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Genotyping of HBV using type specific primer PCR and restriction fragment length polymorphism-PCR

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Key words: HBV: Hepatitis B Virus, TSP-PCR: Type Specific Primer-PCR, RFLP-PCR: Restriction Fragment Length polymorphism-PCR.

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Abstract

Globally, about 2.5 billion people are infected with HBV. In Pakistan, about seven million people are infected with hepatitis B. Hepatitis B is a liver inflammation caused by viral infection and can develop into liver carcinoma if not treated. HBV is categorized into eight molecular genotypes. The main objective of this study was to evaluate the efficacy of most effective method for genotyping as well as genotype distribution in Lahore, Pakistan. 130 HBV DNA positive samples were collected from different hospitals of Lahore, DNA extracted, and was preceded for amplification using type specific primer PCR and RFLP-PCR. Genotype specific amplicons were analyzed on 1.5% agarose gel electrophoresis. Results showed that genotype D was most abundant in Punjab, as first method (TSP-PCR) showed the 90.8% samples were of genotype D and 9.2% samples having the genotype C. Second method RFLP-PCR gave 94.6% samples were genotype D and 5.3% were genotype C after digesting by two enzymes *Psu1* and *Sty1*. According to our sampling data genotyping comparison gave results: TSP-PCR showed that male have 93.50% genotype D and 6.50% genotype C and females have 88.60% genotype D and 11.40% genotype C. Second method RFLP-PCR showed that males have 96.10% genotype D and 3.90% genotype C and females have 92.40% genotype D and 7.60% genotype C. Statically analysis of both methods gave a significant P (> 0.5). It is concluded that both methods are very effective, sensitive and reliable techniques. Genotype D was found most dominant among Punjab population.

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Introduction

Hepatitis B virus infections are a major public health issue because of their prevalence worldwide and about 2.5 billion people are infected with HBV (André 2000). Pakistan is massively affected with HBV with seven million people infected (Khan *et al.*, 2002). Pakistan is in the midway HBV prevalence area with a carrier frequency of about 3–5% (Umar *et al.*, 1999).

Hepatitis is a viral disease caused by the inflammation of liver and characterized by the existence of inflammatory cells in tissues. (Lu and Block, 2004).HBV is a small, double stranded DNA with the genome size of about 3.2kb. The viral DNA translates proteins by four genes as surface protein, viral core protein, X protein (Arzumanyan *et al.*, 2013). Hepatitis B virus occurs in eight different genotypes (A-H) and its prevalence fluctuates by its origin and geographical, these are further categorized into a number of sub genotypes (Lindh *et al.*, 1998; Norder *et al.*, 2004).

The most dominant HBV genotype in Pakistan is genotype D with overall prevalence rate of 65% higher than genotype A (10%), genotype C (7%) and genotype B (5%) (Lu, Chiang *et al.*, 2004). Many studies have showed that not only does HBV genotype affect the significance of the disease but it also impacts the result of therapy with interferon's and pegylated interferon's, with genotype A performing well than genotype D in Caucasians and genotype B doing fine than genotype C in Asians (Zekri *et al.*, 2007).

Hence, the main objectives of this research were; type Specific Primer based PCR and PCR-RFLP based genotyping of all HBV patients, to identify the circulating HBV genotypes in Punjab, Pakistan and their relationships with gender and age.

Materials and methods

Primers designing

The primers used in this study were already reported (Naito *et al.*, 2001) (Table I and Table II).

Table I. List of Type specific primers.

