

STUDY OF GENOTYPES OF HEPATITIS B VIRUS PREVALENT IN KARACHI, PAKISTAN

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ABSTRACT

Hepatitis B virus (HBV) is one of the major causative agents of acute and chronic liver disease worldwide and is believed to be responsible for a million deaths annually. Around 400 million people worldwide carry the virus including 4.5 million only in Pakistan. On the basis of a comparison of complete genomic sequences, HBV has been classified into eight genotypes A-H which show a geographical distribution. Some genotypes are associated with different clinical outcomes. Identification of HBV genotypes is important to begin and follow up the treatment.

Very limited research work on HBV genotyping has been done so far in Pakistan and there is a high level of discrepancy and inconsistency among different groups of researchers. The aim of present study was to determine the prevalent genotypes of HBV in Karachi, Pakistan.

Samples from different physicians and hepatologists of Karachi were sent to PCR and molecular biology lab., KIRAN for HBV DNA detection by PCR methods. A total of 40 HBV DNA PCR positive samples (30 male and 10 female) were tested for genotyping by two step nested polymerase chain reaction (PCR). Genotype D was detected as the most prevalent (92.5 %) genotype followed by mix genotype A & D (7.5 %), no other genotype were found in this limited size of samples.

Key words: hepatitis B virus genotype, HBV genotype D, Pakistan.

INTRODUCTION

Hepatitis B virus (HBV) infection is becoming a serious health threat in many developing countries like Pakistan and it has become the leading cause of acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. In Pakistan Hepatitis B virus (HBV) infection is not only a major health problem leading to significant morbidity and mortality but also catastrophic for its poor economy. In Pakistan, HBV infection rate is increasing day by day. The reason may be the lack of proper health facilities or poor economical status and less public awareness about the transmission of major communicable diseases like Hepatitis B, Hepatitis C and Human Immunodeficiency syndrome (Hanif *et al.*, 2009; Alam *et al.*, 2007a,b).

Genotyping of HBV is essential for characterization of patient groups and for epidemiological studies. The clinical significance of different HBV genotypes has become increasingly recognized in patients with acute and chronic infection. The course of HBV infection depends on several factors such as host genetic factors, age and genetic variability of the virus (Gunther *et al.*, 1999; Hunt *et al.*, 2000). In addition to the epidemiological importance, these genotypes may influence the disease pattern and response to treatment.

Ten HBV genotypes (A-J) have been discovered so far (Zhang and Cao, 2011) and are known to have a distinct pattern of geographic distribution: Genotype A is found in North America and Northern Europe, as well as in some parts of Africa (Bowyer *et al.*, 1997) where as genotype B and C are common in Southeast Asia and genotype D is found universally (Okamoto *et al.*, 1988). Genotype E has been reported from western and Southeast Africa (Bowyer *et al.*, 1997). Genotype F has been detected in South and Central America (Bowyer *et al.*, 1997) and genotype G has been reported in France and North America (Stuyver *et al.*, 2000). In addition to these genotype I (Hannoun *et al.*, 2000) and J (Tatematsu *et al.*, 2009) have been reported as variants different from above mentioned from Vietnam and Japan respectively. The data on distribution of HBV genotype is still emerging in Asia.

Very limited research work on HBV genotyping has been done so far in Pakistan and there is a high level of discrepancy and inconsistency among different group of researchers has been noted (Ahmed *et al.*, 2009). For example, Idrees *et al.* (2004) reported that genotype C is the most pre-dominant, while genotype A was predominant in Sind Province, genotype C in North West Frontier Province (Idrees *et al.*, 2004) and genotypes B and C in Punjab. In another study by Abbas *et al.* (2006) it was reported that genotype D is predominant genotype in Karachi (Abbas *et al.*, 2006). None of the abovementioned studies have performed whole genome or partial genome sequencing of HBV (Ahmed *et al.*, 2009).

In the present study, 40 HBV DNA PCR positive patients from KIRAN Hospital Karachi were selected for genotyping by two step PCR method described by Hideo *et al.*, (2001) with some modifications in cycling profile and PCR constituents.

MATERIALS AND METHODS

Samples from different physicians and hepatologists of Karachi were sent to PCR and molecular biology lab, KIRAN for HBV DNA detection by PCR methods. Genotyping of 40 HBV DNA PCR positive samples were carried out by two step PCR method as described by Hideo *et al.*, (2001). At the same time, data including date, age, sex and location were collected in KIRAN (Table 1). Further testing including liver function test (LFT) and ELISA based detection of Hepatitis B surface antigen (HBsAg) was conducted according to manufacturer's protocols.

