

Genotypes of hepatitis B virus identified in patients tested prior to endoscopy from a Teaching Hospital in the Central Province of Sri Lanka

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Abstract

The present study was carried out to identify the hepatitis B virus (HBV) genotypes in six patients attending the surgical clinic who were positive for hepatitis B surface antigen. DNA was extracted from the serum of patients and subjected to a modified nested PCR to detect a 585 bp region within the S gene of the HBV genome. Positive PCR products were purified and sequenced via a cycle sequencing method. The sequence data were analyzed with reference sequences in the HepSEQ database to identify the particular HBV genotype of the samples. Nested PCR for the S gene of the HBV genome was positive in 2 out of 6 samples. The genotyping and sequence analysis of the PCR products showed HBV genotype A with a homology of 98% to the reference sequences in the HepSeq database.

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Introduction

Hepatitis B virus (HBV) is an enveloped DNA virus that infects the hepatocytes of humans. HBV is transmitted from person to person by sexual contact or mother to baby during delivery or afterwards or from blood products during transfusion or accidental inoculation in a healthcare setting [1]. HBV infection can give rise to either acute infection or chronic disease which can lead to cirrhosis and hepatocellular carcinoma in the long term [1]. The HBV genome is a partially double stranded circular DNA molecule, 3200 base pairs long [2]. Sequencing and phylogenetic studies of the genome indicate the existence of eight distinct genotypes (A-H) and numerous sub types of HBV. These genotypes and sub types have risen from nucleotide substitution mutations occurring at an estimated rate of $1.4-3.2 \times 10^{-5}$ per year due to the low proof reading capability of the viral polymerase during replication. Replication of the HBV genome unlike other DNA viruses occurs via an

intermediate RNA synthesis process involving the action of an error prone viral reverse transcriptase giving rise to mutations at a higher rate compared to that of other DNA viruses. The genotypes of the virus are known to differ from each other by more than 8% of their genome while differences of at least 4% account for their varied subtypes [3]. Identifying the HBV genotypes of chronically infected individuals in a population is useful in determining the available treatment options and predicting the treatment outcomes especially when the chronic HBV infection has evolved to cause the initial fibrotic changes in the liver. DNA sequencing is the gold standard for HBV genotyping [3]. Whole genome sequencing followed by phylogenetic analysis show promising results but these are neither time efficient nor cost effective. A more efficient and accurate method involving the amplification of a target region encompassing genotypic variability, followed by sequence analysis for genotype identification would be a more appropriate method. This study has used such a method by targeting the HBV S gene for genotypic variability.

Methods

A total of six HBsAg positive serum samples collected between 2007- 2011 and stored at -20°C from were analyzed. All samples were from individuals from the Central Province referred from the Surgical Clinic of the Teaching Hospital, Peradeniya prior to endoscopy. Sera of healthy adults that were negative for HBsAg were used as negative controls. Two hundred µl of serum was used for DNA extraction. Manufacturer's instructions (Nucleo Spin® Tissue Kit, Germany) were followed. Sixty µl of DNA was extracted from each sample.

The extracted DNA was then subjected to a nested PCR protocol [4] that was later modified to suit the local laboratory conditions. The first round of PCR was carried out using the forward and reverse outer primers PreS2 (5'-GGGACACCATATTCTTGG-3') and SIR (5'-TTAGGGTTTAAATGTATACCCA-3') to amplify a region between the PreS1 and S genes which would result in a 1025 bp amplicon. A total volume of 20 µl of the

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master mix was used as follows; 5 µl of 5× buffer, 1.25 µl of 2 mM dNTPs, 1.25 µl of 5U Taq polymerase, 6.3 µl of nuclease free water along with 5µl of the extracted DNA template to make up to a total volume of 25 µl. The thermal profile used in amplification was as follows; an initial denaturation of 3 min at 94°C followed by 40 cycles of denaturation, annealing and polymerization at 94°C for 45s, 58°C for 1 min and 72°C for 1 min, respectively. This was followed by a final extension of 72°C for 10 min.

The 2nd round of PCR was carried out using the same master mix composition as for the first PCR, but using different primers YS1 (GCGGGGTTTTTCTTGTTGA) and YS2 (GGGACTCAAAGATGTTGTACAG) that amplify a 585 bp region within the first amplicon (1025 bp). Five µl of the first PCR product was used as the template making a final volume of 25 µl. The thermal profile used was identical to that of the first PCR. The final PCR products were resolved on a 2% agarose gel and visualised.

The purified PCR product was subjected to sequencing using the Genetic Analyzer 3130 (Applied Biosystems; Sequencing Analysing software V5.2). The sequence was compared with reference sequences in the HepSEQ database to identify the HBV genotype of the sample.

Results

Nested PCR gave bands of 585 bp in conjunction with the positive control (Figure 1) for only two of the 6 HBsAg positive patients' samples. Genotyping and sequence analysis carried out on one positive sample revealed a sequence of 517 bps with a 97.49% homology to reference sequences of HBV genotype A. The other positive sample revealed a sequence of 235 bps with a 97.86% homology to reference sequences of HBV genotype A (Figure 2).

