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Research Article

Whole Genome Sequencing of Hepatitis B Virus (HBV) Strains from Myanmar

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Abstract: Hepatitis B virus (HBV) is an important etiological agent of acute or chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. It is estimated that there are currently more than 350 million chronically infected people worldwide and this poses major health problems worldwide, especially in Asian Pacific countries. To date, 10 HBV genotypes, scattered across different geographical regions, have been identified. Complete genome sequences are more reliable for the analysis of genotype and sub-genotyping. In addition to this, certain mutations which may affect the diagnostic detection, drug resistance, disease progression and effective vaccination of HBV can also be detected. In this study we successfully sequenced the 15 full-length genetic sequences (3.2kbp) of HBV isolates from HBV infected Myanmar peoples. Genotyping was done by using HepSEQ web based program, and drug resistant mutations were detected by geno2pheno web based analytical tools. All 15 sequences revealed genotype C and no drug resistant mutations were seen among these isolates. There was also no known vaccine escape mutation among these isolates. This is the first time to sequence the whole genome of Myanmar HBV isolates by Department of Medical Research, and these whole genome sequences will act as baseline data for further genetic studies regarding HBV.

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INTRODUCTION

Hepatitis B virus (HBV) is an important etiologic agent of acute or chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. It is estimated that there are currently more than 350 million chronically infected people worldwide and this poses major health problems worldwide, especially in Asian Pacific countries [1, 2]. Human hepatitis B virus (HBV), which belongs to the genus Orthohepadnavirus and also the prototype member of the family Hepadnaviridae, is a circular, partially double-stranded DNA virus of approximately 3200 bp [3]. This highly compact genome contains the four major open reading frames (ORFs) encoding the envelope (preS1, preS2 and surface antigen HBsAg), core (preCore precursor protein, HBeAg and HBcAg), polymerase (HBPol) and X (HBX) proteins, respectively [4].

HBV replication involves an error prone reverse transcription step. This unique replication strategy accounts for the majority of the point mutations, deletions and insertions observed in the HBV genome. The lengthy evolution of HBV has led to the current existence of various genotypes, sub-genotypes, mutants, recombinants, and even quasi species of HBV [1, 2]. According to the homogeneity of virus sequences, 10 HBV genotypes (A to J) have been defined by divergence in the entire HBV genomic sequences of >8%. Except for the newly identified genotypes I and J, the geographic and ethnic distributions of HBV genotypes and subtypes are well characterized. Genotype D is found worldwide whereas others are restricted to one continent like B and C in Asia, E in Africa, and F and H in the Americas [6].

Some studies have shown that, in certain populations where HBV is endemic, a higher variability of HBV might be expected [8]. HBV genotypes are not only interesting for anthropology and epidemiology but are also useful for clinical reasons. Genotypes C, D and F are on the average more pathogenic than the other genotypes and genotypes A and B respond better to an interferon therapy than genotypes C and D [9]. S gene, coding for surface antigen, is composed pre-S1, pre-S2, and S. S protein is a key viral antigen for binding to cell receptors and facilitating the virus entry [10]. HBV genotyping with the S gene sequence is, in general, consistent

with the genotyping of the full genomic sequence and therefore, HBV genotypes can be assigned based upon S-gene sequences [5, 11]. Sub-genotype classification, however may not be applicable to some HBV strains on the basis of the S region sequence alone [12, 13]. Accordingly, complete genome sequences are more reliable for the analysis of genotype and sub-genotype classification for HBV [13].

The one major antigenic determinant is called the "a" determinant and is located in the amino acid positions between 100 and 160. Mutations inducing a conformational change within the "a" determinant result in a protein with significant 3-dimensional changes in the antigenic epitope. These conformational changes result in HBsAg that is not detected by diagnostic assays and/or by vaccine induced immunity [14]. By getting the whole genome sequence of HBV, we can detect certain mutations which may affect the diagnostic detection, drug resistance, disease progression and effective vaccination.

