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Pattern and molecular epidemiology of Hepatitis B virus genotypes circulating in Pakistan

Zunaira Awan, Muhammad Idrees *, Irum Amin, Sadia Butt, Samia Afzal, Haji Akbar, Irshad-ur Rehman, Saima Younas, Muhammad Shahid, Amreek Lal, Sana Saleem, Bisma Rauff

Division of Molecular Virology, National Centre of Excellence in Molecular Biology, 87-West Canal Bank Road, Thokar Niaz Baig, University of the Punjab, Lahore 53700, Pakistan

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ABSTRACT

The continuously mutating nature of Hepatitis B virus (HBV) is responsible for the emergence of varying genotypes in different regions of the world affecting the disease outcome. The objective of the current study was to find out the pattern of HBV genotypes circulating in Pakistan. HBV genotypes were determined in HBV chronic patients of different age and gender from all the four different geographical regions (provinces) of Pakistan for a period of 2 years (2007–2009). Out of the total 3137 consecutive patients, 300 (175; 58.3% males and 125; 41.7% females) were randomly selected for HBV genotype A through H determination using molecular genotyping methods. Total 269 (89.6%) isolates were successfully genotyped where as 31 (10.3%) samples failed to generate a type-specific PCR band and were found untypable. Out of the successfully genotyped samples, 43 (14.3%) were with type A, 54 (18%) were with type B, 83 (27.6%) were with type C, 39 (13%) were with type D, 2 (0.6%) were with type E, 4 (1.3%) were with genotype F and total 44 (14.6%) were with mixed HBV infections. Of the mixed genotype infection cases, 16 were with genotypes A/D, 9 were B/C, six were A/D/F, five were with genotypes A/F, two were with A/B/D and B/E and one each for A/C as well as A/E genotypes. Four common genotypes of HBV found worldwide (A, B, C & D) were isolated from Pakistan along with uncommon genotypes E and F for the first time in Pakistan. Overall Genotype C is the most prevalent genotype. Genotypes B and C are predominant in Punjab & Balochistan and Khyber Pakhtoonkhwa, respectively whereas genotype A in Sindh.

1. Introduction

Hepatitis B virus (HBV); the prototype member of the family *Hepadnaviridae*, is a circular, partially double-stranded DNA virus of ~3.2 kb genome in size (Magnius and Norder, 1995). HBV is a serious health problem affecting approximately 350 million people (WHO, 2008), and 500,000 to 1.2 million worldwide deaths per year (Qingrun et al., 2007). HBV infection leads to end-stage liver diseases including cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC) (Wiegand et al., 2008).

At present, HBV has been classified into at least 9 genotypes (A through I) based on inter-group divergence of 8% or more in the complete nucleotide sequence (Magnius and Norder, 1995; Okamoto et al., 1988; Norder et al., 1992; Huy et al., 2008; Kanako et al., 2009; Yu et al., 2010). HBV genotypes have a distinct geographical distribution pattern: genotype A is most prevalent in Philippines (Norder et al., 1993b; Kidd-Ljunggren et al., 1995) North-West Europe, North America and in south and east of Africa (Bowyer et al., 1997); genotype B is mostly found in Indonesia, China and Vietnam; genotype C is found in East Asia, Korea, China, Japan, Polynesia and Vietnam; genotype D is predominant in the Mediterranean area and the Middle East extending to India; genotype E is typical for Africa (Kidd-Ljunggren et al., 2002) genotype F is found in central and south America (Norder et al., 1993b; Nakano et al., 2001) and in Polynesia (Couroucé et al., 1983); genotypes G and H are found present in North America and France (Stuyver et al., 2000; Magnius and Norder, 1995; Van Geyt et al., 1998; De-Nys et al., 2003). Two new genotypes I and J have recently been isolated from Vietnam and Japan, respectively (Huy et al., 2008; Kanako et al., 2009).

Different genotypes of HBV are increasingly recognized for their clinical significance and disease outcome. It is evident from several studies that HBV genotypes can significantly influence clinical manifestations, HBeAg seroconversion rates, viremia levels and response to antiviral therapy (Osiowy, 2006; Schaefer, 2005; Abe et al., 2004).

