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ORIGINAL ARTICLE

Serological markers and molecular analysis of hepatitis B infection in a tertiary care hospital at Kathmandu, Nepal

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Abstract

Aim To analyze the serology and molecular markers of the hepatitis B-infected patients from the tertiary care hospital at Kathmandu in Nepal.

Methods A total of 399 blood samples of patients from Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu, were collected. Samples were tested for HBsAg, HBeAg, and IgM anti-HBc using ELISA method. The samples were further categorized as acute and chronic. The genotyping was performed by real-time polymerase chain reaction (real-time PCR) and further validated by sequencing.

Results Out of 399 samples that were collected, 271 and 128 samples were acute and chronic cases respectively. Fifty-six samples were genotyped by qPCR, out of which 40 samples belonged to genotype D, 4 to C/D recombinant, 5 to genotype C, 3 to genotype B, and 4 were genotype A respectively. From these, 15 samples were used for sequencing of P (polymerase) gene and S (surface) genes. Thus, obtained sequences were used to construct neighbor-joining tree using Tamura-Nei model evolution and further validated by Bayesian analysis. A total of four sub-genotypes namely A1, C1, D1, and D5 were detected.

Conclusion Hepatitis B virus infection is a global health problem affecting about 257 million people worldwide. In Nepal, there are few reports on the molecular and phylogenetic analysis of this virus. In this study, we report the circulation of seropositive occult hepatitis as well as CD-recombinant genotype in Nepalese population.

Keywords Chronic hepatitis · Cirrhosis · Genotype · Hepatitis B virus · Hepatocellular cancer · HBV vaccine · Nepal · Occult hepatitis · Portal hypertension · Viral hepatitis

Introduction

Viral hepatitis has emerged as one of the major causes of mortality due to communicable diseases [1]. Worldwide, 257 million people are affected and more than a million deaths have been attributed to cirrhosis, liver failure, and hepatocellular carcinoma (HCC) [2, 3]. Among the responsible pathogens, hepatitis B virus (HBV), a double-stranded DNA virus belonging to *Hepadnaviridae* family, is considered to be one of the main causative agents for liver injury. In conjunction with hepatitis C virus, it accounts for over 90% of the related disability-adjusted life years (DALYs) [4]. According to World Health Organization (WHO), acute hepatitis may be defined as new onset of hepatitis B infection that may or may not be symptomatic. The diagnosis is based on the detection of hepatitis B surface antigen (HBsAg) and IgM antibodies to hepatitis core antigen (anti-HBc). However, chronic hepatitis B may be defined as the presence of HBsAg for

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Bullet points of the study highlights

What is already known?

- The diagnosis of hepatitis B infection is based on the detection of surface antigen (HBsAg), envelope antigen (HBeAg) and IgM antibodies to hepatitis core antigen (anti-HBc).
- Occult hepatitis is considered as an important factor which might lead to hepatocellular carcinoma.
- Nepalese government still struggles to make the vaccine accessible making the treatment and prevention of the disease more difficult.

What is new in this study?

- Our study showed prevalence of genotype D, A and also presence of C and D recombinant strains.
- Our study shows the presence of seropositive occult cases in the tertiary care hospital of Nepal.
- Our study showed the highest reports of the infection from province 2, Nepal.

What are the future clinical and research implications of the study findings?

- The findings of the research could help in viral hepatitis mapping in Nepal.
- The data could also be used for formulating government policies for appropriate vaccination coverage.

6 months or more. Similarly occult hepatitis is defined as HBsAg negative but HBV DNA positive, although at very low levels (invariably < 200 IU/mL); most are also anti-HBc positive [5]. The prevalence of occult hepatitis in a population may further complicate the management of hepatitis B infection as it is considered a risk factor for the development of HCC and of reactivation of the infection [6]. The screening of occult hepatitis is also clinically important for blood donors and for organ transfer [7]. Besides this, the differences in viral genotypes may lead to different clinical outcomes of this disease [8, 9]. Various observations from previous studies have reported that different HBV genotypes induce distinct behaviors in terms of clinical and behavioral parameters [10–12].

