Original Article

Prevalence of Hepatitis B virus genotype D in females in Karachi, Pakistan

Samina Noorali[1], Shazia Tabassum Hakim[2,3], David McLean[1], Shahana U. Kazmi[3], Omar Bagasra[1]

[1]*Department of Biology, Claflin University, 400 Magnolia Street, Orangeburg, South Carolina 29115, United States of America*
[2]*Department of Microbiology, Jinnah University for Women, 5-C Nazimabad, Karachi, Pakistan*
[3]*Department of Microbiology, University of Karachi, Karachi-75270, Pakistan*

Abstract

Background: Hepatitis B virus (HBV) is an etiological agent of acute and chronic liver disease existing throughout the world. The high genetic variability of HBV genome is reflected by eight genotypes (A to H), and each genotype has characteristic geographical distribution, which is important epidemiologically. Previous studies from the province of Sindh, Pakistan, have reported that genotypes A and D as prevalent HBV genotypes. The aim of the study was to investigate the prevalence of HBV genotypes in physically healthy females at two universities in Karachi, Sindh, Pakistan.

Methodology: Blood was collected from a total of 4,000 healthy female volunteer students and serum samples obtained were screened for Hepatitis B surface antigen (HBsAg), and anti-HBs antibodies by immunochromatography and ELISA. Genotyping was conducted for 6 HBV genotypes (A through F). Both genotyping and sequencing data of HBV positive females are described.

Results: Out of 4,000 volunteers, 180 (4.5%) tested positive for HBsAg and 20 (0.5%) were positive for HBs antibodies. All 180 serum samples were genotyped by PCR and sequencing analyses was conducted for 21 samples. Out of 180 HBsAg positive samples, 150 showed a single HBV D genotype infection; 29 showed co-infection of genotypes B and D; and 1 exhibited co-infection of genotypes C and D. Twenty-one representative samples were selected randomly from genotypes B, D, and C for sequencing and each isolate clustered with respective reference genotype sequence, thus validating the genotyping strategy.

Conclusion: Genotype D appears to be the dominant genotype prevalent in Karachi's otherwise healthy female population.

Key Words: Hepatitis B virus genotypes, type-specific primer-based genotyping, sequencing

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Introduction

Human hepatitis B virus (HBV) is an etiological agent of acute and chronic liver disease prevalent throughout the world. The virus belongs to the Hepadnaviridae family. HBV has a circular, partially double-stranded DNA genome of approximately 3.2 kb. The highly compact genome contains four overlapping open reading frames (ORFs) encoding the envelope (pre-S1, pre-S2 and surface antigen HBsAg), core (preCore precursor protein, HBeAg and HBcAg), polymerase (HBPol) and X (HBX) proteins. The NCBI sequences of HBV genotype D shows a 33 bp deletion in the pre-S1 region that explains their smaller genomic size (3182 bp) relative to other HBV genotypes, *i.e.*, A, B, C, E, F, G, and H (3215 bp). Based on genomic nucleotide sequence divergence of greater than 8%, HBV has been classified into eight genotypes labelled A through H [1-4]. The worldwide HBV genotype prevalence follows human migration patterns across the continents with most genotypes showing distinct geographical distribution [5].

Genotype A is mainly prevalent in Northwestern Europe and North America [6]. Genotypes B and C are highly prevalent in Asia [7-10]. Genotype D has been found worldwide [11], but is predominant in the Mediterranean region, the Middle East, and South Asia [12-15]. Genotype E is restricted almost entirely to West Africa [16,17], and genotype F is found in Central and South America [18,19]. Genotype G is found in Europe, the United States, and Central America [3,20] and, genotype H has been found in Central America [4,20].

Previous studies in Pakistan have reported the prevalence of different HBV genotypes in different provinces of Pakistan [21] as well as in Karachi, a major city in the province of Sindh, Pakistan [14]. In this study, we investigated the prevalence of six HBV genotypes (A through F) in 180 HBV positive,

otherwise healthy females in Karachi. Our study shows that the prevalent HBV genotype was D. Further sequencing produced sequences for phylogenetic analysis of preS1 through S genes for 21 samples. The results showed that samples belonging to genotype B, genotype C, and genotype D grouped tightly and separately. Ultimately, the present study shows that genotype D is the prevalent HBV genotype in HBV positive, otherwise healthy females in Karachi, Pakistan.

