

Full Length Research Paper

Molecular characterization of hepatitis B virus (HBV) genotypes in HBsAg positive individuals of Khyber Pakhtunkhwa, Pakistan

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Hepatitis B virus (HBV) is a crucial health problem with up to 350 million people infected globally. The epidemiological significance of HBV genotypes has been well established however, no such large scale data available for HBV genotypes and much little is known about the mixed infection with more than one HBV genotypes. The main aim of the present study was to determine the molecular characterization of HBV genotypes in HBsAg positive patients in Khyber Pakhtunkhwa, Pakistan. A total of 713 HBsAg positive individuals were included in the present study. All the samples were confirmed for HBV DNA with nested polymerase chain reaction (PCR) and HBV DNA positive samples were further processed for HBV genotypes with type specific primers. This study demonstrated that genotype A (33.66%) is the predominant genotype followed by genotype D (29.5%), genotype C (2.10%), genotype F (1.40%) and mixed genotypes A+D (10.52%) while 5.9% of the samples were untypable. Genotype B and E was not found in this study. The current study shows the high frequency of genotype A and a heterogeneous distribution of HBV genotypes. Further study needs to investigate genetic and geographical divergence and characteristics of the virus in this area especially.

Key words: Hepatitis B virus, HBsAg, HBV DNA, genotype, characterization.

INTRODUCTION

Hepatitis B virus (HBV) is the most common infection with approximately one third of the world population has been infected with this infection (Kevin and Leonard, 2011). Chronic HBV infection is common by an estimate in 350 million persons globally, and carriers of HBV are at increasing risk of developing cirrhosis, hepatic decomposition and hepatocellular carcinoma (Jose et al., 2012). HBV is the smallest human DNA virus, with 3200 nucleotides genome (Kao et al., 2011). HBV is transmitted through blood and blood products, although sexual transmission and intrafamilial transmission have also been reported (Rauf et al., 2010).

The evolution of HBV has led to the present existence of various genotypes, sub genotypes, mutants, recombinants, and even quasispecies of HBV (Kao, 2002). At present HBV can be classified into 9 genotypes from A to I (Santos et al., 2010; Yu et al., 2010) based on a nucleotide divergence in the entire genome of at least 8%, with specific and characteristic geographical distributions, but most have a worldwide prevalence because of human migration (Jose et al., 2012). Genotype A can be regarded as pandemic but is most commonly found in Northern Europe, North America and Central Africa, while genotype B predominates in Asia (China, Indonesia and Vietnam). Genotype C is found in the Far East in Korea, China, Japan and Vietnam as well as the Pacific rim and Island Countries, while genotype D, which is also more or less pandemic, is found in the Mediterranean countries, the Middle East extending to India, North America and

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parts of the Asia-Pacific region. Genotype E is related to Africa while genotype F is found predominately in South America, including among Amerindian populations, and also Polynesia. Genotype G has been found in North America and Europe while genotype H has been reported from America (Alam et al., 2007a), but recently two genotypes, "I" in China, Vietnam and Laos (Santos et al., 2010; Kao et al., 2011) and "J" in Japan, were identified (Kao et al., 2011).

According to WHO, Pakistan, falls in the low endemic area of HBV infection with prevalence of 3% infected population. Studies regarding HBV infection from Pakistan focused more towards the HBV prevalence rate, epidemiological issues, genotyping of most prevalent strains and its genetic variability regarding core region (Baig et al., 2007). HBV infection rate in Pakistan is increasing day by day. Awan et al. (2010) reported approximately 38% prevalence with a 4% carrier rate and 32% with anti-HBV surface antibodies by natural conversion (Khan et al., 2011). The reason may be the lack of proper health facilities or poor economical status and less public awareness about the transmission of major communicable disease like HBV, HCV and HIV (Alam et al., 2007a, b). Because HBV genotypic determination is of particular importance for the study of the detection of the virus's origin, course of evaluating HBV, the severity and activity of liver disease, prognosis and response to antiviral treatment, patterns of serological reactivity and replication of the virus, the present study was designed to determine the prevalence of HBV genotypes in the Khyber Pakhtunkhwa Province of Pakistan.

MATERIALS AND METHODS

Study samples

This study was carried out on HBsAg positive patients from September 2011 to January 2012 in seven divisions: Dera Ismail Khan (D.I. Khan), Bannu, Kohat, Peshawar, Mardan, Hazara and Malakand of Khyber Pakhtunkhwa, Pakistan.

