

# Distribution of hepatitis B virus genotypes in general population of Myanmar via Nation wide study

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## Research article

**Keywords:** Hepatitis B virus, Genotype, Sub-genotype, Myanmar

**Posted Date:** October 12th, 2019

**DOI:** <https://doi.org/10.21203/rs.2.11004/v2>

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**Version of Record:** A version of this preprint was published on July 29th, 2020. See the published version at <https://doi.org/10.1186/s12879-020-05269-z>.

# Abstract

Background Hepatitis B virus (HBV) infection is a severe health concern worldwide. HBV is a DNA virus with a rapid rate of mutation. Based on the heterogeneity of the HBV nucleotide sequence, the HBV strains are divided into ten genotypes, A to J, with a characteristic geographical distribution. Identifying and tracking the changes of HBV genotypes is important in epidemiological and transmission studies, predicting the risk for the development of severe liver disease and response to antiviral treatment. The present study was conducted to detect HBV genotypes and sub-genotypes in general population of different states and regions in Myanmar. Methods A total of 5,547 general adult population who residing at seven states, seven regions and Nay Pyi Taw Union Territory were screened for Hepatitis B Surface antigen (HBsAg) by Immunochromatographic test (ICT) in 2015. Of 353 HBsAg positive samples, HBV DNA were detected by using polymerase chain reactions (PCR) targeting the DNA sequences encoding the Pre-S region. A total of 153 PCR positive samples were preceded for genotyping by partial genome sequencing of both directions. The resulting sequences were then edited, aligned and compared with reference sequences using National Centre for Biotechnology Information (NCBI) web based genotyping tool. Results Three HBV genotypes; HBV/ C, HBV/ D and HBV/ B were detected in Myanmar, in which genotype HBV/ C (66.7%) was the most prevalent genotype followed by HBV/ D (32%) and HBV/ B (1.3%) respectively. Sub-genotyping revealed a total of 7 sub-genotypes within genotypes B, C and D: two (B4 and B5) in HBV/ B, three (C1, C5 and C7) in HBV/C and two (D3 and D6) in HBV/ D. Conclusion Genotype HBV/C, sub-genotype C1 was the most predominant genotype distributed in all states and regions of Myanmar. This study was first report on Nation- wide distribution of HBV genotype and sub-genotypes in Myanmar and the findings will be a huge support for hepatitis disease surveillance programme which is the one of the National Priority Diseases in Myanmar.

## Background

Hepatitis B (HBV) is a viral infection that attacks the liver and can cause both acute and chronic disease. An estimated 257 million people are living with hepatitis B virus infection world-wide. In 2015, hepatitis B infection resulted in 887,000 deaths, mostly from complications including cirrhosis and hepatocellular carcinoma [1]. World Health Organization (WHO) estimated that the global prevalence of HBV infection in the general population was 3.5% in 2015. Among those born before the hepatitis B vaccine became available, the proportion of persons living with chronic HBV infection still remains high. Prevalence was the highest in the African (6.1%) and Western Pacific regions (6.2%) and followed by Asia [2]. Myanmar has a moderate to high endemicity of hepatitis B infection. According to the nation- wide seroprevalence survey in 2015, 6.5 % of general population was infected with viral hepatitis B. The prevalence was found to be varied with geographic variation with highest prevalence in Yangon Region (10%) and lowest in Kayah State (4.2%) [3].

The HBV genome comprises a 3.2 kilo base pairs, partially double-stranded DNA that replicates through RNA intermediate anti-genome sequence using its own encoded Reverse transcriptase (RT). HBV-RT does not have proof-reading function. Therefore, the error frequencies are occurred and these error-

prone conditions are similar to those of retroviruses and other RNA viruses [4]. During persistent, long-term HBV infection and under different selected pressures, variants of HBV can emerge. Some variants are able to evade diagnostics and prophylactic and therapeutic measures. The HBV genome encodes viral proteins through four open and partially overlapping reading frames: surface (preS /S), core (preC/C), polymerase (P) and X genes. PreC/ C gene encodes for e antigen (HBe Ag) and core protein (HBc Ag), P gene for polymerase ( reverse transcriptase) and S gene for surface protein [three forms of HBs Ag, small (S), middle (M) and larger (L)] and X gene for a transcriptional transactivator protein[5, 6].

HBV is grouped into many genotypes, according to genome sequence. Ten well-known genotypes (A-J) of the HBV genome have been defined. Some HBV genotypes are further classified as sub-genotypes. HBV sequence is characterized by more than 8% nucleotide differences for genotype, and 4%-8% nucleotide differences for sub-genotype. Over 30 related sub-genotypes belonging to HBV genotypes have been determined to date [7-8].

An earlier classification system divided the HBs Ag into four major serological subtypes, *adw*, *adr*, *ayw* and *ayr*. There is a correlation between HBs Ag subtypes and HBV genotypes. In general, HBV genotypes of A, B, F, G or H have HBs Ag subtype *adw*, those with genotype C have *adr*, and those with HBV/ D and HBV/E have *ayw* [7-8]. Genotype A and D have global distribution but genotype B and C are found predominantly in east and southeast Asia. Genotype E prevails in West Africa. The most divergent genotype F is found exclusively among indigenous peoples in central and south America. Genotype G, found in the USA and France, exhibits some unique molecular structures [6].