Materials and Methods

Study participants

From March 2002 to October 2006, 4,000 blood serum samples were collected from physically healthy female student volunteers, aged 18 to 30 years from two Karachi universities, specifically, the Department of Microbiology, University of Karachi, and the Department of Microbiology, Jinnah University for Women. Signed informed consent forms were collected from all volunteers following Institutional Review Board policies of both universities. All volunteers were referred for health checkups by a medical doctor and were asked about their history of jaundice, blood transfusion, exposure to needles, and surgical and dental procedures. On completion of the medical checkups, volunteers were asked to give 10 ml of blood for different haematological tests, including complete blood picture (CP), haemoglobin percentage (Hb%) and erythrocyte sedimentation rate (ESR), as well as biochemical tests including direct bilirubin, indirect bilirubin, total serum bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). Samples were also subjected to serological analysis for hepatitis B surface Antigen (HBsAg) and HBs antibodies using rapid immunochromatography kits (ICT, Australia and Abbott, USA). A confirmatory test for HBsAg was done by using enzyme linked immunosorbant assay (ELISA, IMX, Abbott, USA). Out of 4,000 samples, 180 samples tested positive for HBsAg. All 6 commercially available vaccines labelled A - F which are in common use in Pakistan to vaccinate people against HBV were purchased from the manufacturers. Hence, a total of 180 HBV positive samples as well as 6 available HBV vaccines (as a positive control) obtained from Karachi were used for genotype evaluation. Specific ethnicity was not determined but we assume these study participants represent collection of different ethnic groups in Pakistan.

DNA extraction and amplification of 180 HBV samples

DNA was extracted from 200 µl serum samples using the PureLink™ Viral RNA/DNA Mini Kit according to the manufacturer's instructions (Invitrogen, CA). Amplification was conducted using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, UK).

Determination of HBV genotypes by nested PCR

The primer sets for first-round PCR and second-round PCR, PCR amplification protocol, and primers for both HBV genome and genotyping amplification for all 180 samples followed previously reported methods [22]. The first round amplification targeted 1,063 bp of HBV genome and this PCR product was used as a template for genotyping different HBV genotypes A to F. HBV A through HBV F genotypes for each sample were determined by separating the genotype-specific DNA bands on 3% agarose gels stained with ethidium bromide (Figures 1 and 2). The sizes of PCR products were estimated according to the migration pattern of a 50 bp DNA ladder (Promega, WI).

HBV genotype analysis based on nucleotide sequencing

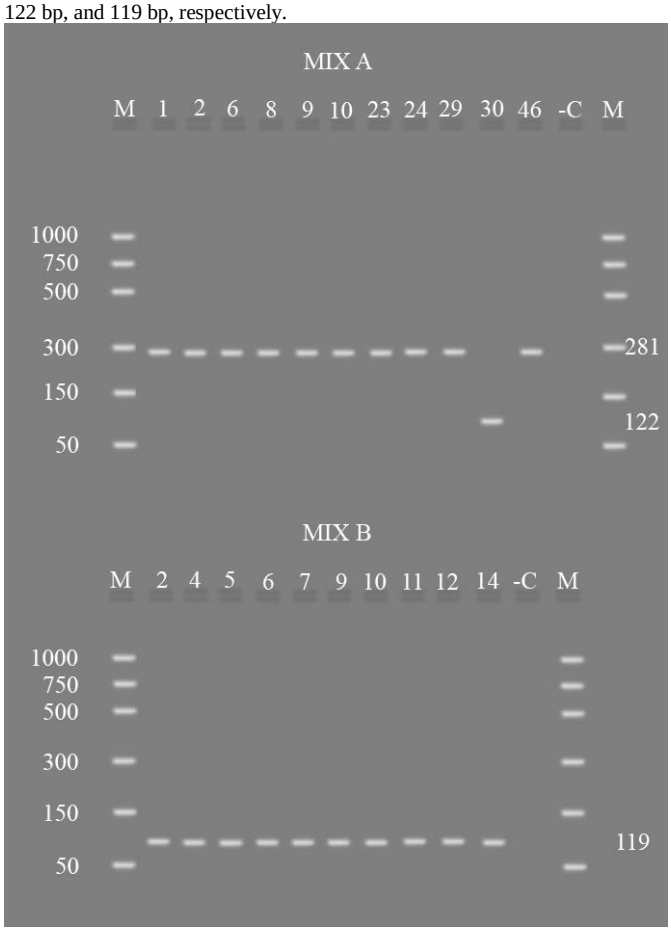
To confirm the PCR genotyping system, the first-round PCR products (1,063 bases) of 21 samples, randomly selected from 180 samples, were sequenced. The PCR products for sequencing were cleaned with Agencourt Ampure and Agencourt CleanSeq (Agencourt Bioscience Corporation, A Beckman Coulter Company, MA). Sequencing used 3.2 pmol each of sense and antisense second-round PCR primers and 70 ng of template. Cycle sequencing was performed with a thermal cycler (DNA Engine, Bio-Rad, CA) using the Big Dye Terminator Cycle Sequencing kit, version 3.1 (Applied Biosystem, CA). Sequence electrophoresis was conducted with an automated DNA sequencer ABI 3130 Genetic Analyzer (Applied Biosystem).

