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Full Length Research Article

HEPATITIS B RESISTANCE TESTING AND GENOTYPE DETERMINATION

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ABSTRACT

Introduction: The aim of this study is to determine the predominant HBV genotypes for a number of samples and controls as well as to identify the mutant genes which thought to be responsible for the HBV antiviral drug resistance.
Methods: A number of samples (8 samples), that were already collected from infected patients by the microbiology staff of the Manchester University hospital for the purpose of the study, are extracted and tested to determine the HBV genotypes and antiviral resistance. The techniques that are used for this purpose in this study are PCR amplification, gel electrophoresis and then sequencing of PCR products by using Applied Biosystems Invitrogen (ABI-3100).
Results: With gel electrophoresis, PCR sample products are compared with the intensity of ladder (marker), and the sequences are analysed by using Bioedit® (Bioinformatics software) and then genotypes of the HBV are retrieved from the NCBI tool.
Conclusion: We found that the infected samples are predominantly returned to viral genotypes D as well as it is noticed that the resistance to the HBV antiviral drugs were related to the replacement of some amino acids in a viral gene by other wild mutant one ,especially at the amino acid codon 180 position there was a mutation where Methionine was replaced with mutant Leucine and also at the codon 204 where Methionine was replaced with mutant Valine.

Key words: Hepatitis B Virus, Hepatitis B Resistance Test, Hepatitis B Virus Genotyping, Antiviral Resistance Testing.

INTRODUCTION

Hepatitis B virus causes a complex liver infection that ranges from acute to chronic hepatitis. The chronic infection may progress into cirrhosis and hepatocellular carcinoma. Therefore, therapeutic treatment is essential to be applied to chronic phase to prevent this progression. Interferon -α with a number of nucleoside and nucleotide analogues are available. However, drug resistance is a major problem, especially when they are used for a long-term. This is mainly due to the selection of a mutation in the viral polymerase gene (Liu and Kao, 2006). Hepatitis B virus is partially dsDNA virus with circular genome that consist of 4 genes encoding the viral envelope, nucleocapsid, polymerase and X protein. In addition, the virus has eight genotypes (A-H) depending on genome sequencing divergence and each with different geographic distribution but the most common genotypes are A, B, C and D (Kramvis *et al.,* 2005; Kramvis and Kew, 2005). Genotyping is performed by examining the DNA sequences of living organism and comparing them to reference sequences to determine genomic difference (genotype) between the organisms (Popa, 2009). Some studies have found that HBV genotypes may have effect on the progression and the outcomes of the chronic infection as well as the antiviral response (Kramvis *et al.,* 2005; Kramvis and Kew, 2005), although, recent evidences are not convincing.

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The importance of genotyping in patient management

Genotyping methods have many advantages in managing the patient. First of all, it is applied for monitoring drug resistance by detecting changes in the genomic sequence in the course of treatment such as Direct DNA Sequencing, RFLP, DNA Hybridization, Clonal Analysis and so on (Sablon and Shapiro, 2005). Furthermore, it is useful to detect some mutations that have a role in viral latency, pathogenesis, virulence, immune escape, and resistance to antiviral therapy (Buti *et al.,* 2005) as well as in viral phylogeny to determine where the organism originate and to identify the way of transmission in families infected with the same genotype (Lin *et al.,* 2005) and also it can be used for epidemic investigation such as in influenza A vaccine which needs updating each year because of high mutation rate. Another advantage is for determining the genetic diversity of other microorganisms such as bacteria and fungi, especially for diagnosis, treatment, and epidemiological analysis of nosocomial outbreaks (Wolska and Szweda, 2012). On other hand, there are some disadvantages, for example, when new viral infection occurs, the sequence databases for this novel virus may not be available, so that genotyping in these situations are inapplicable. Also it might be inappropriate to use in undeveloped countries.

MATERIALS AND METHODS

Materials

Qiagen-QIAamp MinElute Virus Spin Kit®
Amplitaq Gold (Hotstart Taq polymerase
Invitrogen dNTP mixture
PCR and Sequencing primers
Ultrapure DNase- and RNase-free water (Promega)
Gilson pipettes 2-1000 µl
0.2µl PCR reaction tubes
ABI microtitre reaction plate and caps
USB ExoSAP-IT®
Invitrogen E-gel 2% + ethidium bromide
Gel loading buffer
ABI Big Dye Terminator v 1.1 Cycle Sequencing Kit
3M Na Acetate (BDH/Sigma)
70% Ethanol
ABI Hi-Di Formamide

Methodology

Sample Extraction

Qiagen MDX system is used for DNA extraction from serum samples. 300 µl of serum is used and eluted to 100µl buffer.