Sr #	Primer name	Primer sequence	Target gene	PCR product
1	P-1 (sense)	5'-TCACCATATTCTTGGGAACAAGA-3'	S	2.138 Kb
2	S1-2 (antisense)	5'-CGAACCACTGAACAAATGGC-3'	S	
Mix A primers				
3.	BA1R (Sense)	5'-CTCGCGGAG ATTGACGAGATGT-3'	S	68 bp (type A)
4.	BB1R (Sense)	5'-CAGGTTGGTGAGTGACTGGAGA-3'	S	281 bp (type B)
5.	BC1R (Sense)	5'-GGTCCTAGGAATCCTGATGTTG-3'	S	122 bp (type C)
6.	B2 (Antisense)	5'-GGCTCMAGTTCMGGAACA GT-3'	S	Reverse common primer (for A, B, C genotypes).
Mix B primers				
7.	BD1 (Sense)	5'-GCCAACAAG GTAGGAGCT-3'	S	119 bp (type D)
8.	BE1 (Sense)	5'-CACCAG AAATCCAGATTG GGACCA-3'	S	167 bp (type E)
9.	BF1 (Sense)	5'-GYTACGGTCCAGGGTTAC CA-3'	S	97 bp (type F)
10.	B2R (Antisense)	5,-GGAGGCGGATYTGCTGGCAA-3'	S	common primer to 3 (E, F, G genotypes).

Table II. List of of primers of Restriction Fragment Length Polymorphism, RFLP-PCR.

Sr.#	Primer name	Primer sequence	Target gene	PCR product
1.	Outer sense	5'-GGGACACCATATTCTTGG-3'		
2.	Outer antisense	5'-TTAGGGTTTAAATGTATACC-3'	S	1.233 Kb
3.	Inner sense	5'-GCGGGGTTTTTCTTGTTGA-3'		
4.	Inner antisense	5'-GGGACTCAAGATGTTGTACAG-3'	S	585 bp

DNA Extraction

HBV DNA positive serum samples of 130 patients were collected from the different hospitals of Lahore with the consent of patients. DNAs were extracted from Serum samples using kit of Macherey-Nagel, (Germany).

Genotyping

Genotyping of extracted HBV positive DNA were done by two following methods,

Type specific primers PCR

First round PCR

In first PCR outer set of primers were used which were universal primers for HBV named P-1 and S1-2 reaction mixture was prepared containing 25 picomoles / microliter of each primer, 10X of PCR buffer, 50mM of $MgCl_2$, 2.5mM of mixed dNTPs, 5U/ul of Taq polymerase was used and volume made up to 25ul for a single PCR reaction mixture to amplify 5ul of DNA template. Temperature profile used was 95°C of initial denaturation for 10 minutes followed by 35 cycles. These cycles were optimized at these given conditions: denaturation at 94°C for 20 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 1 minute. After 35 cycles final extension was done at 72°C for 7 minutes and hold at 4°C.

Second round PCR

In second round PCR two reaction mixtures were prepared parallel which were Mix A and mix B, using the same components as used in first round PCR reaction, except two ul of DNA template of first round PCR was used and internal sets of primers. Mix A: In mix A four primers were used in which three were forward primers (BA1R, BB1R and BC1R) and one was reverse primer (B2) for three different genotypes, A-B and C. Mix B: In mix B another four primers were used in which three reverse (BD1, BE1, and BF1) and one forward primer (B_2R) was used for other three genotypes which were D-E and F. This temperature profile was modified with two portions, first was initial denaturation 95°C for 10 minutes followed by 20 replications with the following steps, denaturation at 94°C for 30 seconds annealing at 58°C for 20 seconds extension at 72°C for 30 seconds and last step of this PCR portion was final extension at 72°C for 5 minutes, in the next immediate portion for same PCR mixture of A and B the temperature profile was same except the annealing temperature was 60°C for the same time as in the first portion. The temperature difference of two steps of PCR was due to differences in annealing temperature of all the primers of mix A and mix B.

Restriction Fragment Length Polymorphism-PCR

First Round PCR

The first PCR reaction mixture of RFLP-PCR was composed of about 5 microliter of DNA template, 50 mM of $MgCl_2$, 2.5 Mm of dNTPs, 5U/ul of taq polymerase, 25 Pico moles of each outer primers, 10X taq buffer and volume make up to 25 ul with nuclease free water of the reaction mixture. The temperature profile of the first RFLP-PCR was initial denaturation at 95°C for 3 minutes followed by 35 cycles in which final denaturation was at 94°C for 45 seconds, annealing at 53°C for 60 seconds extension at 72°C for 1 minute and 30 seconds and then at the end final extension at 72°C for 7 minutes optimized.

