Original Article

Sub-genotypes of Hepatitis B Viruses Circulating among Chronically Infected Patients in Bangladesh

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Abstract:
High genetic variability divided the hepatitis B viruses (HBV) into different genotypes and sub-genotypes, which considerably differ with respect to geographical distribution, transmission routes, disease progression, responses to antiviral therapy or vaccination, and clinical outcome measures such as liver cirrhosis (LC) or hepatocellular carcinoma (HCC). As limited data is available regarding the sub-genotypes of HBV circulating in Bangladesh, our study demands to determine. A small Bangladeshi cohort was performed in March 2014 and August 2015 with the interview of 172 HBV DNA positive patients from the BSMMU. Among them, a total of 29 patients were randomly selected and HBV DNA samples were isolated. Sequencing had performed by Sanger method. Finally, 226 amino acids (aa) in the small surface (S) gene of HBV DNA sequences were analyzed by bioinformatics tools for determination of sub-genotypes. The results of our present study showed that the frequency of sub-genotypes was C2 in 17 (58.6%), A1 in t 2 (6.9%), D2 in 7 (24.1%) and D1 in 3 (10.3%) isolates. Our study concluded that the most prevalent sub-genotype was C2, followed by D2, and the least dominant sub-genotypes were D1 and A1 in Bangladesh. HBV infection could be controlled and eliminated by the universal vaccination, adequate treatment and vigorous diagnostic assays. To organize of all these measures, an accurate sub-genotyping of HBV is essential. Therefore, HBV categorization is helpful for the improvement of prophylaxis, diagnosis and treatment of HBV infection which will control and eliminate this virus in Bangladesh in future.

Key words: Hepatitis B virus, Sub-genotypes, Chronic HBV infection

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Introduction:
Hepatitis B virus (HBV) is an important infectious agent for the chronic liver diseases, including liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Despite the availability of a safe, effective vaccine against HBV since 1982, HBV infection still remains a major health problem globally. In the world, more than 240 million people are chronically infected with HBV, and more than 7,80,000 people die due to acute and chronic consequences of HBV infection in every year[1]. Bangladesh is an intermediate prevalence area of HBV infection where 5%-6% of apparently healthy individuals are chronically infected with this virus[2-5]. Various studies have shown that HBV is responsible for 31.25% cases of acute hepatitis and 76.3% cases of chronic hepatitis, 61.15% cases of cirrhosis of liver and 33.3% cases of HCC in Bangladesh[2,6-7].

HBV is a DNA virus of the Hepadnaviridae family that contains a partially double-stranded DNA genome approximately 3.2 kb in length. Spontaneously many genotypes and sub-genotypes of HBV emerge over time because of the reverse transcriptase (RT) of this virus does not have proof reading ability. HBV variability also developed from external selection pressures to viral genome by antiviral treatment and universal vaccination. Phylogenetic and evolutionary analyses of complete sequences of HBV genome, this virus divided into eight confirmed genotypes from A to H and two tentative genotypes, such as, I and J. Due to high genetic variability, this viruses are also subdivided into almost forty sub-genotypes till date[8-10].

Genotypes have nucleotide divergence greater than 7.5% [11]. Whereas, "Sub-genotypes" are the further subgroups within the same genotype that have a nucleotide divergence between 4% and 7.5% over the full-length genome with high phylogenetic bootstrap support[12]. Sub-genotypes are again categorized into "Clades" which have nucleotide diversity less than 4% of overall genome sequences[11]. Different sub-genotypes have been differ from transmission routes, disease progression, responses to antiviral therapies and clinical outcomes[13,14].

Since, only one data is available regarding the sub-genotypes of HBV in Bangladesh, thus our study strongly demand to be documented[20]. As different sub-genotypes play important role for the evolution,

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transmission, treatment responses and clinical outcomes of the HBV infection, thus knowledge of sub-genotypes may help to better management of chronic hepatitis B (CHB) patients in Bangladesh and also eliminate HBV infection in future.

Materials & Methods:

Sample selection: This small Bangladeshi cohort was carried out between March 2014 and August 2017. Blood samples were collected from 29 chronic hepatitis B patients (CHB) at the department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh. The patients were selected according to the HBsAg positivity for >6 months and all of them were HBV DNA positive. Molecular studies were done at the Virology Laboratory, BSMMU. The clinical outcomes were observed over the year 2014-2017. This study was ethically approved by the Institutional Review Board of BSMMU (Reference number: BSMMU/2014/106/2) and written informed consent was obtained from all study patients.

