

Hepatitis B virus (HBV) genotypes in a group of Sri Lankan patients with chronic infection

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Abstract. Hepatitis B infection causes a wide spectrum of liver diseases. Previous analyses of hepatitis B virus (HBV) genome have revealed eight HBV genotypes (A-H), with distinct geographical distribution worldwide. The epidemiology of HBV genotypes and their implications for natural history of disease progression and response to anti viral therapy have been increasingly recognized. This study was undertaken to determine the HBV genotypes in a group of Sri Lankan patients with chronic infection who presented for investigation prior to treatment. Genotypes were determined (2007-2009) in 25 patients with evidence of chronic HBV infection. A genotyping system based on multiplex-nested PCR using type-specific primers was employed in assigning genotypes A through F. Genotypes G and H were not determined. Among the 25 patients tested, genotypes B [9 (36%)], C [4 (16%)], D [3 (12%)], A [2 (8%)] and E [1 (4%)] were detected. There was a relatively high prevalence of mixed infections with genotypes B+C (3), A+D (1), and B+D (2), which overall constituted 24% of patients. Although this is a non-representative sample, HBV infections among this group of Sri Lankan patients were predominantly genotypes B, C and D.

INTRODUCTION

Hepatitis B virus (HBV) infection is associated with a wide spectrum of clinical manifestations, ranging from acute hepatitis to chronic hepatitis, cirrhosis and hepatocellular carcinoma. HBV has 8 genotypes designated A to H, distinguished by nucleotide sequence divergence exceeding 8% of the entire genome (Norder *et al.*, 1994; Okamoto *et al.*, 1998). There is a strong association between the genotype and geographical distribution, and increasing evidence that genotype may affect clinical outcome and response to antiviral therapy (Kao, 2002; Miyakawa & Mizokami, 2003). According to studies published on genotype distribution of HBV, genotype A and D are prevalent in Africa, Europe, Middle-east and Central and South-Asian countries (Erhardt *et al.*, 2005). On the other hand, in Asian-

Pacific regions, genotype B and C are common and genotype C is known to have higher disease-inducing capacity through high incidence of core promoter mutations in the infected (Kao *et al.*, 2000; Orito *et al.*, 2001). Further, genotype meta analysis based on published data indicates significant association between HBV genotype and response to treatment in chronic HBV infected patients in that genotype D appears to be the most resistant (sustained virological response (SVR): 20%) and genotype A the most responsive (SVR: 42%) in comparison with genotype C (SVR: 26%) and genotype B (SVR: 28%) (Rajendra *et al.*, 2006).

HBV genotyping by phylogenetic analysis based on nucleotide sequences produces the most reliable genotyping results. However, this is not an appropriate method for large scale genotyping as well as for testing as a routine diagnostic test in a molecular

laboratory with limited resources. On the other hand, the method based on genotype specific PCR is simple, rapid, and affordable in a molecular laboratory in the developing world.

A significant number of research groups have addressed the need to assess the role of HBV genotype in determining clinical outcomes and need to obtain genotype data from all parts of the world. Further, differences between the same genotypes prevailing in the West and in Asia, and accelerated trend of migration causing shift in genotypic prevalence in various countries require further investigations of genetic diversity of HBV in the context of different populations. Therefore, this study was undertaken to determine the HBV genotypes in a group of patients with chronic HBV infection in Sri Lanka who presented for investigation prior to treatment.

MATERIALS AND METHODS

This study included 25 patients with chronic HBV infection who were presented prior to treatment at the Molecular Medicine Unit of the Faculty of Medicine, University of Kelaniya, Sri Lanka for confirmatory diagnosis of HBV infection by qualitative PCR. Patients who had HBsAg for more than 6 months with an abnormal alanine aminotransferase (ALT) level and the presence of anti-HBc-IgG were diagnosed as having chronic hepatitis. All patients had positive HBeAg profiles. Blood samples (5 ml) were collected from patients during the period from 2007 to 2009 for qualitative HBV PCR, serum separated and stored at -20°C until used. An aliquot of separated serum from the recently positive samples were used for the genotyping assay. This method was initially validated using 4 previously characterized genotype specific DNA samples for genotypes A, B, C and D and further by subjecting another 5 HBV DNA positive samples from our laboratory for an inter-laboratory comparison programme for genotyping which generated 100% concordance in test results. The QIAamp DNA extraction kit (QIAGEN GmbH, Germany) was