HBV DNA extraction

Sample preparation from EDTA plasma and serum was performed following the manufacturer's instructions using "INSTANT Virus DNA kit" (AJ Roboscreen, analytikajena Biosolutions, GmbH, Germany) where a synthetic internal control (IC) is included via extraction tubes to control DNA extraction and to indicate the inhibitory effects. DNA was eluted in 60µl elution buffer.

HBV genotyping

Genotyping was done following the two step PCR method described by Hideo *et al.*, (2001) with some modifications in cycling profile and PCR constituents. DNA material extracted for regular PCR detection was used as template in regular PCR after 1:10 dilution. For regular PCR *GoTaq*® Green Master mix (Promega) 10µl, universal primers (P1A and S1-2) 1µl each, ddH₂O 2µl, diluted template 6µl. The cycling conditions were first incubation at 95 °C for 10 min, then 30 cycles of 94 °C for 20 sec, 55 °C for 20 sec and extension at 72 °C for 60 sec. Multiplex nested PCR was performed in two mixes. Mix 1 comprised of primers for genotype A, B and C while mix 2 contained primers for genotype D, E and F. Each mix contained PCR DNA template from regular PCR product 2µl, *GoTaq*® Green Master mix 08µl, primers 1µl each, ddH₂O 8µl. The cycling conditions were first incubation at 95 °C for 10 min, then 40 cycles of 94 °C for 45 sec, 63 °C for 20 sec and extension at 72 °C for 60 sec (ABI 9700). PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and evaluated under UV light (BioRad Gel Doc-XR, USA). The sizes of PCR products were estimated according to the migration pattern of a 50-bp DNA ladder (GeneRuler™ Fermentas) (Fig.1).

RESULTS

A total of 40 HBV DNA positive samples from KIRAN, Karachi were tested for genotyping. The demographic data shows that 30 samples were male and 10 were female patients, age range was 4-70 years with median age of 34.6 years (Table 1). The alanine amino transferase (ALT/SGPT) level of 98% sample was abnormally elevated with mean level of 82 U/L (normal range is 10-40 U/L), all samples were HbsAg positive.

Genotyping:

All samples were tested for HBV DNA by PCR before genotyping by two step PCR method. Among 40 HBV DNA positive samples Genotype D was detected as the most prevalent genotype (37/40) followed by mix genotype A & D (3/40) (Table 1). No other genotype was detected among these samples.

DISCUSSION

The initial studies on HBV genotyping revealed that genotypes B and C are the most prevalent genotypes in Asian regions. It was because of the fact that all such studies were reported from Japan and China where genotype B and C are the most prevalent genotypes. Later on, it was found that all the seven HBV genotypes can be found in Asia (Toan *et al.*, 2006). For instance, the predominant genotypes in India are genotype A and D (Thakur *et al.*, 2002). The predominant HBV genotypes in Afghanistan were found to be genotype D (Amini-Bavil-Olyae *et al.*, 2006). Similarly, Zeng *et al.*, (2004) reported 1.6% patients infected with multiple HBV genotypes. In Pakistan, studies from Karachi showing predominant genotype D (Noorali *et al.*, 2008; Baig *et al.*, 2007; Alam *et al.*, 2007a), study from North West Frontier province also showing genotype D (Alam *et al.*, 2007b) but study from Punjab is showing genotype C as predominant (Idrees *et al.*, 2004). From Islamabad highest prevalence of genotype D followed by genotype B has been reported (Alam *et al.*, 2007b).

Table 1. Demographic, biochemical and HBV DNA PCR (Genotyping) data.