DNA extraction: Blood samples were centrifuged to separate plasma and then HBV-DNA was extracted from 200 µl of this separated plasma using DNeasy Blood and Tissue Kit (QIAGEN, Venlo, Limburg, The Netherlands) according to manufacturer's instruction. Extracted DNA was stored at -20°C until use.

Polymerase chain reaction and DNA amplification: Nested polymerase chain reaction (PCR) was performed to amplify the HBV DNA. A product of 1014 base pairs of small S gene was amplified using 3079-3099 (5′- AGC CCT CAG GCT CAG GGC ATA-3′) / 1163-1140 (5′- CGT TGC CKD GCA ACS GGG TAA AGG-3′) as external primers and 3192-3211 (5′- TCA TCC TCA GGC CAT GCA GT-3′) / 991-972 (5′-GAC ACA CTT TCC AAT CAA TNG G-3′) as internal primers (Biobasic, Canada), which manually described as previously by HPA Document Control System, VW-0461.01, 2007. The amplification products were identified by agarose gel electrophoresis on ethidium bromide staining to observe HBV-DNA bands which was viewed under UV illumination.

Post PCR purification and sequence determination: The corresponding amplicons were purified using the PCR clean-up system by ExoSAP-IT (USB Corp, Cambridge), according to manufacturer's instructions. Internal PCR primers and a BigDye® Terminator v3.1 Cycle Sequencing Kit (California) were used for cycle sequencing reactions and the products were purified by BigDye® XTerminator™ Purification Kit. Sequencing was done in an automatic sequencer (ABI PRISM® 3500xL Genetic Analyzer).

Phylogenetic analysis and determination of sub-genotypes: The electropherogram files of all obtained nucleotide sequences were analyzed using Chromas 2.3 (Technelysium). The sequence similarity of all obtained sequences was searched by NCBI (National Institutes of Health, Bethesda, MD, USA) BLAST (BLASTn). Finally, 678-nt fragment or 226 amino acids (aa) of the HBsAg protein were aligned with the reference sequences collected from NCBI GeneBank using ClustalW program located in the BioEdit 7.0.9.0 suite of programs[15].

Sub-genotypes were determined by an online tool named as: "The Genafor/Arevir–geno2pheno drug resistance tool" (Center of Advanced European Studies and Research, Bonn, Germany; http://coreceptor.bioinf.mpi-inf.mpg.de/). It was also analyzed by phylogenetic tree which was constructed by Neighbor-Joining method[16] using MEGA 6.06 package[17] with bootstrap probability of 1000 pseudoreplicate data sets[18]. Evolutionary distances in the phylogenetic trees were computed using the p-distance method[19].

Results:

A total of 29 patients were included for isolation and sequence analysis of HBV DNA. Among them, 25 (86.2%) patients were male and 4 (13.8%) patients were female with a mean age of 29.8±12 years and age range of 4 to 50 years. According to the treatment history of the study patients, 15/29 (51.7%) patients untreated and n=14 (48.3%) patients were antiviral treated. Their viral load values varied from 9.1×102 to 7.0 ×108 IU/ml, with mean value of 5.5×107 (SD±1.5×108) IU/ml. ALT values varied from 15 to 419 U/l, and mean ALT level was 89.8 (SD ± 74.9) U/l. Among the study population, 22 (75.9%) patients were HBeAg positive and 7 (24.1%) patients were HBeAg negative (Table I).

Phylogenetic analysis revealed that three genotypes present in this study, genotype C, D and A. All the genotype C and A strains showed sub-genotype C2 and A1 respectively. Whereas, in the genotype D strains, two different sub-genotypes were observed: D2 and D1. The frequency of sub-genotypes in this study was C2 in 17 (58.6%), A1 in t 2 (6.9%), D2 in 7 (24.1%) and D1 in 3 (10.3%) isolates (Fig. 1).

In the phylogenetic tree, all the Bangladeshi genotype C2 strains from our study clustered with the South-East Asian strains. Among eleven of them (HBV 1, 2, 3, 5, 6, 16, 17, 18, 20, 21, 22) are closely clustered with similar three Thai and two Malaysian strains. In addition, other two Bangladeshi genotype C2 strains (HBV 7, 9) are also clustered with another Thai strain and one of our Bangladeshi genotype C2 strain of HBV_8 clustered with one Chinese strain. Whereas, our two genotype A1 (HBV 10, 11) strains are clustered with the two Indian and Philippines

strains. Besides that, our five Bangladeshi genotype D2 strains (HBV 13, 15, 27, 28 and 29) are clustered with the two Indian, one Russian and one Japanese strains. However our two Bangladeshi D1 strains (HBV 24 and 25) clustered with two strains from Pakistan and Tunisia.

