide.

Objectives: The aim of this study was to investigate the distribution of HBV genotypes among Azerbaijani patients with chronic hepatitis B, came from the Republic of Azerbaijan country to Iran to receive medical care.

Patients and Methods: One hundred and three patients with chronic HBV infection, referred to hospitals related to Iran University of Medical Sciences and Tehran Hepatitis Center from August 2011 to July 2014, were enrolled in this cross sectional study. About 3-milliliter of peripheral blood was taken from each patient. After viral DNA extraction, HBV genotypes were tested using the INNO-LiPA™ HBV kit (Innogenetics, Ghent, Belgium). HBV genotyping was confirmed using sequencing of hepatitis B surface antigen (HBsAg) and polymerase (pol) regions of HBV.

Results: The mean age of patients was 35.9 ± 11.7 years (19-66). Of 103 patients, 72 (69.9%) were male. In the present study, the predominant HBV genotype was D (93.2%) followed by genotype A (5.8%) and concurrent infection with A and D genotypes (0.97%).

Conclusions: The main and frequent HBV genotype among Azerbaijani patients with chronic hepatitis B virus infection was genotype D followed by genotype A.

Keywords: Hepatitis B virus; Genotype; Patients
2. Objectives

The aim of this study was to investigate the frequency of HBV genotypes among Azerbaijani patients with chronic hepatitis B came from the Republic of Azerbaijan country to Iran to receive medical treatment.

3. Patients and Methods

3.1. Study Population

From August 2011 to July 2014, one hundred and three Azerbaijani patients with established chronic hepatitis B (HBsAg and HBV DNA positive), came from the Republic of Azerbaijan country (one of the Newly Independent States of the former Soviet Union) to Iran for medical care, were enrolled in this cross-sectional study. The patients were referred to hospitals affiliated to Iran University of Medical Sciences and Tehran University of Medical Sciences. Informed consent was obtained from each patient. The present study was approved by the local ethics committee of Gastrointestinal and Liver Disease Research Center (GIDRC) of Iran University of Medical Sciences.

3.2. Collection and DNA Extraction of Specimens

About 3-milliliter of peripheral blood was drawn from all enrolled participants into EDTA-containing vacationer tubes. Plasma was taken and stored at -80°C for later detection. HBV DNA was extracted from 200 μl of plasma samples using DNA extraction kit (Qiagen GmbH, Hilden, Germany) according to the kit instructions. The quality and quantity of extracted DNA was determined with a nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) after DNA extraction.

3.3. Hepatitis B Virus Genotyping

Hepatitis B virus genotypes were analyzed in extracted viral DNA specimens using the INNO-LiPA™ HBV kit (Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. Briefly, HBV-DNA was amplified by nested-polymerase chain reaction (nested-PCR) method. PCR products were hybridized to HBV genotype-specific probes attached to nitrocellulose strips and then the hybrids were revealed with chromogen substrates. The results of HBV genotyping were interpreted using the kit interpretation chart. To confirm the INNO-LiPA HBV genotyping, hepatitis B surface antigen (HBsAg) and polymerase (Pol) regions of HBV from four randomly selected plasma samples were amplified with Ex Taq DNA polymerase [TaKaRa Biotechnology (Dalian) Co., Ltd., Shiga, Japan], 5 μl 10X Ex Taq buffer (Mg2+free), 4 μl MgCl2 (25 mM), 4 μl dNTPs Mixture (25 mM each), and 5 μl viral extracted DNA was used as a template for the first round of PCR and 1 μl of the PCR-amplified product in the first round was used as a template for the second round of PCR. Thermal cycle profiles used for the nested-polymerase chain reaction assay were exactly as described previously (30). PCR products (744 bp) of samples, negative and positive controls and DNA size marker (100 bp) were isolated by 1.5 % agarose gel electrophoresis, stained by ethidium bromide, and then visualized using an ultraviolet (UV) transilluminator. PCR-amplified products in the second round (744 bp) were purified using high pure PCR product purification kit (Roche Diagnostic GmbH, Mannheim, Germany) and then subjected to bidirectional sequencing with inner primers by dye termination method using the sequencer ABI version 3730 XL. The nucleotide sequences of HBsAg region of HBV announced in the present study were submitted to GenBank with the accession numbers from KM035536 to KM035539.