Second Round PCR

In second nested PCR, the template was used the product of first PCR which was 2 ul and rest of the PCR component's concentrations were same as in first PCR of RFLP-PCR. Temperature profile used is given below.

Restriction digestion of the second PCR product

Restriction digestion of 129 samples was carried out; each sample was separately restricted using both *StyI* and *PsuI* fast digest enzymes of thermo scientific lot # 00195582 and 00195619 respectively with fast digest buffer. For each sample, an eppendorf was taken and 7.5µl amplified product, 1.5µl fast digest buffer and 0.5µl restriction enzyme.

Gel analysis of 1st and 2nd PCR product

The band pattern product were detected on 1.5% agarose gel under UV light.

Statistical Analysis

The data obtained from this study were statically analyzed for significance through SPSS software.

Results

Type specific primers PCR method

First Round PCR

The universal outer set of primers was used to amplify the S gene for type specific primers PCR method. 1st round PCR showed 1.063kb fragment of DNA after running the PCR product on agarose gel electrophoresis. The first PCR was analyzed on agarose gel which is shown in Fig. 1.

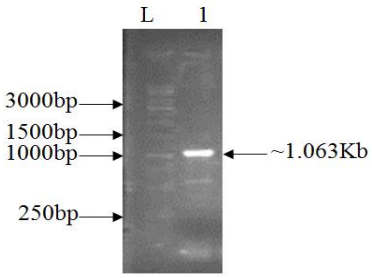

Fig I. 1st Round PCR, here L: 1 kb DNA ladder (GeneRuler by Fermantas), 1: Amplicon of S gene using outer set of primer.

Second round PCR

The product of 1st PCR was used as a template for 2nd PCR amplification using specific primers for specific PCR in two reaction mixtures named as mix A and mix B. The product of second PCR was analyzed on agarose gel electrophoresis and showed that few samples amplified in mix A only of DNA length of about 119 bp which is genotype C (Fig.II).

Mix B showed amplification in maximum samples giving the DNA of fragment length of about 122 bp which is genotype D, most common genotype in province Punjab of Pakistan (Fig. III).

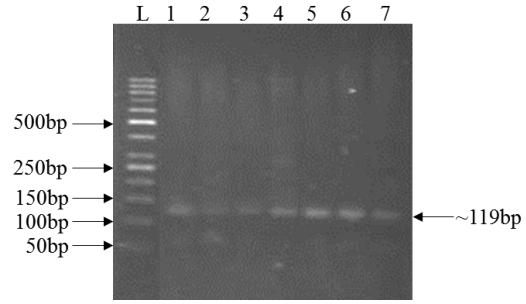

Fig II. 2rd round PCR, L: 50 bp DNA ladder (Gene Ruler by Fermantas), Lane 1-7: Amplicons of S gene using mix A primers.

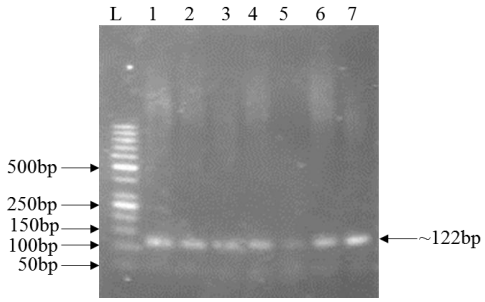

Fig III. 2nd round PCR, L: 50 bp DNA ladder (Gene Ruler by Fermantas), Lane 1-7: Amplicons of S gene using mix B primers.

