



Diversity of Hepatitis B genotypes in Nepal and updated Phylogenetic Tree: a Pilot Survey in 2012

Damodar Paudel,¹ Myo Nyein Aung,^{2,3} Pattaratida Sa-nguanmoo,⁷ Sushma Suvedi,⁴ Anuj Paudel,⁵ Dipendra Sharma,⁶ Yong Poovorawan⁷

¹Mid-western regional police hospital, Nepalgunj, Nepal, ²Department of Public Health, Juntendo University School of Medicine, Tokyo, Japan, ³Boromarajonani College of Nursing, Nakhon Lampang, Lampang, Thailand, ⁴Nepalgunj Medical college, Nepalgunj, Nepal, ⁵Prawash medical college, Palpa, Nepal, ⁶Sechenkaruna Hospital, Baudha, Kathmandu, ⁷Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Thailand.

ABSTRACT

Introduction: Hepatitis B virus (HBV) is a current global health problem. HBV genotypes influence the treatment and long term outcome of HBV infected patients. Moreover, HBV genotypes differ in various region of the world. Such data was reported haphazardly but yet to be comprehensive for Nepal. This study attempted to find out the diverse hepatitis B genotypes in Nepal.

Methods: A convenient serum sample of 58 HBsAg positive patients from different parts of the country mainly from Nepalgunj, Palpa and Kathmandu were screened for hepatitis B genotype. Sequencing was done and Phylogenetic tree was created.

Results: Among 58 samples, 23 were genotype D, 17 were genotype A and 18 were C/D recombinant. Phylogenetic trees were created by distance-matrix and neighbor-joining analyses after bootstrapping to 1000 replicates.

Discussion: HBV genotypes A and D are the most common genotype in Nepal. Horizontal transmission is common in these genotypes. C/D recombinant genotype may be transmitted from Tibetan people living in Kathmandu. Prophylactic major controlling, horizontal and cross border transmission could be effective.

Conclusions: Three major genotypes of HBV in Nepal were found to be A, C and D. Despite being a low prevalence area, Nepal has a diversity of hepatitis B genotypes

Keywords: *genotypes; HBV; nepal.*

INTRODUCTION

Each year three million chronic infected patients and one million annual deaths by Hepatitis B(HBV) related liver disease, indicates an urgent necessity of better ways to prevent the complication of HBV.¹ It is the tenth leading cause of death and main contributor of hepatocellular carcinoma, which ranks fifth leading cause of cancer in the world.²

Indefinite symptoms, delayed diagnosis and consequent progression to complication lead to death among silent HBsAg positive carriers.^{3,4,5} Genotyping is critically necessary for therapeutic decision making as well as

Correspondence: Dr. Damodar Paudel, Department of Orthopaedic, Mid-western regional police hospital, Nepalgunj, Nepal. Email: damodarpaudel@gmail.com, +977-9841381132.

for preventive and control measures.

Among HBV genotype A to I (9), genotype C, A, A, D and A+D is consecutively prevalence in Tibet Nepal, southern and northern India.^{4,6,7,8} The report on Geographical distribution of HBV genotypes in Nepal is still lacking. The objective of the study is to find out the genotypes and to plot them in Phylogenetic tree.

METHODS

Site: This study was conducted as a multiple center research in Nepal

Study design: This is a cross-sectional study based on different regions of Nepal.

Study population: Patients attending the Nepalgunj, Palpa and Kathmandu hospitals with the evidence of HBV infection by positive HBSAg were conveniently included. Patients gave informed consent and hospital authority allowed collection of samples.

Criteria for inclusion:

Steps of sample collections: --3ml each of peripheral venous blood from the routine collection of blood sample for medical check- up was used. Serum was separated and 1.5 ml serum sample were stored at -70°C. Specimen transfer chain was maintained at -25°C and sent to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, and Bangkok, Thailand.

Sample: 1.5 ml each of serum samples stored in -70°C was used for the extraction of viral DNA.

Centre for genotyping: the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, and Bangkok, Thailand.

Method of DNA extraction: Phenol/chloroform/isoamyl alcohol extraction.

Ethical clearance: This study was approved by institutional review board of Mid-Western Regional Police Hospital (MWRPH). The hospital director of Nepalgunj Medical College and, Palpa Medical College, and Sechen Hospital, Kathmandu, allowed collecting the samples. Informed consent was obtained from each patient after providing information concerning this research in the native language.