3.4. Hepatitis B Virus DNA Detection in Plasma Samples and Sequencing of Hepatitis B Surface Antigen (HBsAg) Region

Hepatitis B surface antigen (HBsAg) region of HBV was amplified by nested-PCR method. Briefly, A set of nested primers from the HBsAg region of HBV was used, including an outer primer pair of 947-base span: sense primer S1 (5’-CTGTGATGAGAGTGTGTTG-3’; 56-75), and antisense primer S2 (5’-CCAGGAAATTGTTGACAATCAT-3’; K=GT; 1003-979) and an inner primer pair of 744-base span: sense primer S6 (5’-GGCAGGAAATTGTTGACAATCAT-3’; 857-832) (29). The first and second rounds PCR were performed in a 50 μl mixture reaction containing 2.0 U Ex Taq DNA polymerase [TaKaRa Biotechnology (Dalian) Co., Ltd., Shiga, Japan], 5 μl 10X Ex Taq buffer (Mg2+free), 4 μl MgCl2 (25 mM), 4 μl dNTPs Mixture (25 mM each), and 1 μl of each outer primers, and 5 μl viral extracted DNA was used as a template for the first round of PCR and 1 μl of the PCR-amplified product in the first round was used as a template for the second round of PCR. Thermal cycle profiles used for the nested-polymerase chain reaction assay were exactly as described previously (30). PCR products (744 bp) of samples, negative and positive controls and DNA size marker (100 bp) were isolated by 1.5 % agarose gel electrophoresis, stained by ethidium bromide, and then visualized using an ultraviolet (UV) transilluminator. PCR-amplified products in the second round (744 bp) were purified using high pure PCR product purification kit (Roche Diagnostic GmbH, Mannheim, Germany) and then subjected to bidirectional sequencing with inner primers by dye termination method using the sequencer ABI version 3730 XL. The nucleotide sequences of HBsAg region of HBV announced in the present study were submitted to GenBank with the accession numbers from KM035536 to KM035539.

3.5. Hepatitis B Virus DNA Detection in Plasma Samples and Sequencing of the Polymerase (Pol) Region

Polymerase region of HBV was amplified using PCR method. Briefly, A set of primers was used for amplification of the polymerase region of HBV, including a primer pair of 454-base span: sense primer Pi (5’-GTATTCCCATCCCATCCATCC-3’; 599-619) and antisense primer Pj (5’-CAAGGGAAGATACCGAACATT-3’; 1053-1033) (31). Polymerase chain reaction (PCR) was performed in a 50 μl mixture reaction containing 1.5 U Ex Taq DNA polymerase [TaKaRa Biotechnology (Dalian) Co., Ltd., Shiga, Japan], 5 μl 10X Ex Taq buffer (Mg2+free), 3.5 μl MgCl2 (25 mM), 4 μl dNTPs Mixture (25 mM each), and 15 pmol of each outer primers. Five microliters of viral extracted DNA was used as a template. Thermal cycle profiles used for the reaction were described previously (31). PCR products (454 bp) of samples, negative and positive controls and DNA size marker (100 bp) were isolated by 2% agarose gel electrophoresis, stained by ethidium bromide and then visualized by an UV transilluminator. The PCR-amplified product was purified using high pure PCR product.
purification kit and then subjected to bidirectional se-
quencing with inner primers by dye termination method
using the sequencer ABI version 3730 XL. The nucleotide
sequences pol region of HBV announced in the current
study was submitted to the GenBank with accession
numbers from KM035540 to KM035543.

3.6. Statistical Analysis

Statistical analyses were performed using SPSS version
16 software (SPSS Inc., Chicago, IL, USA). Descriptive analy-
ses as well as Student’s t-test were used. P < 0.05 was con-
sidered statistically significant.

4. Results

One hundred and three patients with hepatitis B virus
infection were recruited in this cross-sectional study. The
mean ± SD age of studied subjects was 35.9 ± 11.7 (range: 19-66 years). Of 103 patients, 72 (69.9%) were male. The
demographic characteristics, laboratory parameters
and distribution of HBV genotypes in all participants are
presented in Table 1. HBV genotypes of the study popula-
tion were determined as follows: genotype D in 96 (93.2%)
patients, genotype A in 6 (5.8%) and mixed HBV infection
(A and D) in 1 (0.97%) patient. The frequency of genotype
A and D in patients younger than 40 years were 8.1% and
91.8%, respectively, which was not statistically signifi-
cant. HBV genotypes of subjects’ plasma specimen were con-
fi rmed via nucleotide sequencing of the HBV HBsAg and
pol regions.

