

Hepatitis B Virus Subtype Distribution in Bangladeshi Chronic Carriers

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Abstract

Background : Hepatitis B virus (HBV) infection is one of the major global health problems and this virus has many variants that differ from epidemiological distributions, transmission sources, clinical outcomes, diagnostic assays as well as therapeutic responses. HBV was also divided into 9 different subtypes which has distinct geographical distributions and important for the epidemiological studies and vaccine development.

Objective : Bangladesh is an intermediate prevalence region for HBV infection, however, very little is known about the incidence of HBV subtypes in circulation. Therefore, we investigated the HBV subtype distribution in Bangladesh.

Method : A small cohort was performed on between March 2014 and August 2017 with 172 HBV DNA positive patients from the BSMMU. From them, 29 HBV DNA samples were isolated for sequencing by Sanger method. HBsAg subtypes were determined by identifying of 226 amino acid (aa) positions in the small surface (S) gene.

Result : Our study showed that HBV subtype adr_q+ was in 17 (58.6%) isolates, ayw₃ in 8 (27.5%), ayw₂ in 2 (6.9%) and adw₂ in 2 (6.9%) isolates.

Conclusion : We conclude subtype adr_q+ predominant in Bangladesh. This was followed by ayw₃, while adw₂ and ayw₂ were least dominant. Subtype adw₂ and adr_q+ strains are found to be related with more complication, therefore, patients infected with these HBV strains need to be careful monitoring to assess their clinical outcome in future.

Key words : HBV, subtype, clinical complications.

DOI: <http://dx.doi.org/10.3329/nimcj.v9i2.38908>

Northern International Medical College Journal Vol. 9 No. 2 January 2018, Page 286-290

Introduction

Hepatitis B (HBV) virus infection is one of the major global health issue and approximately more than 240 million people are chronic carrier with this virus.¹ These chronic HBV carriers are often suffered from liver cirrhosis (LC) and hepatocellular carcinoma (HCC).² As a result, HBV infection leads to 780,000 annual deaths by its acute or chronic consequences.¹

Bangladesh is an intermediate prevalent zone for HBV infection.³ The rate of healthy HBV carrier in this country is about 5%-6% and most of them even not know that they are infected with this virus.^{4,5} In addition, about 6-8 million of younger Bangladeshi peoples are chronically infected by HBV.^{6,7} Analysis of previous studies revealed that HBV is responsible for acute hepatitis (31.25%), chronic hepatitis (76.3%), LC (61.15%) and HCC (33.3%) in Bangladesh.^{3,8}

HBV is classified as a DNA virus of the Hepadnaviridae family. It contains a very short

double-stranded DNA genome of nearly 3200 base pair (bp) lengths that arranged in four overlapping open reading frames (ORFs), such as, polymerase (Pol) gene, envelop/surface (S) gene, core/precore (C/PC) gene and X ORFs gene. Many HBV genotypes, subtypes, different HBV genomic mutants and recombinants emerge over time due to the presence of reverse transcriptase (RT) enzyme which does not have any proof reading ability. In addition, external selection pressure by antiviral agents and vaccine causes many viral variants. Till date, HBV classified into 10 genotypes, named as A to J and also 40 subgenotypes.^{9,10} These 10 genotypes have nucleotide differences more than 7.5%.¹¹ Whereas, "Sub genotypes" are subgroups within the same genotype that contains nucleotide difference as 4% to 7.5%.¹² "Clades" are further division of sub genotypes based on less than 4% nucleotide differences.¹¹

Before the definition of genotypes, HBV were classified as serotypes (subtypes) according to

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serological differences, based on immune reaction by amino acid pattern at specific location of "a" determinant which leads to the 9 different subtypes designating adw2, adw4, adrq+, adrq-, ayw1, ayw2, ayw3, ayw4 and ayr.¹³ Subtypes correlate broadly with genotypes. Some subtype can be found in more than one genotype, which confer additional heterogeneity within the genotypes. Subtypes are important for the epidemiological studies and vaccine development.

Therefore, the determination of HBV subtypes will be helpful in epidemiological surveillance as well as development of the appropriate vaccine in Bangladesh. This will help to control of HBV infection in Bangladesh in near future.

At present, the HBsAg used in all vaccines contains the adw subtype of HBsAg. Whether this vaccine is appropriate for our population or not our is unknown as limited data available regarding the HBV subtypes in Bangladesh. Therefore, our study focused to determine the HBV subtype distribution in Bangladesh.

Materials and Methods

Patients

This small cohort was done on between March 2014 and August 2017 with chronic hepatitis B patients at the department of Virology, BSMMU, Dhaka, Bangladesh. The selection criteria were

- more than 6 months HBsAg positivity along with HBV DNA positive which was detected by real-time PCR.