METHODS

Extraction of HBV DNA and amplification

A total of 58HBSAg positive samples were collected from Nepalgunj, Palpa and Kathmandu under the supervision of experienced health personnel's.

HBSAg was tested by the micro particle enzyme-immunoassay. Positive serum was separated and kept frozen at -70°C in each center. Viral DNA was extracted by using phenol/chloroform/isoamyl alcohol extraction method. Briefly, 100 µl plasma of each sample were incubated with lysis buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% SDS) and 20 mg/ml proteinase K. The DNA in the sample was subsequently extracted in phenol/chloroform/isoamyl alcohol followed by ethanol precipitation. The pellet was re-suspended with 30 µl distilled water. The polymerase chain reaction (PCR) was done under standard conditions: pre-cycle 94°C/3 min, denaturing 94°C/30 sec, annealing 55°C/30 sec, extension 72°C/1.30 min, 40 cycles, final extension 72°C/7 min. a standard operating procedure was strictly applied to prevent cross contamination. First round PCR reaction mixture was comprised of 4 µl re-suspended DNA, 10 µl PerfectTaq™ Plus MasterMix (5 PRIME GmbH, Hamburg, Germany), 0.5 µl of 25 mM PreS1F+ (5'-GGG TCA CCA TAT TCT TGG GAA C-3', nt2814-2835), 0.5 µl of 25 mM R5 (5'-AGC CCA AAA GAC CCA CAA TTC-3', nt1015-995) and distilled water to 25 µl. Second round PCR was distinguished into 2 sets of primers as overlapping fragments for complete S gene. Set A was comprised of 0.5 µl of 25 mM PreS1F+ and 0.5 µl of 25 mM R4 (5'-ATG GCA CTA GTA AAC TGA GCC-3', nt689-669). Set B was comprised of 0.5 µl of 25 mM F4 (5'- GTC CTC CAA TTT GTC CTG G-3', nt348-366) and R5. The reaction mixture and thermal condition of the second round PCR was conducted as the first round PCR.

Genotyping and sequencing

Nested PCR products were checked on 2% agarose gel electrophoresis. PCR products in gel slices were purified by HiYield™ Gel/PCR Fragments Extraction Kit (Real Biotech Corporation, Taipei, Taiwan). Purified products were subjected to determine the nucleotide sequences by direct sequencing method (1st Base DNA Sequencing Service, First BASE Laboratories SdnBhd, Selangor DarulEhsan, Malaysia). Nucleotide sequences were proved by the Basic Local Alignment Search Tool (Blast) on NCBI webpage (available on: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and edited on the Chromas Lite version 2.01 (Technelysium Pty Ltd., Helensvale, Australia). Edited nucleotide fragments were assembled by CAP3 Sequences Assembly Program (available on: <http://pbil.univ-lyon1.fr/cap3.php>) and subjected to classify their respective genotypes by phylogenetic analysis method. All sequences were compared with each published human HBV genotype (A-J) available on GenBank database using ClustalX version 2.0.10

(European Bioinformatics Institute, Cambridge, UK) and BioEdit Sequence Alignment Editor version 7.1.3.0 (Ibis Biosciences, Carlsbad, CA). Phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA) software version 5.2.2 (The Biodesign Institute, Tempe, AZ) rely on neighbor-joining analyses after bootstrapping to 1000 replicates. Uncompleted S gene sequences were genotyped by Viral Genotyping Tool on NCBI webpage (available on: <http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>).

HBV DNA quantitative measurement

SYBR® Green based quantitative real-time PCR was used to determine the HBV DNA concentration. The condition and reaction mixture followed to those described in the previous study.¹⁸

RESULTS

All 58 samples were HBsAg positive. Mean age of the male was 39.9 years and of the female was 31.6 years. Twenty six samples were real time conventional PCR positive. After nested PCR, HBV DNA was successfully amplified from 42 samples. Among the 42 positive samples, complete S part of the viral DNA were easily obtained for 28 samples by direct sequencing using different primer pairs. For the rest of 14 samples complete scene was not amplified and was classified by viral genotyping tool on NCBI.

Table 1. Characteristics of the sample.

