



Analysis of Complete and Partial Genome Sequences of Hepatitis B Virus and Determination of its Genotypes and Sub-Genotypes from Pakistan

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ABSTRACT

Hepatitis B virus has been classified into eight genotypes from A to H which have variety in geographical distribution. HBV/D is reported to be the most prevalent genotype in Pakistan. Sub genotypes D1 and D3 were reported in a study from Karachi. Present study was designed to investigate the genotypes and sub-genotypes of HBV with sequencing of S gene fragment as well as complete viral genome of samples from Islamabad, Pakistan. A total of 22 HBV sample were genotyped by type specific primer PCR method. Complete viral genome of 4 samples and the S gene fragment of all samples was subjected to direct sequencing. Genotypes and sub-genotypes were confirmed by phylogenetic analyses of the sequences. Employing type specific primer PCR method, 14 (63.6%) samples were detected with HBV/D infection while a mix infection with HBV/A and HBV/D was detected in 8 (36.4%) samples. However on direct sequencing of S gene fragment, all 20 of the sequenced samples were identified as only genotype D and not genotype A or mixed, while complete genome sequences of 4 were identified as sub-genotype D1. Thus this study provides evidence for prevalence of D genotype more specifically sub-genotype D1 in Islamabad. Furthermore, it is observed that sequence based results are more reliable as compared to type specific PCR based method.

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Authors' Contributions

MM and AR conceived and designed the study. MMA, NZ and MM collected and analyzed data. MM wrote the manuscript. AK, MAB and MQ supervised the work.

Key words:

Hepatitis B virus, HBV genotype, sequencing, complete genome, Pakistan

INTRODUCTION

Hepatitis B virus genome is greatly variable having diverge genetics in different regions of the world. On the base of at least 8% divergence in its genome, HBV is segregated into eight different genotypes named from A to H (Okamoto *et al.*, 1988; Naumann *et al.*, 1993; Norder *et al.*, 1994; Stuyver *et al.*, 2000; Arouz-Ruiz *et al.*, 2002; Huy *et al.*, 2008; Olinger *et al.*, 2008; Kanako *et al.*, 2009). The genotypes have specific pattern of distribution in geographic regions and ethnic groups of the world (Stuyver *et al.*, 2000; Kidd-Ljunggren *et al.*, 1995; Bowyer *et al.*, 1997; Datta, 2008; Bonino *et al.*, 2010; Nakano *et al.*, 2001).

Currently, HBV genotyping is not used as a diagnostic test before the treatment of chronic HBV patients in Pakistan and the phylogenetic data of HBV strains in Pakistan is not sufficient. According to the

available studies, genotype D is predominant in Pakistan along with a little proportion of genotype A (Hanif *et al.*, 2013; Alam *et al.*, 2007; Noorali *et al.*, 2008; Baig *et al.*, 2009; Ali *et al.*, 2011). Mix infection with genotypes A and D is also highly prevalent in Pakistan (Hanif *et al.*, 2013; Alam *et al.*, 2007; Baig *et al.*, 2009; Awan *et al.*, 2010; Abbas *et al.*, 2006). Major HBV genotypes are also classified in to sub-genotypes on the base of divergence between 4-8% in the complete viral genome (Yousif and Kramvis, 2013). Genotype D is further classified into six sub-genotypes which are confirmed so far (Yousif and Kramvis, 2013). Sub-genotypes D1 and D3 of genotype D have been reported from 11 and 2 samples respectively by the only study from Karachi, a city of Pakistan (Baig *et al.*, 2009).

Clinical significance of HBV genotypes has been studied in various regions of the world. The genotypes affect disease progression, patients' viral load, HBsAg sero-clearance, HBeAg sero-conversion and response to antiviral therapy in many studies (Norder *et al.*, 2004; Cao, 2009; Erhardt *et al.*, 2005; Bonino *et al.*, 2007; Chan *et al.*, 2009). Along with genotypes of HBV, sub-

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genotypes are also significant clinically and, though still not well established, have their impact on course and treatment of the infection (Yin *et al.*, 2008). HBV genotypes and sub-genotypes are needed to be explored, especially in most of the northern and northwestern regions of Pakistan. The present study was designed to identify the sub-genotypes of HBV circulating in the city of Islamabad, Pakistan by sequencing of partial as well as full length HBV genome.

MATERIALS AND METHODS

Samples

A total of 22 samples were collected from patients visiting Nuclear Medicine, Oncology and Radiotherapy Institute (NORI), Islamabad, during the period of January 2012 to April 2012. The samples were the aliquots of plasma or serum originally collected for routine diagnostic purposes. The sera were separated and stored below -70°C until further analysis. All the patients signed an informed consent and the study was approved by institutional ethics committee.

