HBV Genotyping and Phylogenetic Analysis through Multiplex PCR Using Type Specific Primer Pairs in Pakistani Population

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Abstract

Background: Hepatitis B virus causing acute and chronic hepatocellular carcinoma relics a serious health issue affecting more than 350 million people worldwide. Geographically, nine genotypes of HBV have been identified so far. Four open reading frames named (ORF P, S, F and X) renders it to specifically targets hepatocyte cells. Additionally, reverse transcription process present in the life cycle of HBV caused four order higher mutation rates as compared to adenovirus. The structural and functional differences between HBV genotypes are the mainstay to severity, complications, treatment and possibly vaccination against the virus. Hepatitis B genotyping is important for assessing its clinical implications and geographical distribution, but the sub-genotypes have been found useful for determination of specific genomic markers related to hepatocarcinogenesis and medical therapies. In Pakistan, there is no reported data available on molecular evolutionary analysis of HBV. A study was, therefore, much needed to evaluate the spectra of dominant strains prevalent here.

Objectives: This study was aimed to figure out the prevalence of HBV genotypes among population in Pakistan. In this research, we focused to find out the new strains and sub genotypes of HBV. Pakistan is one of the HBV endemic countries but there is no data on subgenotypes and their recombination or the phylogenetic relatedness of the virus endemic in the country. The present study was carried out in this perspective. This study will help future researcher to study some other aspects of HBV related strains in Pakistan and also about its medical therapies.

Place and duration of study: The present study was carried out at Polymerase Chain Reaction (PCR) lab, Nuclear Medicine, Oncology and Radiotherapy Institute [NORI], Islamabad, and the samples were collected between September 2012 to February 2014.

Study design: A total of 450 Hepatitis B surface antigen HBsAg and HBV DNA positive samples were collected in the first step during the period of September 2012 to February 2014 and sent to Nuclear Medicine Oncology and Radiotherapy Institute [NORI], for further analysis. All the samples were genotyped by type specific nested PCR primer pair method for 8 HBV genotypes from A through H. The patients were randomly selected irrespective of their age and gender and a written consent (parental consent in case of less than 18 years of age) was obtained. The study was approved by ethical review committee. In our research, we have used the type specific primer technique to find out the dominant strain in Pakistani populations and furthermore its phylogenetic analysis with reference sequences retrieved from NCBI databases.

Results: Our results showed a direct relationship between viral load and identified genotypes and pinpointed that genotype D is the most pre-dominant genotype and prevailing in high proportions among all genotypes in Pakistani population. Mix HBV genotype infections of genotype A with B & C, D with B and C constitute about 17 percent of all the samples. Subgenotypes of different HBV strains have been identified on the basis of differences in the complete nucleotide sequences. Positive PCR results were repeated twice for confirmation. Furthermore, phylogenetic analysis revealed a homology between our reported sequences and reference sequences. The phylogenetic method showed that genotypes/sub-genotypes varies in the geographical areas and correlate strongly with ethnicity.

Conclusion: Genotype D was identified and come out to be the most dominant genotype in Pakistani community showing sub-genotypes of D1 & D3 in our study. Genotype A and D are present as co-infection with each other and contributed as the second prevailing genotypic group. Additionally, phylogenetic analysis revealed that genotypes varies in the geographical areas and correlate strongly with reference sequences from NCBI.

Introduction

Hepatitis B virus (HBV) is one of those agents causing acute and chronic hepatitis in human beings. HBV infection relics a serious and universal health issue throughout the world, with more than 350 million people are chronically infected from this disease throughout the world [1].

HBV genome is of approximately 3,200 base pairs having partially circular duplex DNA molecule. The circularity of HBV DNA is maintained by 5' cohesive ends. The mRNA is synthesized by negative DNA strand of HBV. Four overlapping open reading frames are present in HBV genome with condensed coding region as well. These ORF are named as P, S, F, and X. The first ORF P codes for viral polymerase and terminal protein present on negative strand (DNA polymerase activity,

Reverse transcriptase and RNase H). Open reading frame S also known as pre-S, codes for those type of surface viral glycoprotein such as HBsAg, hepatitis B antigen surface that's commonly combine with cell receptor and facilitate ingress of virus into the cells [2].

Nine genotypes of HBV have been recognized on the basis of divergence in the whole DNA genome sequence of HBV [3]. HBV Genotypes show a prominent geographical pattern in their distribution. Genotype A is predominant in North America and Northern Europe as well as in the same parts of Africa. Genotypes B and C are more regular in the region of South East Asia [4-6]. Genotype D is found commonly all over the world [5]. Genotype E is detected from western and southern Africa while F genotype is reported in south and Central America [7]. Genotype G has been detected in America and France [8].

HBV infection leads to hepatocirrhosis and liver cancer. Reverse transcription process present in the life cycle of HBV causing four orders higher mutation rate as compared to other adenoviruses and it's because of lack of proof reading activity of reverse transcriptase enzyme of virus [9]. Genomic wide association study (GWAS) reported a new genetic locus contributing in HBV associated hepatocellular carcinoma (HCC) [10]. Locus (rs 17401966) a single nucleotide polymorphism (SNP) on chromosome 1p36.22 is the member of kinesin family named (KIF1B) and is present in the intronic region of the gene. Two SNPs adjacent to this locus (rs 12734551, rs 3748578) showed association to hepatocellular carcinoma risk (HCC) within hepatitis B infected patients.

These SNPs were found to be in linkage disequilibrium state (LDS) with locus rs 17401966 [10]. Cytogenetic studies also confirmed this association between 1p36.22 chromosomal region and numerous different types of cancers including basal carcinoma by the frequent loss of chromosomal region [11,12].