Characteristics	n	p-value
Total	58	
Male : Female	33:25	
Mean age(SD) (yrs; M : F)	39.9(15.3) : 31.6(14.3)	0.04
HBV DNA positive (Total : M: F) ^o	42: 26: 16	0.247
Real time PCR Positive (Total: M: F)	26: 17: 9	0.292
Nested PCR Positive	17: 9: 8	0.469
Genotype (D: A: suspected C/D recombination)	23: 17: 2	0.066
HBV DNA concentration(SD) (copies/ml; M: F)	5.4×10 ⁸ (1.5×10 ⁹): 3.5×10 ⁸ (1.0×10 ⁹)	0.739

The measurement of HBV DNA concentration in the one step real time sybr green PCR assay showed the result of mean viral load: 5.4×10⁸ (1.5×10⁹) to 3.5×10⁸ (1.0×10⁹) for males to females.

Among the 42 Positive samples 23 samples were HBV genotype D, 17 samples were HBV genotype A and 2 samples were HBV C/D recombinant genotype.

*One sample - Real time PCR positive but could not amplify by conventional PCR

Table 2. HBV genotype distribution

	Genotype				Total
	Unfound	A	C/D	D	
Palpa + Kathmandu	4	1	2	6	13
Nepalgunj	12	16	0	17	45
Total	16	17	2	23	58

Different genotypes were detected in the samples from different places, Genotype A and D in Palpa, Nepalgunj and Kathmandu, while C/D recombinant genotype was detected only in Kathmandu.

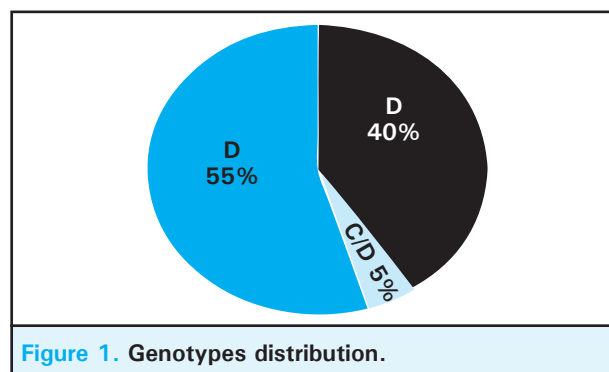


Figure 1. Genotypes distribution.

The Nepal 10 strain was a C/D recombinant genotype and it differed from the genotype A and D of other Nepalese strain. Genotype A1 of Nepal was more similar to that of Bangladesh and Philippines, while A2 was more similar to genotype A2 of Japan, Germany, Canada and Russia. Genotype A1(strain Nepal 31,Nepal 41,24,64,50,6,9,7,47) are similar to the A1 genotype of the Philippines, while strain 36,16 and 18 are more similar to Bangladesh A1 strain. Almost all A1 strain of Nepal showed 88% similarity with Malawi A1 strain. Basically, HBV E genotype of Ghana and Sweden are nearby the Genotype D of Nepal. Even some sequences from Germany and Italy are similar to the genotype D of Nepal.



Figure 2. Phylogenetic tree was obtained from the aligned sequences combining with all type of human hepatitis B sub-genotype.

Genotype D strain of Nepal, strain 12, 55 are similar with German D genotype strain. While strain Nepal 60 and 57 are more similar with D strain of Italy, France and Russian strain of D genotype. These strains are 93 % similar with Nepal 21 strain from where the strain Nepal 28 was evolved. Nepal 28 strain of D genotype is evolved to more similar strain 22, 32, and 14. Nepal strain 40, 38, 1, 53, 11, 42, 73 are more similar. Nepal 73 is more similar with 81, 79 and 72, while Nepal 81 and 79, is 88% similar with Nepal 72 strain.

DISCUSSION

In our study we explored genotype A, D and C/D recombinant genotype which is slightly different from the finding in the previous study as authors explored A, B, C, and D genotypes.⁴ The result is little bit various, may be due to the less number of distribution of

Genotype b in Nepal.