5. Discussion

Hepatitis B virus is a typical example of a virus with vari-
ous genotypes and different geographic distributions
worldwide. A genetic classification based on the com-
parison of HBV complete genomes defined ten genotypes
(from A to J) (5-7). Molecular variation of the virus genome
led to the emergence of HBV genotypes (32). The outcome
of chronic hepatitis B virus infection is associated to vari-
able viral factors such as HBV viral load, HBV genotypes,
HBV variants and hepatitis B e antigen (HBeAg) status
(33). The current study was performed on 103 Azerbaijani
participants with chronic HBV infection, came from the
Republic of Azerbaijan country to Iran to receive medical
care, to determine the distribution of HBV genotypes in
their plasma specimens. According to obtained results,
the frequency of HBV genotypes of the study population
was found as follows: genotype D was the most dominant
(93.2%), followed by genotype A (5.8%) and concurrent in-
fection with HBV genotype A and D in 1 (0.97%) patient.
Determination of HBV genotype is important to clarify
the virulence, pathogenesis and rout of transmission of
the virus (34). The former Soviet Union was considered as
an area with high endemicity of hepatitis viruses and the
present frequency of these infections are unknown in this
region (28). Little is known about the distribution of HBV
genotypes in the Republic of Azerbaijan country. This was
the preliminary study performed on Azerbaijani patients,
so we are unable to compare the results of this study with
any other investigation. The most frequent HBV genotype
in various areas of the former Soviet Union is genotype D
as follows: Armenia (95.5%) (32), Belarus (88.6%) (25), Esto-
nia (81.0 %) (26), Latvia (72.1%) (27), Russia (93.0%) (24), Ta-
jikistan (94.1%) (35), Ukraine (52.4%) (36) and Uzbekistan
(87.0%) (28), which are consistent with the frequency of
HBV genotype of the current study. Distribution of HBV
genotypes and subtypes in some countries of the former
Soviet Union (24-28, 32, 35, 36, Non-Arab countries includ-
ing Afghanistan (35.7%) (23), Iran (100.0%) (19), Pakistan
(58.5%) (22), Turkey (100.0%) (20) and Arab countries such
as Iraq (100.0%) (21), Jordan (100.0%) (37), Kuwait (78.7%)
(38), Oman (76.5%) (39), Saudi Arabia (81.4%) (40) and the

Table 1. Demographic Characteristic, Laboratory Parameters and Hepatitis B Virus Genotypes Distribution Among Azerbaijani Pa-
tients a,b

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>31 (30.1)</td>
<td>72 (69.9)</td>
<td>103 (100)</td>
<td></td>
</tr>
<tr>
<td>Age, Years</td>
<td>36.1 ± 13.9 (19-66)</td>
<td>35.9 ± 10.7 (21-64)</td>
<td>36.0 ± 11.7 (19-66)</td>
<td>0.885</td>
</tr>
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</table>

Laboratory Parameters

<table>
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<tr>
<th>Parameter</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT, IU/L</td>
<td>87.0 ± 72.6 (29.0-269.0)</td>
<td>63.5 ± 38.1 (24.0-299.0)</td>
<td>70.6 ± 52.0 (24.0-299.0)</td>
<td>0.546</td>
</tr>
<tr>
<td>AST, IU/L</td>
<td>70.7 ± 57.0 (11.0-172.0)</td>
<td>48.9 ± 35.8 (19.0-182.0)</td>
<td>55.2 ± 44.1 (11.0-182.0)</td>
<td>0.269</td>
</tr>
<tr>
<td>Viral Load, IU/mL</td>
<td>5155.0 (122-55024405)</td>
<td>3785.0 (244-6321290000)</td>
<td>4350.0 (122-6321290000)</td>
<td>0.678</td>
</tr>
</tbody>
</table>

Type of HBV Genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>28 (90.3)</td>
<td>68 (94.4)</td>
<td>71 (94.2)</td>
</tr>
<tr>
<td>A</td>
<td>3 (9.7)</td>
<td>3 (4.2)</td>
<td>6 (5.8)</td>
</tr>
</tbody>
</table>