A total of 172 patients were interviewed with a pretested questioner at BSMMU. From them, only 29 patients were selected for molecular study from March 2014 to December 2014. Then the patients were yearly once followed upto August 2017.

Blood samples were collected from these 29 selected patients in a microcentrifuge tube containing anti-coagulant EDTA 5%. Then plasma was separated from centrifugation of blood. The separated plasma was aliquot in a centrifuge tube and kept at -20°C until testing.

The study was ethically approved by the Institutional Review Board of Bangabandhu Sheikh Mujib Medical University, Dhaka, Bangladesh and written informed consent for participating in this study was obtained from all patients.

Isolation of HBV DNA

Extraction of HBV DNA was done from 200 µl of plasma using DNeasy Blood and Tissue Kit (QIAGEN, Venlo, Limburg, The

Netherlands) according to manufacturer's instruction. Extracted DNA was stored at -20 until PCR.

Amplification of viral DNA

HBV DNA was amplified using nested PCR that targeted a 1014-bp region of the S gene. Amplification was performed using the following primers: 3079-3099 (5'-AGC CCT CAG GCT CAG GGC ATA-3') / 1163-1140 (5'-CGT TGC CKD GCA ACS GGG TAAAGG-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), as described previously¹⁴. After both reactions, a 1014-bp fragment was obtained and detected by ethidium bromide staining in an agarose gel.

DNA purification and sequencing

Exonuclease Clean-up of nested PCR Products were done by ExoSAP-IT (USB Corp, Cambridge), according to manufacturer's instructions. For cycle sequencing reactions, internal PCR primers and a BigDye® Terminator v3.1 Cycle Sequencing Kit (California) were used. The product of cycle sequencing was purified by BigDye® X Terminator™ Purification Kit. Sequencing was carried out in an automatic sequencer (ABI PRISM® 3500xL Genetic Analyzer).

HBV DNA sequence analysis

The electropherogram files of the all 29 obtained HBV DNA forward and reverse nucleotide sequences were examined and edited using Chromas 2.3 (Technelysium). The nucleotide sequences for sequence similarity searches were performed using the NCBI (National Institutes of Health, Bethesda, MD, USA) BLAST server on Gene Bank database. Sequences were aligned by using the Clustal W program located in the BioEdit 7.0.9.0 suite of programs.¹⁵ Finally, 226 aa of 678 nucleotide (nt) fragment in the HBV S gene (nt 155-835) of HBV were analyzed. Numbering was done according to BioEdit alignment with the NCBI Reference Sequence no: NC_003977.1.

Phylogenetic analysis and HBV genotyping

The HBV genotype was identified by phylogenetic tree analysis. The sequences from the present study were compared to GeneBank sequences in a BLAST search. The nucleotide sequences were aligned pair wise using Clustal W program. Phylogenetic tree was constructed according to Neighbor-Joining method using MEGA 6.06 package.¹⁶ The bootstrap probability at each branching point was calculated with 500 pseudoreplicate data sets.¹⁷ Evolutionary distances in the phylogenetic tree were computed using the p-distance method and are in the units of

the number of amino acid differences per site.¹⁸ All positions containing gaps and missing data were eliminated. There were a total of 226 amino acid positions in the final dataset. Genotyping was also done by using the three online genotyping tools.^{19,20,21}

Determination of HBV subtypes

Determination of the HBsAg subtypes were done from the HBV DNA sequence of the HBsAg protein based on identifying amino acids positions at 122 [Lys(K)/Arg(R) for d/y determinants], 160 [Lys(K)/Arg(R) for w/r determinants], 127 [Pro(P)/Thr(T)/Leu(L)-Ile(I) for w2/w3/w4], and in the case of Arg(R) at 122, Pro(P) at 127, Lys(K) at 160, also at positions 159 [Ala(A)/not Ala (A) for ayw1/ayw2 and ayw4] and 140 [not Ser(S)/Ser(S) for ayw2/ayw4].²²⁻²³

Statistical analysis

Statistical analysis of data was performed using the SPSS software package 17.0 (IBM SPSS Statistics for Windows,

the study population, 22 (75.9%) patients were HBeAg positive and 7 (24.1%) patients were HBeAg negative (Table-1).

Table 1 : Comparison of demographic and laboratory data of 29 study patients
Demographic and laboratory data of total 29 study patients

| | |
|--|--|
| Study group, n (%) | 29 |
| Male, n (%) | 25 (86.2%) |
| Female, n (%) | 4 (13.8%) |
| Age in years, Mean ± SD (Range) | 29.8±12(4-50) |
| Serum ALT (U/l), Mean ± SD (Range) | 89.8 ± 74.9(15-419) |
| *HBV viral load (IU/ml), Mean ± SD (Range) | 5.5×10 ⁷ ± 1.5×10 ⁸ (9.1×10 ² -7.0×10 ⁸) |
| Plasma HBeAg status, | |
| Positive | 22 (75.9%) |
| Negative | 7 (24.1%) |

Note: *HBV viral load in plasma.