The regions where deletion occurs commonly are pre core genes, pre S region, and the region of X gene involved with basic core promoter region (BCP) [13]. These deletions are frame shift deletions and are believed to be linked with the progression of viral hepatitis. These deletions are frequently detected by sero-conversion from HBAsg to anti-HBe [14]. Nucleotide deletions of 1762 and 1764 in the codon region of 130 and 131 of X gene are reported to be common in hepatocellular carcinoma patients [15,16]. Although, deletions also occurred in the pre S region leading to the production of truncated HBV surface proteins that get accumulated in endoplasmic reticulum (ER). The pressure within ER increases due to which expression of cyclin A and host apoptosis suppressor cyclooxygenase-2 occurs [17,18]. In one study, they showed miR-15b, an important miRNA during HBV infection and hepatocellular carcinoma development, directly binds hepatocyte nuclear factor 1 (HNF1) mRNA, a negative regulator of HBV Enhancer I, to attenuate HNF1 expression and resulting in transactivation of HBV Enhancer I. This process leads to the enhancement of HBV replication and expression of HBV antigens, including HBx protein, finally leading to the down-regulated expression of miR-15b in both cell lines and mice in a long cascade of events [3].

Hepatitis B virus, the main form of liver cirrhosis has been detected by various HBV detection techniques which differ from each other according to specificity and sensitivity. Among these detection techniques, one reliable method used for HBV detection is from sequencing and phylogenetic analysis.

Various methods for hepatitis B virus genotyping have been reported separately from nucleotide sequencing, including melting curve analysis [19,20], line probe assays [21], PCR restriction fragment length polymorphism (PCR-RFLP) [22], type specific primers PCR technique [23], Hybridization assays and serological assays of DNA [21] and enzyme linked immunosorbant assay-based method [24]. HBV DNA can also be sequenced through commercial available sequencing kit (TRUGENE HBV Genotyping Sequencing Kit; Siemens Medical Solutions Diagnostic, USA) [25].

Up to 2007, the total numbers of HBV sequences available in public genomic databases were 866 deposited in GeneBank, EMBL and DDJB. All these sequences were contributed by 46 countries and the rate of infection and epidemic of HBV appears more severe in Asia. China contributed much more in increasing public genomic databases due to largest infected population [26].

In this study, an efficient, more precise and more elaborated Real Time PCR based method was used so that we can find out HBV DNA quantification (viral loads) and genotyping with differentiation through A to G. The results showed uniformity with current viral load quantitation, proficient genotyping and sequencing. The PCR method provides a useful means of rapid and inexpensive diagnosis of HBV genotypes with different geographical distributions.

Material and Methods

Blood sample collection

3-5 ml blood was collected from selected 450 HBV positive patients within standard EDTA collection tubes. The whole blood was stored not longer than 6 hours at 4-25°C. The patients were randomly selected irrespective of their age and gender and a written consent (parental consent in case of less than 18 years of age) was obtained. The study was approved by ethical review committee of the institution.

Plasma separation

The whole blood samples were centrifuged for 5 minutes at 12,000 rpm (Clinical Centrifuge, Model CH80-2, Germany) at room temperature. Plasma was separated using filter barrier tips. Plasma was transferred into sterile eppendorf tubes after proper labelling and stored at -20°C for further process.

HBV DNA quantification

Isolation of viral DNA from plasma: The viral DNA was extracted from plasma by using AJ ROBOSCREEN INSTANT virus DNA kit (GmbH, Germany) according to the manufacturer protocol. The HBV DNA quantification kit ROBOGENE is proposed for quantification of hepatitis B virus (HBV) DNA through real time PCR from human EDTA plasma sample. The extracted viral DNA was stored at -80°C for long time storage before initiation PCR.

Viral load quantitation: Viral load quantification was done by RoboGene® HBV Quantification kit (AJ Roboscreen, analytikajena Biosolutions, GmbH, Germany) at NORI. This allows amplifying all known HBV genotypes (A-I) with parallel efficiency by probes applying and primers specific for a subsequence of HBV-S gene encoding HBsAg. Upstream probe is labelled with 5'-end reporter dye (FAM for HBV DNA: Yakima Yellow for IC DNA) and downstream probe is labelled with quencher dye. A three step PCR protocol was followed: Taq activation step at 95°C for 4 min, then 50 synthesis cycles at 57°C for 1 min, melting at 95°C for 30 s, stem formation with fluorescence detection at 45°C for 30 s using Rotor- Gene™3000, Corbett Research, Australia. Data collected in the form of sigmoid growth curves was analyzed according to manufacturer's instructions keeping in view the parameters of slope correction and value, thresholds, CT value of IC DNA and R value, HBV DNA concentration which is expressed in IU/

ml. Quantitation standards ranged from 5×10^2 to 5×10^7 IU/ml were provided along with the kit (Tables 1 and 2).

Agarose gel electrophoresis: For the purpose of electrophoresis, we prepared agarose gel with 2% concentration. Run HBV DNA fragments were separated by means of agarose gel electrophoresis via agarose at concentrations of 2% w/v in 1x TAE buffer. The gel was run in 1x TAE buffer for 45 minutes at 100 V. The size of DNA fragments were compared with GeneRulerTM 50bp DNA ladder (MBI Fermentas, Life Sciences, Catalogue # SM 0373).

DNA sequencing: Selected samples were run on agarose to identify the genotyping. These were then amplified in higher volume 50 μl and were run on 2% agarose gel. The bands were visualized under gel documentation system (BioRad Gel Doc-XR, USA) and were cut using personal protective equipment under UV. DNA was eluted from the gel using GeneGETTM Genomic DNA purification mini kit (Fermentas Biosciences).