Few studies on HBV genotyping with a very small number of samples are available from Pakistan for a specific geographical region. No study on HBV genotyping pattern representing all the geographical regions of Pakistan is available; therefore, we

* Corresponding author. Tel.: +92 42 5293141; fax: +92 42 5421316.
E-mail address: idreeskhan@cemb.edu.pk (M. Idrees).

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initiated the current study with the purpose of finding out the pattern of common HBV genotypes circulating in this region of the world.

2. Materials and methods

2.1. Sample collection

A total of 5229 HBsAg ELISA positive blood samples from HBV carrier subjects were received at Division of Molecular Diagnostics & Virology Division between December 2007 and September 2009 for the detection of HBV DNA by PCR. Out of these, total 3137 (59.9%) were found positive by SmartCycler II Real-time PCR (Cephied, USA) and 2092 (40%) were found HBV DNA negative. Of the HBV DNA PCR positive samples, total of 300 samples were selected randomly for HBV genotype analysis. Out of the selected HBV DNA positive samples, 36 samples were from Khyber Pakhtoonkhwa, 222 belonged to Punjab, 16 samples from Balochistan and 26 sera were of Sindhi patients. Liver function tests such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase and bilirubin levels of all the samples had already been estimated using Auto-analyzer (Hitachi, Tokyo, Japan).

2.2. ELISAs for HBsAg and HBeAg

All the patients were screened for HBsAg and HBeAg using 3rd generation enzyme-linked Immunosorbant Assay (ELISA) (DRG Instruments, Germany) kits using the methodology described by the manufacturers.

2.3. HBV qualitative PCR and viral load

HBV qualitative PCR and viral load was done using SmartCycler II Real-time PCR (Cepheid, USA) using HBV DNA qualitative/quantitative kits (Sacace Biotechnologies, Italy) according to the kit protocol. The viral load equal to or less than 2.0 M copies/mL was considered as with 'low viral load'.

2.4. HBV genotyping

HBV genotypes A through G were determined by using HBV genotype-specific primers for the surface gene of HBV as previously described by Naito et al. (2001) and Liu et al. (2008) with little modifications. For genotype H determination the method described previously by Arauz-Ruiz et al. (2002) was utilized. Briefly, the HBV genome was amplified by nested PCR using two universal forward and reverse primers. The first PCR was carried out in a tube containing 10 μl of a reaction mixture (1× PCR buffer containing 2.5 mM $MgCl_2$, a 100 μM concentration of each of the four deoxynucleotides (dNTPs), 10 pM of each outer forward and reverse primers and 1 U of Taq DNA polymerase enzyme). The thermocycler (ABI PCR system 2700) was programmed to initially incubate the samples for 2 min at 95 °C, followed by 35 cycles consisting of 95 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min. The first round of PCR was followed by two different mixtures containing type-specific inner primers. The two second-round PCRs were performed for each sample, with the common universal sense primer and mix I for types A through C and the common universal antisense primer and mix II for types D through F. Two microlitre aliquot of the first PCR product were added to three tubes containing the second sets of each of the inner primer pairs, each of the deoxynucleotides, Taq DNA polymerase, and PCR buffer, as in the first reaction. These were amplified for 35 cycles with; preheating at 95 °C for 2 min and then 35 amplification cycles of 95 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min. Genotypes of HBV for each sample were identified by genotype-specific DNA bands on 2% agarose gel, stained with ethidium bromide, and evaluated under UV transilluminator. The estimation of the sizes of PCR products was done according to the migration pattern of a 100-bp DNA ladder (Gibco BRL Life Technologies). Mix I allows for the specific detection of PCR products for types A, B, and C, mix II allows for detection of types D, E, and F. Similarly nested PCR was done separately for the specific detection of PCR bands for genotypes G and H.

2.5. Sequence analysis

Genotypes were further conformed by HBV S-gene sequencing using ABI PRISM 3100 Genetic Analyzer (Applied Biosystem Inc., 850 Lincoln Center Drive, Foster City, CA 94404) in both directions. Sequences of isolates were aligned with representative sequences for each HBV genotype selected from the GenBank database with the help of the Multalign program. The phylogenetic analysis of HBV isolates was performed with MEGA 3.0 software and phylogenetic trees were constructed by the neighbor-joining method by using the bootstrap-resampling test from the MEGA program (1000 bootstrap replications).