Myanmar is one of the most ethnically diverse countries and bordered by Bangladesh and India at the western border and China, Laos and Thailand at the eastern border, Thailand at the southern border and China at the northern border. The major genotype in China and Thailand is genotype C and Genotype D is the most dominant genotype in India [4, 11-14].

There were limited studies in Myanmar on HBV serotypes and genotypes. Previous studies in HBV serotypes and genotypes distribution in Myanmar was carried out but mainly on specific population. A study in 2012 reported the distribution of HBsAg subtypes among HBV carriers in Yangon as *adr* (93.2%), *adw* (4.85%) and *ayw* (1.94%) [12]. A hospital-based study showed prevailing HBV genotypes in chronic liver diseases patients was HBV/C followed by HBV/A and there were also mixed genotypes and untypable genotypes [13]. Sa-Nguanmoo et al., (2010) also found that HBV/C (97.5%), HBV/B and HBV/D (1.25% each) among Myanmar migrant workers [14]. Recently, Latt AZ and group reported on whole genome sequences of 15 isolates from Myanmar HBV carriers patients revealed that all are genotypes C with sub-genotypes C1[15].

The genotypes and certain sub-genotypes have distinct geographical distribution and are important in both clinical manifestation of infection and response to antiviral therapy. Moreover, HBV genotype/ sub-genotypes and genetic variability of HBV are also useful in epidemiological and transmission studies, tracing human migrations, in predicting the risk of development of severe liver diseases and response to antiviral therapy [16]. There is no large scale study on geographical distribution of HBV genotypes in

Myanmar and this is the first report on Nation-wide distribution of hepatitis B genotypes and sub-genotypes.

## **Methods**

### **Study Site and study population**

A cross sectional survey was conducted in 18 townships of all States and Regions of Myanmar from May to October, 2015. Eighteen townships were selected from 7 States (Kachin, Kayah, Kayin, Chin, Mon, Shan and Rakhine), 7 Regions (Bago, Sagaing, Magway, Ayeyarwady, Tanintharyi, Yangon and Mandalay) and Nay Pyi Taw Union Territory. A total of 5,547 subjects (aged between 15 to 80 years, both sexes) were participated in the survey.

### **Sampling procedure and recruitment**

Two-stage cluster sampling method was used and selection of primary sampling units (PSUs) was performed that one township was randomly selected, which is considered to have average level of viral hepatitis B infection in all States and Regions of Myanmar. Selection of secondary sampling units (SSUs) was performed that from each selected PSU (township), 10 wards and villages were selected according to probability to population size. From each selected SSU (ward/village), 30 households were selected using systematic random sampling. The sampling frame for this sampling is the list of households available from the Basic Health staff. One eligible participant aged between 15 to 80 years in the selected households was recruited by random sampling.

### **Screening of HBV infection and sample collection for genotyping study**

HBV screening was carried out at the field sites using SD Bioline HBsAg WB (Cat. No 01FK10W, Standard Diagnostic, Inc., Korea) according to manufacturer's instruction. HBs Ag positive subjects were invited for counseling about consequences of infections, treatment options and further testing for HBV genotyping. The field investigators explained the purpose and procedure of the study and informed consent was obtained from each subject before the 2 milliliter venous blood samples were taken. Sera were separated and transported to the Department of Medical Research for further genotyping testing. A total of 353 HBsAg positive subjects, 147 males and 206 females with the mean age of 35.5 years (SD=10.8) were included in this genotyping study.

### **Confirmation of HBs Ag positive serum samples**

HBs Ag positive serum samples by ICT (Immuno-chromatographic Test) were further confirmed by commercially available immunoassay kit, HBs Ag ELISA 3.0 assay (Cat. No 01EK10, Standard Diagnostic Test Kit, SD, Korea). The tests were performed according to the manufacturer's instruction.

### **Viral DNA extraction**

The ELISA confirmed HBs Ag positive were undergone viral DNA extraction which was performed with the QIAamp DNA Mini kit (Qiagen, Inc., Hilden, Germany), according to the manufacturer's instructions.

### **Amplification of pre-S gene of HBV by PCR**

The HBV PreS gene was amplified with nested PCR using PF-PR and NF-NR primers sets (PF 5' TTG GAC TCA CAA GGT GGG AA3'; PR 5' GTC CAC CAC GAG TCT AGA CTCT 3'; NF-5' TCA TTT TGT GGG TCA CCA TAT 3'; NR-5' CTG TAA CAC GAG CAG GGG T3'). Five microliter ( $\mu$ l) of extracted HBV DNA was amplified in a PCR mix including Tris HCL buffer, 2 mM Magnesium Chloride, 0.1 mM dNTPs, 2 units taq polymerase (Cosmo) and 0.25  $\mu$ M each of the primers. The PCR thermal cycling profile was; 5 minutes at 94°C then 30 cycles including 30 sec at 94°C, 30 sec at 51°C and, 45 sec at 72°C, and then 10 min at 72°C. Negative samples after first round PCR were amplified in nested PCR using second round primer set and a thermal profile like the first round but repeated for 35 cycles instead of 30 with annealing 54°C. After confirming by gel electrophoresis, the products were purified with SV column PCR purification kit (GeneAll Biotech, Korea) according to the manufacturer's instruction.