A total of 713 blood samples were collected from HBsAg positive male and female patients, with age 01 to 70 years. Informed consent forms were signed and collected from all volunteers following Institutional Review Board policies of the respective institutes. A 3 ml blood sample was collected in a vacutainer from each patient involved in the study. Sera were separated and stored at -20°C in the Molecular Parasitology and Virology Laboratory, Department of Zoology, Kohat University of Science and Technology Kohat, Pakistan for further processing. For reducing contamination, standard procedures were strictly followed. For the detection of HBV DNA and HBV genotyping all the samples were analyzed.

Biochemical analysis

The liver function tests (LFTs) especially Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), were performed (two readings for each patient) for six months using Microlab 300 (Merck USA) using ALT and AST kit (Diasys Diagnostic System Germany) as described in manufacturer's manual.

HBV DNA detection

DNA extraction

DNA was extracted from 100 µl of HBsAg positive serum, using GF-1 nucleic acid extraction kit (Vivantas USA) according to manufacturer's instructions.

DNA amplification and detection

PCR reactions were carried out in a thermal cycler (Nyxtechnik USA) with 5U *Taq* DNA polymerase (Fermentas USA). The first round of amplification was performed with 5 µl of extracted DNA by using an outer sense primer and an outer antisense primer specific to the surface gene of HBV. Another round of PCR was carried out with inner sense primer and inner antisense primer. Amplified products were subjected to electrophoresis in 2% agarose gel and evaluated under UV transillumination. The 185 bp specific amplified HBV DNA product was determined by comparing with the 50 bp DNA ladder (Fermentas USA), used as DNA size marker.

HBV genotyping

For HBV genotypes determination, the same procedure was followed as described by Naito et al. (2001).

RESULTS

713 HBsAg positive individuals with age of 1-70 years including 489 (68.6%) males and 224 (31.42%) females (Male to Female ratio 2.18:1) were analyzed in this study. Of the total, 419 male and 173 female were confirmed for HBV DNA while in 70 male and 51 female patients HBV DNA was not detected. The confirmed 592 HBV DNA samples were further processed for genotyping. The gender wise distribution of genotypes in all the HBV DNA positive patients of Khyber Pakhtunkhwa is shown in Table 1.

Out of the 592 HBV DNA positive samples analyzed, 550 (92.91%) showed genotype specific bands for genotype A, C, D, F and A+D, while the remaining 42 (7.09%) were untypable. The HBV infection in this study in HBsAg positive patients were attributed predominantly to viral genotype A constituted 240 (33.66%) of the total individuals. Genotype D was the second prevalent with 210 (29.5%), followed by genotype C 15 (2.10%) and genotype F 10 (1.40%). Mixed genotypes A+D were detected in 75 (10.52%) samples (Figure 1), while genotypes B and E were not found in this study. The highest prevalence of genotype A (70%) was found in Mardan Division and that of genotype D (60%) in D.I. Khan Division while the untypable (15%) patients were mostly found in Peshawar Division and the mixed genotype was not found in Mardan Division. The genotype C was found in Hazara Division (5%) and Malakand Division (10%) while genotype F (10%) was found in Hazara Division only (Table 1).

The prevalence of genotypes was assessed further with respect to patient's age. The high frequency of all genotypes, A (39.58%), D (40.48%), F (50%) and A+D

Table 1. Gender and division wise distribution of HBV genotypes in HBV DNA positive patients of Khyber Pakhtunkhwa, Pakistan.

Genotype gender	D.I. Khan n (%)	Bannu n (%)	Kohat n (%)	Peshawar n (%)	Mardan n (%)	Hazara n (%)	Malakand n (%)	Total n (%)
Genotype A								
Male	-	30(12.5)	45(18.75)	25(10.42)	45(18.75)	5(2.08)	12(5.0)	162(27.4)
Female	10(4.17)	-	10(4.17)	20(8.33)	25(10.42)	5(2.08)	8(3.33)	78 (13.2)
Genotype C								
Male	-	-	-	-	-	5(33.33)	7(46.67)	12(2.03)
Female	-	-	-	-	-	-	3(20.0)	3(0.51)
Genotype D								
Male	45(21.43)	25(11.9)	10(4.76)	10(4.76)	-	25(11.9)	35(16.67)	150(25.34)
Female	15(7.14)	15(7.14)	5(2.38)	-	-	15(7.14)	10(4.76)	60(10.14)
Genotype F								
Male	-	-	-	-	-	5(50.0)	-	5(0.84)
Female	-	-	-	-	-	5(50.0)	-	5(0.84)
Mix genotype A+D								
Male	10(13.33)	10(13.33)	10(13.33)	10(13.33)	-	10(13.33)	10(13.33)	60(10.14)
Female	5(6.67)	-	-	5(6.67)	-	-	5(6.67)	15(2.53)
Untypable								
Male	2(4.76)	1(2.38)	3(7.14)	10(23.81)	9(21.43)	3(7.14)	2(4.76)	30(5.1)
Female	-	2(4.76)	-	5(11.9)	3(7.14)	2(4.76)	-	12(2.03)

n = 592 [Male 419 (70.8%) and Female 173 (29.22%)].

