Original Research Article

IDENTIFICATION OF HEPATITIS B VIRUS GENOTYPES AMONG YEMENI PATIENTS WITH CHRONIC HEPATITIS B INFECTION

ABSTRACT

Background and aims: Hepatitis B virus (HBV) infection is a serious public health problem worldwide. Hepatitis B virus genotyping is an important tool in epidemiological studies to determine the type and duration of treatment and to predict the outcome of chronic infections, such as cirrhosis and hepatocellular carcinoma. This study aimed to determine the prevalence of hepatitis B virus genotypes among Yemeni patients with chronic hepatitis B (CHB) and to evaluate some of the associated risk factors. **Methods:** Fifty patients (38 males, 12 females) with chronic hepatitis B from Al-Thawra Modern General Hospital, Al-Kuwait University Hospital, and AL-Gomhoria Hospital were included. HBV DNA was first detected by conventional PCR then HBV genotypes were determined using nested and multiplex PCR. **Results:** Mixed HBV genotypes (A+B+C+D+E), (A+B+C+D+E+F), and (A+B+C+D) were found to be the most prevalent (60 %), it is followed by genotype D (16 %), genotype B (16%) and genotype A (8%), whereas C, E, and F genotype were not found individually among the study population. Blood transfusion was significantly associated with mixed infection ($\chi 2 = 13.06$; $p = 0.005$). **Conclusions:** In conclusion, this study demonstrates the general prevalence of hepatitis B virus genotypes among HBV-infected Yemeni hepatitis B patients who seek medical attention in a hospital. In mono-genotype HBV infection, genotype B and D were the most prevalent genotypes. In HBV mixed genotype infection, the A/B/C/D/E genotype was the most prevalent in the study area. In the future, clinical trials and treatment regimens should be assumed individually based on genotype to effectively manage chronic HBV infection. To this end, a prospective nationwide population study of HBV genotype distribution and clinical outcomes is recommended.

Keyword: chronic hepatitis B, HBV genotype, nested-PCR, prevalence , Yemen

INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem. HBV is a partially double-stranded DNA virus that belongs to the Hepadnaviridae family, in the Orthohepadnavirus genus[1]. It has been estimated that about two billion people worldwide have a proof of past or present infection with HBV and more than 358 million people have chronic lifelong infection and about 887 000 people die every year due to the consequences of hepatitis B[2,3]. The endemicity of hepatitis B virus was estimated in Yemen, where the prevalence of positive HBsAg in the general population and HCWs ranged from 8% to 20%, among infants, it was 4.1%, and up to 50% of health workers and populations usually had prior serological evidence of Hepatitis B virus infection in old reports. On the other hand, recent studies indicated that the rate of HBsAg, which ranges from 0.7-2% among the general population and to 4% among risk groups such as HCWs, as well as HBV decreased more among children [4-11].

Though HBV contains DNA genome, it replicates via an RNA intermediate and due to lacking of proofreading activity for spontaneous error of viral reverse transcriptase, nucleotide mutations of HBV genome lead to the occurrence of various genotypes and subtypes4. Currently, ten HBV genotypes (A-J) and twenty-four sub-genotypes (A1–A3, B1–B5, C1–C6, D1–D6 and F1–F4) are reported [13].

HBV genotypes show a distinct geographic and ethnic distribution. Genotype A is the most commonly distributed genotype in Europe, USA, Canada, Brazil, India, Central African countries, Tunisia and Benin [14-16]. Genotype B is predominant in Taiwan, Philippines, Japan, Hong Kong, China, Thailand, Indonesia, Vietnam, and USA[15-18]. Genotype C is prevalent in Australia, Melanesia, Micronesia, Polynesia, Indonesia, China, Hong Kong, Korea, Taiwan, Vietnam, Thailand, Japan, India, Solomon Islands, Brazil and USA. Genotype D is widespread in Mediterranean region, Spain, Czech Republic, Russia, Turkey, Albania, Afghanistan, South Asia, Middle East, Iran, Solomon Islands, Tunisia, Micronesia, Polynesia, Melanesia, Brazil and USA [18-20]. Genotype E is found endemically in Western Africa while genotype F is widely distributed in new world countries. Genotypes G has been reported from France, and Germany and North America. Genotype H is recorded from Central America, South America and Mexico while Genotype I was isolated in Vietnam and Laos[21-23].

