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Correspondence

Detection & characterization of genotype A1 of hepatitis B virus from central India

Sir,

Despite the availability of effective vaccine, about 1.5 million new hepatitis B virus (HBV) infections are documented annually across the world[1]; further, it is estimated that around 296 million people are living with chronic hepatitis B infection, leading to 820,000 fatalities in 2019[1].

HBV belongs to *Hepadnaviridae* family, and it has ~3.2 kb partially dsDNA that codes for three structural (HBsAg, HBcAg and Pol) and two non-structural proteins (HBeAg and HBxAg)[2]. Owing to low fidelity rates of reverse transcriptase, high selection pressure, use of antiviral drugs *etc*. the virus displays high genetic diversity[3]. Based on nucleotide diversity of eight per cent among HBV genomes, the virus is divided into 10 different genotypes, which are further divided into different sub-genotypes with 4-8 per cent differences[4]. HBV is also classified into distinct sub-types, based on amino acid positions in surface antigen[5].

Distribution of genotypes shows geographical patterns across the globe and within the countries[4]. Though limited, molecular studies have documented circulation of genotypes D, A and E in northern, genotypes D and A in western, genotype D in eastern and genotypes D, A and C in southern parts of India, with overall dominance of genotype D[6]. Rarely, genotypes such as B, C, E and I are also reported[7]. The infecting genotype and the sub-type are known to influence the disease progression, treatment response and clinical outcome[4]. Moreover, it has been shown that HBV-vaccinated individuals respond unequally to different genotypes[3]. These facts make it important to monitor circulating genotypes and sub-types in different areas.

In the past, the circulation of genotype D sub-genotype D1, D2, D3 and D5 has been documented from central India[6]. During the routine molecular surveillance at Jabalpur, central India, a patient infected with HBV genotype A was detected, and the objective of this study was to characterize this genotype detected from central India.

The State-level Virus Research and Diagnostic Laboratory (VRDL) at the ICMR-National Institute of Research in Tribal Health, Jabalpur, India, has mandated to provide diagnosis and monitor viruses of public health importance circulating in central India. Samples referred along with clinical information are tested following syndrome-based approach. To detect hepatitis B infection, the serum samples of patients suspected of viral hepatitis are subjected to HBsAg ELISA using commercially available kit (J. Mitra & Co., New Delhi, India; Cat. No. IR020096). Positive samples having adequate volume and sufficient clinical information are subjected to partial *S* gene PCR and sequencing for the identification of genotype as described by Gandhe *et al*[8].

In this prospective study (January 2020 to December 2021), during routine surveillance work, one sample sequence showed that it belonged to genotype A. Full genome amplification of this sample by PCR in ABI GeneAmp 9700 PCR-Thermal Cycler, USA, using six sets of overlapping primers. The sequencing and phylogenetic analysis was done as described earlier was carried out by Shivlata *et al*[6]. The HBsAg sub-typing was done as described by Purdy *et al*[5]. For phylogenetic analysis of HBV full genome sequences, 48 reference sequences of different genotypes from A to J and subgenotypes of A (A1-A7) were retrieved from GenBank database and a phylogenetic tree was constructed by employing the Neighbor Joining method in MEGA (Molecular Evolutionary Genetics Analysis) software version 5.05 by applying 1000 bootstraps to determine genotype/subgenotype. The full genome sequence was submitted to GenBank (Accession Number OK274310).

This study was approved by the Institutional Ethics Committee (No: NIRTH/IEC/2229/2018) and written informed consent was obtained from all the patients.

The Basic Local Alignment Search Tool analysis of the full genome sequence revealed that the HBV detected belonged to genotype A, sub-genotype A1 (Figure); based on the amino acid positions in S protein, the sub-type of the sample was found to be *adw2*. The sequence showed 99.38 per cent similarity with sequence submitted from Dhaka, Bangladesh (LC519823), 99.32 per cent from Kolkata, India (KT366471.1) followed by sequences from Arunachal Pradesh, India (KF214663.1): 99.10 per cent; New Delhi, India (AY373432.1): 98.60 per cent; Lucknow, India (KT151615.1): 98.67 per cent.

The full length sequence analysis showed several nucleotide and amino acid substitutions of clinical importance (Table). Clinically relevant mutations were observed in the basal core protein/pre-core region of HBV sequences. Mutations occurring in enhancer 1 X promoter region and S1 region were also determined. Interestingly, mutations such as T1041G, A2720G and T3111C which are sub-genotype A2/A3[9] specific were found in the sequence. On analysis of protein sequence of the open reading frames (ORFs), it was observed that many nucleotide substitutions showed their effect at amino acid level (Table).

