
Circulating genotypes of hepatitis B virus in Arunachal Pradesh

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**Background & objectives:** There is a paucity of information on distribution of hepatitis B genotypes from northeastern part of India. Arunachal Pradesh, one of the northeastern State of India bordering Bhutan, China and Myanmar, reported abnormally high numbers of hepatitis B surface antigen (HBsAg) positive cases in one of its districts during January-June 2005. We conducted this study in the subsequent months (August-December 2005) to know the prevalent genotypes by a rapid and specific method based on type-specific primers in Upper Dibang valley of Arunachal Pradesh.

**Methods:** A total of 438 randomly selected individual were screened for HBsAg positivity. Of the 93 HBsAg positive individuals, 36 HBsAg and HBV DNA positive samples were processed for HBV genotyping using type-specific primer based nested PCR (TSP-PCR). Representative samples were retested with RFLP-PCR based genotyping and nucleotide sequencing.

**Results:** Of the 36 samples, 29 (80.1%) could be genotyped by the TSP-PCR based method used. The predominant genotype was genotype A (41.6%) followed by genotypes C (27.8%) and D (11.1%). Seven isolates (19.9%) could not be genotyped by this method.

**Interpretation & conclusions:** The presence of genotype C in this part of the country needs attention as genotype C takes a more aggressive disease course. Also, detection of genotype C in this isolated community bordering Tibet suggests viral gene flow from Tibet or other South-east Asian countries where genotype C of HBV is predominant.

**Key words** Arunachal Pradesh - genotype C - hepatitis B - HBV

Hepatitis B virus (HBV) is a major public health problem with over 360 million chronically infected people worldwide and accounting for about 600,000 deaths from HBV-related liver disease or hepatocellular carcinoma annually. Though a safe and effective vaccine has been available for more than 20 yr, it is not effective for established infections. Recently, HBV genotypes have attracted increasing attention since they influence the activity and outcome of HBV-associated chronic liver disease, as well as the response to antiviral therapies.

There is paucity of data regarding HBsAg prevalence of HBV in north east (NE) India. There are pockets of high HBsAg cases especially among the isolated tribal communities in India. An isolated tribal
region situated at an altitude of over 1800 meters above sea level in Arunachal Pradesh bordering Tibet was investigated during August-December 2005 and the prevalent genotypes were characterized. The Idu Mishimi tribe of Arunachal Pradesh mostly inhabits this region. Idu tribe migrated from Tibet long back. HBV genotype C and C/D hybrids are common in Tibet\(^5\). However, genotypes A and D are predominant in India\(^6\), so we studied this remote tribal community for genotype distribution.

The main objective of the study was to know the circulating genotypes of HBV in this remote tribal community of the northeast, as no data were available on the prevalent HBV genotypes.

**Material & Methods**

The study was conducted between August to December 2005 and samples were collected from subjects enrolled for the study from Anini, the district headquarter of Upper Dibang valley of Arunachal Pradesh. Upper Dibang valley with an area of 9000 sq km has roughly a person per sq km with an approximate native population of 7152 (as per 2001 census) of mostly Idu Mishmi’s, including Anini, which has an approximate four thousand local residents. The route of this Mongoloid tribe can be traced to the Lhoba tribe of Tibet and they migrated to India long back and have remained a closed community for several centuries.

The study protocol was approved by the ethical committee of Regional Medical Research Centre, Dibrugarh. A total of 438 randomly selected individuals from the community between 2 to 56 yr (to cover approximately 4 per cent of world’s Idu Mishimi populations) with unknown HBsAg status were screened for HBsAg (EQUIPAR HBs Ag ELISA kits, Italy); and interviewed after obtaining a written and informed consent, and documented in a structured questionnaire to record the demographic information and clinical history. Non tribals were excluded from the study. Venous blood (5 ml) was withdrawn in a K3 EDTA tube from each person and plasma was separated, transported and preserved at -20°C till analyzed. From the 93 HBsAg positive samples, randomly 36 HBsAg and HBV DNA positive samples were processed for HBV genotyping.

DNA was extracted from 100 µl of plasma using a commercial blood DNA extraction kit (E.Z.N.A. Blood DNA kit, Omega Bio-tek, USA).

The strategy described by Naito et al\(^7\) for classifying six genotypes from A-F with type-specific primers (TSP-PCR) was utilized. In brief, 10 µl of extracted DNA was subjected to 40 cycles of first round PCR using primers 5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (nt 2823-2845, universal, sense) and 5'-CGA ACC ACT GAA CAA ATG GC-3' (nt 685-704, universal, antisense) amplifying a 1063 bp region of S-gene\(^7\).