Different HBV genotypes are also associated with distinct clinical phenotypes and prognosis. The rate of chronicity following acute genotypes A and D infection were reported to be high compared with genotypes B and C [1,14-26]. Infection with genotype C alone was found to be associated with significantly higher risks of liver cirrhosis and hepatocellular carcinoma compared to genotype B infection [27,28]. In contrast, Genotype B infection has a slower progression to liver cirrhosis than genotype C [29]. In addition, the response to antiviral therapy, particularly to interferon, is related to HBV genotypes. Patients with genotype A have been reported to

be more sensitive for treatment by interferon α as compared to those infected with genotype D [30]. HBV genotype B develops antiviral resistance more than genotype C[31]. This study aimed to determine the prevalence of hepatitis B virus genotypes among Yemeni patients with chronic hepatitis B (CHB) and to evaluate some of the associated risk factors.

Materials and Methods

Study population

Fifty patients with CHB were enrolled in this cross-sectional study. Sample size was calculated by using Epi Info version 6 based on population size 1000000 and 2% prevalence of CHB [32]. Patients were randomly selected from Al-Thawrah Modern General Hospital, Al-kuwait University Hospital and AL-Jomhori Hospital in Sana'a-Yemen from December 2016 to June 2017.

Inclusion criteria: Both males and Females infected with HBV for more than six months. Their HBs Ag test was positive but their HBc IgM test was negative.

Exclusion patients: Excluded patients were acute hepatitis B (Anti-HBc IgM test was positive), have mixed infected with HBV and HCV, any patient treated with antiviral of HBV, and patients who had liver cirrhosis or hepatocellular carcinoma.

Specimens collection: Five ml venous blood were collected from each patient. Two ml of whole blood was collected in an EDTA tube for detection of HBV- DNA while three ml was put in a plain tube for detection of Hepatitis B surface Ag (HBsAg) and liver enzymes AST and ALT. Demographic data were collected at the time of sample collection using a predesigned questionnaire.

Detection of Hepatitis B surface antigen and anti-HBc-IgM: HBsAg and anti-HBc IgM in patient serum were detected by ELISA method (Closed system-Abbott diagnostic). Samples that were positive for HBsAg and anti-HBc IgM negative were enrolled in this study.

Estimation of the serum levels of alanine and aspartate transaminase: Serum level of AST and ALT were measured using Enzyme kinetics method (kit -AGAPPE, spectrophotometer-Bayer Diagnostic RA-50Clinical chemistry-Ireland).

Determination of HBV- DNA by conventional PCR:

a-Virus DNA Extraction and PCR amplification

DNA of HBV was extracted using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) in accordance with the manufacturer's instructions. Extracted DNA was stored at -20°C for later analysis. HBV-DNA was then amplified using 1508 bp of P through S genes using universal primers, (FA2F) sense primer was reported by (S1-2) antisense primer that was described by Naito *et al*, 2001, shown in table (1) using AccuPower® ProFi Taq PCR PreMix (bioneer Korea-Bio metar system, Germany)[33]. The PCR program was run for one cycle as: initial denaturation at 94°C for 5 minutes, 35 cycles consisted of denaturation at 94 °C for 30 sec, annealing at 57°C for one minute followed by extension at 72 °C for 1.5 minutes. The final extension was 72 °C for 5 minutes.

Table (1) Universal Primer sequences used for HBV detection

Name primer	Sequences	Position
FA2F	5`- GCGTCGCAGAAGATCTCAAT -3`	2413–2432
S1-2-R	5`-CGA ACC ACT GAA CAA ATG GC-3`	685–704

PCR amplicons were electrophoresed on a 1% agarose gel, stained with ethidium bromide to visualized viral DNA under UV light.

b-Determination of HBV genotypes

Genotyping system was based on nested PCR, using type specific primers for determination of six genotypes A through F of HBV, using (AccuPower® Gold Multiplex PCR PreMix from Bioneer-Bio metar system, Germany). The nested PCR primers were designed based on the conserved nature of the nucleotide sequences in regions of the P through S genes. The genotypes can be determined according to differences in the sizes of amplified DNA, in respective of the six HBV genotypes table (2). Two nested PCRs were performed in different mixtures for each sample: mix 1 (sit 1) applied for identification of genotypes A, B, C with B2 sense universal primer and mix 2 (set 2) for genotypes D, E, F by B2R antisense universal primer. The nested PCR mixture made of 1µL aliquot of the first-round of PCR product in each of mix A and mix B, 1µl of each type specific primers (10 p mole) and 11µl ddH_2O. The nested PCRs were amplified for 40 cycles with the following parameters: initial denaturation at 95°C for 10 minutes, 20 cycles of amplification at 94°C for 20 seconds, 58°C for 20 seconds, and 72°C for 30 seconds, and 20 cycles of 94°C for 20 seconds, 60°C for 20 seconds, and 72°C

for 30 seconds. Amplicon products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and evaluated under UV light.