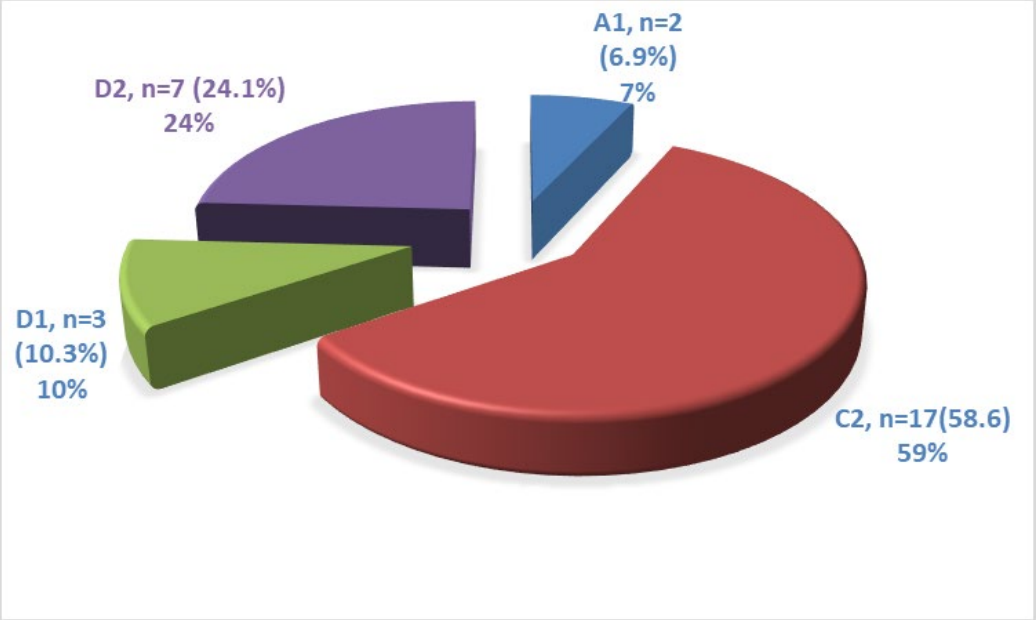

Figure-1: Pie chart showing the Frequency of HBV genotype, n (%).

Observation of the clinical outcomes of study patients infected with different sub-genotypes of HBV showed that the mean viral load of sub-genotype C2 positive patients was 6.2×107±1.7×108 IU/ml with mean ALT level was 102.2±94.1 U/l. Whereas, the mean viral load of sub-genotype D2 positive patients was 7.8×107±1.7×108 IU/ml with mean ALT level was 80±28.9 U/l and viral load of genotype D1 was 2.2×105±1.3×105 with mean ALT level was 80.5±43.1. In addition, mean viral load of sub-genotype A1 positive patients was 8.1×105±9.6×105 IU/ml with mean ALT level was 50±36.8 U/l. After observation of the study patients from March 2014 to August 2017, we observed that HCC was developed in 2 patients infected with sub-genotype C2 strains. Both of the sub-genotype A1 positive patients had shown advance liver diseases: one patient developed HCC and another patient complicated with LC (Table-I).

Table-I: Observation of clinical outcomes of study patients infected with different sub-genotypes of HBV from March 2014 to August 2017.

HBV Sub-genotypes	Viral load, Mean±SD (IU/ml) (2014)	SGPT, Mean±SD (U/l) (2014)	Observed Clinical Outcome = Number of patients (2014-2017)
Sub-genotype C2	$6.2\times10^{7}\pm1.7\times10^{8}$	102.2±94.1	HCC=2
Sub-genotype D2	$7.8\times10^{7}\pm1.7\times10^{8}$	80±28.9	ND
Sub-genotype D1	$2.2\times10^{5}\pm1.3\times10^{5}$	80.5±43.1	ND
Sub-genotype A1	$8.1\times10^{5}\pm9.6\times10^{5}$	50±36.8	HCC=1, LC=1

Discussion:

