Pak. j. life soc. Sci. (2013), 11(2): 112-117 E-ISSN: 2221-7630;P-ISSN: 1727-4915

Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

RESEARCH ARTICLE

Hepatitis B virus Genotypes from Clinical Sample of Hepatitis B Antigen Positive Patients by Using PCR Method in Kohat Region of Khyber Pakhtunkhwa, Pakistan

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ARTICLE INFO
Received: Oct 19, 2012
Accepted: Apr 21, 2013
Online: Apr 25, 2013

Keywords
DNA
Genotype
Hepatitis B Virus
PCR

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ABSTRACT

Hepatitis B is an infection of Hepatitis B Virus which is the burning health problem in Khyber Pakhtunkhwa causing high morbidity and mortality. A total of 84 HBsAg samples were subjected to PCR for DNA detection and genotyping. Of them, 75 (89.28%) were found HBV DNA positive and 9 (10.71%) were negative. The highest prevalence was found in the group 31-40 years (33.33%) followed by the group 21-30 years (29.76%), 41-50 years (21.42%) and above 51 years (15.47%). The lowest prevalence was found in the group <20 years (0%). In risk factors, the blood transfusions 25.00%, surgery 19.04% and hospitalization were 22.61% in the total population. In case of hygienic conditions, the community belonging to poor hygienic conditions was found to be more frequently infected 67.85% as compared to the good (32.14%). Genotype A was most common in all age groups while C, D were comparatively less frequent. Males were found to be more frequently infected (69.33%) as compared to females (30.66%) with a positivity ratio of 2.26: 1, respectively. It is concluded that genotype A, C, D and F are the most common genotypes in Kohat region of Khyber Pakhtunkhwa. PCR is more sensitive and specific method for HBV detection.

INTRODUCTION

Hepatitis B infection is a serious problem and more than 350 million people have chronic infection worldwide (McMahon, 1985). At the beginning the infection was called serum hepatitis (Barker et al., 1996). Hepatitis B infection causes progressive liver infections, including liver cirrhosis and hepatocellular carcinoma (HCC), which caused about 1,000,000 deaths occur annually from HCC (Ljunggren et al., 2004). HBV is a contagious disease that may transmit vertically from mothers to their newborns or horizontally by means of blood products and body secretions.

Hepatitis B is caused by the infection of Hepatitis B Virus (HBV), was discovered in 1966 (Beasley et al., 1981) and belongs to *Hepadnaviridae* family (Blum et al., 1983). HBV is an enveloped DNA containing virus, having 3,200 bp in its genome, which is uniquely organized in a partly double stranded, circular pattern (Lau et al., 1993). The genome has four overlapping open reading frames in which several genes overlap and use the same DNA to encode viral proteins. The outer coat contains embedded proteins which are associated with the viral binding and entry to its host cells (Locarnini, 2004).

Genotyping can provide a valuable tool in tracing the molecular evolution, patterns and modes of spread of HBV. It is known that different genotypes have different disease severity, duration, complications and response to treatment as well as vaccination. Therefore, genotyping of HBV is important in determining the route and pathogenesis of the virus (Norder et al., 2004).

Conventional enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used methods

for detecting and measuring markers of disease. After one week of the initial infection HBV DNA can be detected by PCR (Hollinger and Liang, 2001). HBV genotypes can be identified by genotype specific PCR method such as multiplex PCR and by direct sequencing. The complete genome of HBV can be amplified by using nested PCR (Chen et al., 2006). HBV genotypes show a distinct geographical distribution between different regions and even within the same region, and are proving to be an invaluable tool in tracing the molecular evolution, patterns and modes of spread of HBV. HBV genotype A is mainly found in Northwestern areas of Europe and North America (Norder et al., 1993). Genotype B and C strains are prevalent in the population of Southeast Asia (Theamboonlers et al., 1999).

HBV is a serious problem worldwide and also in Pakistan and HBV genotypes appear have showed varying geographic distribution. In Pakistan, HBV infection rate is increasing day by day. The reason may be the lack of proper health facilities, carelessness or and less public awareness about its transmission modes (Alam et al., 2007**).** Keeping in view the importance of the virus and its health hazards, the present study was designed to determine the prevalence of HBV and its genotypes in Kohat region, Khyber Pakhtunkhwa, Pakistan.