Subtypes were determined by analyzing the following amino

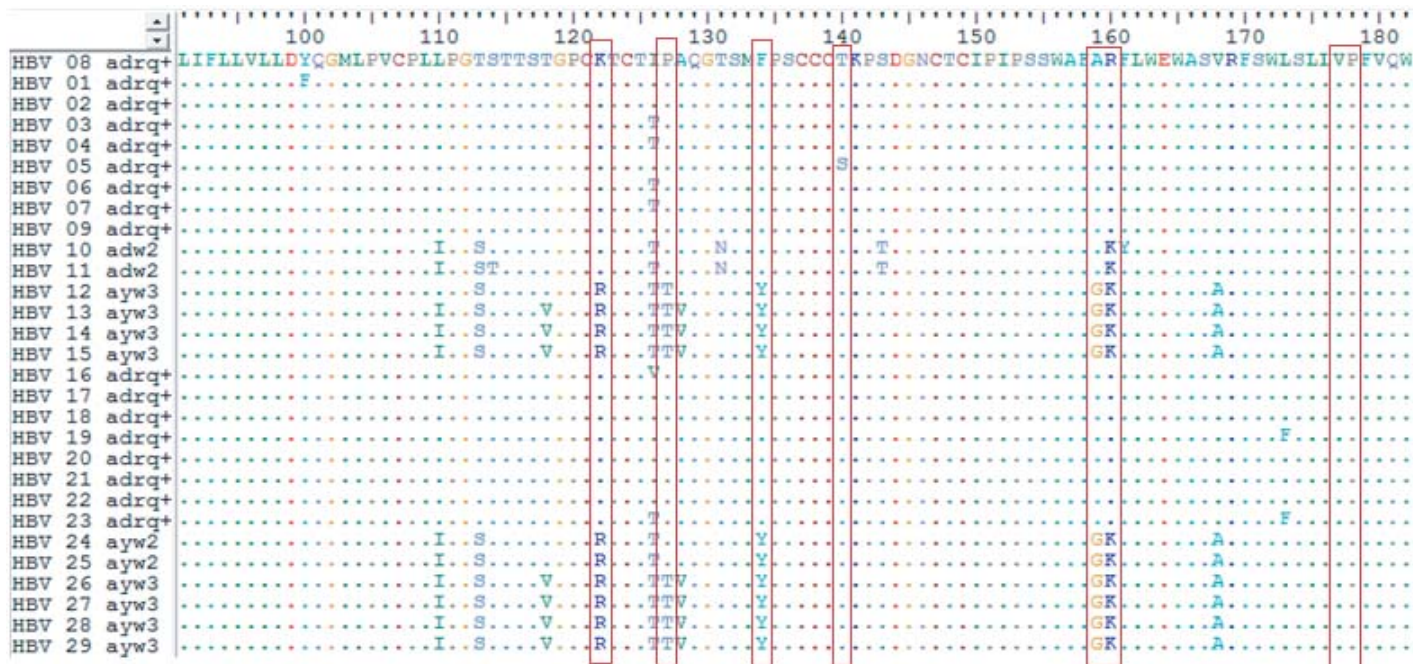


Figure 1: HBV subtype determination* Red boundaries in figures show amino acid positions that were analyzed.

Armonk, NY, USA).

Results

A total of 29 patients were selected for sequencing of HBV DNA and sequence analysis. 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. 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

acids alignment of the small S gene of HBV from our study results (Figure 1).

In this study, the observed frequency of HBV subtypes among study patients were adrq⁺ in 17 (58.6%) isolates, ayw 3 in 8 (27.5%), ayw2 in 2 (6.9%) and adw2 in 2 (6.9%) isolates (Figure 2). In association of genotypes with subtypes, genotype C of HBV isolates showed all were adrq⁺ subtype. Similarly, both genotypes A isolates found adw2 subtype. Whereas, genotype D showed two different subtypes: ayw3 in 8 (27.5%) and ayw2 in 2 (6.9%).

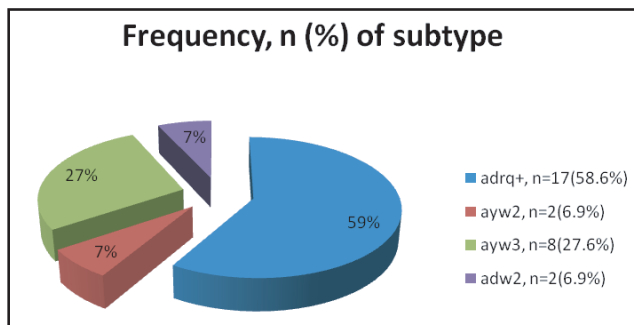


Figure 2 : Frequency of subtype, n(%)