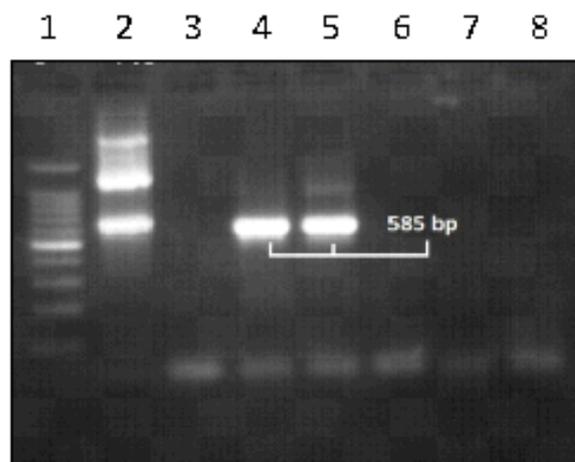


Figure 1. Agarose gel profile of PCR products from 5 HBsAg positive serum samples. Lane 1 is a 100 bp marker and lane 2 is the HBV positive control. Lane 3 (negative control, lanes 4 and 5 are samples that were positive by PCR showing a band size of 585 bp). Lanes 6, 7 and 8 are samples negative for PCR.

Discussion

Nested PCR was positive for 2 of the 6 samples with the expected band size of 585 bps and the other samples did not give a positive PCR result suggesting that though these samples were positive for HBsAg they were negative for HBV DNA. This might be due to the high number of HBsAg particles but lack of HBV in the sera [5]. Another reason for this finding would be that the blood samples from the corresponding patients would have been obtained in the latter rather than early phases of chronic HBV carriage where the levels of HBV DNA are likely to be

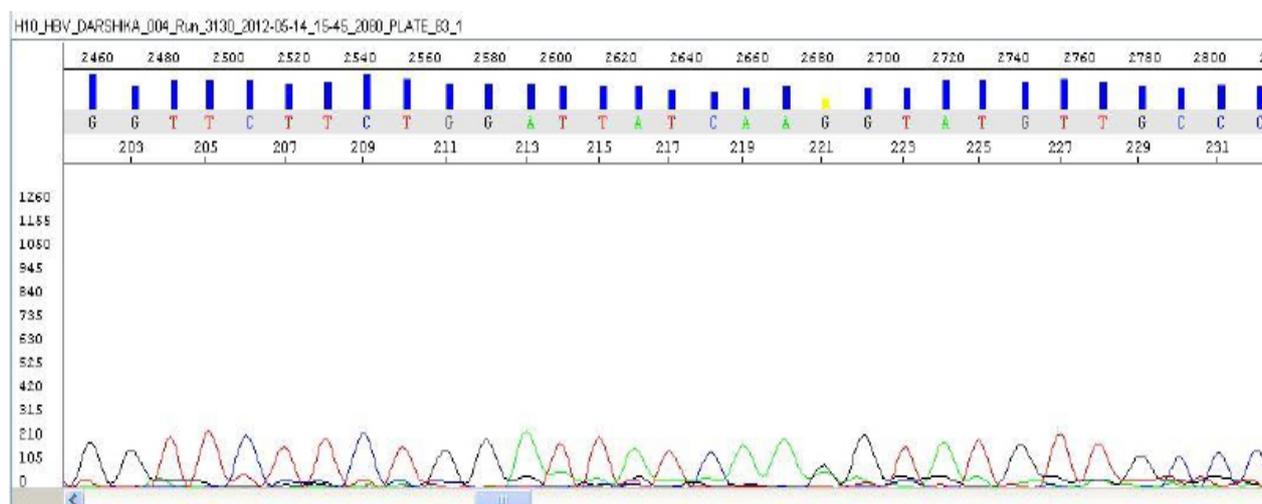


Figure 2. Electropherogram of HBV DNA of positive sample 5.

low in the serum due to clearance by the immune system resulting in negative PCR [6].

Genotyping and sequence analysis results carried out on the PCR positive samples 4 and 5 revealed sequences of 517 and 235 bps which were smaller than the expected target sequence size of 585 bp and this might be due to the physical stress exerted on the PCR products during purification prior to sequencing that may have resulted in degradation of the PCR products. Sequence analysis of both PCR products gave a homology of almost 98% to HBV genotype A instead of the expected 100%, this 2 to 4% heterogeneity may be attributed to different recombinants, quasi-sub-genotypes or differences in the A1 and A2 sub-genotypes. It is also worthy to note that the HepSEQ tool used here utilizes only the polymerase or surface genes for genotype identification that allows the identification of any escape mutants, but is less accurate for detecting any recombinants if they are present in the sample sequences [7].

According to previous studies genotype A of HBV is pandemic and is predominantly found in the USA, Northwest Europe, Africa and Asia and this remains consistent with results of the current study [3]. However, a study done in Colombo, Sri Lanka on prevailing HBV genotypes in a small cohort of patients with chronic hepatitis B indicates that HBV genotype A (8%) occurs at a lower rate than HBV genotype B (36%), C (16%) and D (12%) [8]. Findings from this study are different to results of our study. Both studies did not include representative samples and these findings may indicate a possible difference in HBV genotypes in different areas in the country. This point emphasises the need for identification of prevalent genotypes in different localities of the country which would lead to better understanding of the spread and distribution of HBV genotypes in Sri Lanka.

The study shows HBV genotype A in the small sample tested, the findings are similar to that of many other studies that claim HBV A to be the predominant circulating genotype in South Asia. Furthermore, the study can be used as a basis for future HBV characterisation,

genoty-ping and epidemiological studies in Sri Lanka.

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Conflicts of interest

We declare that there are no conflicts of interest.

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