MATERIALS AND METHODS

Samplings

84 blood samples (57 males and 27 females) were taken from HBs Ag positive patients in different areas of Kohat. All the samples were processed in the molecular Parasitology and virology laboratory Zoology Department Kohat University of Science and Technology Kohat. A proforma was designed to collect desired information from all the patients.

DNA Extraction

DNA was extracted from 200 µL of blood using DNA extraction Kit (GF-1 Viral Nucleic Acid Extraction Kit, Vivantis USA) as per the manufacturer protocol

1. HBV DNA Detection

a) 1ST round PCR (regular PCR)

The extracted DNA was then amplified with Regular PCR using a sense and an antisense primer specific for surface gene of HBV. A single PCR reaction of 20 µL consisted of the following reagents:

4.1 µL of *Taq* buffer, 2.4 µL of $MgCl_2$, 1.2 µL of dNTP (500 mM), 1.1 µL of each primer10pM (P1(sense) & S1-2 (antisense), 5.6 µL of distilled water was mixed gently with 0.5 µL of *Taq* DNA polymerase. A total of 20 µL of reaction volume was prepared for a single reaction and then 4µL of the extracted DNAwas added to the corresponding tubes. Reaction was run in thermal cycler (NyxTechnik USA) using the program of cycling 1 of initial denaturation at 95 °C for 10 minutes, 35 cycle of each having 94°C/25 sec, 52°C/25sec and 72°C/1minutes and finally 72°C for 1minute (Naito et al., 2001) .

b) 2nd round PCR(nested PCR)

Amplified product was re-amplified nested PCR using the pair of primers internal to the first one (Naito et al., 2001). A mixture was prepared in same way as for regular PCR except the primer. The PCR cycling conditions were the same as for regular PCR.

c) Electrophoresis

DNA bands were resolved on a 2% agarose gel prepared by adding 2g of agarose powder in 0.5 X TBE buffer boiled in microwave oven and cooled to room temperature. Then 3 µL of ethedium bromide was added. 3 µL of loading dye was added and mixed well with amplified product and was loaded in gel. The DNA bands were compared with a DNA ladder marker of 50 bp (Farmentas, USA). The bands were resolved under UV illumination using gel documentation system.

2. HBV Genotyping

The HBV DNA positive samples were selected after conformation by nested PCR for HBV genotyping using the procedure of Naito et al., 2001. The following steps were followed for HBV genotyping.

a) Regular PCR

The DNA of the positive samples was then amplified with Regular PCR using a sense and an antisense primer specific for surface gene of HBV. A single PCR reaction of 20µL consisted of the following reagents:

4.1 µL 10X of *Taq* buffer, 2.4 µL of $MgCl_2$, 1.2 µL of dNTP (500mM), 1.1 µL of each primer10pM (P1 & S1-2), 5.6 µL of distilled water was mixed gently with 0.5 µL of *Taq* DNA polymerase. A total of 20 µL of reaction volume was prepared for a single reaction and then 4µL of the extracted DNAwas added to the corresponding tubes. Reaction was run in thermal cycler (NyxTechnik) using the following program of cycling 1 of initial denaturation at 95^0c for 10 minutes, 35 cycle of each having 94^0C/25 sec,55^0C / 25 sec and 72^0C /1minutes and finally 72^0C for 1minute .

b) Multiplex PCR

For each sample, Genotypes were identified using specific primers from the core region of the HBV genome for the amplification of the sixth (A-F) most common types (Naito et al., 2001). For each sample, two different products of second round PCR were carried out with different sets of primers for convenience of amplification and product identification. The procedure was adopted for genotyping of Naito et al., 2001. The following specific primers were used for regular PCR.

1. Universal Sense Primer 5′ TCACCATATTCTTGG GAACAAGA-3′
2. Universal antisense Primer 5′-CGAACCACTGAA CAAATGGC-3′

Primers used for Mix-A

1. Genotype A-E specific, sense primer 5′-GGCTCA AGTTCAGGAACAGT-3′
2. Genotype A-specific, antisense primer-5′CTCGCG GAGATTGACGAGATGT-3′
3. Genotype B specific, antisense primer 5′-CAGGTT GGTGAGTGACTGGAGA-3′
4. Genotype C specific, antisense primer 5′-GGTCCT AGGAATCCTGATGTTG-3′

