ORIGINAL ARTICLE

Detection Different of Genotypes of Hepatitis B virus by Using Genotype- Specific Primers and its Clinical Correlation

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ABSTRACT

Aims: To study the frequency and clinical correlation of Hepatitis B for common genotypes of hepatitis B virus by PCR.

Methods: HBV genotype was determined from 206 serum samples from chronically infected patients with HBV. DNA was extracted from HBsAg and HbeAg positive cases and their genotype were identified by using type specific primers.

Results: All the genotypes except genotype E were identified in the patients. Genotypes B, C and A, were the predominant ones, appearing in 48 samples (23.3%), 57 samples (27.6%) and 43(20.8%), while genotypes, D and F were appearing in 21(10.1%) and 4(2%) of samples, respectively. However, 33(16.2%) samples were determined as mixed genotype. At baseline, patients infected with HBV genotype C were generally younger with a borderline female predominance. Seroconversion from HbeAg-to anti Hbe positively occurs much earlier in genotype B than genotype C carriers. Higher the HBV-DNA levels have been detected in patients infected with genotype C compared with other four genotype.

Conclusion: Genotype of HBV plays a major role in predicting the response to various therapies and that this should be taken as a variable before initiating any treatment. Therefore larger depth studies are necessary in regions where HBV is hyperendemic.

Key words: Genotype, Hepatitis B, Hepatocellular carcinoma, Liver enzymes, PCR, Primers, Virus.

INTRODUCTION

Hepatitis B virus (HBV) is a major health problem globally casting an enormous burden on health care system and a major source of patient's misery. Hepatitis B virus (HBV) is one of the major aetiological agents of acute and chronic liver diseases worldwide including fatal fulminant hepatitis, cirrhosis and it is also an important cause of hepatocelluar carcinoma, one of the most common human cancers and causes of death worldwide[1,2,3]. Chronic hepatitis B is a serious clinical problem in Pakistan[4] and more than 350 million people of the world are chronic carries of the virus[5].

HBV is classified into seven genotypes according to the phylogenetic analysis of its genomic sequences. The first four genotypes (genotypes A.D) were first described by Okamoto and colleagues[6]. Six years later, two additional genotypes[7] (genotypes E, F), and most recently genotype G and H , were described[8,9].

Hepatitis B virus isolated have been classified into eight genotypes, designated A to H and four major serotypes ayw, ayr, adw and ad, based on greater than 8%nucleotide variations over the entire genome[6,10,11,12]. The infection is associated with a wide clinical spectrum, ranging from acute or fulminant hepatitis to various forms of chronic infection, including asymptomatic carrier status, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)[13,14,4]. In Pakistan, it has been estimated that 4.5 million are carriers and as Pakistan remains intermediate HBV prevalence area with a carrier rate of 3-4%[15].

The study was carried out to have a clear picture of prevalence of HBV infection and distribution of genotyping in acute and chronic liver disease. We initiated a study to find out the common HBV genotypes present in Pakistan especially in Karachi. For this purpose we used type-specific genotyping system[16] though nucleotide sequence analysis followed by phylogenetic analysis is the most reliable and certain genotyping results, however, this is not an appropriate method for large-scale genotyping[17,18,19,20].

MATERIALS AND METHODS

The total number of blood samples (n=1204) of hepatitis B surface antigen (HbsAg) was collected for the detection of different HBV genotypes from different parts of Pakistan. The n=206 HBV DNA positive serum samples were used for the evaluation