Nepal is a landlocked country with Himalayan region in the north bordering China and Terai region in the south bordering India. The country has committed to the sustainable development goals (SDG), one of which being the elimination of the epidemics including that of viral hepatitis. But with no national plans for the eradication of this disease, the government is still struggling to make the vaccine accessible; under such situation, the treatment and prevention of the disease more difficult [13, 14]. Besides this, the study of hepatitis B markers in hospital patients has not been adequately studied. However, there are data available regarding the prevalence of this infection in high-risk group such as people who inject drugs, patients having jaundice and HCC, and among sex-workers [15–17].

Sukraraj Infectious and Tropical Disease Hospital is one of the oldest and largest national referral hospitals in Kathmandu, which receives cases not only from the valley but also from all over Nepal. Although hospital patients are not the ideal population for epidemiological studies and might overestimate the condition, such information can contribute in the knowledge regarding mapping of viral hepatitis in Nepal. The objective of our study was to investigate the prevalence of serological markers and to further genotype, sub-genotype and phylogenetically analyze such samples in an attempt to reflect the situation of viral hepatitis in Nepal.

Methods

Collection of human serum

The sera samples from 399 individuals were collected randomly from patients visiting Sukraraj Tropical Infectious Disease Hospital, Kathmandu, from January to August 2018. Informed consent was taken from all the patients, and the ethical approval was taken from the Nepal Health Research Council, Nepal (Reference number: 138/2018). Each sample was coded and transported to the research laboratory at Central Department of Biotechnology, Tribhuvan University, Kirtipur. Upon arrival, processing of the sera samples for different tests was done.

Demographic information and serological tests

A total of 399 sera samples were collected. Contributing information on hepatitis B infection such as gender, age, and permanent and current address as well as travel history of the

patients were collected through a questionnaire. HBsAg, HBeAg, and anti-HBcAb were detected by enzyme-linked immunosorbant assay (ELISA) according to the manufacturer's instructions (Autobio Diagnostics Co. Ltd., Zhengzhou, China). The details are provided in the supplementary file.

Biochemical markers

The biochemical profiles for alanine aminotransferase (ALT) and total bilirubin of the seropositive samples were recorded from the available data.

HBV DNA extraction

Out of 399 samples, HBV DNA was extracted randomly from 56 serum samples that were tested positive by immunoassay method. The various categories obtained were HBs Ag+only, HBs+/HBeAg+, HBsAg+/HBeAg+/IgMAnti Hbc+ and HBsAg-/IgManti HBc+ve. DNA extraction was done according to the manufacturer's instructions (QIAamp DNA mini kit, QIAGEN, Hilden, Germany).The details are provided in the supplementary file.

Viral load detection and genotyping by qPCR

Real-time PCR for determination of viral load and genotyping was performed according to manufacturer instructions. Viral load was estimated by real-time polymerase chain reaction (real-time PCR) PCR (Corbett Research RG6000 PCR, Sydney, Australia) using Step one Real time PCR kit (Arths^{R} HBV RG PCR Kit Handbook (catalog no. 4506265), Qiagen, Hilden, Germany). Viral load detection and genotyping by qPCR details have been shown in the supplementary file.

Sequencing

The study included the seropositive occult cases with hepatitis B based on serology, biochemistry, viral load, and genotyping. Fifteen samples which were HBsAg -/IgM anti-Hbc + with a viral load of less than 200 IU/mL (1000 copies/mL) but ALT level below 40 were selected and sent for sequencing in India. Sequencing was performed at Chromous Biotech Pvt. Limited, Yehalanka Bangalore, India. Sequence and phylogenetic tree analysis methods have been described in the supplementary file.

Statistical analysis for demography and serology

This was done according to IBM Statistical Package for Social Science (SPSS) software version 13.0 (IBM, Armonk, New York, US), and ArcGIS version 10.5 (Esri, Redlands, CA, US).

Results

Demographic analysis

The study included 399 patients who visited Sukraraj Tropical Infectious Disease Hospital from January to August 2018. Based on the information regarding the location, the province-wise distribution of the patients is shown in Fig. 1.

All sera tested for HBsAg, HBeAg, and anti-HBc for HBV infection showed the males 261 (65.6%) to be higher than females 138 (34.6%). Among different age groups, the highest positive cases were from 19 to 28 years of age (31.3%) followed by 29 to 38 year (27.8%). Out of 399 cases, 271 were acute and 128 were chronic cases.