Sequence analysis: Purified samples of selected genotypes were analyzed by sequencing using ABI automated sequencer. The sequences of nucleotide were viewed using software version 2.0 of chromas. Electropherograms of each sample were identified first, analyzed and ambiguities in the data sequences were resolved by comparison of the results of forward and reverse primers with those sequences of HBV obtained from NCBI database.

BLAST analysis: The sequences were submitted as query sequences to BLAST (on line available program at website http://www.ncbi.nlm.nih.gov/BLAST/). After then, different countries sequences were aligned with our subject sequences to obtained the define length of our subject sequence. The BLAST was done to identify the similarity of HBV sample sequences with the reported databases.

Reference sequence retrieval: Reported reference sequences of HBV were retrieved for construction of phylogenetic tree. From phylogenetic tree, the verification of unknown sample can be easily found. Following accession numbers and website was used to retrieve reference sequences. Website: http://www.ncbi.nlm.nih.gov). Sequence of each genotype was retrieved to construct a phylogenetic reference tree (Table 3).

Primers	Sequences (5′-3′)	Base Position	Polarity
PI-A	TCACCATATTCTTGGGAACAACA	2823–2845	Sense
SI-2	CGAACCACTGAACAAATGGC	685–704	Antisense

Table 1: Primers Sequences Used In Study for the Amplification of Hepatitis B Virus (First Round PCR).

Primers	Sequences (5′-3′)	Base Position	Polarity
	Mixture A		
B2	GGCTCMAGTTCMGGAACAGT	67–86	Sense
BAIR	CTCGCGGAGATTGACGAGATGT	113–134	Antisense
BBIR	CAGGTTGGTGAGTGACTGGAGA	324–345	Antisense
BCIR	GGTCCTAGGAATCCTGATGTTG	165–186	Antisense
	Mixture B		
BD1	GCCAACAAGGTAGGAGCT	2979–2996	Sense
BE1	CACCAGAAATCCAGATTGGGACCA	2955–2978	Sense
BF1	GYTACGGTCCAGGGTTACCA	3032–3051	Sense
B2R	GGAGGCGGATYTGCTGGCAA	3078–3097	Antisense

An "M" represents a nucleotide that could be either an A or a C; a "Y" represents a nucleotide that could be either a C or a T;

Table 2: Sequences of Primers for HBV Genotyping Used By Nested PCR (Second Round PCR).

Phylogenetic tree analysis: Phylogenetic tree from HBV sequences were generated by neighbor joining algorithm, using CLC workbench software (www.clcbio.com). The sequences were aligned to draw a consensus sequences and those consensus sequences were submitted to NCBI. The phylogenetic tree was constructed from reported sequences. The amino acid sequences were analyzed for substitution of conservative and non-conservative mode by using software CLC workbench, parentage of sequence comparison of both nucleotides and amino acids were done by using cluster W software.

Results

Viral load quantitation results

Viral load quantification was done by RoboGene® HBV Quantification kit (AJ Roboscreen, analytikajena Biosolutions, GmbH, Germany) at NORI. A three-step PCR protocol was followed using Rotor- Gene™3000, Corbett Research, Australia. Data collected in the form of sigmoid growth curves was analyzed keeping in view the parameters of slope correction and value, thresholds, CT value of IC DNA and R value, HBV DNA concentration was measured in IU/ ml (Tables 4 and 5).

Standard curves and STD ranges along with given concentration and calculated concentrations are given below in table 4. The viral load was calculated based on fluorescence based detection of probes that were labelled with different reporter dyes.

The following table (Table 6) shows the viral load calculated through Real Time PCR using eight standard along with given concentration and calculated concentrations. The gender description and age are also mentioned. It indicate that the high viral load exist in sample collected for genotyping purpose.

First round PCR was performed using outer specific primers PI-A and SI-2, maintaining thermo-cycler parameters. The amplified products were run on 2% agarose gel with GeneRuler™ 100bp DNA ladder (Fermentas, Life Sciences) and observed under GEL DOC XR System (BIO-RAD, Applied Bioscience). The following figures show the results of first round PCR (Figures 1A and 1B).

Sr. No	Accession No	HBV Genotype	Country Origin	Sr. No	Accession No	HBV Genotype	Country Origin
1	AM282986	A	USA	21	JN604278	D	USA
2	JX507200	B	Panama	22	JN370953	D	USA
3	JN104438	C	USA	23	GU570525	E	UK
4	JQ514458	D	India	24	GQ161830	E	UK
5	AY303894	E	Gambia	25	GQ161805	E	UK
6	JX849636	F	Italy	26	GQ161818	E	UK
7	CS409748	G	USA	27	GQ161822	E	UK
8	CS388980	H	USA	28	JX868564	D	Pakistan
9	DQ297903	A	India	29	JX868564	D	Pakistan
10	JQ514338	D	India	30	FJ670514	D	Pakistan
11	JQ514406	C	India	31	EF584653	D	Pakistan
12	JQ514438	D	India	32	FJ670507	D	Pakistan
13	JX090637	D	Russia	33	HQ385240	D	Sudan
14	EU594418	D	Russia	34	AB104722	D	Egypt
15	EU594414	D	Russia	35	AB104715	D	Egypt
16	JX090722	D	Russia	36	HQ385233	D	Egypt
17	JX090648	D	Russia	37	AB263405	D	Mongolia
18	EU769261	G	USA	38	AB263412	D	Mongolia
19	EU769256	A	USA	39	AB263410	D	Mongolia
20	JN604206	D	USA	40	AB644317	D	Indonesia

Table 3: Reference Sequences of HBV Pre-S1 Region.