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of genotyping of HBV. The HbsAg by ELISA (DRG Diagnostic, Germany), were performed. The HBV DNA was extracted from 200ƒÊl serum samples using Gentra DNA extraction kit (Puregene DNA D-5000, Gentra Systems, Minnesota, USA) according to the kit protocol. The extracted DNA pellet was resuspended in DNA hydration solution present in the kit and two rounds of PCR amplifications where the second round primers was hemi nested to the first round PCR at primers were designed for S gene. The typical amplification was performed in 20ƒÊl reaction volume to extracted DNA and Taq polymerase for 35 cycles at 94 oC for 45 seconds, 55 oC for 45 seconds and 72 oC for 1 minute with extension for 10 minutes at 72 oC. All precautions were carried out to avoid contamination during PCR as well as negative and positive control serum was also included in each run. All the positive HBV PCR samples were selected for genotyping analysis. The HBV-DNA genotypes A through H were determined by using HBV genotype .specific primers for the Hbs Ag surface gene with slight modification (20). In first round of PCR amplification, the primers used were pairs of outer primer and in second round inner primer pairs were used on the basis of the conserved nature of nucleotide sequences in regions of the pre-S1 through S genes, irrespective of the eight HBV genotypes. Two primers P1(sense) and S1-2 (antisense) were universal outer primers(1,063 bp). Primer B2 was used as the inner primer (sense) with a combination called mix 1 for genotypes A, B and C. Primer B2R was used as the inner primer (antisense) with a combination called mix 2 for genotypes D, E, and F. Mix 3 contained one universal sense primer specific antisense primer for genotype G and H. These primer combinations for second round PCR were designed on basis of differences in the sizes of the genotype.

The first PCR was carried out in a tube containing 20ƒÊl of a reaction mixture made up of the following components: 10pmol of each outer primers, 500ƒÊM of four deoxyucleotides, 2Uof Taq polymerase (Promega, USA), 10 x PCR buffer containing 1.5mM Mg Cl2. The thermal cycler (Master Gradient PCR System, Eppendorf AG, Germany) was programmed to first incubate the sample for 2minutes for 95oC followed by 35 cycles consisting of 94o C for 45 seconds, 50o C for 45 seconds and 72oC for 1and half minute with final extension for 7 minutesat 72oC. The second PCR was performed for each sample with common sense primer B2 and mix 1 containing primers for type A through C and common antisense primer B2R and mix 2 containing primers for type D through F. Genotypes of HBV for each sample were determined by identifying the genotypespecific DNA bands. The two different second-round PCR products from one sample were separately electrophoresed on a 2% agarose gel, stained with ethidium bromide, and evaluated under transilluminator. The sizes of PCR product were estimated according to the migration pattern of a 50-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. The identification of different HBV genotype found during our study are shown in figure 1 and 2.

RESULTS

The blood samples were collected for the diagnosis of Hepatitis B virus from different parts of Pakistan as shown in map of Pakistan (figure 3). The clinical course of infection with HBV varies, depending on the one hand on the patients age and immune response and on the other hand the status of the disease were done according to their ELISA results and divided into different infectious status.

The total number of samples for detecting Hb s Ag were n=1204; out of which n=829 are negative cases and 375 are positive cases as shown in table 1. The PCR was performed on all the positive cases of Hbs Ag and only (n=205) were HBV-DNA detected as shown in table 2. For genotype study all the HBV-DNA positive cases were further analyzed for HBV genotype by using specific-primers for core region (Fig 1-2). The pie diagram (figure 4) shows different genotype found in Pakistan during this study.

The data was evaluated in figure 5 and was analyzed that genotype A, B and C were predominated ones and genotype D and F was also found in Pakistan, but only in four cases. Genotype G and H were not identified during this study.

Table 1: Active HbsAg infection in ELISA Positive Male & Female patients (n=1204)

	Male	Female	Total
No. of samples	721	483	1204
Negative	231	360	519
Positive	252	123	375
Percentage	34.95	25.46	31.15

Table 2. HBV-DNA Detection by PCR done on positive male & female patients (n=375)

	Male	Female	Total
No. of samples	252	123	375
Negative	135	35	170
Positive	117	88	205
Percentage	46.43	71.54	54.93