Serological analysis

The samples tested for HBV infection based on HBsAg positive were found in 88.97% ($n = 355$) while the HBeAg+/ HBsAg+ was found in 31.58% ($n = 126$). Besides this, 18.05% ($n = 72$) were HBs+/HBeAg+/IgManti-HBc+. However, 9.02% ($n = 36$) were HbsAg negative but anti-Hbc positive.

DNA extraction, viral load, and genotyping

Among the 399 samples, all 56 randomly tested for HBV DNA by qPCR were positive. The viral loads ranged from 1.1 IU/mL to 4×10^9 IU/mL. Out of 56 samples, 41 had viral load more than 10 million IU and 15 had a viral load of less than 10 IU/mL. Forty samples belonged to genotype D, 4 to C/D recombinant, 5 to genotype C, 3 to genotype B, and 4 genotype A.

Sequencing

Sequencing of polymerase (P) and surface regions (S) of the seropositive occult cases confirmed four sub-genotypes namely A1, C1, D1, and D5 (Table 1). However, three (8815, 36318, 36372) of them turned out to be CD recombinant genotype. The best nucleotide identity match with the database ranged from 89.7% to 100%.

Phylogenetic analysis

Phylogenetic tree was constructed using sequences obtained in this study along with sequences obtained from GenBank. Initial neighbor-joining tree was constructed, under Tamura-Nei evolution model, validated by bootstrap method ($n = 1000$) [18]. All the genotypes of HBV from this study clustered together as expected. CD recombinant clustered with genotype C indicating it had backbone of genotype C with recombinant segment from genotype D. Sequences of different isolates from this study