PCR amplification

HBV PCR reaction mixture was prepared for all 4 samples and controls as the following: Volumes shown are for 1 reaction:

Water	42.75µL
10_X PCR buffer	5µL
dNTP mix (2.5 µM each)	1µL
Forward Primer FP (5µM)	0.5µL
Reverse primer RP (5µM)	0.5µL
Ampli Taq Gold 5U/µL	0.25µL
Total	50µL

45µL pipetted into a 0.2µL PCR tube and then 5µL of extract was added to reaction mixture and finally transferred to a 9700 thermal cycler and run at the following thermal condition for **35** cycles:

94C	2min
94C	1min
55C	1min
72C	1min
72C	5min

Gel electrophoresis

Gel electrophoresis was performed on PCR products (amplicons) using 2% agarose gel (E-Gel) as follows:

1. 10µL sample transferred into 0.5µl Eppendorf tube.
2. 1µL loading buffer then added to each sample (cresol red) with 10µL sterile distilled water.
3. 20µL of each sample was loaded on to a 2% E-gel.
4. 5µL DNA molecular weight marker mixed with 15µL of water as loaded onto the gel. (total 20µL).
5. Run for 15-30minutes and finally transilluminator was used to visualise and record the results.

Molecular techniques

UBS ExoSAP-IT method was used to clean up PCR product but because our product had low intensity in comparison to DNA ladder on agarose gel (Figure 1), it was directly mixed with UBS ExoS AP-IT without dilution in RNase-free water. Then after, 2nd PCR was applied for sequencing reaction to amplify DNA. Later, Ethanol/Sodium Acetate method was used to clean up the sequencing product (to remove extra primers, enzymes…etc). Finally, the product was sequenced by using ABI-3100 Genetic Analyser.

RESULTS

After performing of PCR and Gel Electrophoresis, the intensity of PCR products are compared with marker and they are found to be positive confirming the infectivity of samples (Figure 1). Then by using Bioedit software for the sequenced products, the consensus sequence were obtain from query sequence to test the antiviral resistance as well as the NCBI genotype tool was used to determine the predominant HBV genotype by comparing the obtained consensus sequence with the reference sequence.

Figure 1. An example shows the intensity of infected sample product in well 9 and 10

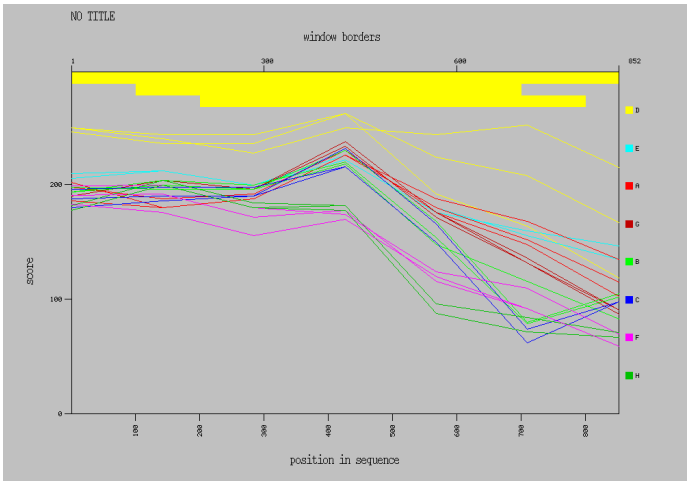

Figure 2. An example shows Hepatitis B genotypes and the predominant type D

Table 1. Resistance patterns of different antiviral drugs used for the treatment of chronic hepatitis B (Bömmel *et al.*, 2015)