### **Determination of HBV Genotypes by direct sequencing of preS gene**

The purified PCR products were subjected to sequencing by chain termination method using commercially available Kit (Big Dye Terminator Cycle Sequencing Kit, Applied Biosystems). Briefly, 2  $\mu$ l of purified DNA was mixed with 1.85  $\mu$ l of 5x sequencing buffer, 0.25  $\mu$ l of Big dye terminator, 0.5 $\mu$ l of 0.125  $\mu$ M primer (Forward or Reverse) and 5.4 $\mu$ l of water. The thermal profile used was; 35 cycles of 60 sec at 96°C; 05 sec at 50°C and 3 min at 60°C. The 3500 XL Genetic Analyzer (Applied Biosystems) was used for Sanger sequencing method [17].

### **Determination of HBV genotypes and sub-genotypes**

HBV genotypes were determined using the sequences of pre-S/S genes with NCBI Web based HBV Genotyping Tool (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>)[18]. HBV DNA sequences were aligned with reference sequences using CLUSTAL method (MedAlign, Lasergene, DNASTAR Inc., Madison, WI) and manually edited the sequences with BioEdit Sequence Editor (version 7.2.5) and phylogenetic relationships were estimated by neighbor-joining method [19]. For determination of sub-genotype, the study sequences were aligned with published sequences representing all known HBV sub-genotypes. Multiple sequence alignment was performed using the built-in ClustalW integrated in MEGA 4.20 [20]. HBV sub-genotypes were determined by phylogenetic analysis in MegAlign software using the neighbour-joining method with a bootstrap test. The nucleotide sequences in this study have been deposited in the NCBI Gene Bank database. (Accession number: MH816993-995 and MH925817-925683)

### **Statistical analysis**

All statistical analysis was performed using with Statistical Program for Social Science Software (SPSS) 23.0 software for Windows (SPSS Inc., Chigago IL., USA). Comparison between categorical variables was

tested by chi-square test. A P-value (two tailed) of less than 0.05 was considered to be statistically significant. In this genotyping study, distribution of HBV genotypes were compared among 5 geographical areas as central, east, north, south and western part of Myanmar. Mandalay, Magway, Nay Pyi Taw, Bago, Ayeyarwady and Yangon regions were collectively described as central area, Shan and Kayah states as eastern area, Kachin state and Sagaing regions as northern area and Mon and Kayin states, Tanintharyi region as southern area and Rakhine and Chin states as western area. HBV sub-genotypes results were analyzed collectively.

## Results

### Distribution of HBV genotypes among the study population

All 353 HBs Ag positive serum samples were positive by HBV ELISA. Of confirmed 353 HBs Ag positive samples, 153 (43.3%) were PCR positive and proceeded for genotyping procedures. The above mentioned 153 PCR positive samples (69 males and 84 females), mean age  $34.0 \pm 11.54$  years were included for genotype analysis. Three major genotypes- C, D and B were found in this study population. HBV genotype C (n = 102; 66.7%) was found to be the predominant circulating genotype (p=0.002), followed by genotype D (n = 49; 32%) and B (n = 2, 1.3%) shown at table 1.

### Distribution of HBV genotypes in five geographical areas

Distribution of different HBV genotypes was described in different regions of Myanmar (Table 2, Figure 2). HBV genotype C was predominant in all areas except western area. Genotype B was found in two areas (north and central area) with only 1.3 percent of HBV isolates. Genotype D was major genotype (59%) for western part of Myanmar which is bordered with India and Bangladesh. Among the 35 subjects from the eastern area, HBV genotype C was predominant, identified in 26 (74.3%) of subjects. Differential genotype distributions are seen on western and eastern areas of Myanmar.

### HBV sub-genotypes in Myanmar

Among the 102 genotype C, the distribution of sub-genotypes were HBV/C1 (90.2%) followed by HBV/ C5 (5.9%) and HBV/ C7 (3.9 %). A total of 49 genotype D samples, majority of the samples, 45 (91.8 %) were clustered into the sub-genotype HBV/D3 and (8.2%) was sub-genotype HBV/D6. Only two HBV isolates were genotype B in our study population which belongs to sub-genotype B4 and B5 in each isolate (Table 2, Figure 2).

Genotyping of 153 HBV isolates (Accession number: MH816993-995 and MH925817-925683) were determined by constructing phylogenetic tree (Fig.3). The test sequences were grouped with reference sequences (additional file 1) according to their genotypes and sub-genotype. The genotypes of these sequences were also determined by NCBI genotyping tool which gave complete fidelity findings with the phylogenetic results.

Genotype D study sequences were clustered into sub-genotype D3 by reference sequences of genotype HBV/ D sub-genotype D1 to D8 retrieved from GenBank data base together with genotype D study sequences to construct phylogenetic tree by neighbor joining

Method using the maximum composite likelihood method to calculate evolutionary distance (Fig.4) and Genotype C sequences were clustered into sub-genotype C1, C5 and C7 by sub-genotype reference sequences (Fig.5).