3. Results

Fig. 1 shows the patients 'disposition and distribution of HBV genotypes in Pakistan. During the course of this study, total 5229 HBsAg positive samples were received from four different geographical regions (provinces) of the country. Out of these 5229 samples, total 2092 samples were found negative by HBV qualitative PCR and so were excluded. Of the PCR positive samples, about 10% constituting 300 samples were randomly selected from each geographical area for genotyping analysis. During the course of this study we were able to genotype 269 (88.6%) of HBV DNA positive samples and only 31 (14.6%) of the samples were found untypable. Out of the typable patients, 43 (14.3%) were of type A, 54 (18%) were of type B, 83 (27.6%) were of type C, 39 (13%) were of type D, 2 (0.6%) samples were of genotype E, 4 (1.3%) were of genotype F and 44 (16.4%) were of mixed HBV infections. No sample was found having HBV-G or HBV-H in this study. Mean age of the genotyped patients was 29 ± 10. Majority of our patients were male (n = 175; 58.3%) and were young people (18–30 years of ages). Most of the patients (n = 188; 62.6%) had low viral load (<2 million copies/mL) and abnormal ALT levels (77 ± 7.6; at least two times greater than normal value).

Table 1 shows the distribution of HBV mixed genotypes in the current study. Out of the mixed HBV infection cases, 16 were of genotypes A/D, 9 were of B/C, 6 patients were of A/D/F, 5 were with genotypes A/F, 2 each of A/B/D and B/E, and one patient sample each for A/C and A/E genotypes.

Table 1
HBV mixed genotypes (N = 44).

Mixed with	No. of isolates	Percentage
A + B + D	2	4.5%
A + D + F	6	13.6%
A + C	1	2.2%
A + D	16	36.3%
A + E	1	2.2%
A + F	5	11.3%
B + E	2	4.5%
B + C	9	20.4%
C + D	2	4.5%

Out of 300 genotyped samples, 44 (14.6%) had more than one genotype. Of these mixed HBV infection cases, 16 had genotypes A/D, 9 B/C, 6 A/D/F, 5 A/F, 2 each A/B/D and B/E, and one each had A/C and A/E genotypes.

