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## VIRAL HEPATITIS

# Distribution of hepatitis B virus genotypes: Phylogenetic analysis and virological characteristics of Genotype C circulating among HBV carriers in Kolkata, Eastern India

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**CONCLUSION:** The present study reports the genotypic distribution and the characteristics of partial genome sequences of HBV/C isolates from Eastern India. Low genetic diversity and confinement of HBV/C in Eastern India possibly indicate a recent, limited, spread in this region. Genotype C with T1762/A1764 mutation has been reported to increase the risk for hepatocellular carcinoma; therefore genotype C carriers in Eastern India should be carefully monitored.

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**Key words:** HBV genotypes; HBV/Cs, Eastern India; T1762/A1764 mutation

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## Abstract

**AIM:** To evaluate the genotype distribution of hepatitis B virus (HBV) in Eastern India and to clarify the phylogenetic origin and virological characteristics of the recently identified genotype C in this region.

**METHODS:** Genotype determination, T1762/A1764 mutation in the basal core promoter (BCP) and A1896 mutation in the precore region of 230 subjects were determined by restriction fragment length polymorphism method (RFLP) and the result was confirmed by direct sequencing.

**RESULTS:** The predominant genotypes D (HBV/D) and A (HBV/A) were detected in 131/230 (57%) and 57/230 (25%) samples. In addition, genotype C (HBV/C) was detected in 42/230 (18%) isolates. Surface gene region was sequenced from 45 isolates (27 HBV/C, 9 HBV/A and 9 HBV/D). Phylogenetic analysis revealed that all of the HBV/C sequences clustered with South East Asian subgenotype (HBV/Cs). The sequence data showed remarkable similarity with a Thai strain (AF068756) (99.5% ± 0.4% nucleotide identities) in 90% of the genotype C strains analyzed. T1762/A1764 mutation in BCP region, associated with high ALT was significantly higher in HBeAg negative isolates than HBeAg positive isolates. Frequency of A1896 mutation leading to HBeAg negativity was low.

## INTRODUCTION

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family of enveloped viruses with double-stranded DNA genome of nearly 3200 bp lengths. The HBV genome consists of four major overlapping open reading frames named surface (S), core (C), polymerase (P), and X.

HBV that infects humans has been classified into mainly eight genotypes, A-H based on the sequence divergence over the entire genome exceeding 8%<sup>[1-4]</sup> and S gene sequence analysis<sup>[5]</sup>. HBV genotypes have distinct geographical distributions and according to various studies seem to have different biological properties affecting, thus, the clinical outcome of HBV disease. Subgroups have been identified within different HBV genotypes, on the basis of > 4% (but < 8%) difference in the complete nucleotide sequence. In HBV genotype A, two subgroups have been defined; one is prevalent in Europe (Ae), and the other is prevalent in Africa and Asia (Aa)<sup>[6]</sup>. Similarly, genotype C has been classified into four subgroups with characteristic

geographical distributions. Subgroup C1 (Cs) is common in Southeast Asian countries like Thailand, Myanmar and Vietnam, C2 (Ce) in East Asian countries like Japan, Korea and China<sup>[7,8]</sup>, C3 in Oceania comprising strains specifying *adrq*-, and C4 specifying *aym3* is encountered in Aborigines from Australia<sup>[9,10]</sup>.

Based on antigenic typing and further analysis of sub determinants HBV has been classified into 9 subtypes<sup>[11]</sup>. Subtypes correlate broadly with genotypes. Some subtypes can be found in more than one genotype, which confer additional heterogeneity within the genotypes.

It is now recognized that mutations in the basal core promoter (BCP) and precore region regulate hepatitis B e antigen (HBeAg) expression. It was reported in an *in vitro* study that the double mutations in BCP A1762T and T1764A (T1762/A1764A) down regulate precore mRNA and slightly increase the efficiency of pregenome mRNA and core mRNA<sup>[12]</sup>. Recently HBV genotypes have been partially clarified as influencing the clinical manifestation of chronic liver disease in hosts. A higher disease inducing capacity of HBV/C than HBV/B has been observed in Asia. Moreover, HBV/C with T1762/A1764 mutation has been reported to increase the risk for hepatocellular carcinoma<sup>[13]</sup>.

India is a vast country with an ethnically diverse population. With more than 40 million carriers of HBV, this is the major etiology of chronic liver diseases in India. Analyses of genomic sequences of HBV isolates from India are limited. Most reports are from Western India and Northern India, where genotypes D and A are found<sup>[14,15]</sup>. Eastern India is a geographical area where genotypes D and A of mainland India and genotypes B and C of China and Southeast Asia converge. Recently genotype C has been reported from Eastern India<sup>[16-18]</sup>. However, data regarding genotype distribution as well as molecular and virological characteristics of genotype C in Eastern India remain undefined.