On observation of the study patients from March 2014 and August 2017, we found that 2 patients positive with adrq⁺ strains developed HCC. Both of our adw2 infected patients had died: one patient from HCC and another patient by LC. (Table 2)

Table 2 : Clinical complications of patients infected with different subtypes of HBV on between March 2014 and August 2017.

| Genotype/Subtype (2014) | Complications (2014-2016) |
|-------------------------|---------------------------|
| adrq ⁺ | HCC=2 |
| ayw2, ayw3 | ND |
| adw2 | HCC=1; LC=1 |

Note : HCC : Hepatocellular Carcinoma; ND: not detectable; LC : Liver Cirrhosis

Discussion

The extent of HBV replication among chronic hepatitis B patients is very high, about more than 10⁸ to 10¹¹ viral particles per day.²⁴ As RT of Pol has no proof reading capacity, HBV replication is also associated with a high mutational rate of 10⁵ substitutions/base/cycle.²⁵ Therefore, all possible single base changes in the HBV genome are generated daily, therefore many genotypes and subtypes of HBV and also different type of genomic mutations emerge.

As the distribution of subtypes of HBV in our country is very little known, thus we demand it for search. Therefore, this study will support to develop appropriate vaccine for Bangladeshi population.

Our present study showed that most prevalent HBV subtypes were adrq⁺ followed by ayw3 (8/27.6%). Whereas, adw2 (2/6.9%) and ayw2 (2/6.9%) were less dominant subtypes. Former study by Rahman et al also showed that the prevalent HBV subtypes were adr (41%), followed by adw2 (28.2%) and ayw3 (25.6%), and less dominant was ayw2 (5.1%) in Bangladesh.²⁶

In correlation with genotypes and subtypes, we found all the genotype C showed subtype adrq⁺ and genotype A had adw2. Whereas, genotype D belonged to two different subtypes: ayw3 (8/27.6%) and ayw2(2/6.9%).This findings was consistent with the previous studies reported from Bangladesh and India.^{13,26}

One study from Shaha et al showed that subtypes associated with genotype D were ayw3 (64.3%) and ayw2 (35.7%), and subtypes adw2 and adr were specific for genotype A and C in Bangladesh respectively.²⁶ Whereas, Rahman et al showed that the subtype adr completely restricted on genomic group C, while, the subtype adw2 found within the both genotype A and C, and the subtype ayw were mostly found among the genotype D.¹³ In comparison of the geographical distribution of HBV, we found that genotype A (subtypes adw2 and ayw1)is most prevalent in North America and North Western Europe. Genotypes B (subtypes adw2, adw3 and ayw1) and C (subtypes adw2, ayw3, adr and ayr) are highly prevalent in East Asia. Genotype D (subtypes adw3, ayw2, ayw3 and ayw4) is most prevalent in the Mediterranean and the Middle East. Genotype E (subtype ayw4) found in West Africa. Genotypes F (subtypes adw4 and ayw4) and H (subtype adw4) are found in Central and South America. Genotype G (subtype adw2) is found in the United States and Europe.^{12,27}

Further observation of the clinical complications on the year of March 2014 to August 2017, we found that HCC developed in 2 patients positive with adrq⁺ strains. Both of the patients infected with adw2 strains had died from the complication of HBV infection: one patient from HCC and another patient by LC. Therefore, we suggested that the patients infected with adw2 and adrq⁺ strains need to be careful monitoring to assess their clinical outcome in future.

Conclusion

In conclusion of our study, the prevalent Bangladeshi HBV subtype was genotype adrq⁺, this was followed by ayw3, while adw2 and ayw2 were present in limited number of patients. Subtype adw2 and adrq⁺ strains are found to be related with more complication, therefore, patients infected with these HBV strains need to be careful monitoring to assess their clinical outcome in future.

Limitations of Study

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

Recommendations

Larger sample size should be required for statistical validity.

Acknowledgments

We appreciate the excellent technical and scientific assistance of Dr. Mohammad Enayet Hossain, Dr. Safiullah Sarkar, Dr. Tasnim Azim, Razibur Rahman, Rashedul Hassan, Sanjoy Mukharjee, Sadia Ahmed, Farzana Ahmed, Warda Hauque, Subarna Barua and A.M Ramim from the Virology Laboratory, icddr,b, Dhaka, Bangladesh. Special thanks to Dr. Mamun-Al-Mahtab from the Bangabandhu Sheikh Mujib Medical University, Dhaka,

Bangladesh and Prof. Jalaluddin Ashraf Haq, Prof. Md. Shah Alam and Dr. Masuda Mohsena from Ibrahim Medical College, Dhaka, Bangladesh. My thanks also go to the laboratory technicians and other staffs of the Department of Virology, BSMMU and icddr,b for their co-operation.

Conflict of Interest : No conflict of interest to declare.

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