This study focused on whole genome sequencing and mutation analysis of hepatitis B virus in Myanmar hepatitis B virus carrier patients.

MATERIALS AND METHODS

Samples

Fifteen blood samples were collected from HBsAg positive persons attending the Hepatitis B virus carrier clinic, Department of Medical Research. Hepatitis B carriers were defined as persons positive for Hepatitis B surface antigen (HBs Ag) for more than six months.

DNA extraction

HBV DNA from the plasma was extracted using QIAmp Blood Mini Kit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions.

Primers

Primer sets were designed in Primer3 to cover the whole genome of HBV. Primer sets used in this study were shown in Table-1, Table-2.

Table-1. Oligonucleotides Used in First Round PCR

Oligonucleotide Identity	Sequence, (5'-3')	Reference
HB8F	TTCACCTCTGCCTAATCATC	Sugauchi *et al.* (2001) [15]
HB6R	AACAGACCAATTTATGCCTA	Sugauchi *et al.* (2001) [15]

PCR Amplification and DNA sequencing

Approximately 3.2 kb length of whole genome of HBV was amplified using 10 sets of oligonucleotides. All oligonucleotides used in this study are listed in Table 1 and Table 2. A fragment of 3.2 kb was amplified in the first round PCR using sense HB8F and antisense HB6R. A second round of PCR using 9 sets of oligonucleotides (HBV1F-HBV1R, HBV2F-HBV2R, HBV3F-HBV3R, HBV4F-HBV4R, HBV5F-HBV5R, HBV6F-HBV6R, HBV7F-HBV7R, HBV8F-HBV8R, HBV9F-HBV9R) were performed on the 3.2 kb fragment to produce 9 overlapping fragments that contributed to full length whole genome sequence when aligned. All amplification reactions were carried out in a Veriti 96-well Thermal Cycler (Applied Biosystem, USA). The first round of PCR was undertaken for 35 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min) followed by an extension reaction at 72 °C for 5 min. The second round PCR was performed for 30 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) followed by extension at 72 °C for 5 min. First round PCR reaction was composed of 12.5 uLof 2x MiFi Mix, 1.0 uLof each oligonucleotides (10 uM), 5.5 uL sterile dH2O, and 5 uL of extracted HBV DNA. The second round PCR reaction was composed of the same reagent concentrations for each of 9 sets of oligonucleotides, except that only 2 uL of the first round PCR product was used as template.

The PCR products were purified by using Montage PCR filter plates [Merck Millipore MultiScreen PCR96] according to manufacturer's protocol. For cycle sequencing reactions, same PCR primer pairs and a Big Dye Terminator v3.1 Cycle Sequencing Kit was used. Purification of Cycle sequencing products was done by ethanol purification method. Sense and antisense strands were sequenced by ABI3500xL Genetic Analyzer at Department of Medical Research.

Table-2. Oligonucleotides Used in Second Round PCR

Oligonucleotide Identity	Sequence, (5'-3')
HBV1F	AGC ACA TTC CAC CAA GCT CT
HBV1R	GTC CCG TGC TGG TAG TTG AT
HBV2F	ATC CTG CTG CTA TGC CTC AT
HBV2R	CAA GGT ACC CCA ACT TCC AA
HBV3F	TAT TGG GGG CCA AGT CTG TA
HBV3R	AGA GGA GCC ACA AGG GTT C
HBV4F	TCT GAA CCT TTA CCC CGT TG
HBV4R	GTG CAG AGG TGA AGC GAA GT
HBV5F	GGA CTC TAC CGT CCC CTT CT
HBV5R	GAT CCC GAA TAG ACG GAA AG
HBV6F	TGT TCA AGC CTC CAA GCT GT
HBV6R	AGA TAG GGG CAT TTG GTG GT
HBV7F	CAT GGG CCT AAA AAT CAG ACA
HBV7R	TTT GGT ACG GTT AGG ATA GAA CC
HBV8F	TCC CTC TTT TCC TCA CAT TCA
HBV8R	TCC CAC TCC TAC CTG GTT TG
HBV9F	TTC TGT TCC CAA TCC TCT GG
HBV9R	GAT GCG GTG CTC TCC ATA TT

Ethical Statement

This study was considered as ethical because informed-consent were obtained after explaining the nature and detailed procedure of the study, blood samples were taken in a minimum amount under strict aseptic precautions. All samples were identified as code system, without using real personal identity. This study was ethically approved by Ethic Review Committee, Department of Medical Research, Ministry of Health and Sports. Ethical approval number is (3/ Ethics 2015).