Therefore the present study was undertaken to investigate the distribution of HBV genotypes in Eastern India and the molecular and virological features of HBV/C circulating in Eastern India.

## MATERIALS AND METHODS

### Patients

A cross sectional study was performed on 230 HBV DNA positive serum samples from patients with HBV infection who were referred to Indian Council of Medical Research (ICMR) Virus Unit for HBV DNA detection. Among them, 200 samples came from the outpatient clinics of Kolkata hospitals during the period of March 2001 to February 2004. All patients were known to have been positive for surface antigen (HBsAg) for > 6 mo. In addition 30 HBsAg positive asymptomatic carriers found during a community based epidemiological point prevalence study carried out by Institute of Post Graduate Medical Education & Research (IPGMER) in the rural areas, about 150 km away from Kolkata, were also included in this study<sup>[19]</sup>. These samples were sent to our Unit for HBV DNA detection and for genotyping.

Only pretreatment samples were included in the study. Informed consent was obtained from the patients, and the Institutional Ethical Committee approved of the study protocol.

### Serological testing

HBsAg, HBeAg, antiHBe were tested by using commercially available enzyme linked immunosorbent assay kits (Organon Teknika, Boxtel, The Netherlands).

### HBV DNA preparation and amplification

The sera were stored at -80°C until analysis. Viral DNA was extracted from 200 µL serum by phenol/Chloroform extraction after incubation with Proteinase K<sup>[20]</sup>. HBV DNA was detected using in-house nested polymerase chain reactions (PCR) targeting the DNA sequences encoding the surface and the precore/core regions by the method described earlier<sup>[16,17]</sup>. Instructions to prevent cross contamination were followed strictly<sup>[21]</sup> and the results were considered valid only when they were consistently obtained in duplicate.

### Genotyping and Sequencing of HBV

HBV genotyping was done in 230 HBV DNA isolates by Restriction Fragment Length Polymorphism (RFLP) using the methods described earlier<sup>[5]</sup> and reconfirmed on the basis of phylogenetic analysis of the 440 nt fragment of S gene of 45 randomly chosen isolates. Of these 45 isolates, 27 were from genotype C, 9 from genotype A and 9 from genotype D. The nested PCR products were sequenced from both directions on an ABI Prism 377 (Applied Biosystems, Foster City, California, USA), using the PCR primers. BCP and precore core region were sequenced using primers as described earlier<sup>[17]</sup>.

Subgenotypes of HBV/C were assigned as described previously, C1 (Cs), C2 (Ce)<sup>[7,8]</sup> and C3, C4<sup>[10]</sup>.

### Phylogenetic analysis

For sequence alignment as well as phylogenetic analysis, we selected the GenBank sequences with the best and the high scoring matches with our sequences in a NCBI BLAST search. Sequences were edited, aligned and analyzed using Bioedit version 7.0.4.1<sup>[22]</sup>. Genetic distances were calculated using the Kimura two parameter algorithm and phylogenetic trees were constructed by the neighbor joining (NJ) method. To confirm the reliability of the pair wise comparison and phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 1000 times. Phylogenetic analysis was done using MEGA version 2.1<sup>[23]</sup>.

## RESULTS

The clinical and demographic characteristics of 230 HBV carriers are shown in Table 1. Among them 200 patients (age between 5-68 years, 77 HBeAg+ and 123 HBeAg-) were native resident of Kolkata and its neighborhood, while 30 (age between 2-50 years, 3 HBeAg+ and 27 HBeAg-) were from rural areas. All the patients were ethnic Bengali. Median serum HBV DNA load of the patients was 5.37 (range 5.15-9.15) log copies/mL.

**Table 1 Clinical and demographic characteristics of 230 HBV carriers from Eastern India**

|                  | HBV carriers from    |                         | Total         |
|------------------|----------------------|-------------------------|---------------|
|                  | Kolkata<br>(n = 200) | Rural areas<br>(n = 30) |               |
| Age (yr)         | 32.56 ± 12.98        | 26.03 ± 14.14           | 31.71 ± 13.29 |
| Sex (M/F)        | 173/27               | 21/9                    | 194/36        |
| Genotype (A/C/D) | 57/40/103            | 0/2/28                  | 57/42/131     |
| ALT (IU/L)       | 83.24 ± 75.15        | 37.63 ± 21.73           | 77.29 ± 72.15 |
| HBeAg positive   | 77                   | 3                       | 80            |
| HBeAg negative   | 123                  | 27                      | 150           |