Table (2): Primer sequences used for HBV genotyping by nested PCR

Name primer		Sequences	Position
B2- sense	Set 1 (A,B,C)	5`-GGC TCM AGT TCM GGA ACA GT-3	67–86
A- antisense		5`-CTC GCG GAG ATT GAC GAG ATG T-3	113–134
B- antisense		5`-CAG GTT GGT GAG TGA CTG GAG A-3`	324–345
C- antisense		5`-GGT CCT AGG AAT CCT GAT GTT G-3`	165–186
D- sense	Set 2 (D,E,F)	5`-GCC AAC AAG GTA GGA GCT-3 `	2979–2996
E- sense		5`-CAC CAG AAA TCC AGA TTG GGA CCA-3 `	2955–2978
F- sense		5`-GYT ACG GTC CAG GGT TAC CA-3`	3032–3051
B2R		5`-GGA GGC GGA TYT GCT GGC AA-3`	3078–3097

STATISTICAL METHOD

Data analysis was done using SPSS program version 20 (SPSS Inc., Chicago, IL, USA). Quantitative data were presented as means and stander divisions whereas nominal data was presented as numbers and percentages. Chi-square test was used for verifying existence of associations. P values ≤0.05 were considered statistically significant.

RESULTS

The mean age of HBV patients was 32.64± 7.67 years. Most of CHB patients (24, 48%) were at age group 20-30 years old and only two (4%) CHB patients at age group >50 years old. Majority of CHB patients were males (38, 76 %) table (3). Distribution of HBV genotypes among study population is shown in table (4). Out of 50 patients, 30 patients (60%) had mixed genotypes, followed equally by genotype B and genotype D (8, 16%) and finally genotype A (4, 8%). Genotypes C, E, and F were not found alone but found in combinations with other genotypes. Mixed genotypes included A+B+C+D+E (20, 66.67%) followed by A+B+C+D+E+F genotypes (6, 20%) and finally genotypes A+B+C+D (4, 13.33%.). The association between HBV genotypes and certain risk factors is shown in table (5). The association between the blood transfusion and HBV genotypes was found to be statistically significant (χ 2= 13.06; p = 0.005). However, the surgical and dental procedures had no association with HBV genotypes in study groups (χ 2= 3.96; p = 0.27; χ 2= 1.39; p = 0.71, respectively).

DISCUSSION

HBV infection is a major health problem in Yemen with intermediate to high endemicity of hepatitis B[34]. HBV genotypes have attracted more attention since they may affect the disease progression and outcomes of HBV-related chronic liver disease, in addition to patient's response to antiviral treatments [35.] Therefore, this study focuses on evaluating the prevalence of the HBV genotype in Yemen. This molecular genotyping of hepatitis B virus was the first of its kind in Yemen using a polymerase chain reaction (PCR)-based method, and no data on hepatitis B virus genotypes and mutations in hepatitis patients have been previously reported. However, there was a previous study in genotyping of HCV conducted in Yemen [36].

The mean age of the studied group is 32.64 ± 7.67 years, which means that the registered patients were born before the implementation of the national program for neonatal hepatitis B vaccination in Yemen. The majority of CHB patients were predominantly men (38, 76%) versus women (12, 24%). The tendency of hepatitis B infection to be more common in males than females may be because males are exposed to risk factors more frequently than females. Other studies from Yemen[7-10], Saudi Arabia [37], Bahrain[20], Rwanda [38] and Pakistan[39] reported that hepatitis B infection is more prevalent among males than females.