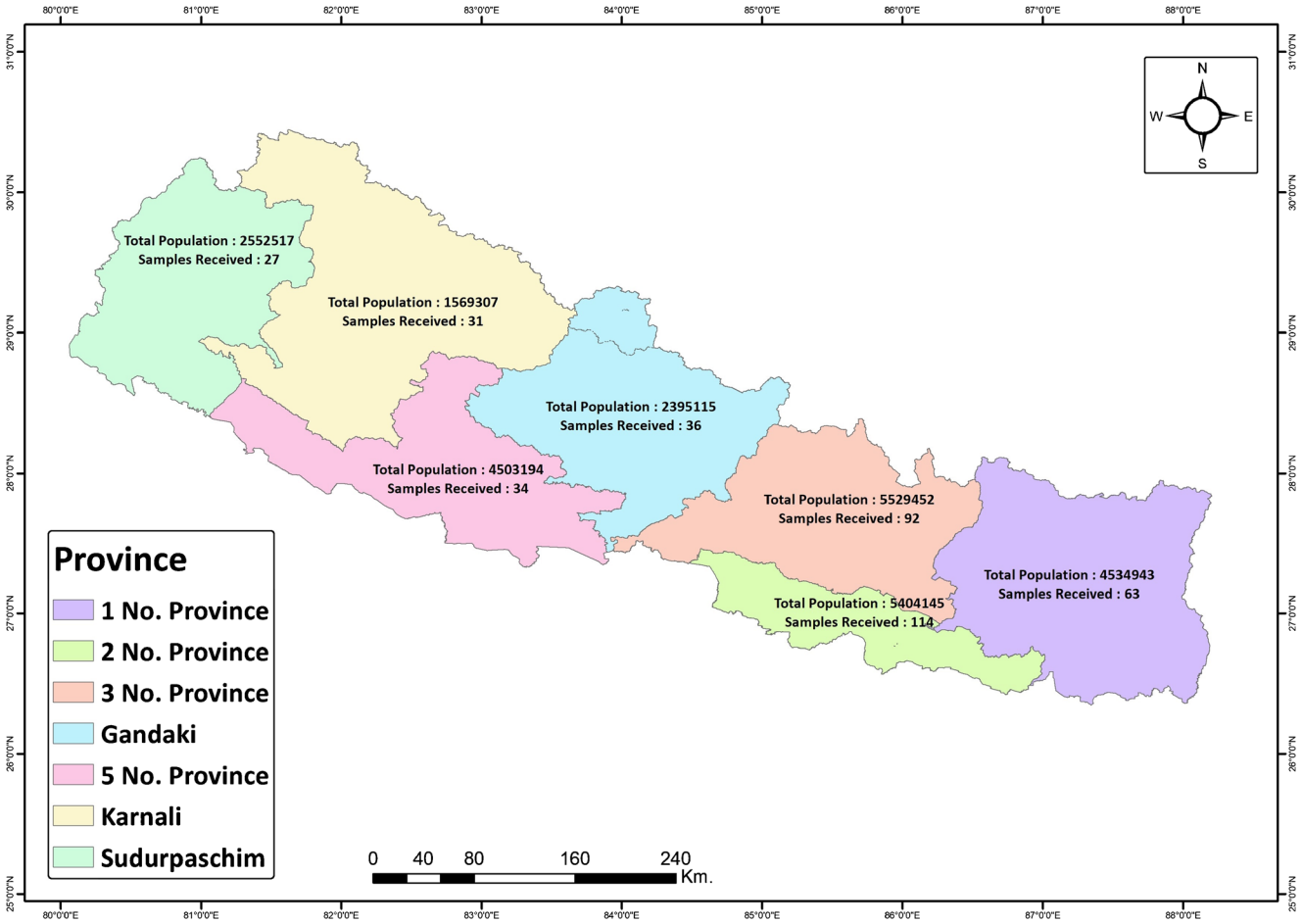

Fig. 1 Province-wise total population and the received sample population

Table 1 Genotype as determined by sequencing and using online tools (https://hbvdb.ibcp.fr/)

S. No.	Sample ID	Genotype	Sub-genotype	Accession No.
1	36572	A	A1	MK138689
2	36662	A	A1	MK138690
3	8436	A	A1	MK138688
4	8998	A	A1	MK138687
5	33290	C	C1	MK138691
6	440	C	C1	MK138692
7	1012	D	D1	MK138682
8	429	D	D1	MK138681
9	32970	D	D4/D5	MK138685
10	33666	D	D4/D5	MK138684
11	5461	D	D4/D5	MK138686
12	7426	D	D4/D5	MK138683
13	8815	CD	CD	MK138695
14	36318	CD	CD	MK138693
15	36372	CD	CD	MK138694

seemed to cluster together, indicating same origin for all those isolates.

Further validation of phylogenetic tree was performed by constructing tree using Bayesian tree inference method with MrBayes v3.2.2 (http://nbisweden.github.io/MrBayes/). Rooted tree was constructed under GTR+I+ substitution model with nst = 6 and uniform starting rate of substitution after 1 million generation and two simultaneous runs. Sampling of the tree was performed after every 1000 generation, and all the trees were summarized under 25% burning (number of trees = 3602). There appears to be very distinct population of genotype D in Nepal, indicated by very distinct clade in the tree. The clade containing genotype D appears to be much originated long time back indicated by branch length (branch time) (Figs. 2 and 3). Further, genotype C (C1) and genotype CD also formed their own separate clade and are close to Southeast Asian countries (China, Japan, and Myanmar). That indicates probable origin of genotype C circulating in Nepal. The clade patterns and branch length/time indicate recent diversification of genotype A1, which are spreading rapidly all over the world, namely Europe and