Genotyping using type specific primer method

The DNA was extracted using QIAamp® DNA blood mini kit (Qiagen, Hilden, Germany) from 200µl serum following manufacturer's protocol. DNA was eluted in 50 µl PCR grade water and stored below -70°C until further analysis.

Type specific primer- PCR method (Naito *et al.*, 2001) was used for HBV genotyping at Nuclear Medicine, Oncology and Radiotherapy Institute (NORI), Islamabad, with a few modifications in thermal profile and reagents as described earlier (Hanif *et al.*, 2013).

Genotyping by direct sequencing of S gene

The samples were genotyped by direct sequencing of an S gene fragment of 685 nucleotides. QIAamp MinElute Virus Spin Kit (Qiagen) was used to isolate HBV DNA following manufacturer's protocol. Five µl of extracted HBV DNA was amplified in a PCR mix including Tris HCL buffer, 2 mM Magnesium Chloride, 0.1 mM dNTPs, 2 units Dream Taq polymerase (Fermentas) and 0.25 µM each of the primers, LAMes and Pol4as (Table I). The PCR thermal cycling profile was; 3 minutes at 94°C then 35 cycles including 30 sec at 94°C, 30 sec at 55°C and, 30 sec at 72°C, and then 7 min at 72°C. Negative samples after first round PCR were amplified in semi-nested PCR using internal HBV reverse primer POL181as (Table I) and a thermal profile like the first round but repeated for 20 cycles instead of 35. After confirming by gel electrophoresis, the products

were purified by ethanol precipitation protocol (Beckman Coulter, USA) and subjected to sequencing by chain termination method using commercially available Kit (Beckman Coulter, USA). Briefly, 2 µl of purified DNA was mixed with 8 ul of DTCS Quick Start Master Mix, 4 µl of 1.6 µM primer (LAMes and POL181as) and 6 µl of water. The thermal profile used was; 30 cycles of 20 sec at 96°C; 20 sec at 50°C & 4 min at 60°C. The instrument CEQ 8000 XL Analysis System (Beckman Coulter, USA) was used for sequencing.

Complete genome sequencing

Complete HBV genome was sequenced for 4 samples. The genome was sequenced in 7 different overlapping fragments. Each fragment was sequenced using separate pair of primers according to the method described above. The sequences of primers are given in Table I. All the sequencing and phylogenetic analysis was performed at Hepatology Laboratories, University Hospital of Pisa, Italy.

Analysis of sequences

Initially sequences were observed and corrected manually in Chromas-Lite, Version 2.1.0. Initial alignment, editing and joining of different genome fragments was performed by Bio-edit and Multalin online tools. The sequences were finally analyzed with MEGA version 4.0 to obtain the phylogenetic trees using maximum composite likelihood with 500 bootstraps replication in neighbor joining method. Number of base differences per sequence and number of base differences per site were calculated by the distance analyses conducted in MEGA4 using complete deletion option in which all the positions containing gaps and missing data were eliminated from the dataset. There were a total of 3092 positions in the final distance analyses dataset.

RESULTS

Genotyping

Out of the total 22 samples, genotype D was identified in 14 (63.6%), while 8 (36.4%) samples were found to be infected with a mixture of genotypes A and D (A+D) using type specific primer PCR method.

On direct sequencing genotype D was detected in 20 (91%) samples out of the total 22 samples, while sequencing was not successful for 2 samples. Neither of the samples was detected with genotype A or the combination of A+D. In phylogenetic analysis, all the 20 sequences were clustered with the sequences of genotype D and none of them was clustered with sequences of other genotypes (Fig. 1).