Primers used for Mix-B

1. Genotype D-F specific, antisense primer 5′-GGAG GCGGATTTGCTGGCAA-3′
2. Genotype D specific, sense primer 5′-GCCAACAA GGTAGGAGCT-3′
3. Genotype E specific, sense primer 5′-CACCAGAA ATCCAGATTGGGACCA-3′
4. Genotype F specific, sense primer 5′-GTTACGGT CCAGGGTTACCA-3′

i. Mix-A Composition

A 4.1 µL 10X of *Taq* buffer, 2.4 µL of $MgCl_2$, 1.2 µL of dNTP (500 mM), 1.1 µl Sense primer (B2) (10 pM), 1.1 µl Anti sense primer (BA1R) (10 pM), 1.1 µl sense primer (BB1R) (10pM), 1.1 µl Anti sense primer (BC1R) (10 pM) and 5.6 µL of distilled water (Naito et al., 2001).

ii. Mix-B composition

A 4.1 µL of Taq buffer, 2.4 µL of $MgCl_2$, 1.2 µL of dNTP (500mM), 1.1 µl Sense primer (BD1) (10pM), 1.1 µl Anti sense primer (BE1R) (10pM), 1.1 µl sense primer (BF1R) (10pM), 1.1 µl Anti sense primer (B2R) (10pM) and 5.6 µL of distilled water.

All the above components were mixed gently. A total of 20 µL of reaction volume was prepared for a single reaction and 0.5 µL of *Taq* DNA polymerase was used. The samples were run in thermal cycler (NyxTechnik) using the program of cycling condition, Cycle of 1 of initial denaturation at 95^0C for 10 minutes, 20 cycle of each having 94^0C/20 sec, 58^0C/20sec and 72^0C/30 sec. and finally 20 cycle of each having 94^0C/20 sec, 60^0C/20sec and 72^0C/30 seconds.

Gel Electrophoresis and Results Interpretation

PCR products were electrophoresed in 2% agarose gel prepared in 0.5 X TBE buffer (boiled for 3 min in a microwave oven and cooled to 55^oC), adding ethidium bromide (0.5 µg/ mL) stained and evaluated under ultra violet UV light. The specific DNA of amplified product was determined by identifying the DNA bands of a specific genotype (Key Below) comparing with 50-bp DNA ladder (Fermentas Germany), used as DNA size marker.

HBV Genotype	Product (base pair)
A	68
B	281
C	122
D	119
E	167
F	97

(base pair already detected in Fig 1)

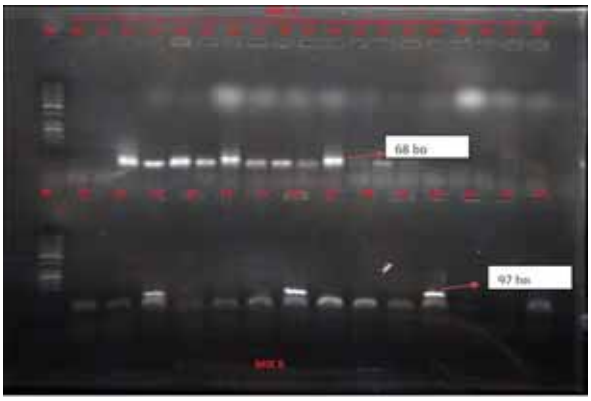

Fig. 1: Genotype specific bands in 2% Agarose gel using 100bp Marker

Fig. 2: Showing the genotype A in the Mix-A amplified product using 50bp marker

RESULTS

A total of 84 HBsAg samples were subjected to PCR for DNA detection and genotyping. Of them 75(89.28%) were found HBV DNA positive and 9 (10.71%) were HBV DNA negative. The age range was from <20 to >51 years. In term of age distribution the subjects were grouped into <20 years (0%), 21-30 years (29.76%), 31-40 years (33.33%), 41-50 years (21.42%)and >50 years (15.47%). The table 1 shows that the highest prevalence was found in the group 31-40 years (33.33%) followed by in the group 21-30 years (29.76%), 41-50 years (21.42%),>51 years (15.47%). no infection was found in the group <20 years (0%).To show the socioeconomic conditions the subjects were divided into upper level (monthly income >Rs 80,000), middle level (monthly income >Rs 40,000) and lower level (monthly income <40,000).The lower level was found to be more frequently infected (64.28%) as compared to middle (15.47%) and higher level (20.23%). In risk factors the blood transfusions were 25.00%, surgery were 19.04% and hospitalization were 22.61% in the study population In case of hygienic conditions the study subjects belonging to poor hygienic conditions were found to be more frequently infected 67.85% as compared to the good hygienic condition (32.14). The general characteristics of the study subjects are given in the (Table 1).