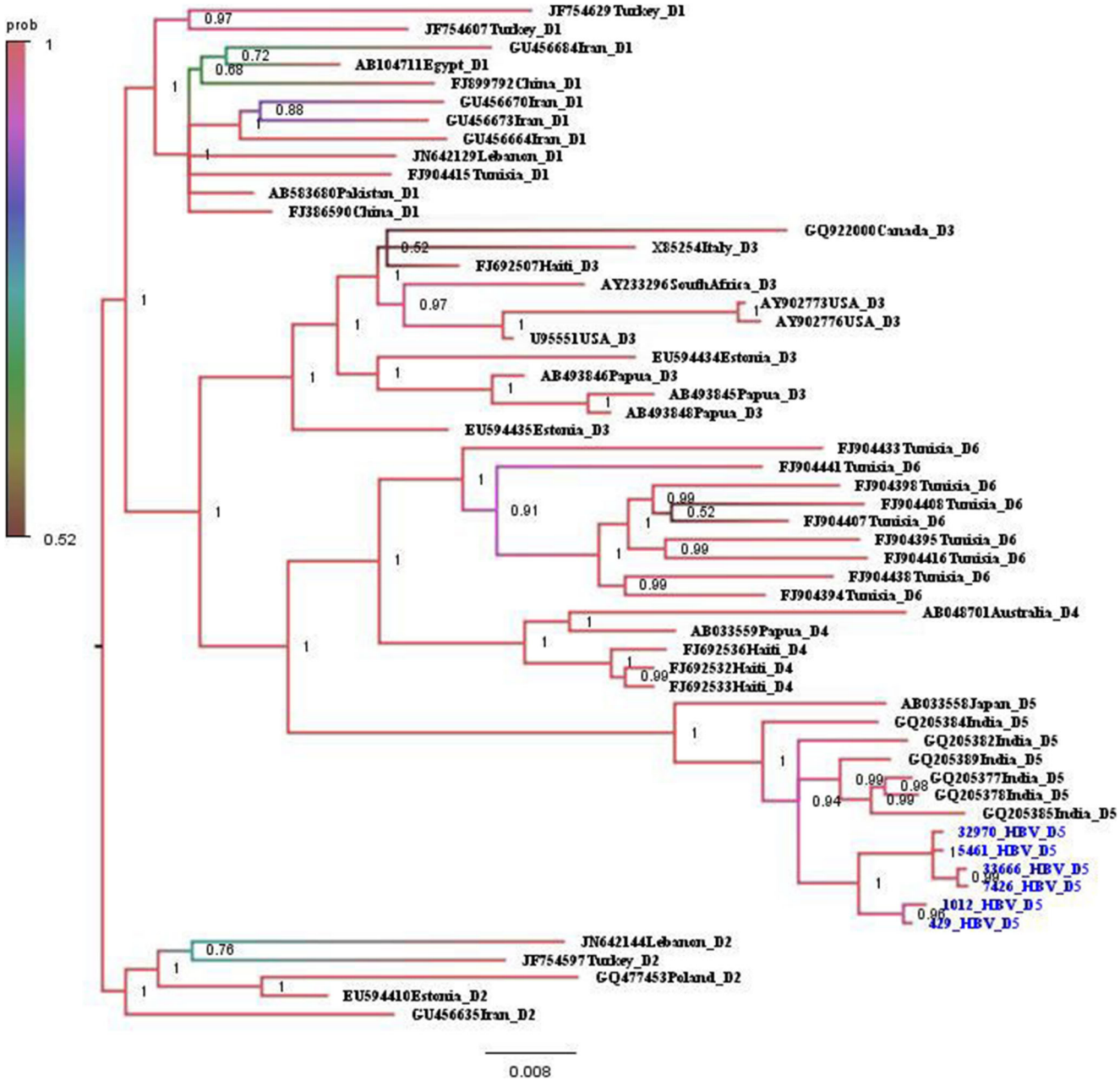

Fig. 2 Neighbor-joining tree drawn to the scale, built under Tamura-Nei evolution model using Geneious R11. Tree was validated by bootstrap method ($n = 1000$). All the sequences were obtained from NCBI GenBank and were re-labeled in format-genotype_accession no._country of origin. Samples were labeled as-sample ID_HBV_genotype. A gradient color is applied according to node age and branch time. Samples are labeled in red color while reference sequence is in blue and others in black color

Asia. From the tree it can be inferred that genotype D is probably the most ancient and prevalent genotype in Nepalese population, whereas genotype A1, C1, and CD are probably most recent introduction. Neighbor-joining tree (Fig. 2) and Bayesian tree (Fig. 3) both have similar clade topology. In both the trees, genotype CD recombinant is having backbone of genotype C as they are being clustered with genotype C despite being CD recombinant.

Among the six sub-genotypes of HBV D genotype, the sub-genotype D5 formed a separate clade from other genotypes having strains isolated mostly from India. The 6 sequenced samples of D genotype were found to be that of sub-genotype D1 and D5 (32970, 15461, 33666, 7426, 1012 and 429_HBV).

Both trees obtained from neighbor-joining and Bayesian analysis gave similar tree (Figs. 2 and 3). All