**Table 2 Characteristics of 42 HBV/C isolates of Eastern India**

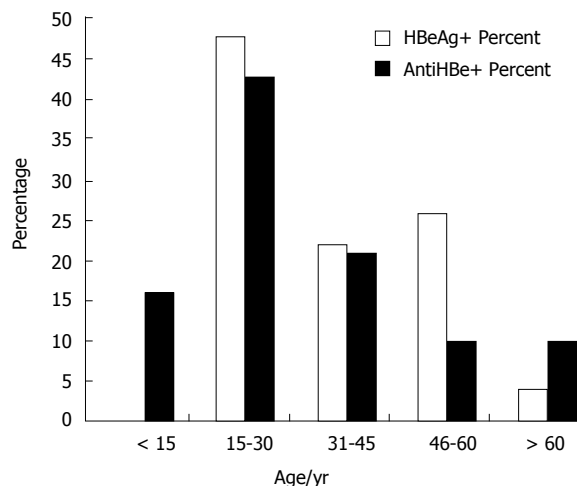
|                    | HBeAg positive<br>(n = 23) | HBeAg negative<br>(n = 19) |
|--------------------|----------------------------|----------------------------|
| Age (yr)           | 32.67 ± 13.04              | 32.47 ± 13.01              |
| Sex (M/F)          | 19/4                       | 15/4                       |
| ALT (IU/L)         | 75.13 ± 39.50              | 85.84 ± 54.14              |
| Subtype adrq+/adw2 | 23/0                       | 18/1                       |
| T1762/A1764        | 12 (52%)                   | 17 (89%)                   |
| A1896              | 2 (9%)                     | 4 (21%)                    |

**Genotype distribution**

Three HBV genotypes (A, C and D) could be detected among the 230 HBV DNA positive samples studied by RFLP method. Majority of the samples belonged to Genotype D, 131/230 (57.0%), followed by genotype A, 57/230 (24.8%) and 42/230 (18.2%) isolates were identified as genotype C, the majority of which (40 of 42) were from outpatient clinics of Kolkata. Out of the 30 samples from incidentally detected asymptomatic carriers from rural areas, only 2 (2/30, 6.7%) were of genotype C, while the rest were of genotype D (93.3%) (Table 1). The banding pattern of all the genotype C samples was similar to that of genotype C pattern found among Southeast Asian carriers by Lind *et al* 1997.

**Characteristics of 42 HBV/C isolated from Eastern India**

Among the 42 HBV/C strain, 23 (53%) were HBeAg positive and rest 19 (47%) were antiHBe positive. In order to clarify the clinical characteristics of HBV/C carriers in this region clinical and laboratory data between HBeAg positive and antiHBe positive patients were compared (Table 2). There was no significant difference between the mean age of HBeAg positive and antiHBe positive group. The mean ALT level was slightly high in antiHBe positive (85.84 ± 54.14 *vs* 75.13 ± 39.50) cases but the difference was not statistically significant. T1762/A1764 double mutation in the BCP region was more frequent in antiHBe positive than HBeAg positive group (17/19, 89% *vs* 12/23, 52%) with elevated ALT level, whereas the frequency of A1896 mutation that creates a stop codon in the precore region was low 6/42 (14%). To examine the correlation between age and HBeAg/antiHBe status, the age specific prevalence of the HBeAg/antiHBe status in 42 HBV/C