No.	Colour	Name	Type	Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	% Var
1	(red)	STD1	Standard	15.51	50,000,000	48,386,650	3.2%
2	(red)	STD2	Standard	15.51	5,000,000	48,386,650	3.2%
3	(red)	STD3	Standard	21.88	500,000	522,595	4.5%
4	(red)	STD4	Standard	15.51	125,000	48,386,650	3.2%
5	(red)	STD5	Standard	25.19	50,000	49,712	0.6%
6	(red)	STD6	Standard	27.09	12,500	12,890	3.1%
7	(red)	STD7	Standard	31.71	2,500	482	3.6%
8	(red)	STD8	Standard	15.51	500	2,500	3.2%
9	(yellow)	NTC	NTC				

Table 4: STD Ranges with given and calculated concentration (no of copies/ul).

Cycle	Cycle Point
Hold at 95°c, 4 min 0 sec	
Cycling (50 repeats)	Step 1 at 95°c, hold 15 sec
	Step 2 at 45°c, hold 15 sec, acquiring to Cycling A ([Red][4][4],[Green][1][1],[Yellow][2][2],[Orange][3][3])
	Step 3 at 57°c, hold 40 sec

Table 5: Cycling Profile

Serial No	PRN No	Gender	Age	Allotted Tube No	Viral Load
1	015956/12	Female	24	3	$5.7×10^8$
2	026283/12	Male	22	5	$4.2×10^6$
3	026047/12	Female	50	6	$6.1×10^5$
4	024232/12	Male	47	7	$1.6×10^7$
5	026137/12	Male	19	8	$1.2×10^8$
6	009221/12	Female	55	10	$2.5×10^7$
7	026652/12	Male	03	11	$3.7×10^7$
8	017661/10	Female	43	12	$1.2×10^5$
9	026986/12	Male	15	13	$1.4×10^7$
10	027188/12	Male	21	14	$7.7×10^6$
11	027957/12	Male	30	16	$4.0×10^8$
12	028112/12	Male	10	17	$1.4×10^8$
13	028422/12	Male	22	18	$6.6×10^7$
14	029997/12	Male	32	19	$1.4×10^9$
15	030043/12	Female	28	20	$1.8×10^9$
16	030282/12	Male	13	21	$1.9×10^9$
17	007728/10	Female	20	22	$1.9×10^7$
18	029722/12	Female	38	23	$1.1×10^9$
19	030578/12	Male	15	24	$2.6×10^8$
20	030691/12	Male	30	25	$1.3×10^9$

Table 6: Viral Load calculated by Real Time PCR.

Nested PCR product results

For finding out the genotypes of various HBV samples, we performed the nested PCR using inner primer pair common with the universal sense primer B2 with mixture I for genotypes A to C. The common antisense primer B2R is used with mixture B to identify genotypes

D, E and F. The nested products of PCR were run on 2% agarose gel with GeneRuler™ 50 bp DNA ladder (Fermentas, Life Sciences) run as marker. The 69 bp and 119 bp DNA bands were obtained in mixture A and B respectively (Figures 2A and 2B)

Relationship between viral load and genotypes

A clear relationship was observed between the viral load and identified genotypes. The genotypes were found by multiplex PCR using type specific primers pair. The Table 7 shows that genotype D is prevailing in high proportion among all genotypes in Pakistan. In some samples, mixed genotype A+D was also observed. The high viral load was reported from dominant genotypes D and A+D. The identified genotypes are further confirmed by sequence analysis.

Construction of phylogenetic tree

To elucidate the results of HBV genotype D, sequencing was performed. The NORI-HBV1 to NORI-HBV10 sequence isolates were subjected to NCBI for blast. The phylogenetic tree of sequences was constructed by use of CLC sequence view software through neighbor joining method compared with forty reference sequences obtained from NCBI. In phylogenetic tree, the sequences are grouped into three clusters (Figure 3). Cluster one contained our reported sequences
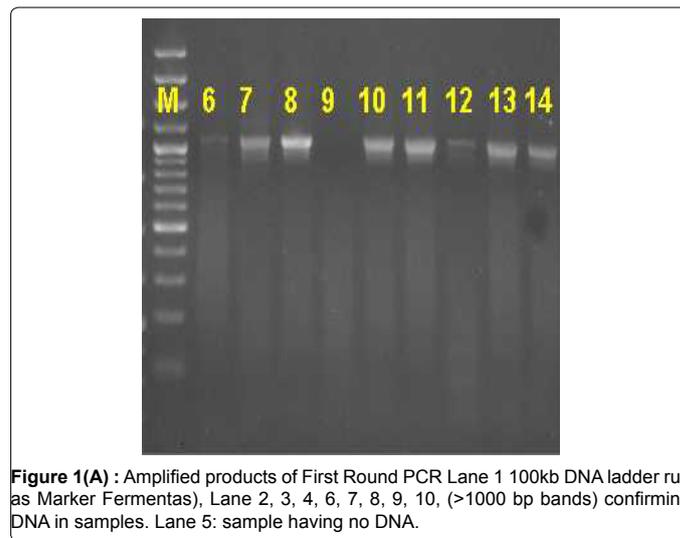