The current study revealed that the majority of Yemeni patients with CHB are infected with multiple HBV genotypes. CHB patients with mixed infection had four (A/B/C/D) to six (A/B/C/D/E/F) different HBV genotypes which might indicate co-infection or superinfection with different genotypes. The most common mixed genotypes were A + B + C + D + E while the least common mixed genotypes were A + B + C + D + E + F. Genotypes B, D and A were found to be mono-infection among CHB patients while Genotypes C, E, and F were only found in combinations with other genotypes. This result was similar to that described by Rashid and Saleh, 2014 [40], who found all Iraqi patients in their study had mixed infections[40]. However, our result differs from that reported from different countries around the world which reported that many patients are affected mainly by one genotype [16,41-45]. For example, a study from Saudi Arabia, the country bordering Yemen, found genotype D to be the most common genotype among Saudi patients with CHB[46]. It also differs from that reported in the UAE which reported that many Emirati patients with viral hepatitis are commonly infected with

either genotype D or A[47]. Moreover, the Egyptian study revealed that all CHB patients had genotype D in which sub-D1 genotype was dominant[48].

With regard to risk factors, blood transfusion was found to be significantly associated with transmission of HBV genotypes in which patients may be exposed to co-infection or superinfection by transfusion of contaminated blood. No surgical history or dental operation was found to be significantly associated with HBV genotypes infection. A blood transfusion may result in mixed infection in recipients if blood from donors who are carriers of hepatitis B virus are not tested or tested using low-sensitivity laboratory techniques[8-10].

Limitation of this study was the relatively small sample size. Only 50 patients were tested for HBV genotypes, which may not represent the accurate picture of HBV genotypes among HBV patients.

CONCLUSIONS

In conclusion, this study demonstrates the general prevalence of hepatitis B virus genotypes among HBV-infected Yemeni hepatitis B patients who seek medical attention in a hospital. In mono-genotype HBV infection, genotype B and D were the most prevalent genotypes. In HBV mixed genotype infection, the A/B/C/D/E genotype was the most prevalent in the study area. In the future, clinical trials and treatment regimens should be assumed individually based on genotype to effectively manage chronic HBV infection. To this end, a prospective nationwide population study of HBV genotype distribution and clinical outcomes is recommended. Also testing blood donors with highly sensitive tests is necessary to avoid co-infection and super-infection with HBV.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available. Anyone interested can get upon reasonable request from corresponding author.

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AUTHORS' CONTRIBUTIONS

EMA, AMA, AMO, HAA and SSB contributed equally to the design, implementation, statistical analysis and manuscript drafting. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

No conflict of interest associated with this work.

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Table 3. Characteristics of chronic hepatitis B patients

		No.	%
Gender	**Males**	38	76
	Females	12	24
	Total	50	100
Age group (year)	Age mean ± SD = 32.64 ± 7.67		
	20 – 30	24	48
	31 – 40	21	42
	41 – 50	3	6
	>50	2	4
	Total	50	100
AST	Normal	20	40
	High	30	60
ALA	Normal	20	40
	High	30	60

*SD: standard deviation

Table 4: Distribution of HBV genotypes in CHB* patients

Genotype	Frequency	%
A	4	8
B	8	16
D	8	16
Mix	30	60
Total	**50**	**100**
Mix		
A+B+C+D	4	13.33
A+B+C+D+E	20	66.67
A+B+C+D+E+F	6	20
Total	**30**	**100**

Table 5: Association between HBV genotypes and risk factors among CHB patients

Risk factor		Type of genotypes								Total		χ 2*	*P***
		A		B		D		Mix					
		No	%	No	%	No	%	No	%	No	%		
Blood transfusion	**Yes**	4	20	2	0	2	10	8	40	20	40	13.06	0.005
	No	0	0	6	20	6	20	22	73.3	30	60		
	Total	**4**	**8**	**8**	**16**	**8**	**16**	**30**	**60**	**50**	**100**		
Surgical procedure	**Yes**	0	0	2	20	0	0	8	80	10	20	3.96	0.27
	No	4	10	6	15	8	20	22	55	40	80		
	Total	**4**	**8**	**8**	**16**	**8**	**16**	**30**	**60**	**50**	**100**		
Dental procedure	**Yes**	0	0	0	0	0	0	2	100	2	4	1.39	0.71
	No	4	8.3	8	16.6	8	16.6	28	58.3	48	96		
	Total	**4**	**8**	**8**	**16**	**8**	**16**	**30**	**60**	**50**	**100**		

χ^{2*}: Fisher exact ** *P*-value: probability value $p \leq 0.05$ (Significant)