Sequence analyses

Reference DNA sequences of genotypes A to H (Figure 3) and putative HBV genotype specific sequences from 21 samples were aligned using CLUSTAL W (version 1.8). Distance matrices of the resulting multiple alignments were estimated by the eight parameter method [HKY, 23], by the Jukes-Cantor distance [24], and by the Kimura two-parameter distance [25]. Phylogenetic trees were constructed from these matrices by the neighbour-joining method [26]. All distance matrices and neighbour-joining trees were

computed using Phylo_Win (version 2.0) [27] and the resulting trees were displayed by TreeView (version 32). Phylogenetic tree nodes were tested by bootstrap analysis [28] of 500 replicates.

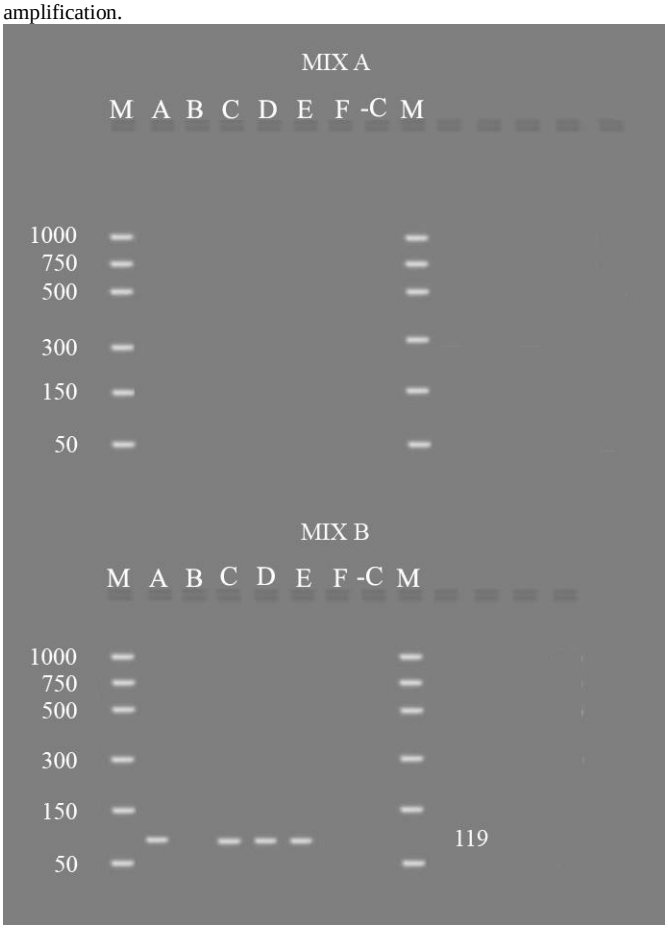
Figure 1. Electrophoresis patterns of PCR products from 3 different HBV genotypes as determined by PCR genotyping system. Genotypes B, C, and D showing amplification product of 281 bp, 122 bp, and 119 bp, respectively.