Figure 1(A) : Amplified products of First Round PCR Lane 1 100kb DNA ladder run as Marker Fermentas), Lane 2, 3, 4, 6, 7, 8, 9, 10, (>1000 bp bands) confirming DNA in samples. Lane 5: sample having no DNA.
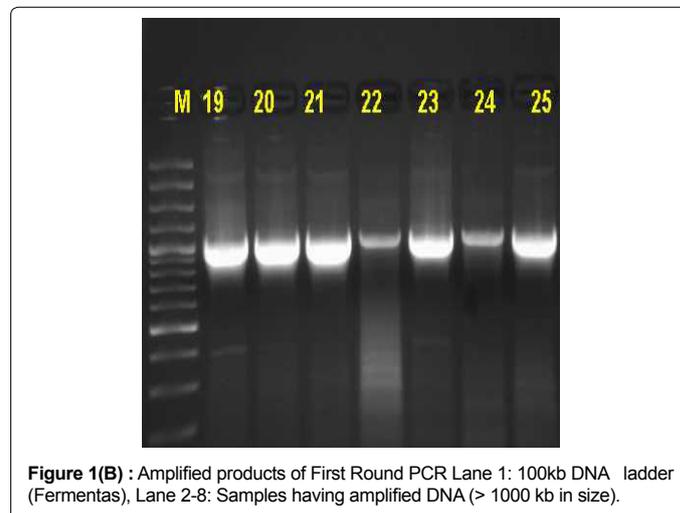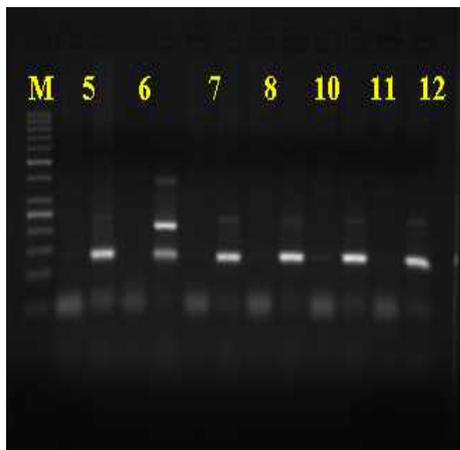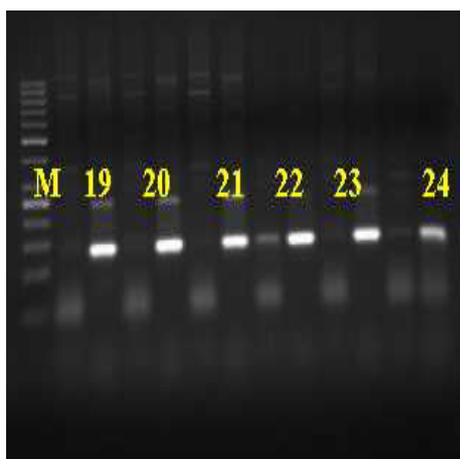

Figure 1(B) : Amplified products of First Round PCR Lane 1: 100kb DNA ladder (Fermentas), Lane 2-8: Samples having amplified DNA (> 1000 kb in size).

Figure 2(A) : Nested PCR Products (Second Round PCR) Lane 1: GeneRulerTM 50bp DNA ladder (Fermentas, Life Sciences) run as marker. Mix I & 2 run side by side. Lane 2, 4, 6, 8, 10, 12: PCR product of HBV positive samples with 119bp size fragments (Genotype D).

Figure 2(B) : Nested PCR Products (Second Round PCR) Lane 1: GeneRulerTM 50bp DNA ladder (Fermentas, Life Sciences) run as marker. Mix I & 2 run side by side. Lane 2-12: PCR product of HBV positive samples with 119 bp size fragment (Genotype D).

Serial No	Allotted Tube No	Sequencing No	Colour	Viral Load	Results
1	3	HB31	Red	$5.7×10^8$	**A&D**
2	5	HB32	Red	$4.2×10^6$	**D**
3	6	HB33	Red	$6.1×10^5$	**D**
4	7	HB34	Red	$1.6×10^7$	**D**
5	8	HB35	Red	$1.2×10^8$	**D**
6	10	HB36	Red	$2.5×10^7$	**D**
7	11	HB37	Red	$3.7×10^7$	**D**
8	12	HB38	Red	$1.2×10^5$	**D**
9	13	HB39	Red	$1.4×10^7$	**A&D**
10	14	HB40	Red	$7.7×10^6$	**A&D**
11	16	HB41	Red	$4.0×10^8$	**A&D**
12	17	HB42	Red	$1.4×10^8$	**A&D**
13	18	HB43	Red	$6.6×10^7$	**A&D**
14	19	HB44	Red	$1.4×10^9$	**D**
15	20	HB45	Red	$1.8×10^9$	**D**
16	21	HB46	Red	$1.9×10^9$	**D**
17	22	HB47	Red	$1.9×10^7$	**D**
18	23	HB48	Red	$1.1×10^9$	**D**
19	24	HB49	Red	$2.6×10^8$	**D**
20	25	HB50	Red	$1.3×10^9$	**D**