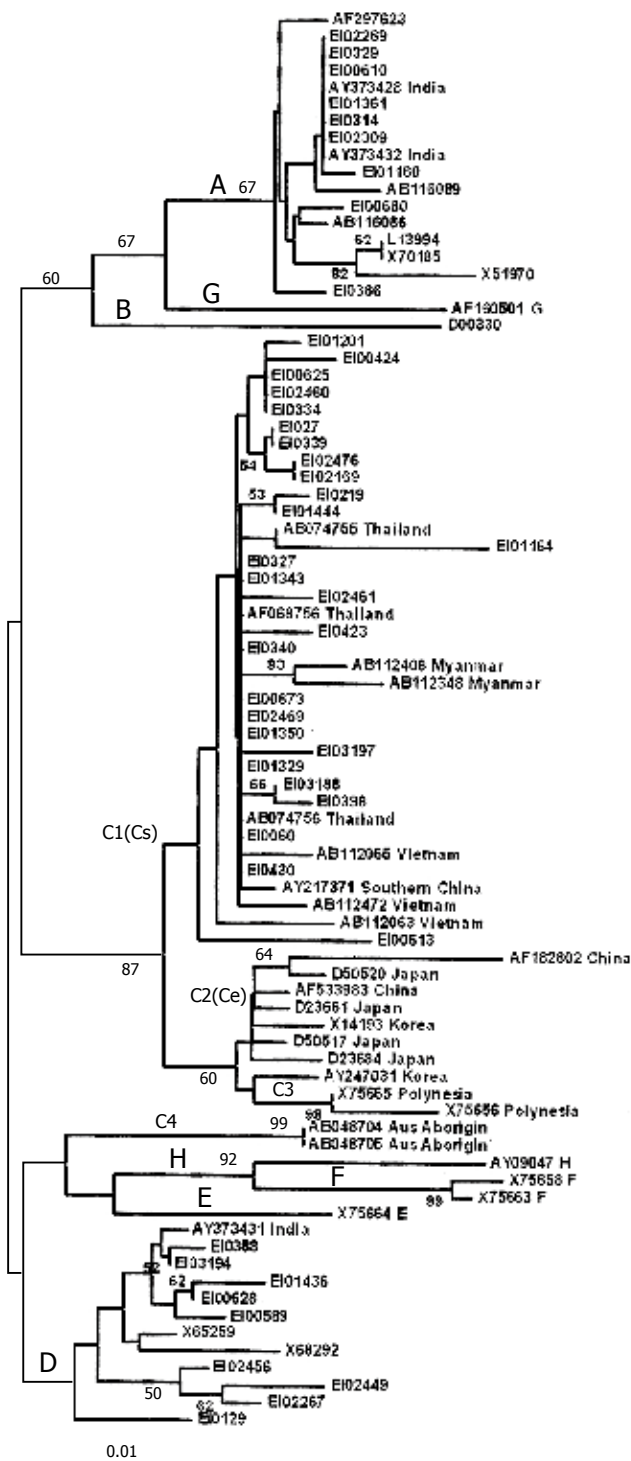
**Figure 1** Age specific prevalence of HBeAg/antiHBe status in 42 HBV/C strain isolated from Eastern India.

subjects was analyzed (Figure 1). About 19/42 (45%) of subjects were in the age group of 16-30 years irrespective to HBeAg status. After that the infection rate gradually decreased.

**Phylogenetic analysis**

Phylogenetic analysis based on nucleotide (nt.) 256-696 of the Surface (S) gene region of HBV isolates was used to confirm the presence of these three genotypes from 45 isolates. The 45 S gene fragments were analyzed along with 39 reference sequences of different genotypes retrieved from GenBank in the phylogenetic tree presented in Figure 2. Genotypes A with adw2 subtype and D with ayw2 and ayw3 subtype from the present study clustered with the genotype A and D sequences previously reported from India. However, genotype C isolates with adrq+ and adw2 subtype from Eastern India clustered with HBV/Cs subgroup found in South East Asian countries rather than HBV/Ce subgroup found in the Far East like China, Japan, and Korea. Overall percent nucleotide identity (PNI) for HBV genotype C isolates from Eastern India with HBV/Cs varied from 98.4 to 100%. In addition to the above observation, 24 strains were most closely related to the Thai strain, AF068756 in the NCBI BLAST search. The PNI for these 24 sequences with AF068756 was found to be 99.5% ± 0.4%, across the 440 nt fragment in the S gene region. EI01343 and EI01329 were two HBV/C strain isolated from rural areas. Sequences have been submitted under GenBank accession numbers AY879184-AY879228.

Figure 3 represents the phylogenetic relatedness based on core sequences of the HBV/C strain from Eastern India. In this study, the core gene sequences were obtained from 19 HBV/C strain, 12 in this present study (accession no AY967430, AY967435, AY967442, AY967456-62, AY967465, AY967467) and 7 from previous study<sup>[17]</sup>. The phylogenetic tree was also constructed using the core gene sequences (nt. 1900 to 2350) of 39 reference sequences of different genotypes retrieved from GenBank along with 19 HBV/C strain from Eastern India. It was evident that all