employed for DNA extraction from serum samples according to the manufacturer's instructions. A genotyping system based on multiplex-nested PCR using type-specific primers was employed in assigning genotypes A through F based on pre-S1 through S genes of the HBV genome (Naito *et al.*, 2001). The sequences of PCR primers used in this study are shown in Table 1. The P1 and S1-2 were universal outer primers. Primer B2 was used as the inner sense primer with a combination of other anti-sense primers for genotypes A, B, and C in a multiplexing system called "Mix A". Primer B2R was used as the anti-sense inner primer with a combination of sense primers for genotypes D, E and F in a multiplexing system called "Mix B". The genotype specific primers have been designed based on the conserved nature of those sequences within a genotype and poor homology with the sequences derived from other HBV genotypes (Naito *et al.*, 2001). The first PCR was carried out in 25 µl reaction mixture containing 0.25 µM each outer primer, 0.25 mM each dNTP (Promega, USA), 5X PCR buffer containing MgCl₂, 1U of Taq DNA Polymerase (Promega, USA), and 2.5 µl of extracted DNA. The thermocyclic parameters were 95°C for 5 min, followed by 40 cycles consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Two second round PCRs were performed for each sample, one with the common universal sense primer (B2) and type specific primers for genotypes A, B, C in "Mix A" and the other with the common universal anti-sense primer B2R and type-specific primers for genotypes D, E, F in "Mix-B". Reaction mixtures of the second multiplexing PCR systems contained 0.5 µl of the first PCR product, 0.25 µM of each primer, 0.25 mM dNTP, 5X PCR buffer containing MgCl₂ and 1 U of Taq DNA Polymerase (Promega, USA). The cyclic parameters were 95°C for 5 min, followed by 30 cycles consisting of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min. The two different second round PCR products originating from each sample were visualized on an ethidium bromide stained 3% agarose gel. Genotype of each sample was identified by visualizing genotype specific

Table 1. Primer sequences used for HBV genotyping by nested multiplex PCR

Primer	Sequence (position, specificity & polarity)
First PCR	
P1	5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (nt 2823-2845, universal, sense)
S1-2	5'-CGA ACC ACT GAA CAA ATG GC-3' (nt 685-704, universal, anti-sense)
Nested PCR	
Mix A	
B2	5'-GGC TCM AGT TCM GGA ACA GT-3' (nt 67-86, types A to E specific, sense)
BA1R	5'- CTC GCG GAG ATT GAC GAG ATG T-3' (nt 113-134, type A specific, antisense)
BB1R	5'- CAG GTT GGT GAG TGA CTG GAG A-3' (nt 324-345, type B specific, antisense)
BC1R	5'- GGT CCT AGG AAT CCT GAT GTT G-3' (nt 165-186, type C specific, antisense)
Mix B	
BD1	5'-GCC AAC AAG GTA GGA GCT -3' (nt 2979-2996, type D specific, sense)
BE1	5'- CAC CAG AAA TCC AGA TTG GGA CCA - 3' (nt 2955-2978, type E specific, sense)
BF1	5'- GYT ACG GTC CAG GGT TAC CA - 3' (nt 3032-3051, type F specific, sense)
B2R	5'- GGA GGC GGA TYT GCT GGC AA-3' (nt 3078-3097, types D to F specific, antisense)

bands with distinct sizes (Mix A: Type A-68 bp, Type B-281 bp, TypeC-122 bp; Mix B: Type D-119 bp, Type E-167 bp, Type F-97 bp) according to the migration pattern of a 100 bp marker (Promega, USA).

RESULTS

Among the 25 patients tested, genotypes B [9 (36.0%)], C [4 (16.0%)], D [3 (12.0%)], A [2 (8.0%)] and E [1 (4.0%)] were detected (Figure 1). There was a relatively high prevalence of mixed infections with genotypes B+C (3), A+D (1), and B+D (2), which overall constituted 24.0 % of patients (Figure 1). Further, HBV genotype F was not detected in this group of patients and genotypes G and H were not determined.

DISCUSSION

This is the first report describing the HBV genotypes in Sri Lanka. Although this is a non-representative sample, HBV infections among this group of Sri Lankan patients were predominantly genotypes B, C and D. We also found a relatively high proportion of mixed infections. However, although the genotype specific multiplex PCR method described in this study was initially validated by typing previously characterized samples and by

subjecting HBV DNA positive samples for an inter laboratory comparison for genotyping, it was not possible to verify and validate the genotypes of the PCR bands obtained in this study by DNA sequencing due to financial constraints.