Gender Wise Distribution of Patients Positive for HBV DNA

In the whole population, male were 57 (67.85%) and females were 27 (32.14%). Males were found to be more frequently infected (69.33%) as compared to females (30.66%) with a positivity ratio of 2.26: 1 respectively (Table 2).

Age Wise Distribution of Patients Positive for HBV DNA

The age range was from 20 to 60 years. In table 3 the ages were divided into five groups. 26 (34.66%) individual, positive for HBV DNA were belonging to the age group ranging 31–40 years of age followed by the 25 (33.33%) positive individuals of 21– 30 years age, 15 (20%) of 41-50, 9 (12%) of >51and 0 (0%) of age <20. Highest frequency of HBV DNA positive individuals were found in the younger age falling under the 21–40 age groups while very young and very old individuals were very less frequently infected by HBV (Table 3).

Proportion of different genotypes

A total of 84 HBs Ag positive patients were analyzed of which 75 were confirmed for HBV DNA including Genotype A was 34.66%(26/75), B 0%(0/75), C 6.66% (5/75), D 1.33%(1/75), E 0%(0/75) and F 20% (15/75) respectively. Twenty eight of the samples were HBV DNA positive but cannot type by the prescribed system (Table 4).

HBV Genotypes in Different Age Groups

The age range of the subjects was from <20 to >51 years. Genotype A was most common in all age groups while F, C and D were comparatively less frequent. Further details of different age groups along with their corresponding genotypes are presented in the following table.

Table 1: General characteristics of the study population in Kohat region of Khyber Pakhtunkhwa

Character (n)	Male (%)	Female (%)	% age
HBsAg (84)	57 (67.85)	27 (32.14)	100
Age groups<20 (0)	0	0	0
21-30 (25)	18 (72)	7 (28)	29.76
31-40 (28)	19 (67.85)	9 (32.14)	33.33
41-50 (18)	13 (72.22)	5 (27.77)	21.42
>51 (13)	7 (53.84)	6 (46.15)	15.47
Socioeconomic condition (In Rs)			
Upper {>80,000}(17)	13 (76.47)	4 (23.52)	20.23
Middle {>40,000}(13)	8 (61.53)	5 (38.46)	15.47
Lower {<40,000} (54)	38 (70.37)	16 (29.62)	64.28
Risk Factor			
Blood transfusion (21)	7 (33.33)	14 (66.66)	25.00
Surgery (dental & general) (16)	5 (31.25)	11 (68.75	19.04
Hospitalization (19)	6 (31.57)	13 (68.42)	22.61
Hygienic conditions			
Good (27)	21 (77.77)	6 (22.22)	32.14
Poor (57)	36 (63.15)	21(36.84)	67.85

Table 2: Gender wise distribution of patients positive for HBV DNA

Gender	HBV DNA positive (%)	HBV DNA negative (%)
Male	52 (69.33)	5 (8%)
Female	23 (30.66)	4 (14.81%)
Total	75 (89.28%)	9 (12%)

Table 3: Age wise distribution of patients positive for HBV DNA

Age Group (n)	HBV DNA positive (%)	HBV DNA negative (%)
<20 (0)	0	0
21-30 (25)	25 (100)	0
31-40 (28)	26 (92.85)	2 (7.14)
41-50 (18)	15 (83.33)	3 (16.66)
51-60 (13)	9 (69.23)	4 (30.76)
Total (84)	75 (89.28)	9 (10.71)

Table 4: Percentage of detected genotypes of HBV in Kohat Region

Genotype	No. of samples	%age
A	26	34.66
B	0	0
C	5	6.66
D	1	1.33
E	0	0
F	15	20.00
Untyped	28	37.33

Table 5: HBV Genotypes in Different Age Groups

Age Group	No of patients	No: of samples typed (Genotype)	%age
<20	0	0	0
21-30	25	14 (A, F)	56.00
31-40	26	17 (A)	65.38
41-50	15	9 (A, D, F)	60.00
>51	9	7 (A, C, F)	77.77

DISCUSSION

It is estimated that about 350 million people are infected by HBV worldwide. The liver infection and hepatocellular carcinoma are the major clinical aspects of HBV infection (McMahon, 1985). It is a serious problem in developing countries like Pakistan. HBV is classified into eight genotypes (A-H) (Lee, 1997). In the present study the different HBV genotypes of Kohat region of Khyber Pakhtunkhwa, Pakistan were characterized molecularly by multiplex PCR.