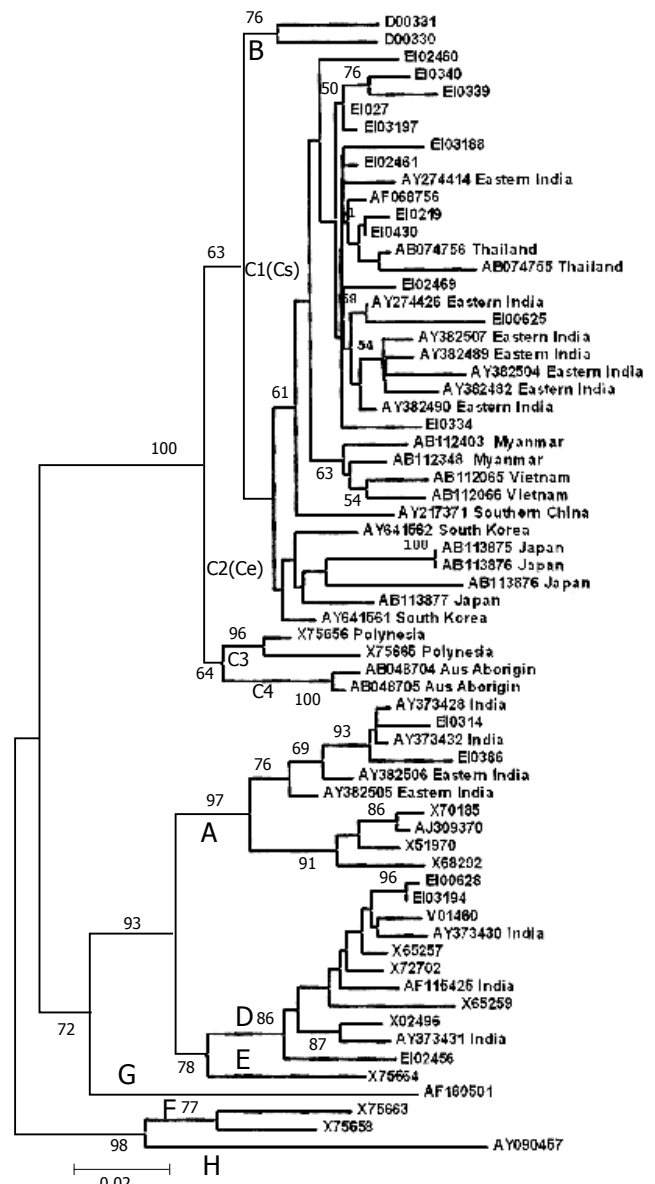


**Figure 2** Neighbor joining phylogenetic of S gene region (codon 35-180) of HBV isolates from eastern India (denoted with EI) along with other genotypes derived from Genbank.

the HBV/C strain isolated from Eastern India clustered with South East Asian strain (HBV/Cs) and also found to be most closely related to Thailand sequences.

**Mutation in S gene region**

Amino acid sequences of the part of S protein (codon 41-180) of 27 HBV/C isolates from our study were compared with sequences retrieved from GenBank. Subtyping of the HBV isolates was done on the basis of presence of amino acid residues at codon 122, 127 and



**Figure 3** Neighbor joining phylogenetic analysis using the core gene region (nt. 1900-2300) of HBV isolates from Eastern India (denoted with EI).

160 of the S gene region (Figure 4A and B). The sub determinant q<sup>+</sup> and q<sup>-</sup> was differentiated on the basis of amino acid residue at codon 177 and 178. Among the genotype C isolates 26 out of 27 isolates had amino acid Lys122 and Arg160 as well as Val177 and Pro178 indicating that they were adr<sup>q+</sup> subtype. However, only one isolate (EI01164) was classified as adr<sup>q-</sup> subtype on the basis of amino acid Lys122 and Lys160 and Pro127 present in the antigenic determinant.

Comparison of amino acid sequences showed that most of the sequences were conserved (Figure 4A and B). Consensus amino acid substitution at codon 53 and 126 was observed within HBV/Cs and HBV/Ce. Out of 27 genotype C sample studied 20 were shown to have Ser to Leu amino acid substitution at codon 53, which was also found in all genotype HBV/Cs sequence from the database. The remaining 7 samples had Ser at 53 codon, which was the characteristic of genotype Ce. Most of the samples from Far East (HBV/Ce) had amino acid Ile at