In Sri Lanka, although the community prevalence of HBV infection is considered low based on serology markers (< 1%) (Premaratne, 2002), the infection still poses a significant threat to individuals in high risk groups such as patients who had undergone haemodialysis, renal transplantations, and multiple transfusions. All patients in the study group are ethnic Sri Lankans whose HBV DNA profiles had become positive by qualitative testing. The patient base of the study group included five patients who have had multiple transfusions while on treatment for cancer or leukemia and two patients who have had kidney transplantations. Of the five patients who have had multiple transfusions while on treatment for cancer or leukemia, one had mixed genotype HBV infection with genotypes "B" and "C".

Using different methods of genotyping, several reports have described high rates of double infection with two different HBV genotypes in many parts of the world (Ding *et al.*, 2003; Chen *et al.*, 2004). However, the epidemiology and clinical significance of such mixed genotype infections is still poorly understood. In chronic HBV patients, super

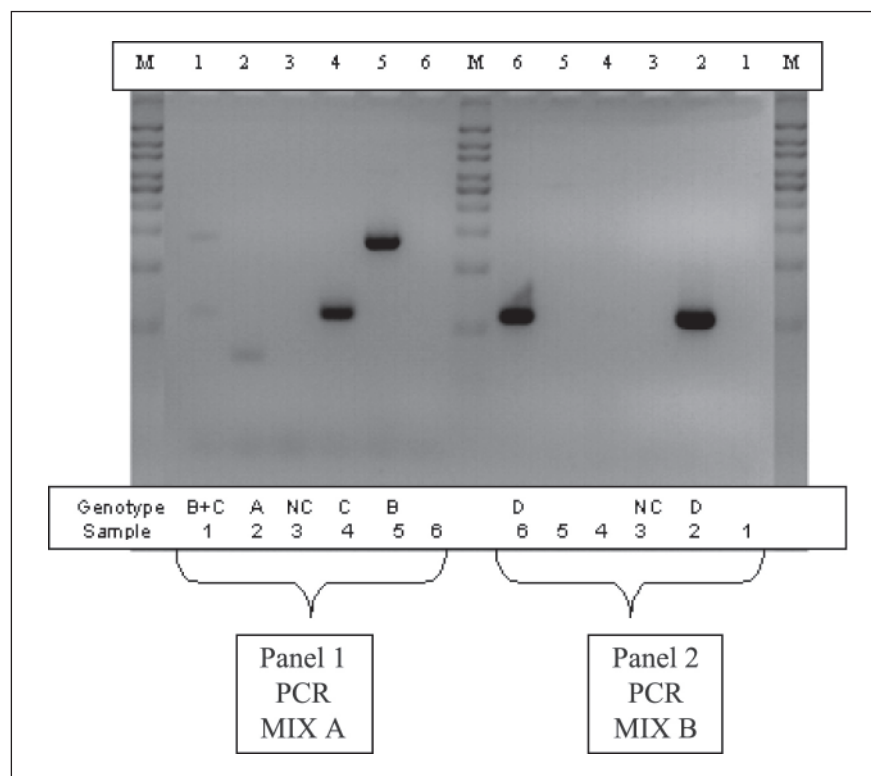


Figure 1. Ethidium bromide stained 3% agarose gel indicating different genotypes corresponding to samples through 1-6; Panel 1 shows genotyping PCR with “mix A” and panel 2 shows PCR amplification with “mix B” for the same set of samples; negative control is denoted by “NC” and the 100 base pair marker is denoted by “M”.

infection with HBV isolates of the same or different genotype has been described by Kao *et al.* (2001), and super infection is accompanied by acute exacerbation of the chronic disease. A study conducted in four regions of China suggested that mixed infection with genotypes B and C might lead to more severe liver damage (Zhu *et al.*, 1993) and 26% of British liver transplant patients were reported to be infected with multiple genotypes (Girlanda *et al.*, 2004). Toan *et al.* (2006) found that chronic infection was more common in patients infected with mixed genotypes. As different genotypes have different pathological and epidemiological profiles, their detection and monitoring would be medically significant. Further, efforts to prevent super-infection or co-infection in patients with chronic hepatitis B should not be overlooked, especially in the case of patients that will receive blood transfusions or kidney transplantations.

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