(40%) was found in the age group of 16-30 years. However, in individuals aged more than 60 years, genotype A 10 (4.17%) and D 15 (7.14%) was found and no other genotype, mixed genotype or untypable was found in this age group. Genotype C 15 (100%) was found only in the age group of 46-60 (Table 2).

DISCUSSION

HBV infection is a global health problem with its

continuously increasing burden on the developing countries like Pakistan (Khan et al., 2011). Very limited data on HBV epidemiology and pattern of transmission representing all the geographical regions of Khyber Pakhtunkhwa is available. To investigate the epidemiological distribution of HBV genotypes in the Khyber Pakhtunkhwa, we applied nested PCR with type specific primers. HBV genotypes have different biological and epidemiological behavior (Attaullah et al., 2011). Since they influence the activity and outcome of

HBV-associated chronic liver disease, as well as the response to antiviral therapies (Zhu and Dong, 2009; Eftikhari et al., 2010; Khaled et al., 2010), their detection and monitoring is more than just academic but also medically significant. Therefore HBV genotyping become a routine exercise in clinical medicine and molecular epidemiology (Khaled et al., 2010). Since several genotypes HBV are very closely associated with the severity, development of severe liver diseases (cirrhosis and hepatocellular carcinoma) and antiviral

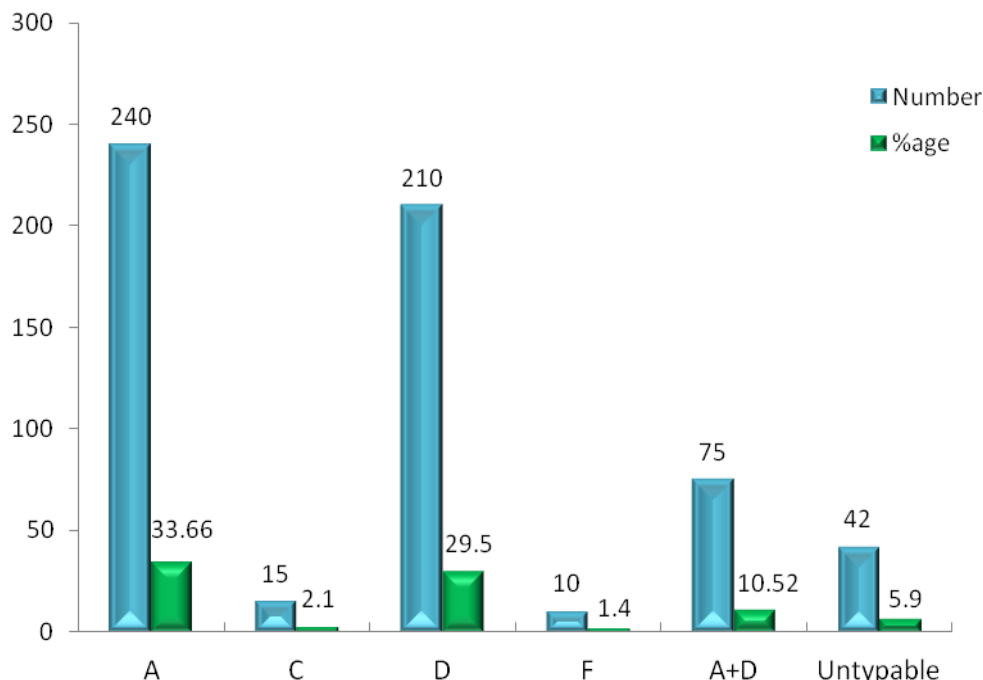


Figure 1. HBV genotypes distribution in HBsAg positive patients.

Table 2. Age wise distribution of genotypes in HBV DNA positive patients (n = 592).

Age (in years)	Genotype						Total n(%)
	A n(%)	C n(%)	D n(%)	F n(%)	A+D n(%)	Untypable n(%)	
1-15	25 (10.42)	-	35 (16.67)	-	10 (13.33)	3 (7.14)	73 (12.33)
16-30	95 (39.58)	-	85 (40.48)	5 (50.00)	30 (40.00)	23 (54.8)	238 (40.20)
31-45	85 (35.42)	-	45 (21.43)	5 (50.00)	10 (13.33)	12 (28.6)	157 (26.52)
46-60	25 (10.42)	15 (100)	30 (14.29)	-	25 (33.33)	4 (9.52)	99 (16.72)
>60	10 (4.17)	-	15 (7.14)	-	-	-	25 (4.00)

therapy. Detection of HBV genotypes is also very important to clarify the pathogenesis, route of infection and virulence of the virus (Dokanehiifard and Bidmeshkipour, 2009).