## Discussion

The genotyping of HBV is important for clarifying the route of infection with and virulence of the virus. In particular, examination of sequence diversity among different isolates of the virus is important since variants may differ in their patterns of serological reactivity, replication of the virus, activity of liver disease, prognosis and response to treatment.

A total of 353 subjects with hepatitis B infection from general population of Myanmar were enrolled in this study. Previously, there is no information regarding the regional prevalence of HBV genotypes from Myanmar. A multi-country study on chronic liver diseases patients, the most common genotype identified in Myanmar was type C [21]. The recent study findings, the major genotype was C which is in accordance with previous HBV studies in Myanmar.

HBV genotypes have been shown to have a distinct geographic distribution. The predominant genotypes reported from Southeast Asian countries are genotype HBV/ C from Thailand, genotypes HBV/C and HBV/ B from Indonesia [28]. In China, HBV/C and HBV/ B were found to be predominant among the Negrito and Mongoloid tribes of these islands. Genotype HBV/A and HBV/ D were the most prevalent genotypes in India [29].

In this study, genotype HBV/D is predominant genotype in western area of Myanmar and type C mostly found in Eastern border area bordered with China and Thailand as well as central and southern area. It's indicated that genotype C is major genotype of Mongoloid tribes and might be spread of root of infection.

Moreover, Paraskevis et. al [22] reported that genotype C is the oldest HBV genotypes and it has highest numbers of sub-genotypes, C1-C16[23-24], reflecting the long duration of its endemicity in humans. In this study, a few numbers of sub-genotypes circulate in different parts of Myanmar but majority was sub-genotype C1. It was quite similar sub-genotypes found in United States- bound refugees from Myanmar and adult immigrant in Australia from Myanmar [25-26].

Regarding the sub-genotypes of HBV/ C is at least two subtypes in Asia and HBV/C1 was found only in Southeast Asia including Vietnam, Myanmar and Thailand, while HBV/C2 was found in east Asia including Japan, Korea and China. In this study, most of the Genotype C was found to be sub-genotype C1 which was equally distributed all geographical areas of Myanmar. Some strains from study subjects showed sub-genotypes C5 and C7 which was in few percentage of study population and found mainly on

central area of Myanmar. Moreover, this result was quite similar to the previous study on 15 isolates from hepatitis carriers which showed that all the HBV isolates were sub-genotypes C1 [15] and it was seemed to be present in quite long time in Myanmar [21]. Presence of multiple sub-genotypes HBV/C indicated that HBV has been spread for a very long duration in Myanmar.

According to recent system and comparative analysis of sub-genotypes D, at least six (D1- D6) can be classified. Among the genotype HBV/D, the sub-genotypes HBV/ D3 was mostly found in this study and it was followed by D6 (Table.1). This is the first report on second most prevalence of genotype HBV/D, sub-genotypes D3 in Myanmar because few percentages of genotype D were reported previously on clinical case study [13]. However, the prevalence of genotype D was higher than previous findings and it might be due to frequent travelling of people from one country to another and migration. In addition, there is no large scale study of HBV genotypes in Myanmar with sequencing method for reliable data and it might be also associated with regional variation. Genotype D was mostly found in western area of Myanmar which was quite near to India and Bangladesh in which Genotype D is more prevalence [27, 28].

In this study, a relatively lower portion (1.3%) of study population is found genotype HBV/B, sub-genotype HBV/B4 and HBV/B5. The previous study in Yangon region also showed that the absence of B genotype was found in their study population [13] and discrepant findings with adult immigrants study in Australia where there was shown 10.5% of adult immigrant from Myanmar was characterized as genotype B[26]. This study showed only 1.3% of study population was found to be HBV/B genotype which indicated that low prevalence of this genotype was circulating in the country.

Geographic distribution of HBV genotypes may be related to route of exposure. For example, genotypes B and C are more common in high-endemic regions of perinatal or vertical exposure, which plays an important role in viral transmission. Other genotypes are primarily observed in regions of horizontal exposure [9-11,29]. Therefore, genotyping provides an epidemiological clue in the investigation of acquisition and the geographical distribution of HBV [9-11,29]. In this study, genotype C is predominant in most regions of Myanmar and vertical transmission is seemed to be the main mode of transmission. As there is no documented study on transmission pattern of HBV in Myanmar, further study will be needed to be clarified.

In an era of frequent international travel and human migration, introduction of new HBV genotype to a community might have far reaching effects, including recombination between genotypes [30] or replacement of one genotype by another [31]. HBV genotype C is associated with delayed hepatitis B e antigen (HBeAg) seroconversion [32], more-active hepatitis[33], lower response to antiviral therapy[31], more advanced liver disease and a higher risk of hepatocellular carcinoma[33], compared with HBV genotype B.