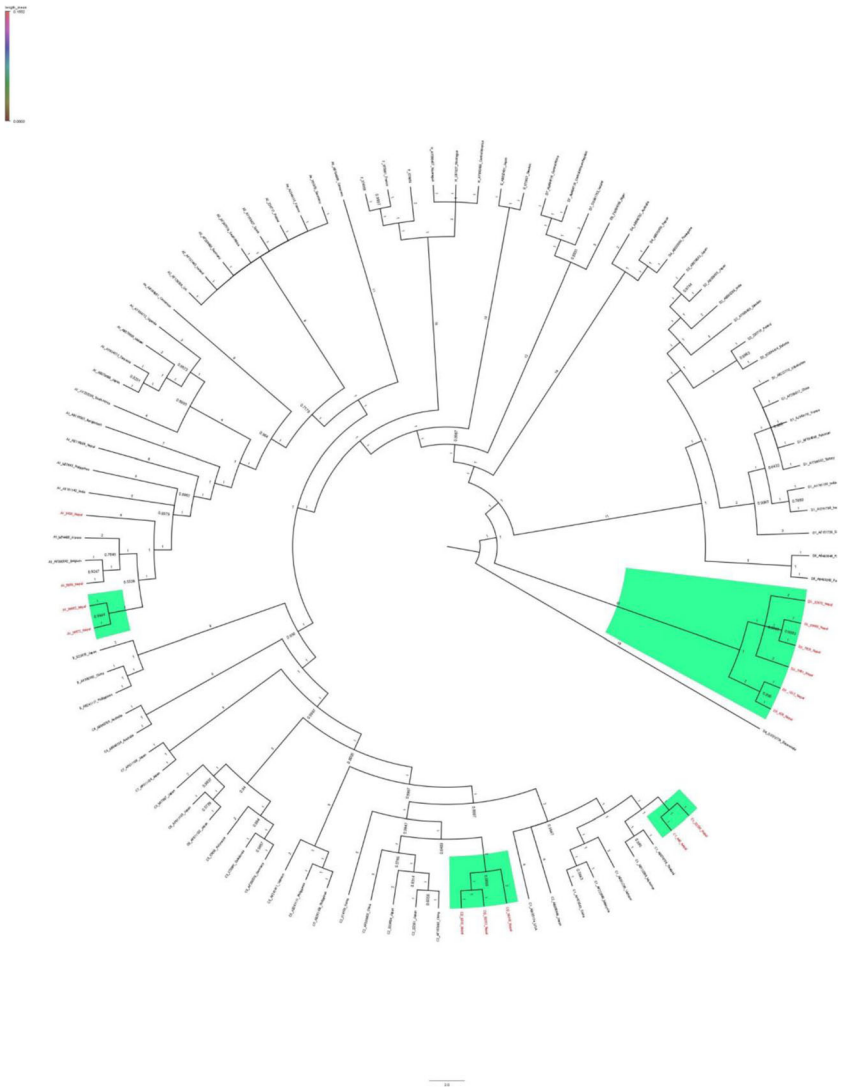

Fig. 3 Phylogenetic analysis through Bayesian inference method (MrBayes v3.2.2) for evolutionary analysis against sequences of hepatitis B virus (HBV) originating from all over the world (downloaded from NCBI GenBank). Samples and reference de-oxy ribonucleic acid (DNA) sequences are presented in the format-genotype_accession no./sample ID_country of origin. Trees were annotated with posterior probability value at node and node age at branch. Branches have been drawn to the scale. All the sequences obtained in this study are highlighted in green color

the genotypes were clustered in their respective group forming distinct cluster. However, CD recombinant was being clustered in C1 genotype's clade but not D or its own unique clade.

Discussion

HBV has been a serious health concern in a low-income countries like Nepal. Nepal does not have a database on the prevalence of this disease in various provinces, and we believe that this work should contribute in viral hepatitis mapping

In the present study we investigated the prevalence of hepatitis B markers HBsAg, HBeAg, and IgM anti-HBc from Sukraraj Tropical Infectious Disease Hospital (STIDH). Our analysis showed the highest reports of the infection from province 2. A similar study of Poudel et al. shows a higher prevalence rate of HBV among male migrant refugees from western Nepal [19]. The other contributing factors being lack of appropriate vaccination coverage, horizontal transmission, and the fact that not all the municipalities have appropriate structure that might contribute in the spread of this infectious disease [14, 20].

Our serological investigation on the prevalence of HBsAg, HBeAg, and anti-HBc shows that males (261, 65.5%) had higher prevalence than females (138, 34.6%). The age group showing highest prevalence rate was 17–28 (31.3%) followed by 29–38 (27.8%) indicating that the most productive group of people are being affected by HBV most. This is supported by a similar study on healthy subjects showing that young males were more affected [21]. We also found 72 (18.05%) samples were HBsAg+/HBeAg+/IgM which is an indicator of recent contact of the hepatitis B virus and active replication. However, 36 (9.02%) samples showed HBsAg-/IgM anti-Hbc +, which indicated occult hepatitis B virus infection.