Hepatitis B genotyping is important for the clinical management of the disease, because HBV genotype is correlated with the replication of the virus, its activity in the liver and treatment outcome.⁹

In our study Hepatitis B is more prevalent in young men than in women the ratio of male to female is 1.13:1. Young female mean age less than male was found infected which is different picture than a study in neighboring country Pakistan.¹⁰

Distribution of different genotype of HBV is different in both genders in our study. As huge percent of female was found genotype D and rest of the female has got the genotype A. There is no mix genotype isolated in female. Most of the male has genotype A, while less of them have Genotype D and mixed infection. Our study prevails that there is prevalence of single Genotype D and genotype A infection in Nepal, which are the predominant genotype in India too but There is no mix infection of HBV genotype A + D or B + D, as in neighboring country India and Pakistan.¹⁰ HBV genotype A and D correlation with age group was not significant in our study.

The geographical distribution of genotype intra-country, A and D is almost the same in ratio in Nepalgunj, the mid-western city of Nepal. Genotype D was dominant in Palpa, a western region of Nepal. The C/D recombinant genotype was found in Kathmandu, the central region of the country. The recombinant strain C/D in Kathmandu may be prevalent in Tibetan migrant as mentioned by the author.¹⁹

A study conducted in New Delhi, India revealed that mix infection of A + D is prevalent in Northern India which is near by our one of the study site. The dominance of Genotype D in Nepal was reported in the previous study too.¹²

HBV genotyping showed distinct geographical distribution within and between the regions. Although the sample size from Kathmandu and Palpa was small, distribution of the different genotypes in the mid-west Nepal (Nepalgunj) would be suggestive of all genotypes representing in the country including genotype A, B, C and D reported by previous publication.¹³

In our study the mean age of the HBV patients is 35 years old, indicating that HBV is a disease of working population in Nepal. Young and sexually active female are more prone to the disease it may be due to their sexual behavior.¹⁴ As genotype D can be transmitted by all routes (parenteral, perinatal, sexual) may be because of which it is distributed in all region.

This study confirmed that HBV genotype A and D are dominant in Nepal.¹⁴ Shrestha et al. 2007 reported by the same observation Genotype of Nepal is more similar with India and Bangladesh. The HBV genotype A of Asian type has older Phylogenetic origin and have infiltrated more deeply in this region.¹⁵ Philippines genotype Aa is more similar to Nepal than Bangladesh and south Africa¹⁶ which suggest that there may be some ship trade between India and Philippines, from where the disease was transmitted to neighboring country Nepal.

In Tibet, HBV genotype C is high prevalent. One of the famous Himalayan tribe known as Sherpa's are the ancestor of Tibetan, they have HBV genotype A reported by the previous study. Nepalese Sherpa who are settled for many 100 years in the base of Himalaya has Genotype A, which is more similar with Japanese genotype Aa⁷ may be suggestive of the disease transmission intra country especially from the urban cities like as Kathmandu.¹³ Asian type of genotype Aa has poor clinical outcome than genotype from Europe Ae.¹⁷

HBV genotype A, which is prevalent in Africa, has 4.5 fold higher risks to get Hepatocellular carcinoma (HCC) than none A genotypes.¹¹ However, another group⁹ reported a controversial result that HBV genotype A has the good clinical prediction than HBV genotype D. High mutant gene could be the early cause of HCC and Nepalese strain of HBV has high deletion mutant of s1 (about 27%). High prevalence of s mutant is in Nepalese HBV could be the cause of HCC, cirrhosis, chronic carrier or asymptomatic carrier of hepatitis B disease.¹³

In our study C/D recombine genotype in Kathmandu may be suggestive that, the transmission of the disease

in Kathmandu is horizontal and vertical both route as mention in previous study.¹⁴ It could be suggestive that Nepal has less prenatal transmission (vertical) than other routes (horizontal).

LIMITATIONS

Even though we took the sample from different sites, the small sample in our study may not represent nation-wide distribution. We took HBSAg positive sample, the finding is not quite different from previous finding⁴ represented by a little big sample size 121 from Kathmandu. Consequently, viral load was not sufficiently high to be sequenced in some samples.

CONCLUSIONS

HBV genotypes in Nepal. It is important for the prevention strategy. The authentic agency will have to focus on horizontal transmission to prevent HBV in Nepal. Moreover, the finding in this pilot study will guide to calculate the sample necessary for bigger nation-wide study. A nation-wide, properly sampled molecular survey of HBV and a prospective trial or cohort study to see the treatment outcome among prevalent genotypes in Nepal are recommended

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