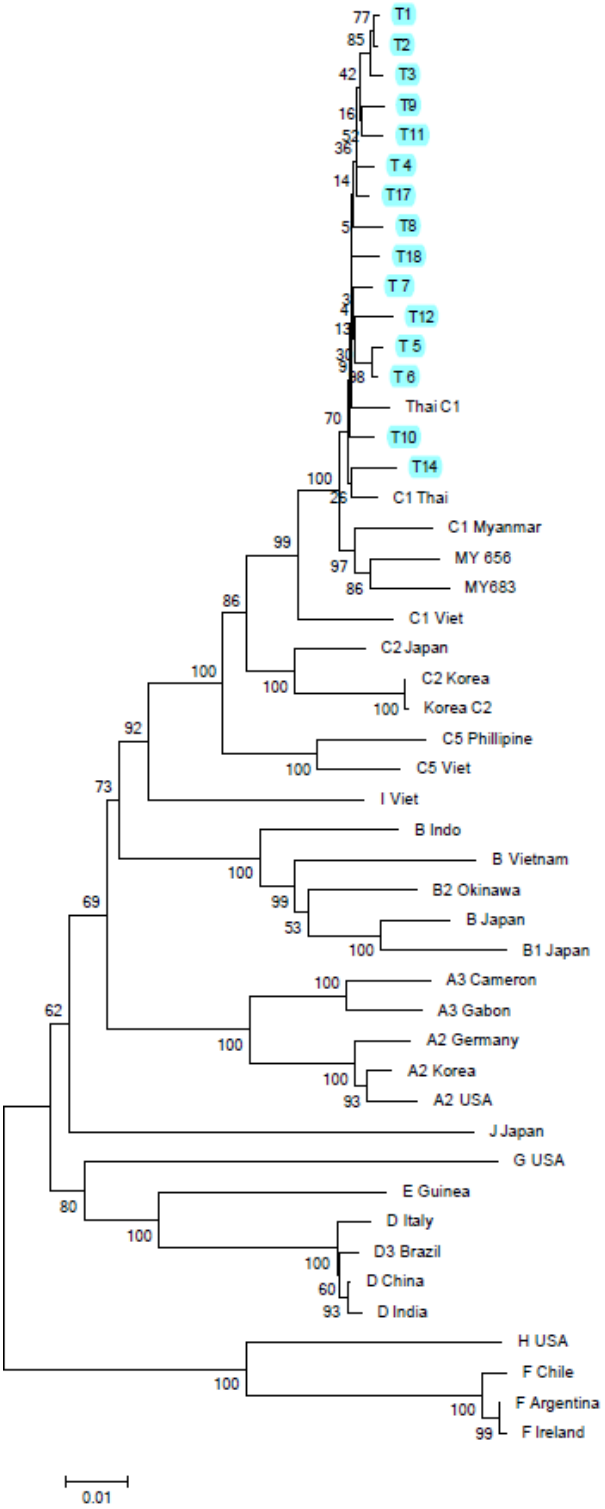

Fig. 1: Phylogenetic analysis of whole genome sequences

Data analysis

The obtained sequences for each isolate were assembled into complete whole genome sequences in DNA Star Lasergene Seqman Pro sequence analysis software [version 7.1.0 (44.1)]. Resulting sequences were multiple-aligned by using a built-in ClustalW implementation in Molecular Evolutionary Genetic Analysis (MEGA 6.06) software. Genetic distances were calculated using the Kimura two-parameter method and phylogenetic trees were constructed by the neighbor-joining method. To confirm the reliability of the pairwise comparison and phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. Phylogenetic and molecular evolutionary analyses were done using MEGA version 6.06 . All sequences were analyzed by HEPSEQ, International Repository for Hepatitis B Virus Strain Data for the presence of drug resistant mutations. All sequences were checked for vaccine escape mutation manually.