Mixed HBV Genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A and D</td>
<td>0 (0.0)</td>
<td>1 (1.4)</td>
<td>1 (0.97)</td>
</tr>
</tbody>
</table>

a Abbreviations: ALT, Alanine aminotransferase; AST, Aspartate aminotransferase.
b Data are presented as No. (%).
Table 2. Distribution of Hepatitis B Virus Genotypes in the Former Soviet Union, Non-Arab and Arab Countries

<table>
<thead>
<tr>
<th>Region/Countries</th>
<th>Genotypes and Subgenotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Most Common</td>
<td>Less Common</td>
</tr>
<tr>
<td>The former Soviet Union</td>
<td>Armenia  D (95.5)</td>
<td>A (4.5)</td>
</tr>
<tr>
<td></td>
<td>Belarus    D (88.6)</td>
<td>A2 (11.6)</td>
</tr>
<tr>
<td></td>
<td>Estonia    D (81.0)</td>
<td>A (18.5)</td>
</tr>
<tr>
<td></td>
<td>Latvia     D (72.1)</td>
<td>A (28.0), E (0.9)</td>
</tr>
<tr>
<td></td>
<td>Russia     D (93.0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Tajikistan D (94.1)</td>
<td>A (5.8)</td>
</tr>
<tr>
<td></td>
<td>Ukraine    D (52.4)</td>
<td>A (14.2), C (4.7)</td>
</tr>
<tr>
<td></td>
<td>Uzbekistan D (87.0)</td>
<td>A (13.0)</td>
</tr>
<tr>
<td>Non-Arab Countries</td>
<td>Afghanistan D (35.7)</td>
<td>C (32.2), A (19.3), B (7.0)</td>
</tr>
<tr>
<td></td>
<td>Iran       D (100.0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pakistan   D (58.5)</td>
<td>A (10), Mixed genotypes A and D (31.5)</td>
</tr>
<tr>
<td></td>
<td>Turkey     D (100.0)</td>
<td>-</td>
</tr>
<tr>
<td>Arab Countries</td>
<td>Iraq       D (100.0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Jordan     D (100.0)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Kuwait     D (78.7)</td>
<td>A (5.0), Mixed genotypes D and A (16.3)</td>
</tr>
<tr>
<td></td>
<td>Oman       D (76.5)</td>
<td>A (18.3), C (1.2), E (1.2)</td>
</tr>
<tr>
<td></td>
<td>Saudi Arabia D (81.4)</td>
<td>E (5.7), A (1.4), C (1.4), Mixed genotypes (10.0)</td>
</tr>
<tr>
<td></td>
<td>The United Arab Emirates D (79.5)</td>
<td>A (18.2), C (2.3)</td>
</tr>
</tbody>
</table>

United Arab Emirates (79.5%) (41) are shown in Table 2. HBV genotypes have a distinct geographic distribution worldwide. Hepatitis B virus genotype D is found all over the world; it is the most prevalent genotype in the Mediterranean basin, the Middle East, north-eastern Europe, northern Africa and the Indian subcontinent, also it has been detected in Oceania (1). Global distribution of HBV genotypes may change over time because of the population migration (32). The origin of HBV D genotype, subgenotypes and their expansion throughout the world is still unknown. Zehender et al. suggested that the Indian subcontinent was the location in which HBV genotype D originated. Their suggestion is supported by the fact that genotype D is the most prevalent in India (1). There are a few data available regarding HBV genotype prevalence in central Asia, but HBV D genotype is the most prevalent genotype in the region (1). In this study, all patients infected by HBV genotype A were younger than 40 years. This is interesting and needs more studies with larger population size. In conclusion, the current study showed that the predominant HBV genotype in Azerbaijanis patients with established chronic hepatitis B is genotype D (93.2%) followed by genotype A (5.3%). The current research was a preliminary study on HBV genotyping. However, it is suggested to perform further investigations to determine HBV subgenotyping in these patients.

Acknowledgements

We wish to thank all the volunteers who generously participated in the present research.

Authors’ Contributions

Farah Bokharaei-Salim designed the present study and responsible for the overall study management. Farah Bokharaei-Salim and Hossein Keyvani organized the study analysis. Farah Bokharaei-Salim, Hossein Keyvani, Seyed Hamidreza Monavari, Maryam Esghaei, Shahin Fakhim, Angila Ataei Pirkooh and Bita Behnava prepared the manuscript. The statistical analyses were performed by Farah Bokharaei-Salim and Shahin Fakhim. All authors contributed to the final version of the manuscript.

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References