In addition, 102/153 (66.7%) of study population are genotype C isolates. Thus, the patients infected with genotype C are needed to be carefully monitored to assess their clinical outcome in future. In the case of the genotype C, in particular C1, for example, an increased tendency for the development of cirrhosis and hepatocellular carcinoma (HCC) has been found for patients of over 50 years of age [37,38]. For the

genotype B, higher rates of seroconversion of HBeAg and HCC have been found in young patients [37, 38].

## Conclusions

Genotype HBV/C, sub-genotype C1 is most predominant type in Myanmar, distributed all part of states and regions and Genotype HBV/D (sub-genotype D3 and D6) is predominant type in Myanmar–India border. This study provides information on geographical distribution of viral hepatitis B genotypes in Myanmar and can contribute to Hepatitis B control measures in Myanmar.

## Abbreviations

HBV: Hepatitis B Virus; HBsAg: Hepatitis B Surface antigen; ICT: Immunochromatographic test; PCR: Polymerase chain reactions; NCBI: National Centre for Biotechnology Information; WHO: World Health Organization; PSUs: primary sampling units; SSUs: Secondary sampling units; SPSS: Statistical Program for Social Science; HBe Ag: Hepatitis B e antigen HCC: Hepatocellular carcinoma

## Declarations

### Ethics approval and consent to participate

The ethics approval and consent to this study was approved by Ethics Review Committee of Department of Medical Research. The approval number is 22/ Ethics 2015, dated 25.3.2015

**Consent for Publication-** Not relevant

### Availability of data and materials

The partial sequences of the 153 HBV isolates were submitted to Gene Bank. The accession numbers of this study isolates were MH816993-995 and MH925817-925863. Original data may be obtained by email to corresponding author.

### Competing interests

The authors declare that they have no competing interests.

### Funding

The study was funded by Pusan National University. There was no role of funding body in design of the study, data collection and analysis.

### Authors' contributions:

YYK-sample collection, proposal writing, study design, study supervision, performing sequencing work, data compilation and analysis and manuscript writing. AAL and MMT participate in proposal writing, sample collection. HOMS, sample collection and performing sequencing work. KSA, proposal writing, study design, study supervision. HMT, proposal writing, study design, study supervision. KZT, proposal writing, study design, study supervision. KTA- performing sequencing. JHC- study supervision, manuscript writing and review. All authors read and approved the final manuscript

## ACKNOWLEDGMENTS

The authors would like to thank Korea International Co-operation Agency (KOICA) and National Research Foundation of Korea for financial support and Clinton Health Care Foundation for permission to continue molecular epidemiology study from Nation- wide sero-survey. The authors also would like to thanks Dr. Wah Wah Aung for editing the manuscript and Dr. Myat Htut Nyunt for helping sequence analysis.

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## Tables

**Table1. Characteristics of subjects and Genotype and sub-genotypes distribution**

Characteristics	Genotype B	Genotype C	Genotype D	Significant
Number of subjects (%)	2	102	49	
n=153 (100%)	(1.3%)	(66.7%)	(32%)	
Age (Mean± SD)	41.5±9.12	33.84±11.72	33.88±10.66	NS <sup>a</sup>
34.0±11.4 Yr				
Gender ( M/ F)	2/0	45/57	22/27	NS <sup>b</sup>
(69/84)				
Sub-genotypes pattern	2	3	2	
	B4,B5	C1,C5, C7	D3, D6	

<sup>a</sup>One way analysis of variance, <sup>b</sup> Pearson Chi Square Test, NS for non significant

**Table2. Area- wise distribution of HBV genotypes in Myanmar**

HBV Genotypes	Total Subjects	Area				
		Southern Area (Mon, Tanintharyi, Kayin states)	Western Area (Chin & Rakhine states)	Eastern Area (Shan & Kaya States)	Northern Area (Kachin states & Sagaing region)	Central Area (Mandalay, Magway, Nay Pyi Taw, Ayeyarwaddi, Bago, Yangon)
		(n=29), n%	(n=17), n%	(n=35), n%	(n=12), n%	(n=60), n%
<b>C</b>	102	21 (72.4%)	7 (41.2%)	26 (74.3%)	11 (91.7%)	37 (61.7%)
<b>D</b>	49	8 (27.6%)	10 (58.8%)	9 (25.7%)	0	22 (36.7%)
<b>B</b>	2	0	0	0	1 (8.3%)	1 (1.6%)
	153		NS			