TSP-PCR was performed in two separate mixes A and B utilizing 1 µl of 1st round PCR product and subjecting to two rounds PCR cycles (20 cycles each) as described by Naito et al\(^7\). In mix A, primers specific for genotype A (5'-CTC GCG GAG A TT GAC GAG A TG T-3' nt 113-134, type A specific, antisense), genotype B (5'-CAG GTT GGT GAG TGA CTG GAG A-3' nt 324-345, type B specific, antisense), genotype C (5'-GGT CCT AGG AA T CCT GA T GTT G-3' nt 165-186, type C specific, antisense) and a common universal sense primer (5'-GCC TCA AGT TCA GGA ACA GT-3' nt 67-86, types A to C specific, sense) were used\(^7\).

In the mix B a common antisense primer (5'-GGA GGC GGA TCT GGT GAG AA-3' nt 3078-3097, specific for types D to F, antisense) along with genotype specific primer D (5'-GCC AAC AAG GTA GGA GCT-3' nt 2979-2996, type D specific, sense), E (5'-CAC CAG AAA TCC AGA TTG GGA CCA-3' nt 2955-2978, type E specific, sense), and F (52 -GTT ACG GTC CAG GGT TCA CA-3 nt 3032-3051, type F specific, sense) were used. Mix A allowed for the specific detection of PCR products for types A (68 bp), B (281 bp), and C (122 bp), and mix B allowed for detection of types D (119 bp), E (167 bp), and F (97 bp)\(^7\). Some of the representative samples were rechecked by another genotyping system using restriction fragment length polymorphism (RFLP) of S gene amplicons with five different restriction enzymes (StyI, BsrI, DpnI, HpaII and EaeI) as described by Zeng et al\(^8\).

Three representative PCR amplified products of S-gene (363 bases) were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an automated DNA sequencer (ABI PRISM 310 Genetic Analyser, Applied Biosystems) at National Institute of Virology, Pune. Nucleotide sequences were aligned using CLC free workbench version 3.2 software (CLC bio, Denmark). Phylogenetic analysis was performed on the three samples comparing with the best and high scoring
matches in a NCBI BLAST search and also with the partial S-gene sequences from China, Thailand, Eastern India, Myanmar, Vietnam, etc., using accession numbers from GenBank database of NCBI. Phylogenetic tree was constructed using neighbour-joining method and for reliability of the pair-wise comparison, bootstrap repetition and reconstruction were carried out 1000 times using the CLC free workbench version 3.2 software (CLC bio, Denmark).

**Results & Discussion**

Of the 36 samples positive for HBsAg and HBV DNA subjected to genotyping, 29 (80.1%) could be genotyped utilizing the TSP-PCR based method. Genotype A with 15 samples (41.6%) showing a 68 bp band in the mix A group was the predominant genotype (Fig.1). Genotype C with a 122 bp band was detected in 10 samples (27.8%). While four samples had genotype D with a 119 bp (11.1%) detected in the mix B. Genotypes B, E or F were not detected. Four out of the six genotype C samples were rechecked with RFLP and found to be genotype C subtype C1. Of 25 individuals in whom the genotypes could be known, a mother and her 4 yr old offspring were genotype C, while in another case the father and his 2 yr old daughter had the same genotype A. Partial nucleotide sequencing of two genotype C and one genotype D (GenBank Accession numbers EF199993, EF199994, EF199995) matched with the result of both TSP-PCR and RFLP-PCR method. Phylogenetic analysis based on partial S-gene sequences (363 bp) of two genotype C isolates clustered with genotype C isolates from eastern India and China, whereas the genotype D isolate clustered with isolate from Andaman and Nicobar islands. In BLAST search the three isolates had 99-100 per cent identity matched with isolates from China, Japan and Korea.

Most of the study subjects were apparently healthy and appeared to be chronic carriers. No overt clinical disease was detected, however, on detailed clinical investigation, signs and symptoms like anorexia, fatigue and history of hepatitis were seen more in HBsAg positive subjects. Source of infection in this isolated community was not investigated in detail with the genotypes. Though, there were not adequate data to prove that vertical transmission could be the predominant transmission pattern, but identical genotyping results between index parent and carrier children pointed towards a vertical transmission pattern. Infection in early life frequently results in persistent infection and clustering of the chronic infection is common within the family.

By tradition, hepatitis B virus is classified into 4 subtypes or serotypes (adr, adw, ayr, and ayw) based on antigenic determinants of the hepatitis B surface antigen. Genotypically, HBV genomes have been classified into eight groups, designated A–H, and based on an intergroup divergence of 8 per cent or more in the complete nucleotide sequence.