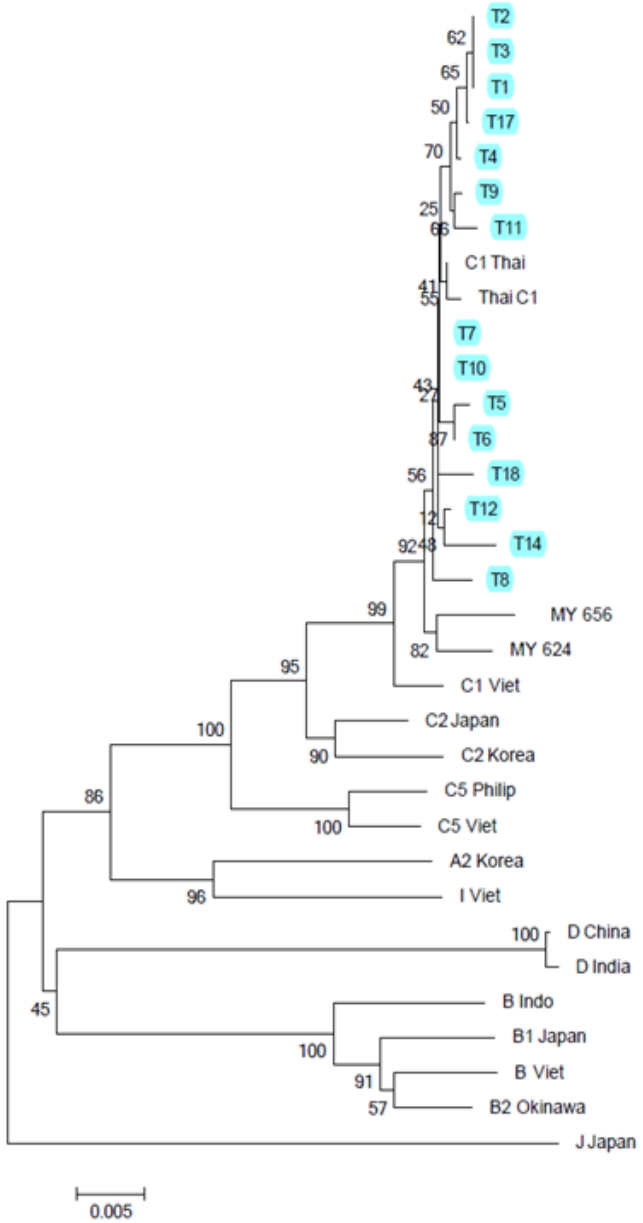

Fig. 2: Phylogenetic analysis of S-gene sequences

RESULTS AND FINDINGS

Nucleotide sequences and phylogenetic analysis

By complete genome sequencing of 15 HBV isolates (T1-12, T14, T17 and T18), we found out that 13 isolates were composed of 3215 base pairs while 3 isolates were composed of 3227 base pairs. All of 15 isolates were within same genotype and sub genotype because inter nucleotides variation was not more than 4% between each genome. Resulting all 15 genome sequences were submitted to

GenBank (Accession No. were KT307718-9, KT364718-21, KT987423-26, KU051423-27). By phylogenetic analysis of 48 whole genome sequences (18 Myanmar isolates consisting 15 from current study and 3 (C1 Myanmar, MY656 and My683) from previous study [7], 18 from other Asian countries, 3 from Europe, 3 from Africa, 3 from USA and 3 from Latin America countries), all 15 isolates from this study were found to be clustered within genotype C and sub type C1 (apart from 15 isolates other 33 sequences were obtained from database entries). Among the Myanmar isolates, three isolates from previous study (2003) [7] were clustered in one branch and 15 isolates from this study (2014) formed another branch. Among the C1 sub genotype, isolate from Vietnam is more close to C2 sub genotype group from East Asian countries. This may due to geographic closeness of Vietnam to East Asia. Since Thailand is neighboring country of Myanmar, isolates from Thailand were more similar to Myanmar isolates (Fig-1).