## Figures

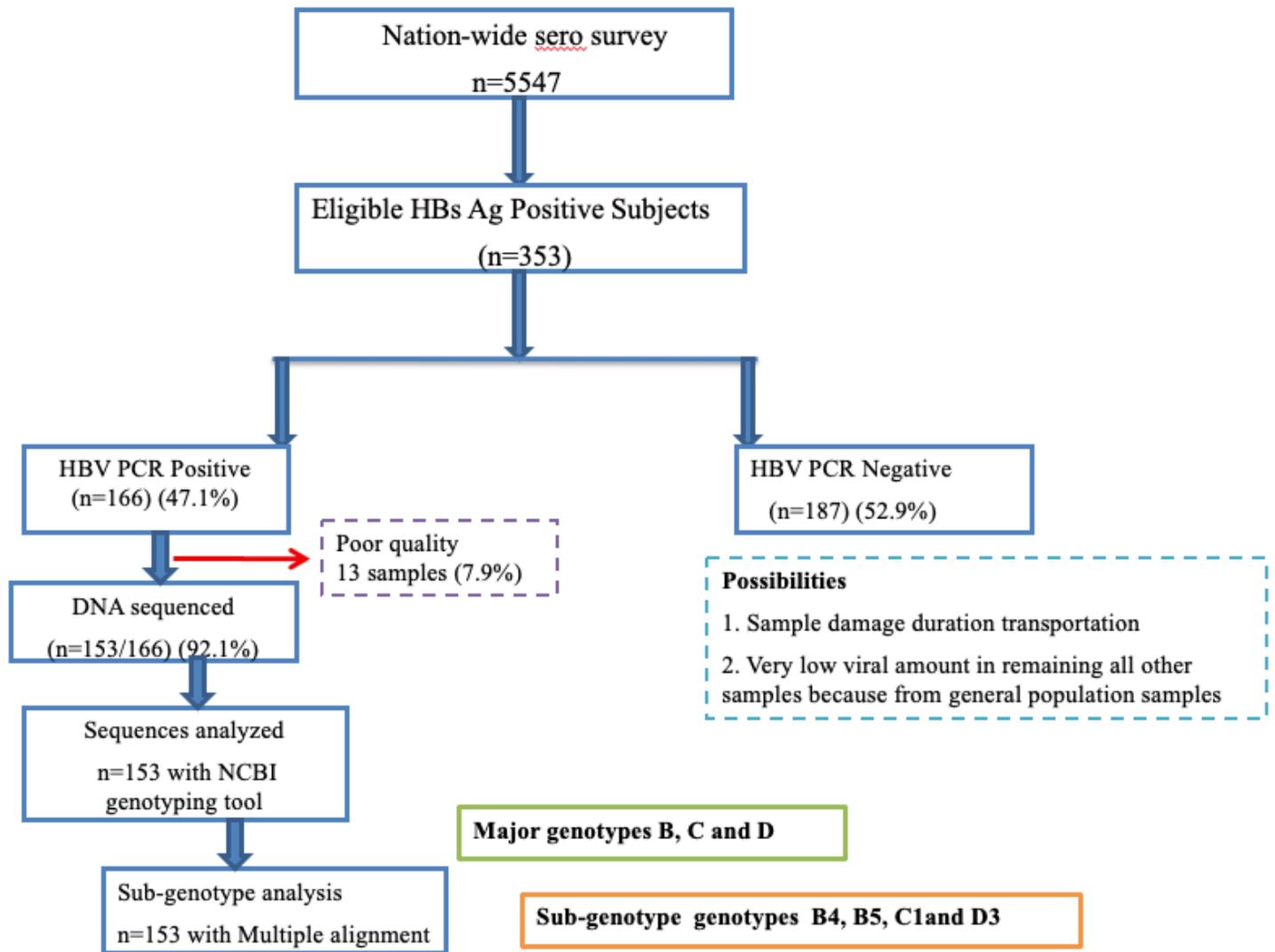
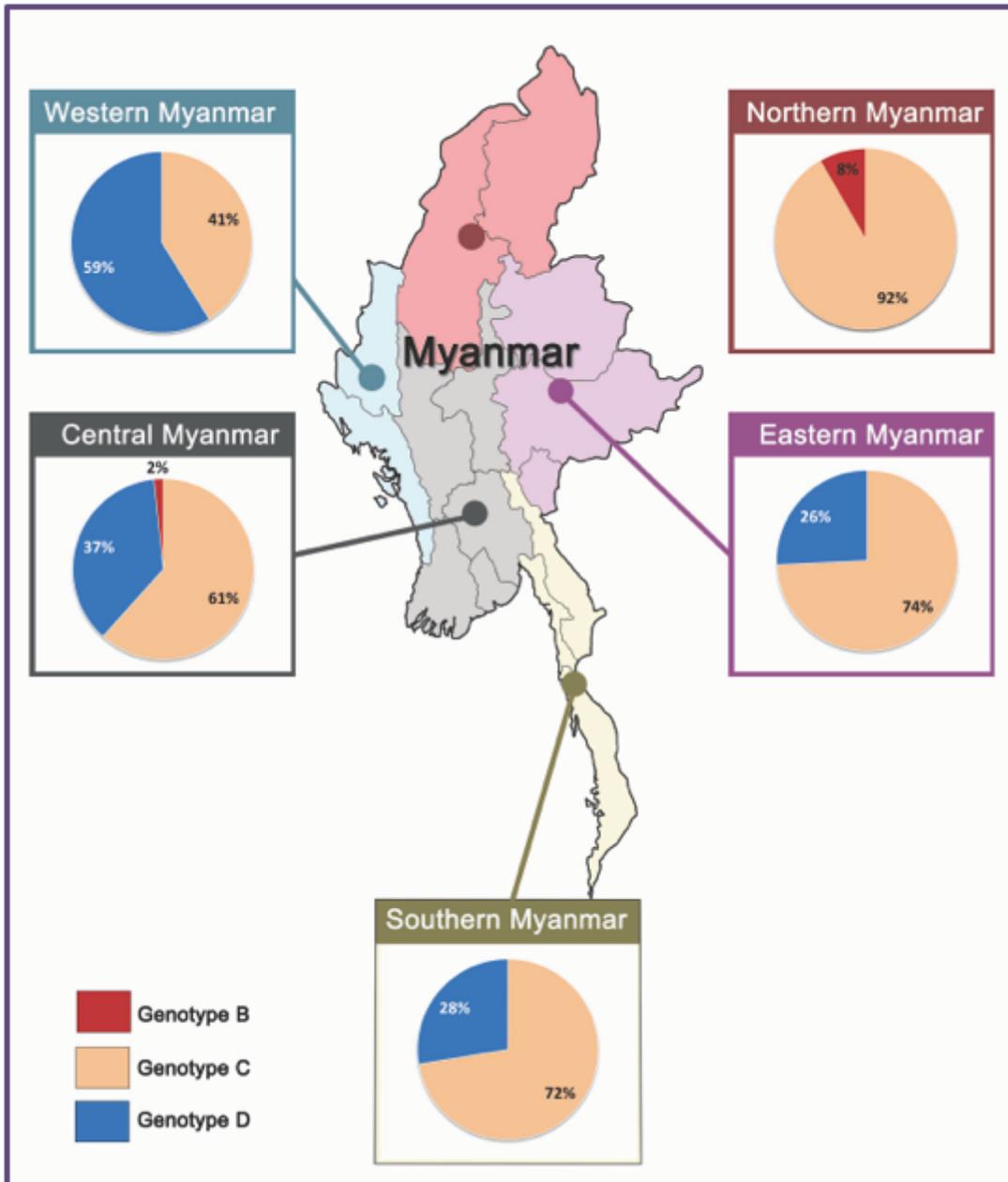