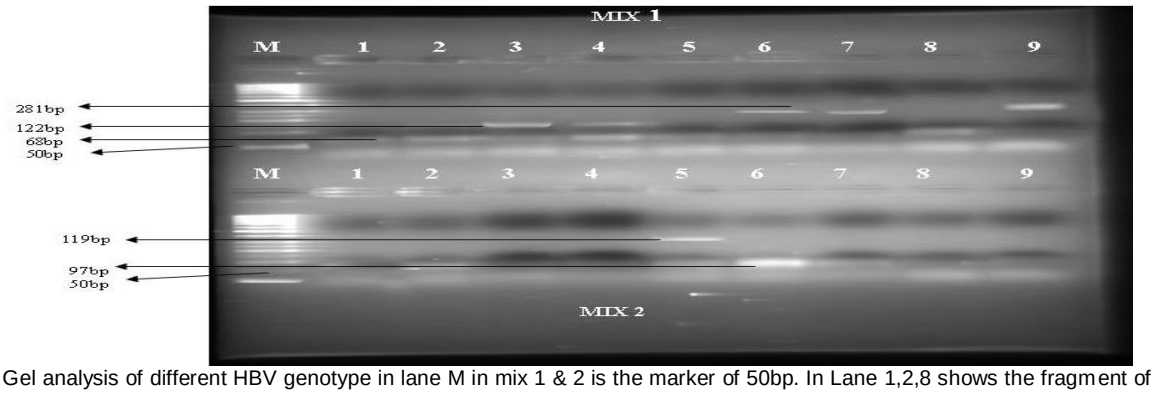

Figure 1. Gel analysis of different HBV genotype in lane M in mix 1 & 2 is the marker of 50bp. In Lane 1,2,8 shows the fragment of 68bp of genotype A, lane 3&4 shows fragment of 122bp of genotype C Lane 6,7 and 9 shows fragment of genotype B at 281bp. In mix 1 genotype A,B & C primers are added. In mix 2, lane 1,2 & 6 indicates band of genotype F97bp whereas lane 5 indicate genotype D with 119bp Lane 1,2, & 6 are mixed genotype.

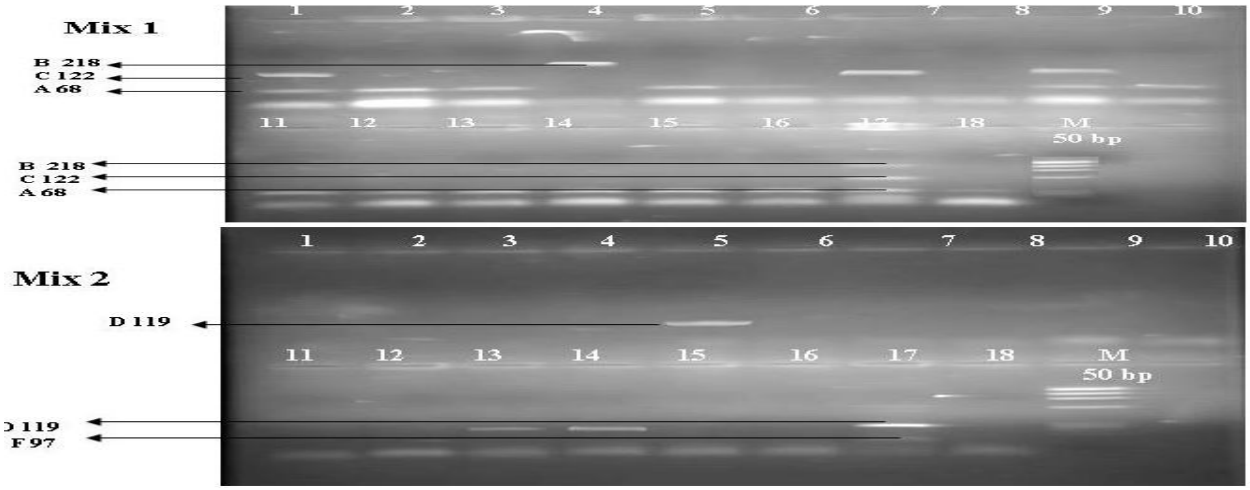

Figure 2. Lane 17 in mix 1&2 shows that all the genotype of HBV present in patient with co infection with HDV found in Karachi