The patient, a 65 yr old male a native of central India, presented to the department of General Medicine, Netaji Subhash Chandra Bose Medical College & Hospital (NSCBMC & H), Jabalpur, with complaints of fever, abdominal distension with abdominal pain, jaundice and melena over one month. He had not travelled to any other States in the last six months. Clinical examination revealed moderate pallor and icterus. His abdomen examination revealed a significantly distended abdomen, with subcutaneous abdominal wall oedema with a positive fluid thrill. On ultrasonographic assessment, liver echotexture was found to be altered suggestive of liver cirrhosis, along with gross ascites and bilateral mild pleural effusion. There was no evidence of hepatocellular carcinoma (HCC) in the patient. Blood investigations revealed an increased ALT (alanine aminotransferase) level of 162.7 U/l with total bilirubin level of 3.87 mg/dl with direct bilirubin of 1.87 mg/dl, and total protein levels were found to be 6.41 g/dl. Persistent viraemia was evident with an increased HBV DNA viral load of 1×10^8 IU/l (tested commercially using kit from Altona: AltoStar® HBV PCR Kit 1.5). Serologically, the patient was negative for HBeAg but positive for anti-HBe antibody, indicating an immune reactivation phase with no virological remission. Complete blood count revealed decreased haemoglobin levels of 8.7 g/dl with a total leucocyte count of 10,300/µl. Serum creatinine was found to be within normal range (1.15 mg/dl). The patient succumbed to the complications of decompensated liver disease after one month of treatment; therefore, the effects of the detected mutations could not be studied further.

This study documents the detection and molecular characterization of genotype A sub-genotype A1 from central India. Earlier studies from this region have documented detection of various sub-genotypes of genotype D[6]. Genotypes A-F are reported from different parts of India; however, genotype D is reported as the most dominant genotype[18]. Genotype A infection is shown to be associated with more severe liver disease than genotype D[19]; the detection of this genotype from the area thus has clinical and epidemiological importance. The patient had no travel history to HBV A1-endemic areas, suggesting that he acquired the infection in the region. Circulation of subtype *adw2* of genotype A1 has been documented from south and north as well as east and west India[8,18,20,21], and our findings are in agreement with those studies.

The genotype A/A1 is known to have clinically important characteristic mutations throughout its genome. We detected various nucleotide and amino acid substitutions/mutations of clinical and epidemiological significance in enhancer I X promoter, basal core promoter, pre-core, core, S1 promoter, RT region, X protein, pre-core and core protein. Important ones are given in the Table.

Our sequence showed the presence of sub-genotype A1-specific pre-core kozak sequence 5'-TCAT-3' at position 1809-1812 when aligned to the reference sequence[9]. However, we detected guanine at 1041 (in the enhancer I X promoter region) instead of thymine which is generally found in genotype A3[9]. Further, two genotype A2-specific mutations (A2720G and T3111C) were also found in our sequence. We also detected mutations G1467A and C1674T in the enhancer I X promoter region reported to cause low-gene expression and increased apoptosis, respectively[10-12], which was observed in this case. The mutation in the basal core region of G1862T (that is a