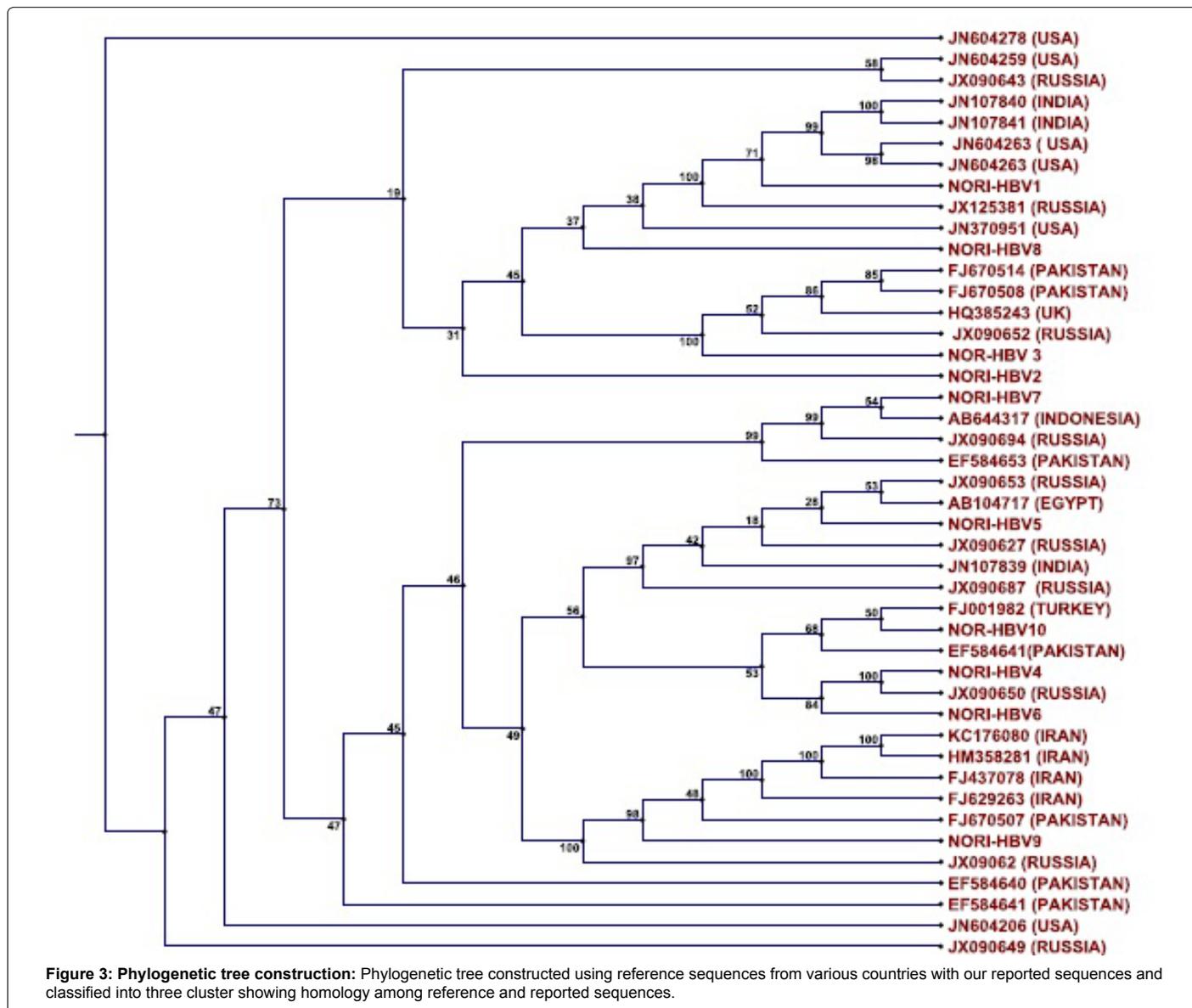**Table 7:** Genotype Find out by Multiplex PCR and its Relationship with the Viral Load.

of reference sequences from USA and Pakistan showing genotype D, E. It contained our six sequences abbreviated as NORI-HBV1, NORI-HBV2, NORI-HBV3, NORI-HBV5, NORI-HBV7, and NORI-HBV8.

Third cluster contained our two sequences abbreviated NORI-HBV4, NORI-HBV6 and aligned with genotype D sequence from USA and Pakistan.

Discussion

The aim of the study was to find out HBV dominant strain and genotyping via a modern, precise and elaborate method of HBV genotyping. The phylogenetic analysis was performed to allow specific fortitude and discrimination of all recognized genotypes. The HBV genotypes are disseminated geographically but its virulence and pathogenicity vary from each region. Finding HBV genotype is important to clarify the infection pathway and virulence of virus. Variants may vary in pattern of serological activity, virus replication and treatment response. In fact, genotype C harbors patient experience a poorer response to treatment than genotype B [27].

In our methodology, genotype A to F could be detected by only single amplification set for each genotype through nested PCR. In our present study, we focused to find genotype within Pakistani community and found that genotype D is the most predominant genotype among all known genotypes. Genotype D is frequently linked by pre core mutations that lead to increase the chances of causing cirrhosis and hepatocellular carcinoma [26]. Mutations resulted in CCAAT motif present over the pre-SI gene caused retention of protein S that becomes the more aggressive type of HBV liver related diseases [28]. Due to the lack of unbiased approach, the mutational extent and functional impact of HBV in HCC progression is not studied. The sequencing technologies provide an opportunity to investigate the global extent of this mutation and functional impact of viral integration in the host genome [29]. Keeping in view the fact that, this type of mutant seems to occur frequently in our population. Current antiviral agents can control

NORI-HBV1, NORI-HBV2, NORI-HBV3, NORI-HBV8 of genotype D and the reference sequences of genotype D from UK, USA, Russia and India. Two sequences NORI-HBV5, NORI-HBV7 of genotype D are present in second cluster and show homology with sequences of genotype D from India, Russia, Indonesia and Egypt. Third cluster consist of reference sequences of genotype D from Turkey, Iran, USA and Russia with our reported sequences of genotype D NORI-HBV4, NORI-HBV6, NORI-HBV9 and NORI-HBV10.

Phylogenetic tree two constructions

Phylogenetic tree two was constructed by taking reference sequences of different genotypes from various countries. These sequences taken from NCBI were blast with our current sequences. All sequences are grouped into three clusters (Figure 4).