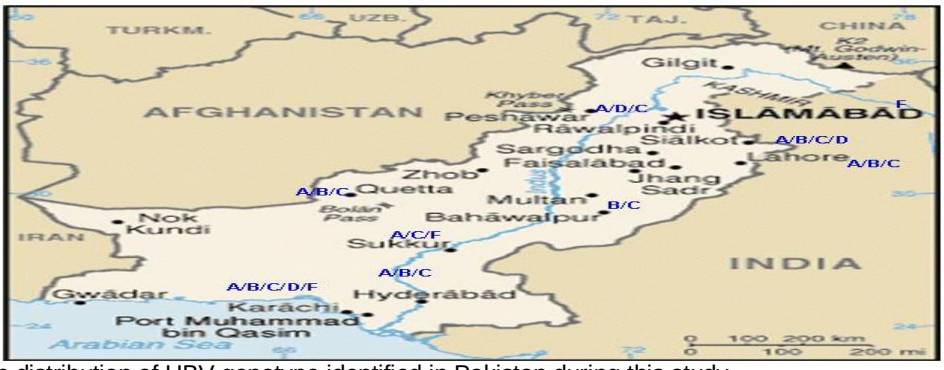

Fig. 3: Geographic distribution of HBV genotype identified in Pakistan during this study.

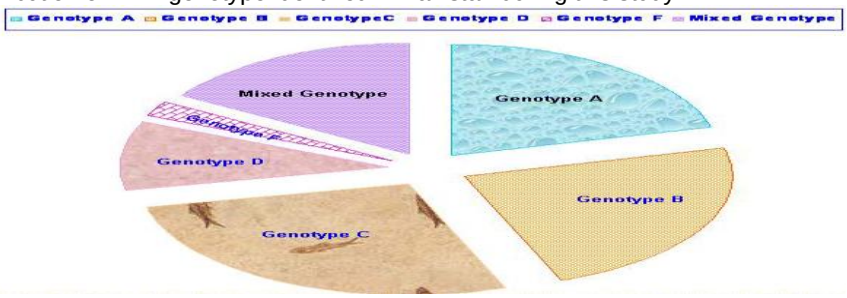

Fig.4: Percentage of different HBV-DNA genotype found in Karachi.