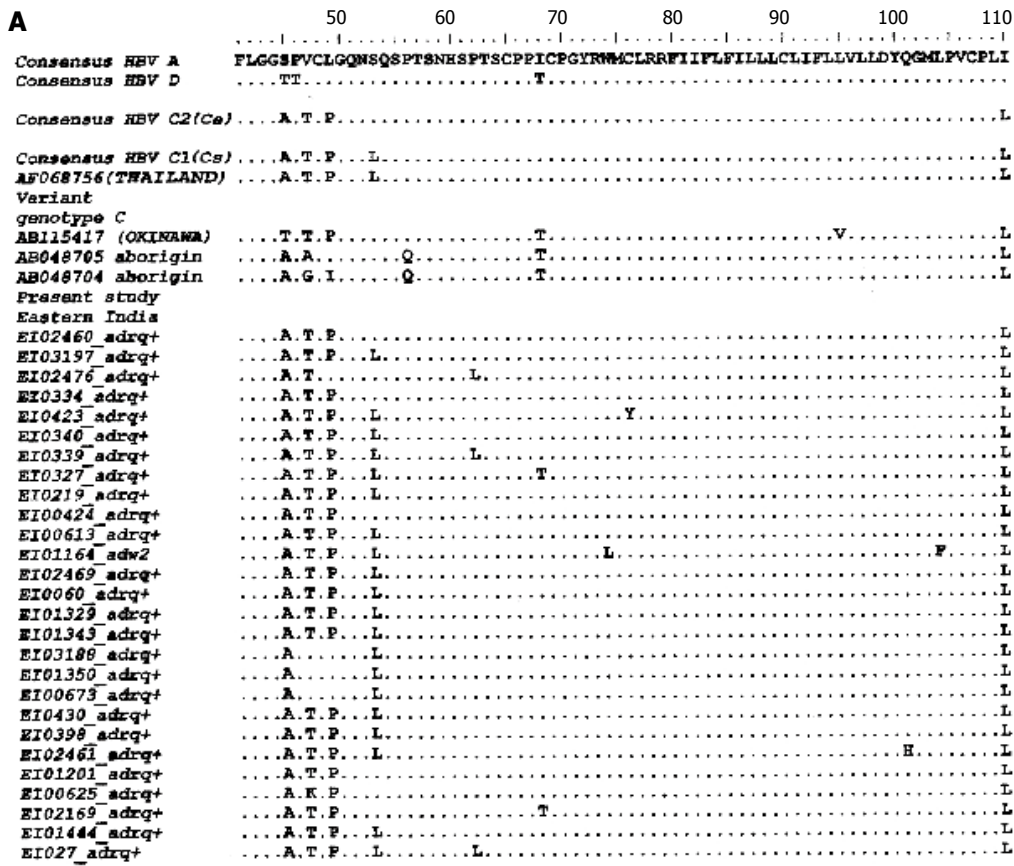
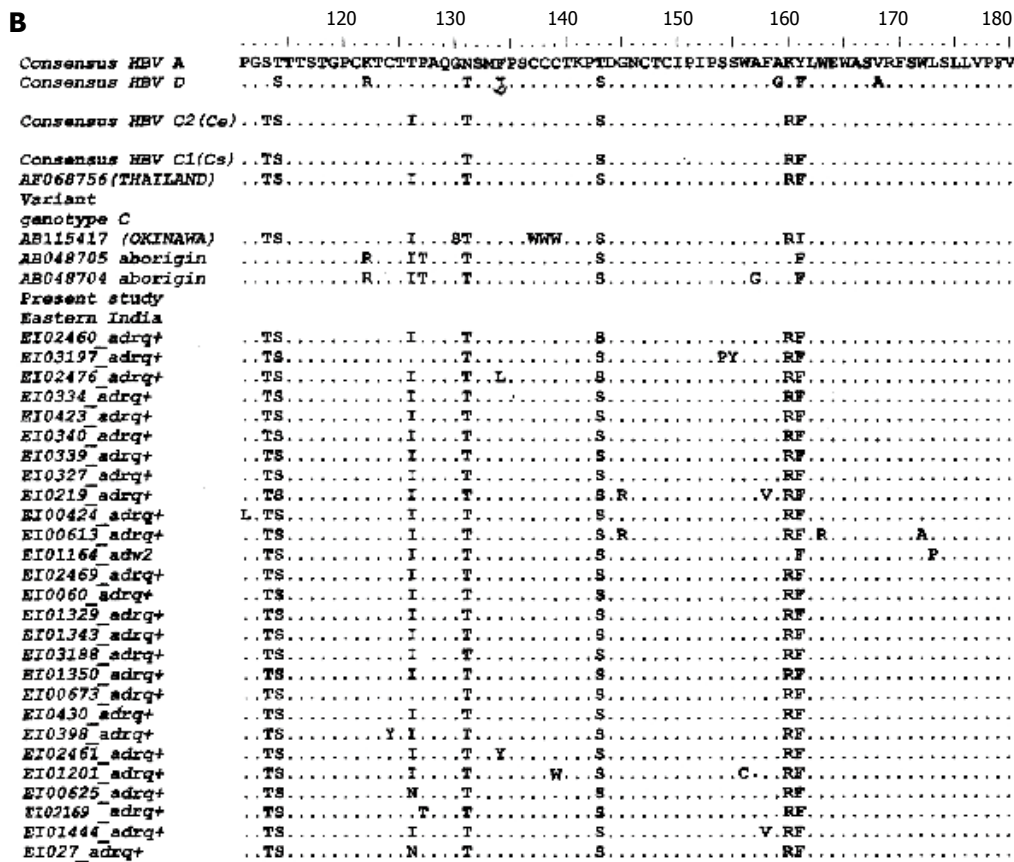