Initially HBV genotypes were analyzed in Japan and China, where genotype B and C were considered as the most prevalent genotypes and predominant of genotypes D in South Asia and the Middle East including India, Afghanistan and Iran (Baig et al., 2009). HBV genotypes show a characteristic geographic distribution with a proposed association with human migration. It is interesting to note that Arians firstly colonized to the North of the Caspian Sea, then migrated to Iran, India and Europe. It might be those people who acquired the virus with the genotype D before their migration and then transmitted the virus generation by generation after their migration. That is why the dominant genotype in India,

Iran and most part of the Europe is D (Jazayeri and Carman, 2009). In countries with high levels of immigration, a variety of genotypes are being reported as all of the known genotypes can be found in the Europe and North America (Kurbanov et al., 2010). The presence of genotypes A and D also reflected the immigrant origins of the population of Buenos Aires, Argentina which is cosmopolitan city and has received immigration from the Mediterranean area (Afghanistan, Iran, Pakistan, Egypt etc), where genotype D predominates.

In fact genotyping can help to trace the migration of ancestors as well as the routes of transmission in accidental exposure to HBV (Poustchi et al., 2007). A study of 39 asymptomatic HBV carriers and 103 liver diseases patients from southern China showed circulation of A, B, C, and D genotypes with 78.9% being genotype C (Kaya et al., 2007). However, in Pakistanis

62% were genotype D, A (14%), C (6%), other genotypes (4%) and recombination (10%). Interestingly, no genotype other than D has been found in Iran. The epidemiological data about HBV genotypes in various Asian countries demonstrated the presence of all seven genotypes, particularly the pre-dominance of genotype D (Jazayeri and Carman, 2009). However, genotype A is distributed globally and is the main genotype found in Europe, North America, Africa and India (Santos et al., 2010). Hepatitis B virus genotype A has been increasing in chronic HBV patients in Japan (Matsuura et al., 2009); some HBV/A isolates have been imported from foreign countries. But unlike previous research, our study shows the dominance of genotype A which is the second most prevalent genotype in Pakistan (Ali et al., 2011). Idrees et al. (2004) reported the high prevalence of genotype A in Sindh province of Pakistan. This is good news because previous studies shows that genotype A is less severe disease and highly responsive to interferon therapy as compared to genotype D and have lower HBV DNA levels (Ali et al., 2011).

It is of much important finding that we have reported such patients infected with multiple (more than one) HBV genotypes in the current study. This is in accordance with a number of very recent studies from different regions of the world. Hannoun found 8% of HBV patients with genotype mixture (Alam et al., 2007b). Leblebcioglu and Erglu (2004) reported that chronic patients are more prone to be infected with more than one HBV genotype than acutely infected patients. Genotypes mixture in HBV patients is also common in Thailand (Jutavijittum et al., 2006). 16% HBV cases were positive for HBV genotype mixture in France (Halfon et al., 2006).

In our study, 42 HBV DNA positive samples remained untypable for HBV genotypes. It may be assumed that such samples represent recombinant or new genotypic variants present in our population that can be resolved after sequencing and further analysis. Because, some minor HBV genotypes as well as novel or distinct genotypic groups may be present in any population besides major genotypes (Bowyer and Sim, 2000).

Regarding the sex distribution of HBV infection there were more male (68.6%) patients than female (31.42%). This was compatible with work of Naz et al. (2002), who reported a high prevalence in males 68.3% than females 31.7%, which is quite comparable with our results. Nwokediuko (2010), Zubair et al. (2010), Moosa et al. (2009) and Awan et al. (2010) also reported a significantly higher infection rate in male as compared to the female. The higher HBV infection in males as compared to female may be due to their being employed outside their homes, visiting barber shops and also their involvement in blood transfusion practices. While women are mostly involved in house hold activities based on the social, cultural and religious preferences and influence.

Prevalence data from individual studies were further segregated into age groups. There was an age effect on

the prevalence of hepatitis B infection. Prevalence rose from 18.33% in children's 1-15 to a peak of 46.67 and 25% in people aged 61-30 and 31-45 years respectively. After this it declined to 6.67 and 3.33% in people aged 46- 60 and >60 years. Alam et al. (2007b) also reported a significantly higher infection in persons with age between 21-40 years followed by 41-60 years age. Very young and old individuals were very less frequently infected by HBV. Castolo et al. (2001) report also supported our finding that prevalence of HBV infection is higher in patients up to the age of 40 years. HBV infection being higher in young's respondents may be due to their greater exposures and interaction in society as compared to children and aged persons.

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