Restriction fragment length polymorphism-polymerase chain reaction

First Round PCR

In this method, using first round we used outer set of primers, same samples which were used in first method, were used again to amplify the S gene which was analyzed on agarose gel and results showed that fragment of S gene was amplified of approximately of 1.233kb shown in gel Fig. IV. The results of first PCR encouraged us to do second round of RFLP-PCR. In this round we used inner set of primers to amplify further specific S gene and analyzed on 1.5% agarose gel and then result viewed by gel documentation system which showed the fragment of 585 base pairs shown in Fig. V.

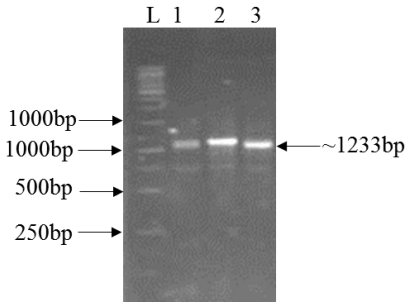

Fig IV. 1st Round PCR, here L: 1 kb DNA ladder (Gene Ruler by Fermantas), Lane 1,2,3: S gene amplicon using outer set of primers.

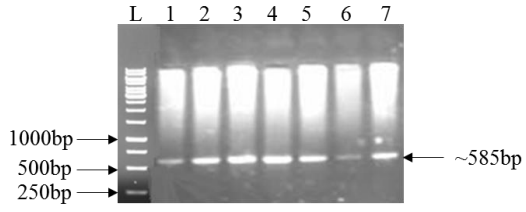

Fig V. 2nd Round PCR, here L: 1 kb DNA ladder (Gene Ruler by Fermantas), Lane 1-7: S gene amplicon using inner set of primers.

Results of Restriction digestion

The next step in this method was restriction digestion of these amplified fragments which was carried out by two different enzymes, restriction was optimized and restriction of each samples with both enzymes were carried out, showing the fragment size of about 252 and 350 bp in genotype C and in second genotype which is D showed *Psu 1* enzymes cut 585 base pairs amplicon into three fragments by cutting it from two restriction sites giving fragment of about 250bp lowest, 300bp middle and 350bp fragment which is shown in Fig. VI and VII.

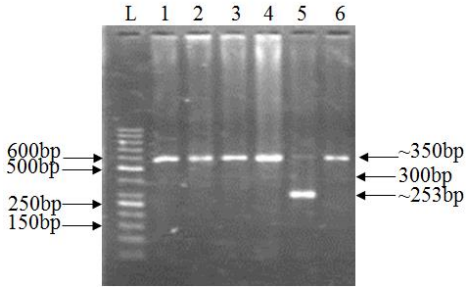

Fig VI. Restriction digestion (4.5), here L: 50 bp DNA ladder (Gene Ruler by Fermantas), Lane 1-6 restriction samples. Lane 1,2,3,4 and 6 showing no restriction with *styI,* Lane 5 showing restriction by *styI*.

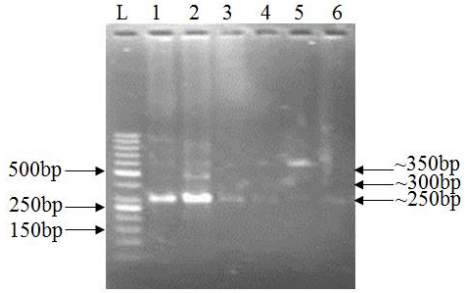

Fig VII. Restriction digestion (4.5), here 50 bp DNA ladder (Gene Ruler by Fermantas), Lane 1-6 showing positive restriction by *PsuI*.

Result Analysis of genotypes based on type specific method and RFLP-PCR

Type specific primer-PCR gave genotype C of 11 (9.2%) sample and genotype D of 119 (90.8%) samples out of 130 samples and the second method which is restriction fragment length polymorphism-PCR showed 7 (5.3%) samples having genotype C and 123 (94.6%) samples having genotype D. There are not very large differences in the results of both methods. Then we apply biostatistics on these results to know what the significant value show (Table III and Fig. VIII).

Table III. HBV Samples data.