Figure 1

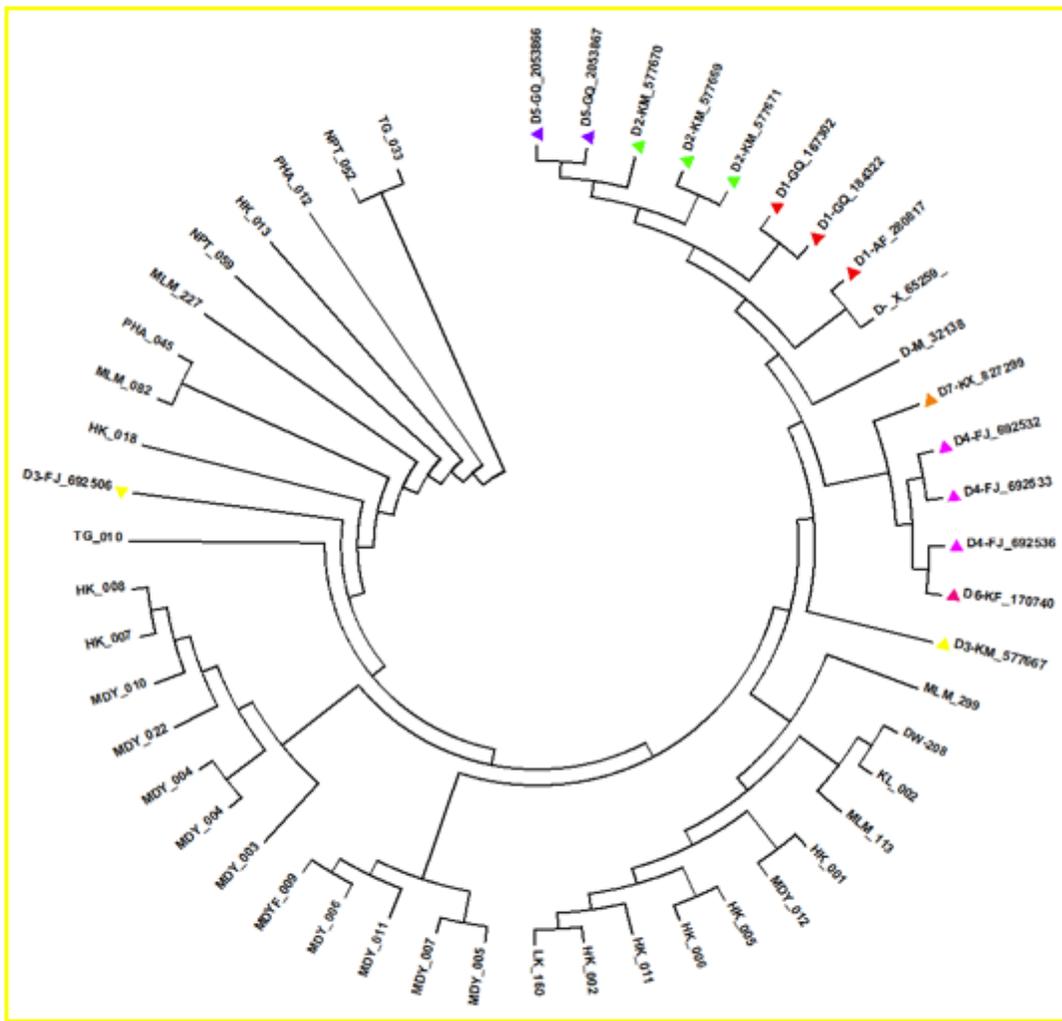
Flow Chart Diagram of HBV genotyping study