Results

Before screening for HBV status, all 4,000 healthy female volunteers from two Karachi universities, (specifically, the Department of Microbiology, University of Karachi, and the Department of Microbiology, Jinnah University for Women) were subjected to routine physical checkups for exclusion criteria. All 4,000 serum samples were screened by immunochromatography for the presence of HBsAg, anti HBs antibodies. Positive results were confirmed by ELISA. Out of 4,000 subjects, 180 (4.5%) tested positive for HB surface antigen (HBsAg) and 3820 (95.5%) were negative by both immunochromatography and ELISA. Out of these 180 (4.5%) individuals who tested positive for HBsAg, 20 (0.5%) were positive for anti-HB surface antibodies.

Figure 2. Electrophoresis patterns of PCR products from 6 different HBV vaccines as determined by PCR genotyping system. Vaccines labelled as A, C, D and E showed amplification of 119 bp corresponding to genotype D. Vaccines labelled as B and F showed no amplification.

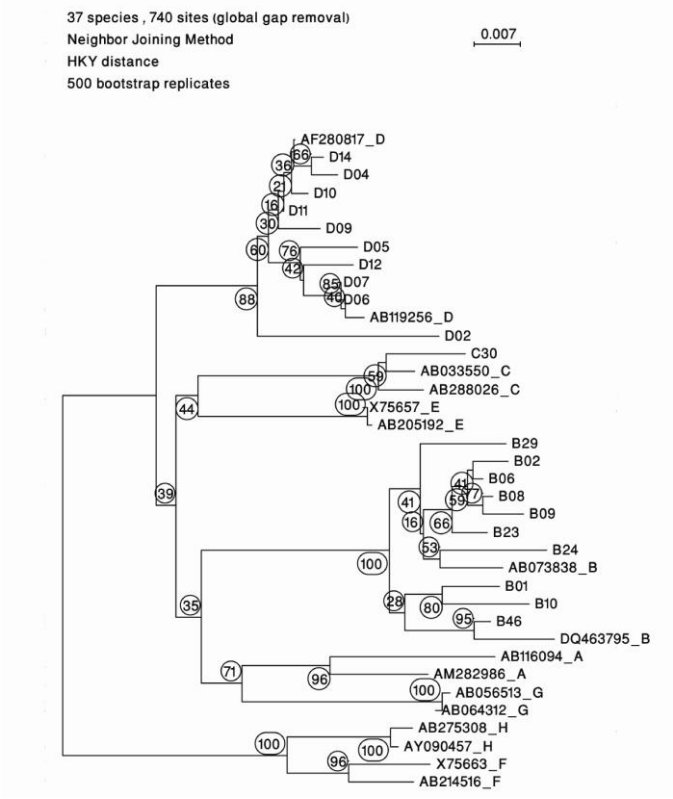


The haematological parameters (WBC count, RBC count, hematocrit and platelet count) of the 180 HbsAg-positive individuals were within the normal range, while mean Hb% was 9.8±1.6 g/dL. Direct bilirubin (0 to 0.3 mg/dL), indirect bilirubin (0.1 - 1.0 mg/dL), total serum bilirubin (0.3 to 1.9 mg/dL), ALT (0 - 36 U/L), AST (0 - 31 U/L) and alkaline phosphatase (20 - 125 U/L) were also within the normal range for 170 HbsAg-positive individuals, except for the raised ALT (>36 U/L) and AST (>31 U/L) levels in 10 participants with a previous history of jaundice who were also positive for HBsAg. All 180 samples that were positive for HBsAg were confirmed for the presence of HBV by PCR.

A total of 180 HBV positive samples and 6 HBV vaccines were genotyped at the Biotechnology Center,

Department of Biology, Claflin University, Orangeburg, SC, USA and Jinnah University for Women, Karachi, Pakistan. Primers of Mix A were targeted to amplify genotypes A, B and C, and the primers of Mix B were targeted to amplify genotypes D, E and F. Out of 180 HBV positive samples, 150 showed a single HBV D genotype infection; 29 showed co-infection of genotypes B and D; and 1 exhibited co-infection of genotypes C and D (Figure 1). Out of the 6 HBV vaccines, those labelled as A, C, D, and E showed amplification for genotype D, whereas vaccines labelled as B and F showed no amplification (Figure 2).