By phylogenetic analysis of 33 large S gene sequences from Asian countries, all 15 isolates from this study were found to be clustered within the same genotype C (Fig-2). There were 2 main branches among the C1 group. Vietnam isolate was in one branch and other C1 isolates were clustered in one branch (the same finding seen in whole genome tree). After phylogenetic analysis based on large S gene sequences genotype A2 isolate of Korea and genotype I isolate of Vietnam were clustered into same root. However in whole genome based phylogenetic analysis, these two isolates were found to be under different roots. Therefore whole genome based analysis is more reliable in genotype classification than S gene sequence alone.

Table.3: Genotype Variants of Polymerase gene among Myanmar isolates based on HepSeq consensus sequence

HBV Isolates	Genotype Variants
T1	N13R, S135A, N248H
T2	N13R, S135A, N248H
T3	N13R, S135A, N248H
T4	N13H
T5	N13H
T6	N13H
T7	N13H
T8	N13H, N121I
T9	N13R
T10	N13H
T11	N13R
T12	N13H
T14	N13H, T128A
T17	N13R,S135A
T18	N13R,S135A, N248H
MY 624	N13H, T38A, P64T, Q125H,D134E, L145V, L155W, S219A
MY 656	N13H, S57F, W58C, N139R
MY 683	N13R, N139H, L229S

HBV isolates for this study; T1-12, T14, T17 and T18
HBV isolates for previous study[7]; MY624, MY656 and MY683

Characterization of the HBsAg

All 15 isolates have Glycine amino acid at position 145, so there were no vaccine escape mutants (G145A) in these sequences. Figure-3 shows the alignment of amino acid sequence from "a" determinant of small HBsAg protein which is the major structural protein of the hepatitis B viral envelope. At amino acid position 117 former Myanmar isolate (2003) has Isoleucine (I) and all current 15 isolates (2014) have Serine (S). At amino acid position 147, former Myanmar isolate (2003) has Glycine (G) and all current 15 isolates (2014) have Cysteine (C) residue.

Characterization of Polymerase Gene

Drug resistant mutations were detected by HepSEQ, International Repository for Hepatitis B Virus Strain Data. Each polymerase gene sequences were analyzed and there were no drug resistant mutations in all 15 isolates. Table-3 shows genotype variants detected by HepSEQ among 18 Myanmar isolates. Most common variant site is amino acid position 13 where all Myanmar isolates have Arginine (R) or Histidine (H) wile consensus sequence has Asparagine (N). One isolate (MY624) from previous study has 8 variants while others have 4 in maximum.
(http://www.hpa-bioinformatics.org.uk/HepSEQ-Research/Public/Tool/genotype_tool.php)

DISCUSSION AND CONCLUSION

HBV genotypes are distributed geographically, but their virulence and pathogenicity differ in each location [16]. Genotyping of HBV is important for clarifying the route of infection and virulence of the virus. In fact, patients infected with genotype C have a more aggressive clinical phenotype than those with genotype B [17]. By means of phylogenetic analysis in the S-region from 33 isolates from Asia, we found that all 15 isolates from this study and 2 from previous Myanmar isolates were genotype C and subgenotype C1. All 18 isolates from Myanmar were in the same branch with Thailand C1 strains but this branch rooted with the Vietnam C1 genotype. This finding was also confirmed by phylogenetic analysis of whole genome sequences of 48 isolates worldwide including Asia isolates. Therefore C1 is still a common subgenotype for more than a decade especially in Myanmar.
After analysis by geno2 pheno online tool, there was no drug resistant mutation among 15 HBV isolates in this study. This analysis was not only directed for secondary or drug induced mutation, but also there may be primary resistance or transmission of drug resistant mutants among the patients [18].