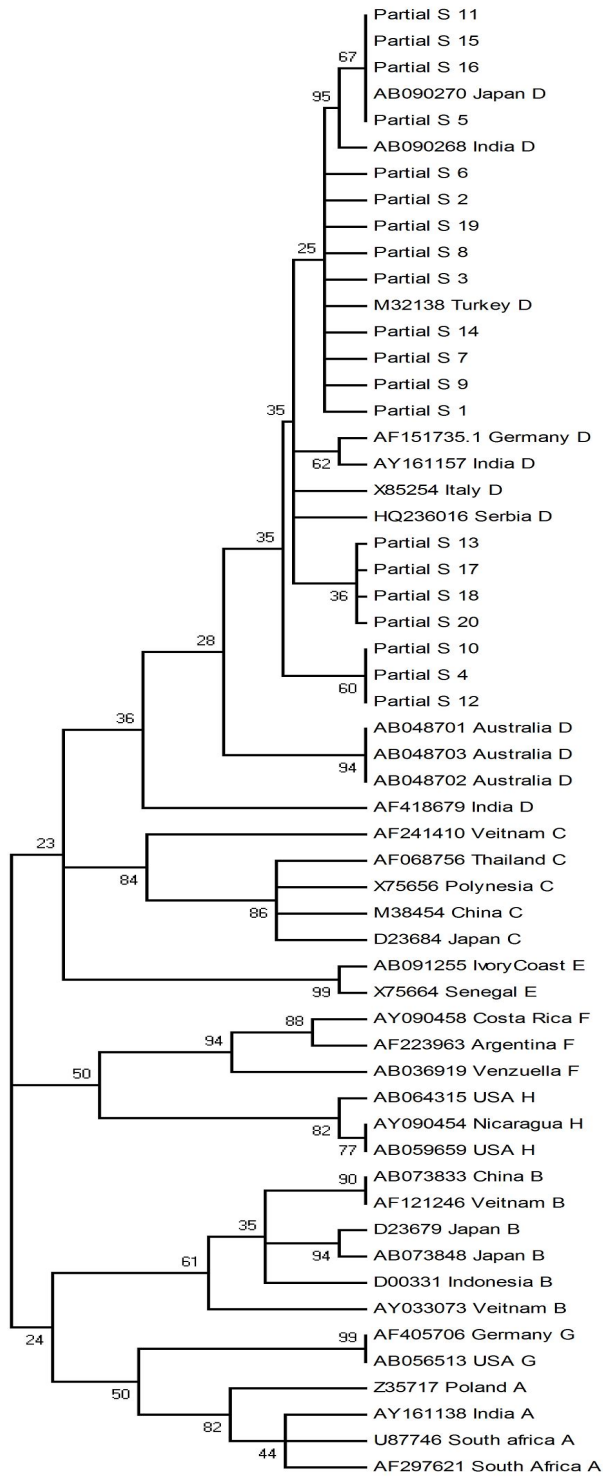


Fig. 1. The phylogenetic tree of 20 partial S gene sequences with reference sequences of all HBV genotypes and sub-genotypes confirming genotype D.