The results of our study showed that three genotypes were present here: genotype C, D and A. Among these three observed genotypes, we found that the genomic group C and A strains was restricted in single sub-genotype C2 and A1 respectively. Whereas, two different sub-genotypes were observed within the genomic group D strains: D2 and D1. The most prevalent sub-genotypes was C2 which was found in 17 (58.6%) isolates, followed by D2 in 7 (24.1%) and D1 in 3 (10.3%) isolates. The least dominant sub-genotype was A1 showed in t 2 (6.9%) isolates. However, a former Bangladeshi study conducted by Rahman et al. reported that all the Bangladeshi genotypes C and A showed sub-genotypes C1 and A1 respectively[20]. However, in case of genotype D, the author found multiple sub-genotypes was present in Bangladesh, such as, D1, D2, D3 and D5 [20]. Phylogenetic analysis from our present study also revealed that the similarity of our Bangladeshi sub-genotype A1 and D2 strains was found with the Indian strains. Bangladeshi A1 strains also similar with strains from Philippines and D2 also with strains from Russia and Japan. In

addition, Bangladeshi D1 strains found to be similar with strains from Pakistan and Tunisia. However, Bangladeshi genotype C2 was found most similar with the Thai and Malaysian strains, and also similar with the Chinese strain. Bangladesh has an important communication though the purpose of business, recreation, treatment and also has good labor market with these countries. Therefore, it can be assumed that population migration may possess important causes of dispersion of HBV sub-genotypes from these countries to Bangladesh or vice versa.

Different sub-genotypes of HBV had shown different clinical outcomes. Observation of the clinical outcomes of the study patients infected with different sub-genotypes of HBV showed that the mean ALT level was higher in patients infected with sub-genotype C2 strains of HBV compared to patients infected with other strains like sub-genotype D2, D1 and A1. We also have observed our study patients from March 2014 to August 2017 and we found that both of the sub-genotype A1 infected patients developed advanced liver diseases: one patient developed HCC and another patient complicated with LC. Previous study from Livingston et al. suggested that in West and South Africa, sub-genotype A1 has a more severe clinical outcome compared to sub-genotype A2 [21]. This author showed that patients infected with this sub-genotype A1 developed HCC at younger age in West and South Africa, whereas patients infected with A2 sub-genotype in Europe had developed HCC mainly at older ages[21]. In addition, Sanchez-Tapias et al. found that European patients infected with A2 sub-genotypes had a mild clinical outcome with high HBsAg and HBV DNA clearance[22].

Further study from Pourkarim et al. reported that more occult cases had been identified in Europe among patients infected with A1 sub-genotypes comparing with A2 or other sub-genotypes[23]. Therefore, sub-genotype A1 prevalent area need more surveillance and patients infected with this A1 strains need careful monitoring to prevent the unwanted clinical outcomes. Besides that, in our present study we also found 2 patients infected with genotype C2 sub-genotypes and had developed HCC. Genotypes B and C were compared with published data found from most studies done in South Asia. From that, one study demonstrated that there was an increased risk for HCC in patients infected with C2 sub-genotype compared to C1 and genotype B sub-genotypes[24].

Another large study showed that HCC developed in a higher percentage of patients infected by C2 or F2 sub-genotypes compared to A2 and B6 sub-genotype or genotype D sub-genotypes[25]. However, further research revealed that sub-genotype B6 infected patients were commonly associated with a mild clinical outcome, while B1 resulted in fulminant and acute hepatitis B infection[26,27]. It was also shown that patients infected by this B1 sub-genotype had developed advanced liver disease at older age compared to patients infected with B2-B5 sub-genotypes[26-28]. Moreover, precore mutations frequently observed in F1 than F2 sub-genotype[29,30]. Therefore, by review these data it is suggested that sub-genotyping is an important surveillance to a country for prevention and control of HBV related health hazards.

Limitations of the Study:

1) The present study conducted with small sample size, so statistical association could not be done adequately.

2) The study had analyzed only small S gene, while accurate sub-genotyping is required full genome sequencing with high bootstrap support.

Recommendations:

To determine the sub-genotyping of HBV, full genome sequence analysis with larger sample size should be required.

Conclusion:

We concluded that the most prevalent HBV sub-genotype was C2, followed by D2 and the least dominant sub-genotypes were D1 and A1 in Bangladesh. Control and elimination of HBV infection requires universal vaccination, effective treatment and a vigorous diagnostic scheme. To organize all these measures from prophylaxis to therapy, it is important to know accurate sub-genotypes of HBV circulating in a region. Thus, applying this HBV sub-genotyping may help for improvement of prophylaxis, diagnosis and treatment of HBV infection that will control and eliminate this virus from Bangladesh in future.

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Conflict of Interest:

No conflict of interest is declared by the authors.

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