Sr. #	Parameters	Variables	Variables
1.	Total samples 130	Strong Positive= 102 78.4%	Weak positive= 28 21.6%
2.	Average Age = 45	Minimum age= 10	Maximum age=80
3.	Gender Male / Female	Male= 77 59.2%	Female=53 40.8%
4.	Genotypes and methods	By type specific primers PCR Genotype C= 11(9.2%) Genotype D=119(90.8%)	By restriction fragment length polymorphism. Restriction by Psu1 Genotype D=123 samples (94.6%) Restriction by Sty1 Genotype C= 7 samples (5.3%)

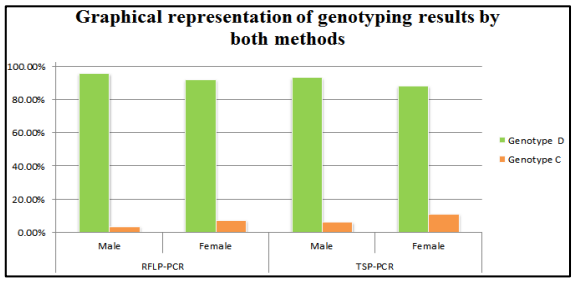

Fig VIII. Graphical representation of genotyping results of gender and of both (TSP-PCR and RFLP-PCR) methods.

Discussion

In current research, focus was on studying the genotypes of HBV. According to results found in this study, genotype D of HBV was detected as the dominant genotype in Punjab Pakistan. HBV has been classified into 8 genotypes (A-H) that show a distinctive geographical distribution (Kramvis *et al.,* 2005). Because HBV is an etiologic mediator of acute and chronic disease all over the world (Yalcinkaya *et al.,* 2005) and also its genotypes might affect mutation patterns in precore and core promoter regions, severity and activity of liver disease, serological reactivity, viral replication, prognosis and antiviral treatment response(Zeng *et al.,* 2004). Detection of HBV genotypes is very important to clarify the pathogenesis, route of infection and virulence of the virus. The genotype-specific primers PCR, it is well recognized that not only greater similarity in the whole sequences but also the matching of the two to three nucleotides at the 3' ends

is one of the necessary factors for specific priming. Based on this information, we designed type-specific PCR primers. Sequences within the same genotype were different by 2 or more nucleotides among the complete sequences of the genotype-specific primer, while the sequence within the different genotypes had a difference of less or equal of 3 nucleotides.

The other genotyping method which is restriction fragment length polymorphism-PCR is also a very sensitive technique in which genotyping is based on the restriction patterns which are found after cutting at specific restriction sites, by different restriction enzymes and it is also very sensitive and effective method(Dokanehiifard and Bidmeshkipour, 2009). In the current study, one method of genotyping, which is based on type-specific primers PCR, by which HBV isolates, can be ordered into genotypes A through F is compared with the second method, which is restriction fragment length polymorphism-PCR. Both methods are highly specific and convenient and will help research hands in managing far-reaching epidemiological studies. We found genotype D most dominant genotype and few patients with genotype C in Punjab Pakistan.

In another study(Mizokami *et al.*, 1999) selected the S gene keeping in mind the whole the fact that the former directly overlaps the active site present in the P gene, which is further encoded in another frame. (Mizokami *et al.,* 1999) studied on the HBV affected people in Pakistan mainly in cities like Faisalabad, Lahore and also in Islamabad. They showed that only C and D genotypes were present in the local population and eventually the adopted algorithm of this study was further used for identification of different genotypes of HBV.

According to Ali *et al.,* 2011 Genotype D of HBV is most prevalent and about 63.71% in Pakistan. Another study carried out by Badar *et al.,* (2012) in Faisalabad Punjab showed the percentage prevalence of genotype C and D of 9.81% and 91.1% using RFLP-PCR method. Our results showed the prevalence of genotype C and genotype D in TSP-PCR method 8.5% and 91.5% and in RFLP-PCR method genotype C and genotype D is 94% and 6% respectively. Our study showed that genotype D is more prevalent in Lahore than Faisalabad and Karachi.

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