	No. of Patients	TOTAL (%)
Total	40	40
Male	30	30 (75%)
Female	10	10 (25%)
Median age (years)		35 (range 7-70)
Median ALT U/L	82	82 (10-488)
S Antigen positive	40	40 (100)
S Antigen Negative	0	0 (0)
HBV DNA positive	40	40 (100)
Genotype D	37	(92.5%)
Mix Genotype A&D	3	(6.5%)

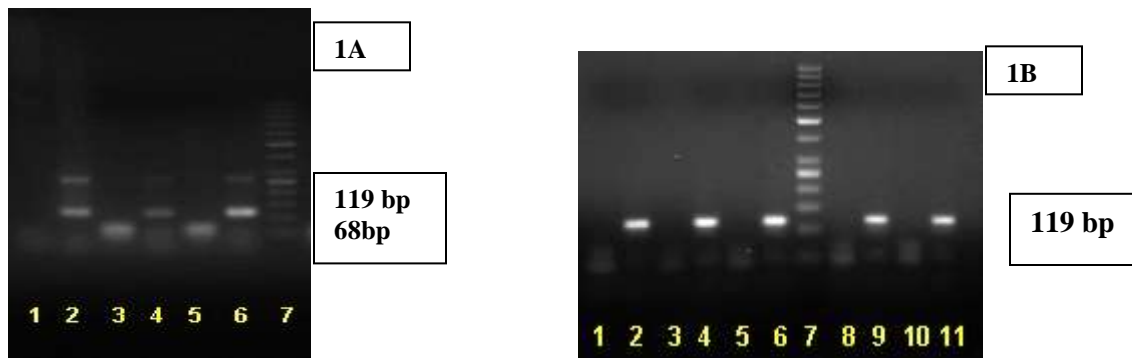


Fig.1A. Lane 1, 3, 5 are mix 1, lane 2, 4, 6 are mix 2, lane 7 is GeneRuler™50bp ladder (Fermentas. #SM0373). Patient 1 (lane 1 & 2), patient 2 (lane 3 & 4) and patient 3 (lane 5 & 6) are of mix genotype of A and D.

Fig.1B. Lane 1, 3, 5, 8, 10 are mix 1 (No band), lane 2, 4, 6, 9, 11 are mix 2 (119bp, genotype D), lane 7 is GeneRuler™50bp ladder (Fermentas. #SM0373).

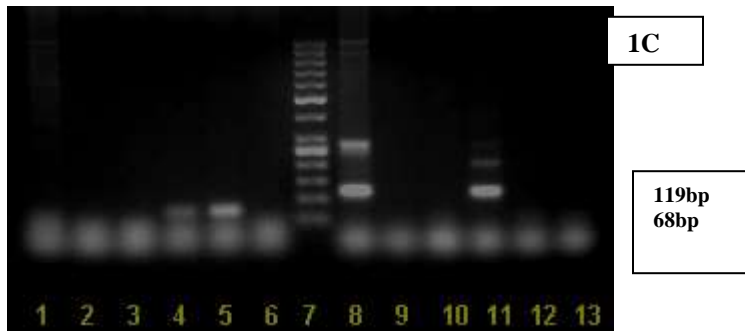


Fig.1C. Lane 1-6 are mix 1, lane 8-13 mix 2, lane 7 is GeneRuler™50bp ladder (Fermentas. #SM0373), Lane 6 and 7 are reagent controls. Lane no. 2 and 9 is negative control.

A 68 bp band is seen in Lane 4 & 5, and a 119 bp band is clear in Lane 8 and 11 showing that patient no. 1 is a D genotype, patient 4 is of mix genotype, and patient 5 is of genotype A.

Fig 1. Agarose 2 % gel electrophoresis of PCR products genotyping of HBV.

Majority of our patients had genotype D (92.5%) and some are coinfecting with type A (7.5%). This pattern is similar when compared to other studies in Pakistan (Noorali *et al.*, 2008; Baig *et al.*, 2007; Abbas *et al.*, 2006; Idrees *et al.*, 2004) except the study from Punjab, Lahore (Idrees *et al.*, 2004). In south east Asia, genotypes B and C are more prevalent (Wong and Chan, 2003) but in India mixed pattern of genotypes A, C and D is observed (Gandhe *et al.*, 2003; Kumar *et al.*, 2005). There are therapeutic implications of these findings as patients with genotype D have more severe disease (Kao *et al.*, 2002) and are less responsive to interferon therapy as compared to genotype A and B (Sablón and Shapiro, 2004; Erhaedt *et al.*, 2000) and have higher HBV DNA levels (Westland *et al.*, 2003).

Genotypes are important for designing effective treatments (Ding *et al.*, 2003) and determining the genotype-specific mutations and antiviral drug resistance. The influence of genotypes on liver disease progression (Sablon and Shapiro, 2004, 2005) has already been recognized, therefore, the determination of prevalent genotypes will certainly help in the treatment of patients, studying the antiviral treatment response in the population and disease progression, and controlling and ultimately eradicating HBV from the Pakistani population.

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