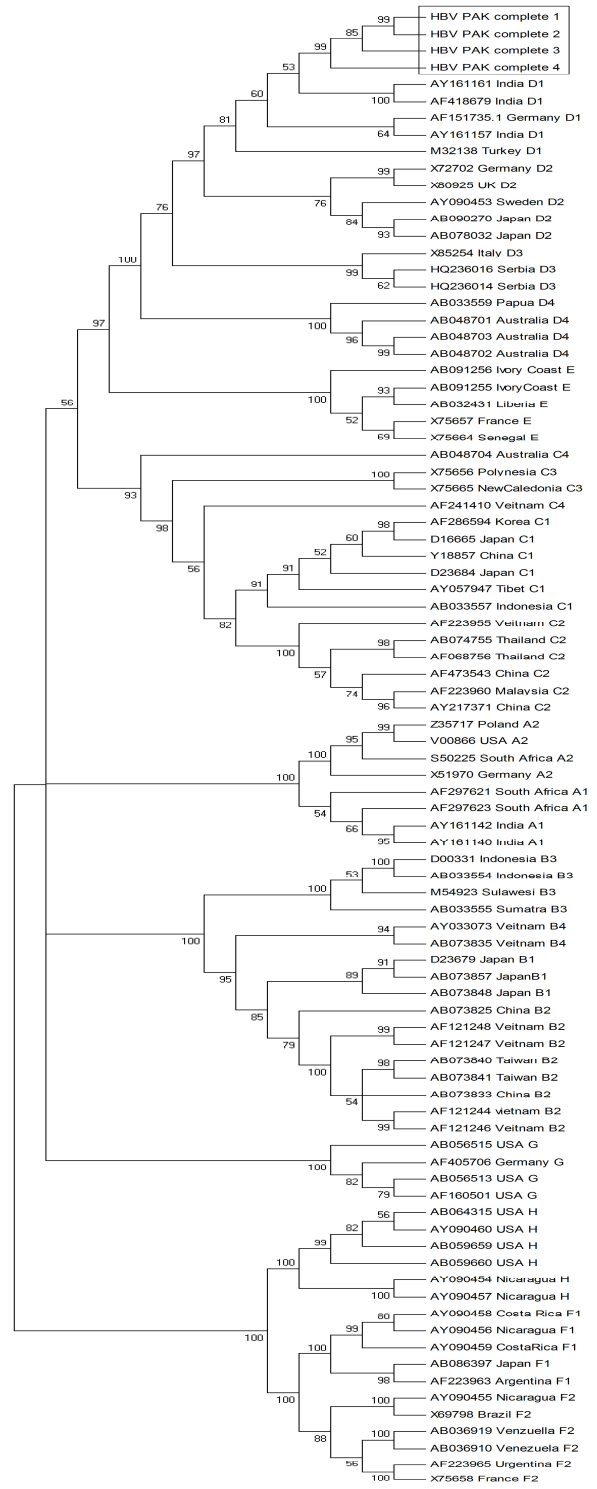


Fig. 2. The phylogenetic tree of 4 complete genome sequences with reference sequences of all HBV genotypes and sub-genotypes confirming sub-genotype D1

Table I.- Primers used for amplification of different fragments for sequencing of HBV genome.

Forward Primer (5'-3')		Reverse Primer (5'-3')	
S1-2	CGAACCACTGAACAAATGGC	P1	TCACCATATTCTTGGGAACAAGA
LAMes	GGATGTGTCTGCGGCGTTT	Pol181as	GACCCACAATTCKTTGACATACTTTCC
		Pol4as	GGCATTAAACGCAGGATAWCCACATTG
Pol2s	GACTATCAAGGTATGTTGCC	antiBC7	GCTAGGAGTTCGCGAGTATGG
P2	GTCACCATATTCTTGGGAACAAG	aP14	TAACACGAGCAGGGGTCCTA
BC9L	TTACATAAGAGRACTCTTGGAC	C6	CGTCTGCGAGGCGAGGGAGT
BC11	TCTCGCCAACCTACAAGGCC	Fullas	AAAAAGTTGCATGGTGCTGG
C5	ACCACCAAATGCCCTATCT	P5S	GGTTGAAGTCCCAATCTGGAT

Table II.- Pair wise distances showing number of base differences per sequence and per site between the study sequences and reference sequences of sub-genotype D1 along with a sequence of sub-genotype H as out-group.

	HBV_Pak_1		HBV_Pak_2		HBV_Pak_3		HBV_Pak_4	
	N/seq	N/site	N/seq	N/site	N/seq	N/site	N/seq	N/site
HBV_Pak_1	00	0.000	16	0.005	26	0.008	30	0.010
HBV_Pak_2	16	0.005	00	0.000	26	0.008	23	0.007
HBV_Pak_3	26	0.008	26	0.008	00	0.000	36	0.012
HBV_Pak_4	30	0.010	23	0.007	36	0.012	00	0.000
AB583679_Pakistan	82	0.027	71	0.023	80	0.026	78	0.025
AB583680_Pakistan	55	0.018	47	0.015	59	0.019	51	0.016
FJ899792_China	63	0.020	59	0.019	67	0.022	63	0.020
AY161157_India	45	0.015	37	0.012	49	0.016	39	0.013
AY161161_India	75	0.024	67	0.022	81	0.026	75	0.024
AF418679_India	67	0.022	57	0.018	69	0.022	65	0.021
AY090460_USA_H	417	0.135	415	0.134	421	0.136	414	0.134

+N/seq= number of base differences per sequence, N/site= number of base differences per site

Analysis of complete genome and sub-genotype

Complete genome sequencing of four samples also reveal and confirm genotype D. The four sequences of complete HBV genome were aligned with 88 reference sequences of all the major genotypes (from A through H) and their sub-genotypes of HBV obtained from gene bank. All the four sequences were clustered on a branch of sub-genotype D1 along with samples from India, Germany and Turkey. All of them were thus identified as sub-genotype D1. The accession numbers of reference sequences and their country of origin are given in the obtained phylogenetic tree (Fig. 2). The complete genome sequences of study samples were submitted to gene bank where they were assigned the accession numbers KF061167 to KF061170.

All the four complete genome sequences were then aligned with closely related sequences of sub-genotype D1, including 2 sequences from Pakistan and a sequence of sub-genotype H as out-group for distance analysis. After elimination of all the positions containing gaps and missing data with the complete deletion option, the final dataset, of which the distance analyses were performed,

was comprised of 3092 positions. A phylogenetic tree was obtained (Fig. 3). Pair wise distance analyses were performed for the number of base differences per sequence and number of base differences per site. Table II describes the distance analyses. The distance analysis revealed clearly the sub-genotype D1. In both number of base differences per sequence and number of base differences per site, all the four study sequences showed closest relation with a sequence of sub-genotype D1 from India (AY161157) followed by a D1 sequence from Pakistan (AB583680) and then a D1 sequence from China (FJ899792). The farthest relation was shown with sequence of genotype H which was taken as out-group sequence (Fig. 3, Table II).