The world can be divided into three areas where the prevalence of chronic HBV infection is high (>80%), intermediate (2-8%), and low (<2%). In Asia and Africa the disease is epidemic and in China it is endemic, in Africa and Asia the infection is mostly restricted to

childhood (Williams, 2006). This infection is prevalent in Asia, Africa, Southern Europe and Latin America (Hong et al., 2009). In most Asian countries, with a general carrier rate of 5-35%, HBV infection is hyper endemic and in North America the infection is mostly found in young adults (Clarke et al., 2005). According to WHO, Pakistan is endemic to HBV infection. About 3% of the country population is infected with HBV (Alam et al., 2007). In the present study a total of 84 HBsAg samples were subjected to PCR for DNA detection and genotyping. Of them 89.28% were found HBV DNA positive.

It is reported that HBV infection is 69.1% in male which is higher than females, 30.9%. In our study, male were 57 (67.85%) and females were 27 (32.14%). Males were found to be more frequently infected (69.33%) as compared to females (30.66%) with a positivity ratio of 2.26: 1 respectively. Our findings are similar with the report of (Baig et al., 2007). Men are frequently infected which may be due the exposure of men to the risk factors. In Pakistan women are mostly limited to their home due cultural and religious preferences.

HBV infection is found to be significantly higher in persons with age between 21–40 years (Alam et al., 2007). In our study the age range was from 20 to 60 years. The ages were divided into five groups. 26 (34.66%) individual, positive for HBV DNA were belonging to the age group ranging 31–40 years of age followed by the 25 (33.33%) positive individuals of 21–30 years age, 15 (20%) of 41-50, 9 (12%) of >51and 0 (0%) of age <20. Highest frequency of HBV DNA positive individuals were found in the younger age falling under the 21–40 age groups while very young and very old individuals were very less frequently infected by HBV. Our findings were almost similar with the findings of years (Alam et al., 2007).

There were four different genotypes detected in the samples. These were A,C, D and F. Out of these three, genotype A was found in greatest proportion 34.66%, while remaining three (F, C and D) were found in 20%,6.66% and 1.33% of cases respectively. According to the report of (Baig et al., 2007) the highest prevalence is of the genotype D followed by genotype B and A. Our report is also in contrast with the findings of (Abbas et al., 2006) which show the highest prevalence of genotype D. research on genotyping in Asia was formerly conducted in Japan and China, therefore, the genotypes of these countries namely B & C were considered as the most prevalent genotypes of Asia. But when research was extended to other countries, predominance of D was found in South Asia and the Middle East such as India, Afghanistan and Iran (Toan et al., 2006).

It is reported that genotypes B andCare major genotypes of HBV distributedin Asian countries (Kwok et al., 1989). Studies have shown that genotypes distribution is a contradictory. Idrees*et al*. reported genotype C as themost prevalent genotype in Pakistan, genotype A as the mostprevalent in Sind Province, genotype C as the most prevalent inNorth West Frontier Province, and both genotypes B and C in Punjab (Idrees et al., 2004). According to Alam et al. (2007) the most prevalent genotypein North West Frontier Province is D (62.5%). In our study genotype A was found in greatest proportion 34.66%, while remaining three (F, C and D) were found in 20%, 6.66% and 1.33% of cases respectively. So according to our study genotype A is most common in our study region and this may be due to small samples size.

In Afghanistan genotype D is the most prevalent (95 %) and genotype is distributed worldwide (Amini-Bavil-Olyaee et al., 2006). Our study is in contrast with these findings showing genotype D the least (1.33%) prevalent in our study area and this may be due to different socioeconomic and geographic conditions. So the prevalence of genotype D is less in Pakistan than in Afghanistan, China and India.

Conclusion

It is concluded from the present study that HBV genotype A, C, D and F are the most common genotypes in Kohat region of Khyber Pakhtunkhwa, Pakistan. Molecular characterization of HBV genotypes by PCR method is the most effective method. It has also been determined that PCR is more sensitive and specific method for HBV detection.

Acknowledgements

The research work is partially funded by the Department of Zoology, Kohat University of Science and Technology, Kohat-Pakistan and HEC funded project entitled “Epidemiology and molecular detection of leishmaniasis in canine and human being in Khyber Pakhtunkhwa” for which I am so grateful that it make possible to complete my work.

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