This study mainly based on TSP-PCR, revealed that genotype A, C and D were the predominantly circulating genotypes in this region of northeast India. Other investigators from New Delhi, using TSP-PCR have found consistent result compared with RFLP-PCR and nucleotide sequencing. This study probably reported for the first time the presence of genotype C of HBV from northeast India. Recent studies from Kolkata have reported the presence of genotype C among chronic carriers with the possibility of viral gene flow from South-east Asian countries. Also, presence of genotype C has been documented from neighbouring Bangladesh. Genotype C is known to take a more aggressive disease course than genotype B. A higher rate of progression to liver cirrhosis has been shown among patients with genotype C than genotype B. Moreover, it is seen in comparative studies of different genotypes with antiretroviral therapies that genotypes play a role in the outcome of antiretroviral therapy in chronic hepatitis B infection. The evidence is stronger between genotypes B and C and response to interferon. There is also a clear association between HBV genotypes with different rates of progression from acute

![Fig. 1. Genotyping of HBV by specific primer based nested PCR. Eight samples run from lane 2 to 9 and 11 to 18. Mix A reveals genotypes A, B & C and mix B reveals genotypes D, E & F. Lane-1 & 20 are 100 bp DNA ladder, lane 2-4 are genotype A, Lane 5-7 are genotype C, Lane 17 & 18 are genotype D.](image-url)
Fig. 2. Phylogenetic analysis of isolates P-89, P-200 (genotype C) and P-105 (genotype D) from Arunachal Pradesh compared with reference strains from GenBank; showing the genotype C and D (from Arunachal Pradesh) clustering with genotype C from eastern India (Kolkata) and with genotype D from Andaman & Nicobar Island, respectively. Phylogenetic tree was based on comparison of 363 bp (codon 284 to 646) of the S-gene and constructed using neighbour joining analysis with 1000 bootstrap replicates using CLC free Workbench 3 software.
to chronic HBV infection\textsuperscript{19}. There is a growing evidence that HBV genotypes may influence HBeAg seroconversion rates (spontaneous seroconversion rate is earlier in genotype B than genotype C) and mutational patterns in the precore and core promoter regions (that abolish or diminish the production of hepatitis B e antigen). Thus, the most common precore mutation, a G to A substitution at nucleotide 1896 (G1896A), which creates a premature stop codon is found in association with HBV genotypes B, C, and D but not genotype A\textsuperscript{17}. Probability of precore mutants among the genotype C and D needs to be accessed in this region.

HBV genotypes have distinct geographical distributions. In India, genotype A and D are the circulating genotype with genotype D being the predominant genotype circulating in western India\textsuperscript{6}. A recent study among patients with chronic liver disease from New Delhi reported predominantly genotype D followed by genotype A\textsuperscript{12}. HBV isolates from one of the primitive tribes of the Andaman and Nicobar Islands showed predominance of genotype D like mainland India with minimal difference in isolates suggesting the likelihood of the introduction of HBV from mainland India\textsuperscript{20}. In our study we found the predominance of genotype A followed by genotypes C and D.

Presence of genotypes A and D concords well with the prevalent genotypes in India\textsuperscript{6,12,18,20}. As around 20 per cent of the isolates could not be genotyped by the method used, the prevalence of genotypes G and H or recombinant forms cannot be ruled out. Moreover, specific primer based PCR genotyping method though may be cost-effective, it may miss out atypical, nonspecific or recombinant forms. Co-infection with HBV of distinct genotypes is not infrequent and found in about 10 per cent of infected individuals, and is responsible for intertypic recombination of HBV genomes\textsuperscript{21}.

While the existence of genotype C in the isolated tribal communities of Idu-Mishmi’s, bordering China preludes to a mix-up or origin of this tribe from China, where genotype C is prevalent\textsuperscript{5,22} (Only three samples were partially sequenced in this study, detailed phylogenetic analysis was not done). As we know from history, the present Idu-Mishmi tribe migrated form Tibet long back and HBV may have circulated within this tribe for centuries. Moreover, due to lack of sequence data from neighbouring regions of Tibet, one cannot implicate that the virus isolates in the present study region had their origins in Tibet or China.

The origin and track of this viral gene flow could be known only with a detailed phylogenetic study among the different tribes of northeast India. Genotyping of HBV till now has remained a research tool, but growing evidence suggests that genotypes influence the activity, and outcome of HBV-associated chronic liver disease, as well as the response to antiviral therapies; thus the need for hepatitis B genotyping in the different hyperendemic population of northeast India is essential.

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References