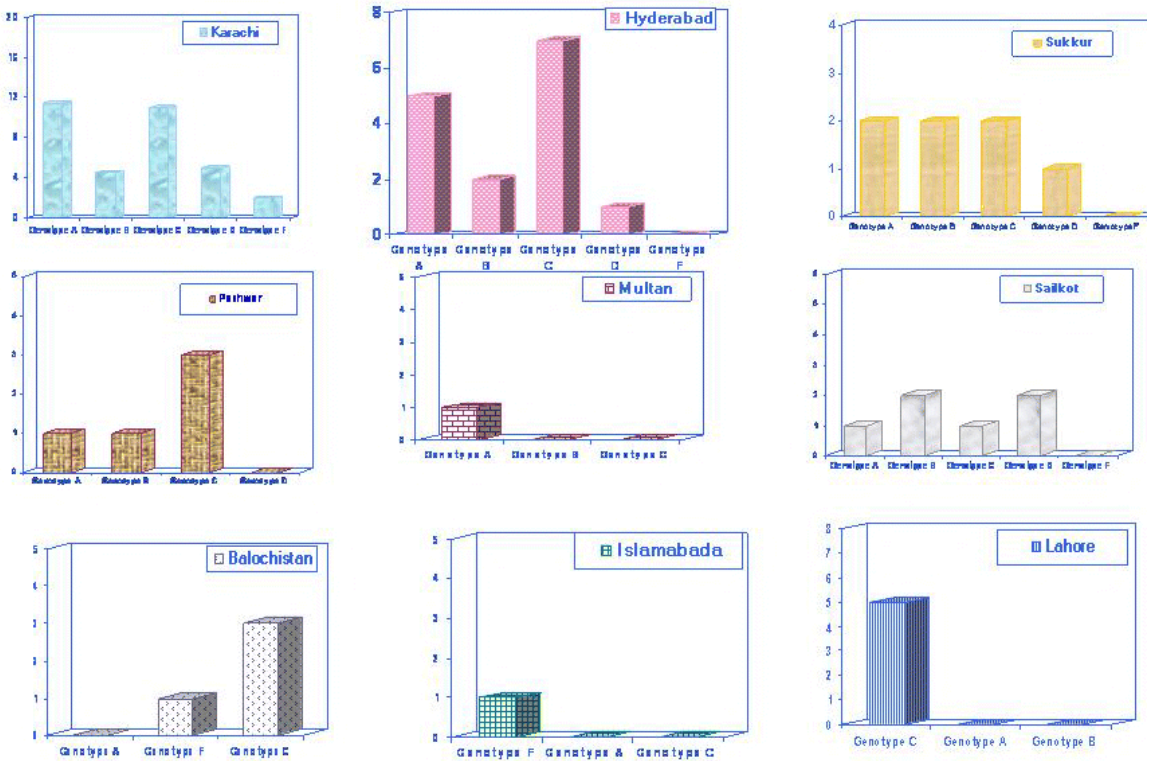

Fig.5: Overview of samples evaluated on HBV genotyping in Pakistan

DISCUSSION

Traditionally, viruses have been classified according to antigenic characteristics, but with recent advances in molecular biology, genotypic classification through the analysis of genomic variation. The genotyping of HBV is important to clarify the route and pathogenesis of the virus. The examination sequence diversity among different isolates of the virus is important, because variants may differ in their patterns of serologic reactivity, pathogenicity, virulence and response to therapy.

The clinical course of infection with HBV varies; depending on the one hand on patient fs age and immune response and on the other hand as increasing evidence is showing on the virus strain infecting the individual. On the whole it has been noted that less than 1% of acute infections lead to fulminant hepatitis and death. Approximately 10% of infected adults become chronic carriers of HBV. Chronic carriers often lack symptoms but may have histological evidence of hepatocellular damage from mild inflammation to cirrhosis and HCC (hepatocellular carcinoma)[21].

During this study five different genotypes were identified i.e. A, B, C, D and F. Taking into account that genotype A strains are mostly found in chronic patients.

In cross sectional study by Mayerat, et al.[22] reported by suggesting that genotype A lead to more often to chronicity as it was found more often in chronic hepatitis patients than genotype D. whereas the opposite situation was found in patients with acute hepatitis.

Karachi being a cosmopolitan city and several migratory movements of different ethnic groups has occurred since the time of Alexander the great. It has been reported that in over time where well-known waves of migration have occurred as other countries the prevalence of different HBV genotypes reflects the origin of the immigrants and other patterns of migration. Bowyer, et.al[23] reported that the prevalent genotype A and D were the exemplified by South Africa, correlating the migration from Northwest Europe, Southern Europe and India. The same genotypes in Argentina, A and D reflect migratory waves from North western Europe, Italy and Spain.(24). Although it shares some structural features with genotype A strains and it is believed to be the original genotype of the New World[23].

HBV genotype F was found in four cases with Hbs Ag was positive, with high viral load titer (figure 5). The most divergent genotype, F, is found in South and Central American[25,26,27]..

During this study we have also come across with mixed genotype. In all the cases the common genotype are B and C. In most of the cases there

were three genotypes. In some cases both HBV and HCV were PCR detected. It was noted that genotype A and C were present in all the cases. In patients with coinfection with HDV genotype A, B, C, D and F were present (figure 2.).

In this study genotyping method based on specific primers for PCR, by which HBV isolates can be classified into genotype A through H was described. To confirm the specificity of the results of PCR typing, phylogenetic analysis in the pre-S1 through S genes of HBV was performed and we confirmed the specificity of the results obtained with our PCR genotyping system. This method is very convenient and will assist research workers in conducting large-scale epidemiological studies. In fact, Stuyver et al. (20), in 2000 reported that recently the identification of a novel genotype of HBV, designated as genotype G.

CONCLUSION

The environmental factors make it difficult to extrapolate findings from onegeographical region to another. Therefore larger depth studies are necessary in regions where HBV is hyperendemic. Traditionally, viruses have been classified according to antigenic characteristics, but with recent advances in molecular biology, the genotyping of HBV is important to clarify the route and pathogenesis of the virus. It is becoming increasingly evident that the genotype of HBV may have a role to play in predicting the response to various therapies and that this should be taken as a variable before initiating any treatment. It also concluded that genotype B and C occurred more frequently in patients with an acute form of liver disease.

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