NORI-HBV9 sequence is present in cluster one and it shows homology with the sequences from India (Genotype D), UK (Genotype E), USA (Genotype A) and Italy (Genotype E). Cluster two comprised

Figure 3: Phylogenetic tree construction: Phylogenetic tree constructed using reference sequences from various countries with our reported sequences and classified into three cluster showing homology among reference and reported sequences.
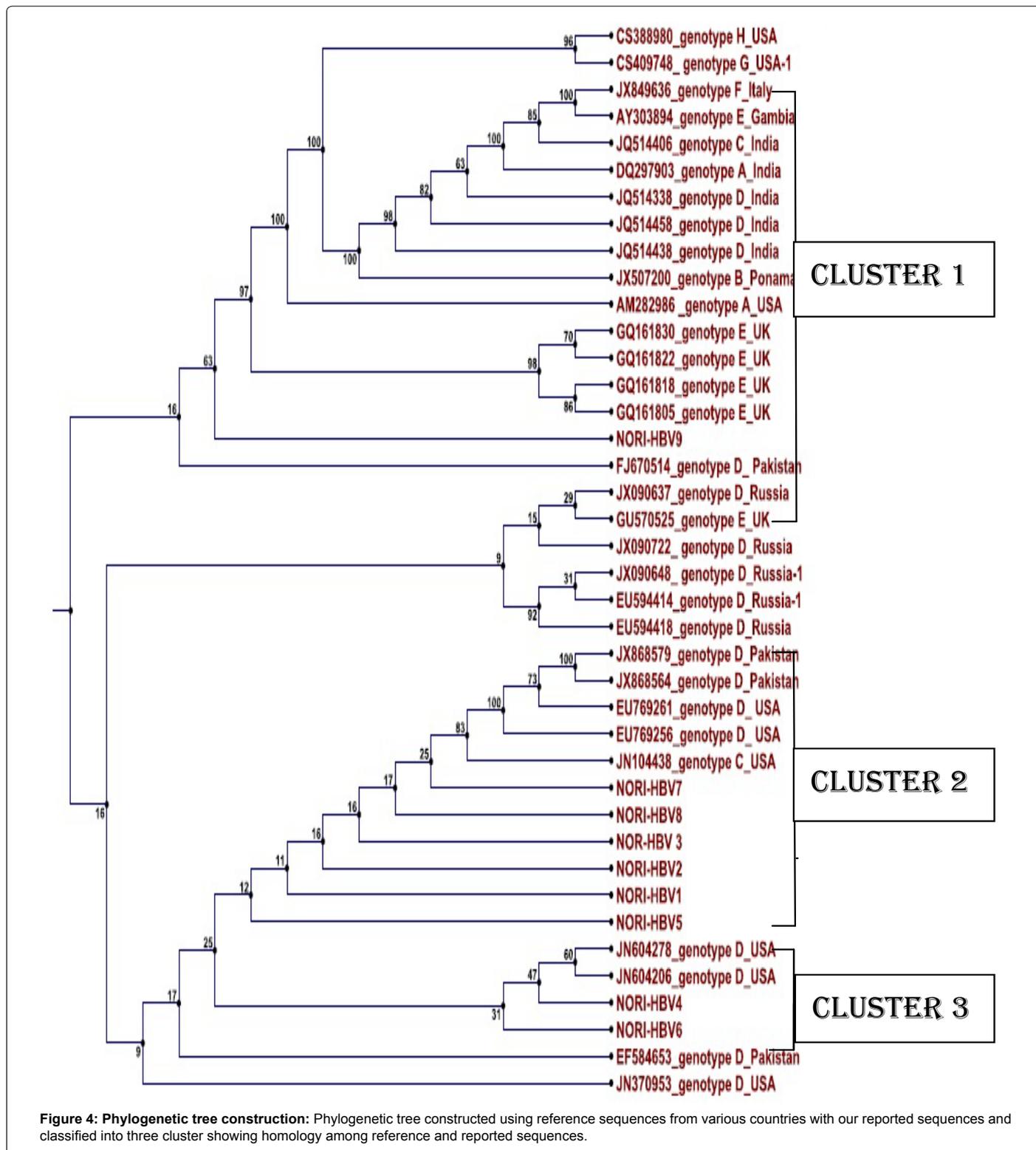
but not eliminate hepatitis B virus (HBV), because HBV establishes a stable nuclear covalently closed circular DNA (cccDNA). Interferon-α treatment can clear HBV but is limited by systemic side effects. Interferon-α can induce specific degradation of the nuclear viral DNA without hepatotoxicity and propose lymphotoxin-β receptor activation as a therapeutic alternative.

Most studies have concluded genotype D, a worse prognosis factor than genotype B and showed the correlation between hepatocellular carcinoma and the rate development of genotype C. In one study, they showed miR-15b which directly binds hepatocyte nuclear factor 1 (HNF1) mRNA, a negative regulator of HBV Enhancer I, to attenuate HNF1 expression and resulting in transactivation of HBV Enhancer I. This process leads to the enhancement of HBV replication and expression of HBV antigens, including HBx protein, finally leading to the down-regulated expression of miR-15b in both cell lines and mice in a long cascade of events [3]. In contrast to the above facts, some findings suggest the role of sub genotype in HCC development. Genotype C infected patients show recurrence of HCC and metastasis form of liver cancer.

Several methods for HBV genotyping including fragment length polymorphism by restriction enzyme (RFLP), direct sequence analysis and enzyme linked immunosorbent assay (ELISA) was used as the gold standard method but is not suitable for finding mixed genotypes [30]. Our multiplex PCR method used has low cost and is more reliable as compared to other methods for HBV genotyping and reported to be superior for the detection of double infections. Specific genotype primers pair was constructed based on sequences of nucleotide between S and pre-SI gene. Chen et al. [31] developed to find genotype A to F and sub genotypes collectively.