Among randomly selected 56 samples from the four serological profiles (HBsAg+only, HBs+/HBeAg+,

HBsAg+/HBeAg+/ anti-Hbc +, and HBsAg−/ anti-HBc +ve), the presence of detectable amount of DNA (< 200 IU/mL) in HBsAg−/anti-Hbc+ further verified the prevalence of occult form of hepatitis B infection in Nepal. Our study shows prevalence of genotype D and genotype A and also presence of recombinant strains which is similar to the study of genotyping performed on the patients suffering from inferior vena cava obstruction and liver cirrhosis and HCC in Nepal [22].

Out of the 56 samples, we randomly selected 15 samples seropositive for occult hepatitis B infection for sequencing and phylogenetic analysis. The obtained sequences were deposited in the NCBI for accession number. The details are shown in Table 1.

The sequence data submitted to NCBI were analyzed for phylogeny, which were in agreement with genotyping result, as all the sequences clustered with their respective genotypes. Reference sequences for representative genotype and sub-type were obtained from NCBI GenBank to construct tree, which showed close relation with the data from around the world. Genotype A1 was very closely related to isolates from France and Belgium. This result showed the presence of crossborder but also the role of international travelers in spread of disease; genotype C1 also was found to be more closely related to isolates from Japan, but genotype D1 and D5 were related to isolate from India, giving hint to their probable origin (Figs. 2 and 3).

CD recombinant genotype however was found to be related to C1 genotype indicating probable divergence of genotype C to genotype D or recombination event might have occurred in S-gene (As S-gene determines genotype).

To further investigate the evolutionary pattern of HBV isolates, phylogenetic analysis using Bayesian was performed with sequences from this study and previous isolates present in GenBank (Fig. 3). In this study, genotype D was found to be the most prevalent genotype, which can be inferred from phylogeny as well. Recombination in viruses is very common and could happen in favorable conditions (most likely co-infection) occurring mainly in surface antigen gene that generates genetic diversity [23].

Here, we investigated the prevalence of serological markers and performed molecular study of HBV in patients who visited STIDH from January to August 2018. Nepal faces major demographic problems like rapid population growth, large migrant influx, and high migration rate from rural to urban areas [24]. In such situation, an idea about the linkage between the health and disease in a population becomes important. Our study showed that males had higher prevalence than females. The age group showing highest prevalence rate was 17–28 (31.3%). We also observed a number of patients (72 [18.05%]) showing HBsAg+/HBeAg+/IgM anti-Hbc +, which is an indicator of recent contact with HBV and active replication of the virus irrespective of the acute or chronic from of the infection. However, 36 of the samples showed HBsAg-/IgM anti-Hbc +, which indicated as seropositive occult HBV infection. The genotype prevalence was found to be genotype D and A followed by recombinant genotype C/D. Sequence analysis revealed the close relation of the virus from India as well as from Japan, France, and Belgium. The subgenotypes found were A1, C1, D1, and D5 along with presence of occult hepatitis B infection among Nepalese population. We detected three CD recombinant in this study, which were very closely related indicating probably single recombination event. In conclusion, we believe that the above data could work as a preliminary base that reflects a pattern of infection that is prevalent in Nepal. Hence, it has become imperative for the concerned authorities to raise awareness among the public for reducing the incidence rate of HBV infection as well as to manage the subsequent disease condition.

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Author's contributions SS performed all the experiments and wrote the manuscript and performed data analysis; SM collected the samples and drafted the manuscript; SBN helped in sample collection and manuscript writing; MS and RN helped with manuscript writing and data interpretation; KDM supervised the work and helped with manuscript writing.

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Data availability The data generated in this study has been kept in the manuscript with sequence information deposited at NCBI. The accession no. has been mentioned in the supplementary file. Further information may be provided upon request to the authors.

Compliance with ethical standards

Conflict of interest SS, SM, SBP, MS, RN, and KDM declare that they have no conflict of interest.

Ethics statement The study was performed conforming to the Helsinki declaration of 1975, as revised in 2000 and 2008 concerning human and animal rights, and the authors followed the policy concerning informed consent as shown on Springer.com. The work was approved by Nepal Health Research Council (Ref No. 138/2018).

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