Figure 3. A phylogenetic tree of the HBV constructed by the eight parameter method [HKY, 23]. Reference sequence tags begin with the accession number and end with associated HBV genotype. For example, AF280817_D is the reference sequence AF280817 that is associated with the HBV D genotype. Sequences generated in this study were tagged with the HBV genotype and are arbitrary numerical sample identifiers. For example, the 10th sample was identified as HBV D and HBV B, labelled D10 and B10, respectively. Bootstrap values at the nodes are encircled.



Twenty-one representative samples were selected randomly from genotypes B (n = 10), D (n = 10), and C (n = 1) for sequencing. Of the 21 isolates, each clustered with respective reference genotype sequence, thus validating the genotyping strategy. Figure 3 shows a phylogenetic tree of 21 HBV samples for the pre-S1 through S genes, constructed using the eight parameter method. None of the sequences were clustered with the sequences of the genotypes A, E or F.

Out of 180 HBsAg-positive samples, 150 were genotype D (83.3%). Co-infection with HBV genotype B and D was seen in 29 (29/180 = 16.1%), and co-infection with HBV genotype C and D (1/180 = 0.55%) was seen in one. Participants who tested positive for HBV were contacted and were advised to seek appropriate medical care for their own benefit and for public health benefit.

Discussion

There is a wide variation in the prevalence of HBsAg worldwide. The carrier rate of HBsAg varies from 0.1% to 0.2% in Britain and in the USA, more than 3% in Greece and southern Italy, and up to 15% in Africa and the Far East [29]. Pakistan is highly endemic with HBV [30]. Studies are too limited to give a clear picture of the prevalence of HBV at the national level, especially among otherwise healthy individuals. Most previous studies targeted different small groups of individuals with some clinical indications, so they do not accurately reflect the overall prevalence in Pakistan.

In Pakistan alone, the prevalence of HBsAg has been reported as 9.97%, 10%, 3.1%, 0.99%, 1.11%, 4%, 3%, 3.2%, 3%, 4.3% and 6.5%, respectively, in different groups of individuals [31,29,32-39]. Our study showed the prevalence of HBsAg among otherwise healthy women to be 4.5%.

HBV genotyping is important to track the route and pathogenesis of the virus. In particular, the variants may differ in their patterns of serologic reactivity, pathogenecity, virulence, and response to therapy. HBV has genetic variations which correspond to their geographic distribution and has been classified into 8 genotypes (A to H) on the basis of whole genome sequence diversity of greater than 8 %.

In this study, genotyping was conducted for 6 HBV genotypes (A through F). This study suggests that the HBV D genotype is ubiquitous (100%) among otherwise healthy females in Karachi, Sindh, Pakistan. Two earlier studies conducted for the prevalence of HBV genotypes in known hepatitis B positive patients in Pakistan report the prevalence of genotypes HBV A (68%) [21] and HBV D (100%) [14] in province of Sindh. Interestingly, these findings respectively contradict and corroborate the HBV genotype distributions reported here as the subjects in this study were asymptomatic. Out of 180 subjects positive for HBsAg, 170 were not aware of their HBV status, except for 10 who did report previous history of jaundice. In

the nearby north Indian population, HBV D was reported as the predominant genotype (75%) in patients diagnosed with chronic liver disease (CLDB) [40].

We also show that out of the 6 commercially available vaccines labelled A - F which are in common use in Pakistan to vaccinate people against HBV, 4 (A, C, D, and E) showed amplification for genotype D, indicating that these vaccines are also targeted against genotype D, which is the prevalent HBV genotype in Pakistan.

We also saw mixed HBV infections of genotypes B and D as well as of C and D (16.1% and 0.55%, respectively). Furthermore, phylogenetic analysis of the pre-S1 through S genes clearly separated genotype clusters indicating that genotypes for HBV can be assigned based on the pre-S1 through S genes.

In conclusion, genotype D appears to be the dominant genotype prevalent in the otherwise healthy female population of Sindh, Pakistan, and genotype B appears to be the next most prevalent genotype.

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Corresponding Author: Omar Bagasra, Department of Biology, Claflin University, 400 Magnolia Street, Orangeburg, South Carolina 29115, USA
Tel: (803) 535-5253; Fax: (803) 535-5776
E-mail: obagasra@claflin.edu

Conflict of interest: No conflict of interest is declared.