INNO-LiPA HBV assay used for genotyping is a very sensitive method detecting mixed genotypes but it's too much expensive and the result may be affected by a single nucleotide change so it cannot be used for epidemiological purposes on large scale investigation in developing countries like Pakistan. The RFLP and MCA also allow simultaneous genotyping and quantification of HBV. However, the observed accuracy of MCA is 92.3% and RFLP is of 87%. The results were affected by

Figure 4: Phylogenetic tree construction: Phylogenetic tree constructed using reference sequences from various countries with our reported sequences and classified into three cluster showing homology among reference and reported sequences.

point mutations that change the melting point leading to uncertain identification of genotype A, C and D.

Usuda et al. [24] developed ELISA technique for the detection of different strains of HBV. Specific monoclonal epitopes were used in this method against pre-S2 region to identify various strain of HBV.

Another method used in most of the clinical laboratories is real time PCR with the help of which both quantification and genotyping can be performed easily. The real time PCR employed fluorescent probes or a dye that firmly attached to the double stranded DNA and aids in the detection and quantification of hepatitis B virus DNA (HBV-DNA). The genotypes are recognized in the samples based on the melting

curve temperature (Tm). Various genotypes show different values of melting curves. This difference is due to the contemporarily relationship between the target DNA and probe or it can be due to the presence of GC content within the hybridization sequence.

Payunporn et al. [19] showed 90.4% similarity between sequencing and RFLP while with melting curve procedure, it come out to be 92.3%. Real time PCR system has high sensitivity, superior to oligonucleotide microarray chips and sequencing in detecting mixed genotypes. But the results can be effected by some complications its harbours. Among them, one is single nucleotide polymorphism (SNP) at primer site affecting the sensitivity of this method. The other is low ability of real time to distinguish mixed genotypes due to close proximity between the values.

Peg interferon therapy (PEG-INF) is mainly used against HBV infection and its treatment. The rate of recovery depends to some extent on the presence of its different types. Various finding reported that genotype B infected patients show better response and early recovery than genotype C. The nucleoside analogues are affected by HBV infection and render it less responsive. In the same way, a genotype infected patients give strong response to peg interferon compared to genotype D. The response rate to peg interferon of genotype B and A increases significantly during short-term therapy treatment.

Various methods for HBV sequencing were proposed with the passage of time. One of them is Whole Genome sequencing method which was a standard method for HBV DNA sequencing. Phylogenetic analysis can be performed very easily through this technique but it was not used very widely due to time consuming procedure and it could only identify the dominant genotype from genotypic mixture. The other major condition holding hurdle was that it required more than 100 clones to be screened. Latter an alternative method was developed to whole genome sequencing method by which a single gene can be identified very easily [32]. This method was highly sensitive and sensitivity depends on size of the target sequence.

Studied carried out in USA used commercial available sequencing kit named HBV TRUGENE Genotyping kit. HBV DNA mutations as well as genotypes both were detected simultaneously by this kit. After DNA extraction, polymerase chain reaction was performed to amplify the antigen surface (HBsAg) gene and polymerase gene overlapping with antigen gene [25]. PCR products were added in four reaction tubes and fluorescent based DNA primers were added in those reaction tubes. Bi- directional sequencing was performed to find out the genotype and mutation within HBV genome. Another study in China used type specific nested PCR assay. Inner and outer primers pair of S region used were SF, PR3 and PF, S4R respectively. DNA Analyzer used in this study was of Applied Bio-system (named Applied Bio-system 3730 XL DNA Analyzer). The sequencing was done very efficiently and MEGA 4.0 software was utilized during phylogenetic analysis. All known sequences are deposited in public genomic databases. Through these sequences, one gets knowledge about the geographical distribution of HBV and the host clinical status. In these databases much contribution are taken part by China.

The multiplex PCR technique is more convenient, precise and elaborates to assist future researchers to use it to identify large scale epidemiological studies within different region of our country by the use of type specific primers. It will also help in the remediation of disease after its identification.

We have not experienced the untypeable results by using type specific primers. The results obtained by genotype specific primers

in separate mixture correlate well with those obtained by sequence analysis of HBV samples. Among all the samples, genotype D was the pre dominant one and was identified with a very high viral load [33]. Mixed genotype A+D was also present with high viral load but its proportion was very low as compared to genotype D.

Moreover phylogenetic analysis of these samples with published sequences confirmed the sub typing of the strain. Data suggested that although gold standard method for determination of HBV genotype is sequence analysis of phylogenetically informative coding region of the HBV genome and comparison to consensus sequences of known genotypes. Since therapy management decisions have been made on the basis of genotype, not subtype, the overall accuracies of both assays have been considered acceptable. But this knowledge is widely used for research purpose and vaccine development. Genomic sequence analysis followed by phylogenetic analysis for assigning genotypes is time consuming and inconvenient for clinical use and not adapted to clinical studies on large scale routine use. However modified genotype specific primer method is one of the fast typing methods and used in large scale for epidemiological studies.

Conclusion

Hepatitis B virus is the main causative agent of liver cirrhosis and hepatocellular carcinoma affecting more than 400 million people worldwide. HBV genotypes are geographically distributed throughout the world with specific epidemiological type. In Pakistan, genotype D is the most pre-dominant genotype among all known genotypes occurring in the population and is frequently linked by pre core mutations, increasing the chances of liver cirrhosis and HCC. The second most prevailing genotype is mixed infection A+D. Moreover, phylogenetics analysis further confirmed the occurrence and phylogenetics significance of the HBV.

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