**Figure 2**

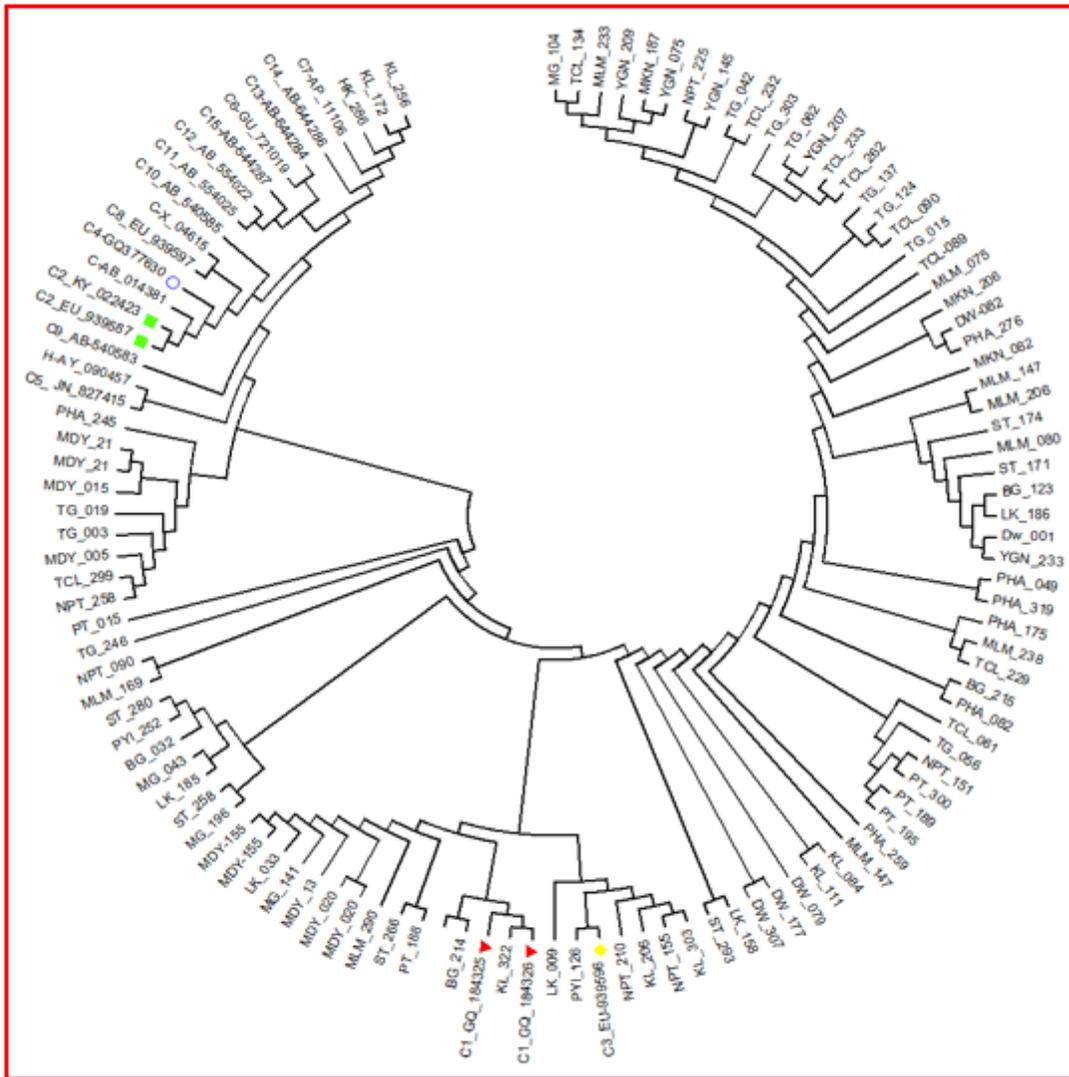
HBV genotype distribution in five geographical parts of Myanmar. The map that was described in figure 2 was drawn by ourselves, own creation.





**Figure 4**

Phylogenetic determination of HBV sub-genotypes of genotypes D. Phylogenetic tree was constructed using 578 bp nucleotide sequences (2860-222) PreS1/ PreS2 region was constructed in MEGA6.0 using neighbour-joining method with bootstrap test of 1000 replicates and maximum composite likelihood model. Genbank reference sequences are shown by HBV subgenotype and accession number. Study sequences were designed by study number.



**Figure 5**

Phylogenetic determination of HBV sub-genotypes of genotypes C. Phylogenetic tree was constructed using 578 bp nucleotide sequences (2860-222) PreS1/ PreS2 region was constructed in MEGA6.0 using neighbour-joining method with bootstrap test of 1000 replicates and maximum composite likelihood model. Genbank reference sequences are shown by HBV sub-genotype and accession number. Study sequences were designed by study number.