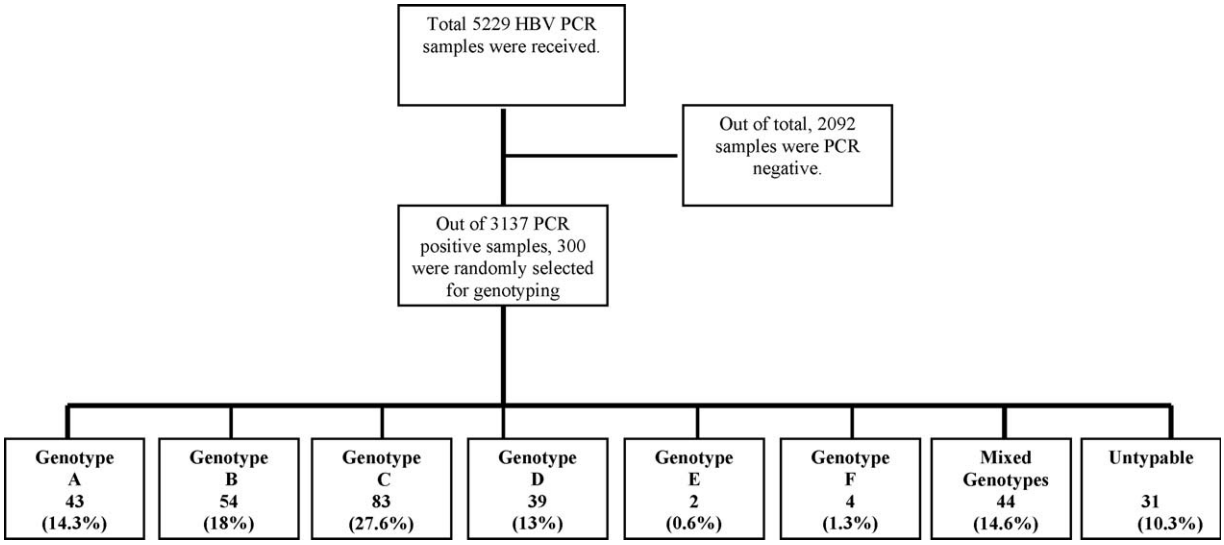

Fig. 1. Patients' disposition and distribution of HBV genotypes in Pakistan. During the course of this study, total 5229 HBsAg positive samples were received from four different geographical regions. Of the PCR positive samples, about 10% constituting 300 samples were randomly selected from each geographical area for genotyping analysis. Total 269 (90%) of HBV DNA positive samples were successfully genotyped. Out of the typable patients, 43 (14.3%) were of type A, 54 (18%) were of type B, 83 (27.6%) were of type C, 39 (13%) were of type D, 2 (0.6%) samples were of genotype E, 4 (1.3%) were of genotype F and 44 (16.4%) were of mixed HBV infections.

Table 2
Geographical distribution of HBV genotypes in Pakistan ($N = 300$).

Area	No. of samples genotyped	Genotype A (%)	Genotype B (%)	Genotype C (%)	Genotype D (%)	Genotype E (%)	Genotype F (%)	Mixed (%)	Untypable (%)
KPK*	36	6 (16.6)	5 (13.8)	14 (38.8)	2 (5.5)	0	0	9(25)	0
Balochistan	16	2 (12.5)	8 (50)	0	0	0	0	5(31.2)	1(6.25)
Punjab	222	25 (11.2)	41 (18.4)	66 (29.7)	29 (13)	2 (0.9)	3(1.35)	26(11.7)	30(13.5)
Sindh	26	10 (38.4)	0	3 (11.5)	8 (30.7)	0	1(3.8)	4(15.3)	0
Total	300	43	54	83	39	2	4	44	31

Area wise distribution of HBV genotypes in all four regions of Pakistan shows that HBV genotype C is the dominant genotype of HBV in Khyber Pakhtoonkhwa, followed by A (16.6%), B (13.8%) and D (5.5%). Similarly genotype C is dominant in Punjab with a rate of 29.7% followed by genotype B (18.4%), D and A (13% and 11.2%, respectively). Twenty-six mixed genotypes were also obtained from Punjab area. Genotype B is dominant in Balochistan with an occurrence of 50% while others are; genotype A (12.5%) and mixed (31.2%).

* KPK, Khyber Pakhtoonkhaw.

Table 2 translates the area wise distribution of HBV genotypes in all four regions of Pakistan. HBV genotype C (38.8%) was the dominant genotype of HBV in Khyber Pakhtoonkhwa area, followed by A (16.6%), B (13.8%) and D (5.5%). Similarly genotype C was dominant in Punjab with a rate of 29.7%. The second most common genotype was B (18.4%) in Punjab. The observed rate of genotypes D and A was 13% and 11.2%, respectively. Twenty-six mixed genotypes were also obtained from Punjab area. Five were with genotypes A/D and two were with C/D. Genotype A (38.4%) was dominant in Sindh area, followed by D (30.7%), C (11.5%) and mixed (15.3%). Genotype B is dominant in Balochistan with an occurrence of 50% while others are; genotype A (12.5%) and mixed (31.2%).

For the confirmation of genotyping results, bands (S-gene; 100 bp to 680-bp depending on HBV genotype) of three genotypes each with B, C, D, and two with mixed genotypes (A/D & B/C) were sequenced in both directions. Sequencing data confirmed the genotyping data as sequencing generated the same results as were generated by the genotyping methods. All the sequences were submitted to GeneBank data bank. The Accession Numbers provided for our nucleotide sequences by the GeneBank are from FJ966112 to FJ966118.

4. Discussion

The purpose of the present study was to investigate the pattern of HBV genotypes circulating in Pakistan because few available studies on the subject have been carried out in only one specific geographical region of the country (city or area); we were interested in investigating the HBV genotype pattern representing all the geographical regions of this country and its comparison with other regions of the world. The clinical, virological, and therapeutic implications of HBV genotypes in patients with chronic HBV infection have now been clarified as several studies have suggested that HBV genotypes are associated with the severity of liver disease including development of cirrhosis and hepatocellular carcinoma (HCC) (Livingston et al., 2007; Kao et al., 2000; Kao, 2007). Thus know how of HBV genotypes present in a population is very important for the best management of the disease (Idrees et al., 2004). In the current study we were able to genotype 90% of our HBV DNA positive samples and only 10% of the samples were found untypable. In our study 14.3% samples were with type A, which is mainly found in Northwestern areas of Europe and North America (Schaefer, 2005; Norder et al., 1993). Type B was 18% whereas the most prevalent genotype was type C (27.6%). Types B and C are dominant genotypes in China (Theamboonlers et al., 1999) which is an important neighbor of Pakistan in the North. Previous reports from Pakistan showed genotype D as the most prevalent genotype however, these studies were from one region of the country (Alam et al., 2007; Idrees et al., 2004). Previous studies published about 10 years back had shown that genotypes B and C strains belong to the indigenous population of Southeast Asia (Okamoto et al., 1988; Theamboonlers et al., 1999). In Japanese homosexual men coinfected with HIV were