Figure 4 A, B: Alignment of amino acid sequences (41-180) of the partial S protein of HBV/C isolated from Eastern India.



codon 126, whereas most of the samples from Southeast Asia had either Asn or Thr at codon 126. However the isolate AF068756 from Thailand had Ile at codon 126. Major-

ity of the genotype C isolates studied by us also had Ile at codon 126. Only three isolates had Thr and two had Asn in that position.

## DISCUSSION

Except for the HBV sequences from two studies that focused on Western and Northern India<sup>[14,15]</sup>, there are hardly any HBV sequences available from our vast country. In this study, HBV DNA sequencing and phylogenetic analysis of S gene and core region established the presence of genotype C among the HBV carriers in addition to HBV/A and HBV/D from Eastern India. This is in contrast to Western and Northern India where genotypes D and A are prevalent<sup>[14,15]</sup>. Moreover, genotype C could be detected both from Kolkata as well as from the incidentally detected asymptomatic carriers from the rural population. Vivekanandan *et al* 2004 reported the presence of genotype C (by RFLP) only among chronic patients who went to their hospital for treatment from Eastern India. However, they could not detect HBV/C among their asymptomatic relatives who were incidentally detected HBsAg positive during blood donation. On the other hand, HBV genotypes found among patients who came from Southern India to that hospital were of genotype D excepting one case. However they have not characterized the molecular features of the genotype C strains of Eastern India. Taking into consideration their report, our findings, as well as other reports from Western and Northern India, it seemed likely that genotype C is at present confined mostly to Eastern India. Similar significant difference in the geographic distribution of HBV genotypes was recently reported from Japan and USA<sup>[24,25]</sup>.

Four subtypes adrq-, ayr, adw, adrq+ were associated with genotype C. Recently, association of serotype ayw3 was also found with genotype C variant, among Australian aborigines<sup>[9]</sup>. In our study, in addition to adrq+ subtype, one (EI01164) of the genotype C HBV isolates showed adw2 subtype. High prevalence of this adw2 subtype within genotype C was already reported from Tibet, East Asia<sup>[26]</sup> which borders Northern India. Genotypes A and D in our study group were of adw2 and ayw2, ayw3 subtype respectively which is consistent with the previous study reported from other parts of India<sup>[14,15]</sup>.

In places where well-known waves of migration have occurred over time, prevalence of HBV genotypes is known to reflect anthropological history of human migration, origin of immigrants and other patterns of migration. Thus, an apparent south-to-north gradient of genotypes C and B in Japan is considered to reflect anthropological history of migration from Asian countries to Japan<sup>[24]</sup>. In contrast, the presence of genotype D HBV among primitive tribes of Andaman and Nicobar islands is thought to be due to introduction of HBV from mainland India in the past century<sup>[27]</sup>. However, carrying the HBV/Cs strain among Jarwas (an isolated tribe from Andaman and Nicobar islands) correlate with the anthropological history of migration of this tribes from Southeast Asia<sup>[28]</sup>.

Population groups of Northern, Western and Eastern India ethnically are of Caucasoid origin, who speak Indo-European languages and show close genetic affinities with populations of Eurasia and Europe<sup>[29]</sup> where HBV genotypes A and D are prevalent. It is therefore not surprising that HBV genotype D and A is most predominant in India. On the other hand genotype C is predominant in countries

neighboring Eastern part of India, where the population groups (of Mongoloid origin) are believed to have originated from Tibeto-Burman language subfamily. Genetic study showed that the Bengali population group of Eastern India had close ethnic affiliation with Caucasoids<sup>[30]</sup> and formed clusters distinctly different from the North East population groups (ethnic affiliation to Mongoloid) who are possibly descendants of ancestral population of China, where genotype C and B are prevalent. Since our study population is not of Mongoloid origin, therefore it is quite unlikely that presence of genotype C in Eastern India reflects the history of population migration long ago.