The emergence of HBV S-gene mutants was first observed in 1988 in Italian vaccinated children's sera with the presence of both HBs antigen and anti-HBs antibodies. S-gene sequence revealed glycine (G) to arginine (A) substitution at position 145, within the a-determinant of S-gene, causing conformational changes that allow for the virus to escape the vaccine-induced response [19]. G145R is the most common mutation observed in the a-determinant (from aa 124 to aa 147) of HBsAg representing the marker of the escape mutant under immunological pressure (passive or active) and capable of determining false negative results with some assays for the detection of HBsAg [20]. When the sequences were analyzed in HEPSEQ Web based tool, there was no vaccine escape mutation.

We successfully sequenced the whole genome of 15 HBV isolates from Myanmar. Our findings support the previous studies which indicated that genotype C1 is the most

prevalent genotype in South-East Asia region especially in Myanmar, Thailand, Laos and Cambodia.

All samples from this study were collected from Yangon region and so we would like to suggest that future study should be done to determine the Nation-wide HBV genotype distribution pattern in Myanmar. This will be a huge support for the hepatitis disease sentinel surveillance program which has become one of the National Health priorities in Myanmar.

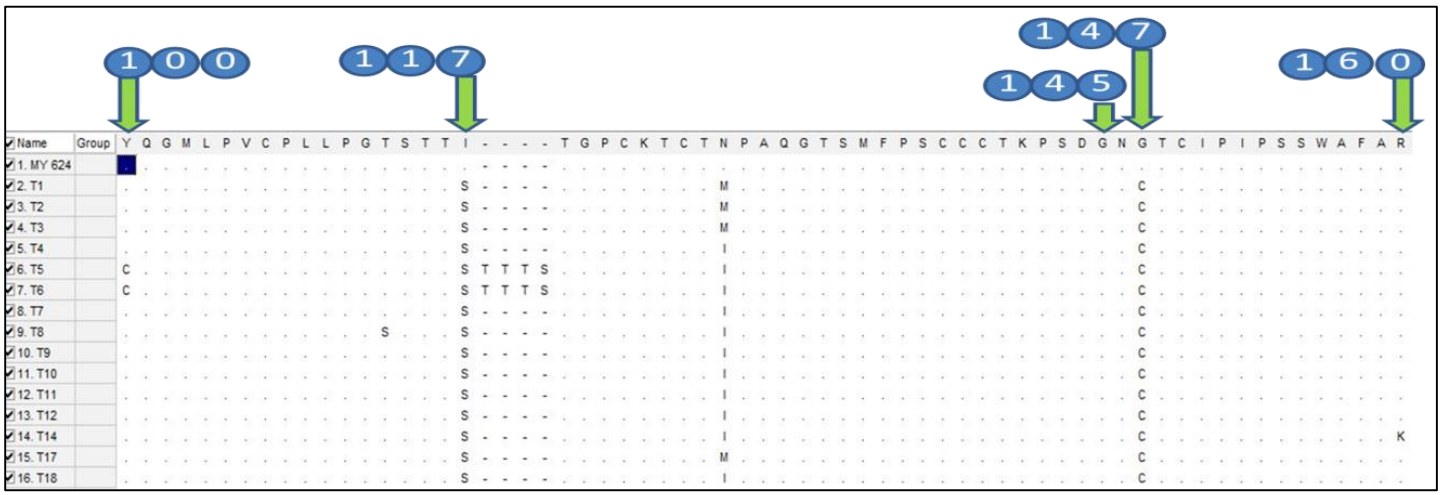

Fig. 3: Alignment of amino acid sequence from "a" determinant of small HBsAg protein

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CONFLICTS OF INTEREST

The authors have reported no conflict of interest.

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