unexpectedly found to harbor HBV genotype A instead of C or B, which are the prevailing genotypes in Japan (Kanako et al., 2009; Koibuchi et al., 2001).

The predominance of genotype C in chronic Pakistani patients is a bad news as this genotype is known to be associated with more severe liver diseases and is more common in cirrhotic patients as compared to other genotypes (Okamoto et al., 1988; Theamboonlers et al., 1999). The genotype C-associated HCC and is difficult to treat. In addition, genotype C is also associated with a higher frequency of HBeAg positivity and HBV DNA level, well marked liver inflammation, and a lower frequency of pre-core mutants as well as a higher frequency of core promoter mutants (Van Geyt et al., 1998; Osiowy, 2006; Schaefer, 2005). Genotype D was observed in 13% of the total isolates that has previously been reported as the most common genotype of Pakistan (Alam et al., 2007; Idrees et al., 2004). This genotype is common in South Europe, Middle East and India (Schaefer, 2005; Abe et al., 2004). Interestingly we were able to isolate 1.3% genotype F for the first time from this region. This genotype has been reported to be the most divergent genotype in American natives and in Polynesia (Magnius and Norder, 1995; Huy et al., 2008; Kanako et al., 2009; Van Geyt et al., 1998; De-Nys et al., 2003). This emergence could be due to the increasing influx of Americans in the region.

Importantly our 14.6% patients had mixed HBV infections. Majorities (36.3%) of the cases with mixed genotypes were due to HBV A + D. Interestingly of the mixed genotype infections, 4.5% had genotype E as one of the genotype. Genotype E is circulating in Africa (Kidd-Ljunggren et al., 2002) and none of our patients visited Africa then how this genotype enters Pakistan? is still question mark. As genotype E is the most similar to genotype D genetically (Norder et al., 1993; Norder et al., 1994) and can be assumed a subset of genotype D (Norder et al., 1994). Mixed HBV genotype infections are present worldwide but their clinical significance are unclear and needs further elucidation. Importantly for our 31 (10%) patients no genotype-specific bands were seen. All these samples were HBsAg and PCR positive however, were HBeAg negative. Some mutations in the HBV genome rendered specific primer annealing and amplification (Idrees et al., 2004) that might be the reason for untypable genotype results. In the current study we were unable to isolate genotype G and H from this region of the world.

Most important finding of the current study is the observation that different genotypes pattern exist in different provinces of this country. HBV genotype C was found as the dominant genotype in the province of Khyber Pakhtoonkhwa that was 38.8%. This genotype has been reported as the predominant HBV genotype in China a neighbor of Khyber Pakhtoonkhwa. This resemblance could be due to free trade between Khyber Pakhtoonkhwa and China. Genotypes C and B were found dominant in Punjab. Genotype A (38.4%) was dominant in Sindh area, which has previously been reported as the dominant HBV genotype circulating in India (Thakur et al., 2005). Majority of our Sindhi patients belonged to Karachi where most of the people had migrated from India in 1947 that might be the reason of genotype resemblance. In Balochistan genotype B was found as the dominant genotype (50%).

The only limitation of the current study is the relatively small sample size even though it is the highest number genotyped from this country so far representing all the four provinces. For this reason, the findings of the present study cannot be generalized to the broader community of Pakistan based on this single study alone and further studies on large sample size are suggested.

In summary, overall genotype C is the most prevalent genotype which is known to be associated with more severe liver diseases such as cirrhosis and HCC. The rate of mixed genotype infection is high as compared to other regions of the world. Different HBV genotype patterns exist in different geographical regions of this country.

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