This is further supported from the fact that when the sequences from the present study were compared to GenBank sequences in a BLAST search, the best matches and the high-scoring matches were from Southeast Asian Countries, especially from Thailand and not from China. Phylogenetic analysis both from Surface and core gene also revealed that almost all sequences from our study group clustered with Southeast Asian HBV/Cs subgroup and not with HBV/Ce found in East Asia and predominant in China. Of the 27 genotype C isolates sequenced, 24 (90%) had percent nucleotide identity (PNI) of 99.5%  $\pm$  0.4%, with Thai genotype C sequence (Accession No. AF068756), in the S gene region.

There are several reports available where low genetic diversity of HBV strains has been considered to suggest limited and relatively recent spread of the strain over the geographical region. Arankelle *et al*<sup>[27]</sup> reported a recent introduction of the virus, on the basis of low genetic diversity of partial S gene (PNI varied from 1.6% to 2.0%), of genotype D isolates from mainland India to tribal population of Andaman and Nicobar islands. For similar reason, HBV/E was considered as relatively recent introduction in West Africa<sup>[31]</sup>. Thus considering low genetic diversity of HBV/C strain in Eastern India with Thai sequences, we presumed that the genotype Cs in Eastern India possibly might have spread from Southeast Asia, particularly from Thailand, rather recently. It is noteworthy that the prevalence of genotype C is much higher in urban population (20% *vs* 6.7%) than in rural population. Moreover, genotype C HBV samples have not been reported from Northern and Western parts of India<sup>[14,15]</sup>. In the Southern Indian Tertiary hospital<sup>[18]</sup>, genotype C was detected in a significantly higher proportion of patients from Eastern India compared with those from Southern India (16.8% *vs* 0.9%,  $P < 0.0001$ ). This selective confinement of genotype C to Eastern India especially to the urban population suggests that perhaps sufficient time has not yet passed since its introduction to Eastern India for its subsequent spread to the rest of the country. However, we must admit that this introduction of genotype C in Eastern India would have been best addressed if sequential study showing changing HBV genotype pattern could have been documented in Eastern India.

Kolkata is the most important port city of Eastern India and proximal to Southeast Asia. Increased trade relationship with Thailand suggests that spread of genotype Cs to Eastern India is possible, but the infectious source and the route of transmission are not clear. Previous reports showed presence of Thai HIV strains as well as Thai

HCV strains<sup>[32,33]</sup> from North Eastern and Eastern India. Heroin trafficking routes have been associated with IDU and HIV infection in Thailand, Myanmar, China and India<sup>[34]</sup>. HBV and HIV share common modes of transmission. There is possibility of co transmission of HBV from Southeast Asia to Eastern India, *via* this route. This might have been followed by spread to general population via unsafe injection practices, which is common in developing countries including India<sup>[35,36]</sup>. The presence of HBV/Cs is known in our neighboring country Bangladesh, however the proportion of HBV/C among HBV carriers in Bangladesh, is unknown<sup>[10]</sup>. People from Bangladesh come to Kolkata for treatment. This population might also have some influence in the presence of genotype C in Kolkata. Whatever may be the source, this study confirmed the presence of Cs in the Eastern Indian population.

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<sup>[37]</sup> or replacement of one genotype by another<sup>[38]</sup>. HBV genotype C is associated with delayed hepatitis B e antigen (HBeAg) seroconversion<sup>[39]</sup>, more-active hepatitis<sup>[40]</sup>, lower response to antiviral therapy<sup>[41]</sup>, more advanced liver disease and a higher risk of hepatocellular carcinoma<sup>[42]</sup>, compared with HBV genotype B. Furthermore, HBV/C with T1762/A1764 mutation in BCP region has been reported to be an increased risk factor for hepatocellular carcinoma<sup>[13]</sup>. In our study, at least 45% of the subjects were in the age group of 16-30 years. In addition, 29/42 (69%) of genotype C isolates had T1762/A1764 mutation in BCP region, most of them associated with elevated ALT. Thus, the patients infected with genotype C need to be carefully monitored to assess their clinical outcome in future.

In conclusion, the present study reports the genotypic distribution and partial genome sequences of HBV/C isolates from Eastern India in addition to most predominant HBV/A and HBV/D. These isolates clustering with HBV/Cs genotype found in South East Asia, is prevalent in considerable proportion (18%) of Eastern Indian HBV carriers. Low genetic diversity and confinement of HBV/C in Eastern India possibly indicates a recent, limited, spread of HBV/Cs in this region. Several studies have suggested that HBV genotype C is associated with more active or severe sequelae of liver disease in Southeast Asia compared with genotype B. Therefore, the presence of HBV genotype C in Eastern India should be carefully monitored by further studies including epidemiological, clinical and virological assessment.

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