DISCUSSION

The present study was designed mainly to explore genotypes/sub-genotypes of hepatitis B virus in Islamabad, Pakistan and then to confirm the mix genotype infection of genotype B and D with direct sequencing of an S gene fragment as well as complete genome of HBV.

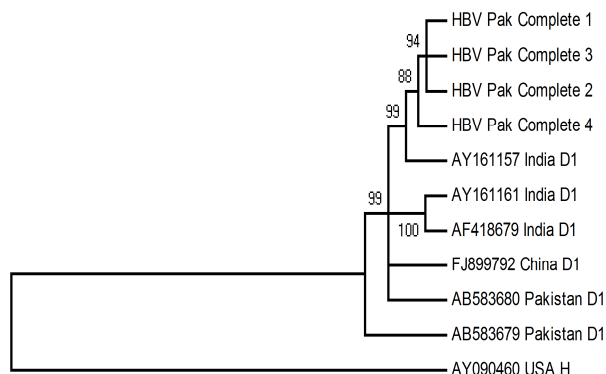


Fig. 3. The phylogenetic tree of 4 complete genome sequences with 7 reference sequences of HBV genotype D sub-genotype D1 including 2 previously published sequences from Pakistan and a sequence of genotype H as out-group.

When genotyped by type specific primer PCR method, the method used commonly to genotype HBV in Pakistan, we found genotype D alone from 63.6% of the samples and mix infection with genotypes A and D simultaneously from 34.4% of the samples. However, with direct sequencing method, we did not identify any sample with dual infection and all the successfully sequenced samples had genotype D sequence. The primer PCR method amplifies different fragments of different genotypes and the primers used are specific to the genotypes and they did not amplify the positive controls of non-specific genotypes when tested for confirmation. Moreover, in many types of confirmatory tests including different types of positive and negative controls, the mix genotype infection was confirmed by the type specific primer PCR method. The mix infection with the combination A+D was also reported by some other studies from Pakistan (Hanif *et al.*, 2013; Alam *et al.*, 2007; Baig *et al.*, 2009; Awan *et al.*, 2010; Abbas *et al.*, 2006) which confirm the presence of this type of infection. The reason may be the sensitivity difference of both the methods used as it is possible that genotype A which was not detected in any of the sample by direct sequencing method, has very less number of copies in the sample as compared to genotype A copies and was, consequently, not detected by the sequencer. The genotype A may have a low replication capacity or at least in the presence of another genotype such as D. This may be because of some competition for the replication machinery inside the cell or for new cell infection. All this is required to be studied in a larger number of samples infected with both genotypes A and D simultaneously.

Sub-genotypes D1, D2 and D4 were recorded in 9, 4 and 7 of our samples respectively by direct sequencing of S gene fragment while D1 was identified from all the four samples subjected to full length genome sequencing. Sub-genotypes D1 and D3 are already reported from Pakistan (Baig *et al.*, 2009). Sub-genotypes D1, D2, D3 and D5 are reported by more than one studies from Eastern India (Banerjee *et al.*, 2006; Datta *et al.*, 2008) while D1 and D2 are also reported from Turkey (Bozdayi *et al.*, 2005). So the presence of D1 and D2 genotypes in Pakistan, where genotype D is the predominant one, is quite likely. The presence of sub-genotype D4 which was identified in 7 out of 20 samples, however is surprising as this genotype was neither previously reported from Pakistan nor from any of the neighboring countries but it is mostly present in Australia, Oceania, Western Africa and South America (Norder *et al.*, 2004; Kwange *et al.*, 2013; Santos *et al.*, 2010). So, a study is needed for the confirmation of this sub-genotype from Pakistani isolates of HBV. This study concludes that genotype D and sub-genotype D1 of hepatitis B virus are dominant in Islamabad. Moreover, mixed HBV genotypes, specifically the mixtures of A and D genotypes detected by type specific PCR method has not been confirmed by direct sequencing method strengthening the reliability of direct sequencing method over type specific PCR method for HBV genotyping.

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Declaration of conflict of interest

The study is a part of PhD thesis of Majid Mahmood. There is no other conflict of interest among the authors.

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