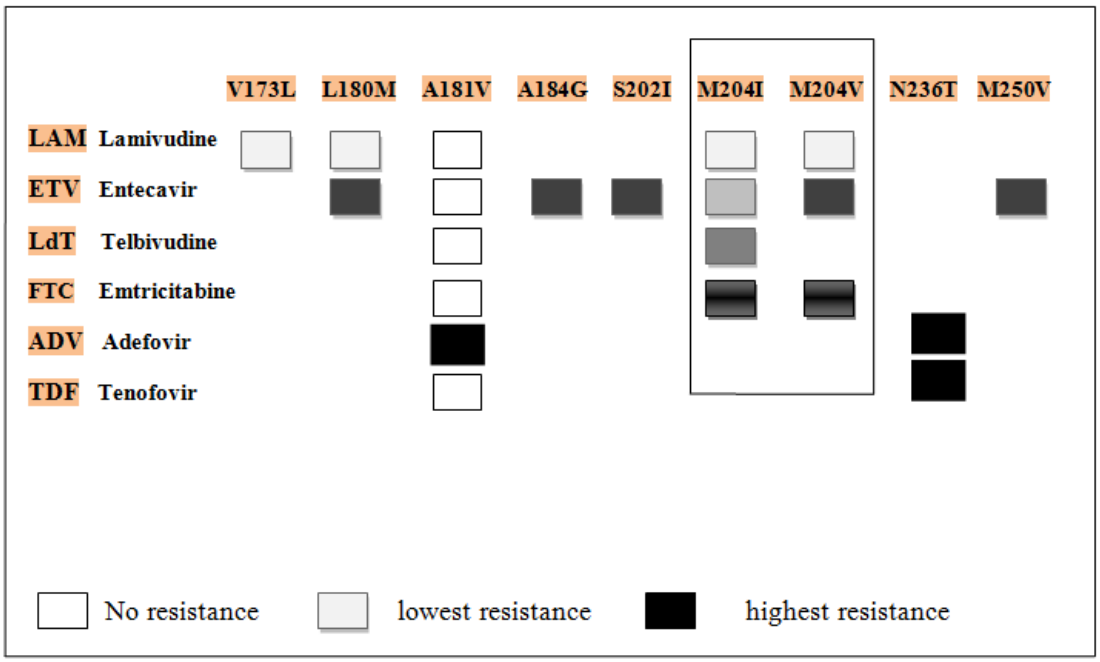

Table 2. Determine for nucleotide positions whether each codon is wild type or mutant (Practical Protocol of MSc Medical Microbiology and Medical Virology, 2013)

Nucleotide position	Codon	Nucleotide	Wild Type Amino Acid	Usual Mutant Nucleotide	Mutant Amino Acid
507	169	ATT ATC	Isoleucine (I)	ACN	Threonine (T)
519	173	GTG	Valine (V)	TTG	Leucine (L)
540	180	TTG or CTG	Leucine (L)	ATG	Methionine (M)
543	181	GCN	Alanine (A)	GTN	Valine (V)
552	184	ACN	Threonine (T)	GGN AGT AGC TCN	Glycine (G) or Serine (S)
582	194	GCN	Alanine (A)	ACN	Threonine (T)
606	202	AGT AGC TCN	Serine (S)	ATT ATC ATA	Isoleucine (I)
612	204	AGT	Methionine (M)	GTG or ATA ATT ATC	Valine (V) or Isoleucine (I)
708	236	AAC or AAU	Asparagine (N)	ACN	Threonine (T)
750	250	ATG	Methionine (M)	GTN	Valine (V)

CONCLUSION AND DISCUSSION

Antiviral Resistance Testing

In this study, it is found that there were some changes in corresponding amino acids that would be responsible for antiviral resistance. As shown in (Table 2), at the amino acid codon 180 position there was a mutation where the wild type Methionine was replaced with mutant Leucine and also at the codon 204 where wild type Methionine was replaced with mutant Valine. In addition, at the codon 202 where the Isoleucine was altered to Glycine but this could be normal since the nucleotide AGT also codes for Serine.

Regarding to the level of drug resistance as shown in Table 1, the mutation at L180M shows high rate of resistance to Entecavir and relatively low resistance rate to Lamivudine whilst it shows no resistance to other antiviral agents such as Adefovir, tenofovir, Telbivudine and Emtricitabine, in other words, these agents are better to be used for treatment of the patient whereas mutation at the M204V codon position shows resistance to Emtricitabine, Entecavir, Lamivudine but at S202I codon only shows resistance to Entecavir while there is no resistance to the remaining agents, that is mean Entecavir is not a drug of choice and its use should be limited [5].

Hepatitis B genotypic determination

Methods have been used for HBV genotyping are including PCR amplification assay by using specific primers and NCBI genotyping tool. This study shows that HBV infected sample is predominantly returned to viral genotypes D (Figure 2). This genotype is characterised globally by its high distribution rate in the Mediterranean area, the near and middle east, and south Asia and also by its high risk rate for producing fulminant hepatitis. It is also the more frequent cause of severe chronic hepatic diseases in comparison to other HBV genotypes. Furthermore, this genotype is also found to be mostly associated with precore mutants that increase the rate of progression to cirrhosis and hepatocellular carcinoma (Bahri *et al.*, 2006).

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