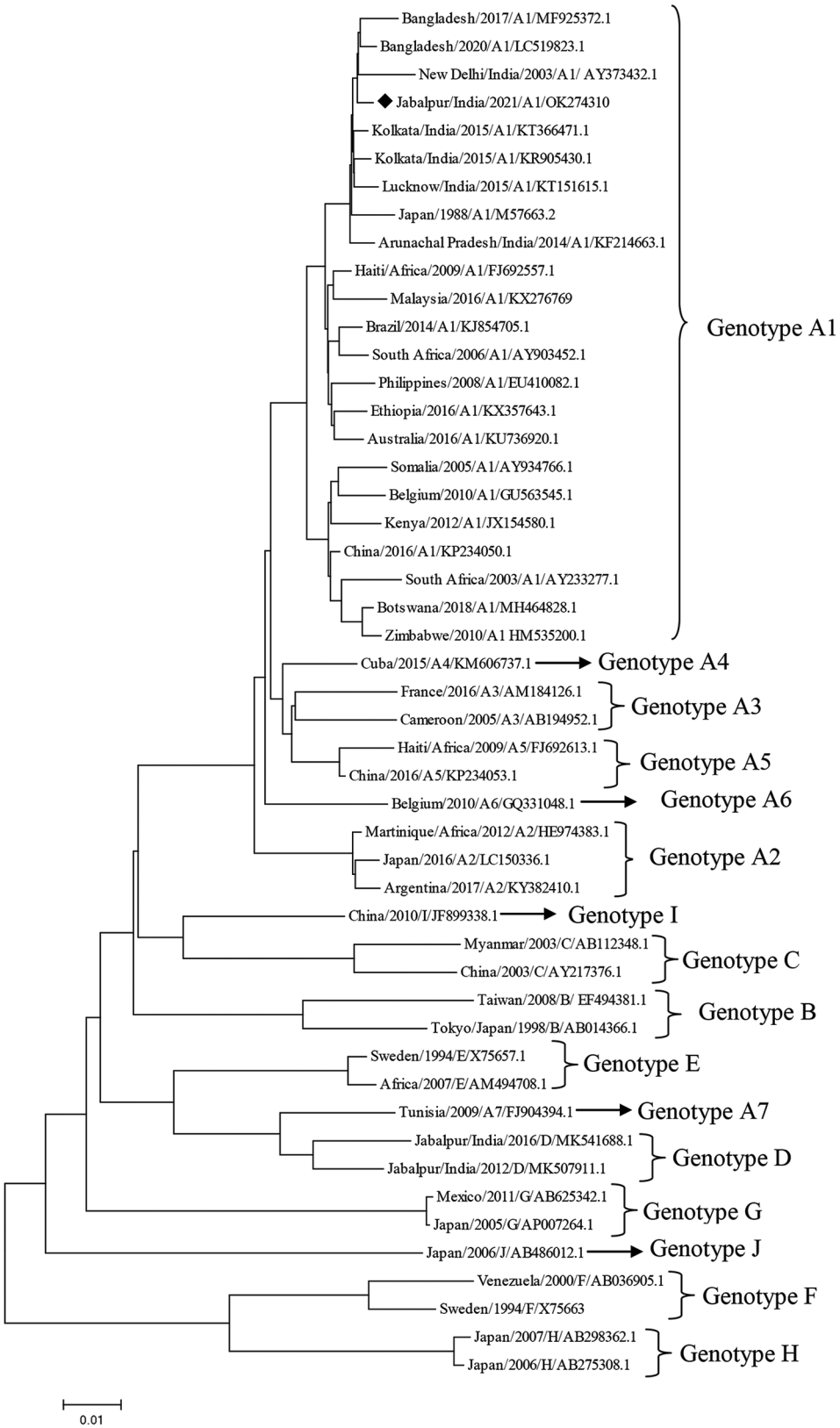

Figure. The phylogenetic tree of HBV sequence detected from central India (marked with ◆: OK274310) was constructed using the neighbour-joining method in MEGA5. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Pattern of labelling City/Country/Year/Genotype/Accession Number. HBV, hepatitis B virus.

Table. Nucleotide and amino acid substitutions detected in a 65 yr old male residing in central India

Nucleotide substitutions		
Region	Nucleotide substitution	Significance
Enhancer I X promoter	T1041G	Sub-genotype A3 specific mutation[9]
	G1467A	Causes A at 31 position of X protein, that results in low gene expression, immune surveillance escape, apoptosis reduction and thus progresses to HCC[10,11]
	C1674T	Causes P101S in X protein[10]
		Increases the expression of *p21* gene, reduces cell cycle and increases apoptosis[12]
Basal core promoter/ pre-core mutation	G1862T	Responsible for causes fulminant hepatitis[3,13]
Pre-core mutation	G1888A	Results in additive decreased expression in HBcAg when G1862T and G1888A occurred together[14]
Pre-core/core mutation	C2078G	Results in limited virion secretion, yet maintaining intracellular DNA replication[15]
S1 promoter	A2720G	Presence of 'G' at position 2720 which has been considered as sub-genotype A2 specific[9]
		Affects *HNF-1* binding site
	T3111C	Presence of 'C' at position 3111 which has been considered as sub-genotype A2 specific[9]
Amino acid substitutions		
Region	Amino acid substitution	Clinical significance
RT region	A7D	Ismail *et al*[16], have reported this mutation, but clinical significance is not clear
	H126Y	Novel mutation associated with drug resistance, presumed to be associated with tenofovir resistance[17]
X region	S31A	Prevalent in patients suffering from HCC[11]
	P101S	Increases the expression of *p21*, reduces cell cycle and increases apoptosis[12]
Pre-core/core protein	V17F	Prevents the synthesis and secretion of HBeAg[13]
Core protein	L60V	Causes significant reduction in secreted virions containing normal or mature genomes with relaxed-circle DNA form[15]

HCC, hepatocellular carcinoma; HBcAg, hepatitis B core antigen; RT, reverse transcriptase; HBeAg, hepatitis B envelope antigen

presumed cause resulting in fulminant hepatitis) was detected in the sequence[3]. The mutation G1888A in pre-core region responsible for decreased expression of HBcAg, was detected.

At amino acid level, the sequence showed a mutation H126Y in HBV pol region which is associated with tenofovir resistance[17]. Mutations in the X region (S31A) responsible for HCC[11] and increased apoptosis (P101S)[12] were detected; however, our patient did not have HCC. The amino acid change at position 17 of pre-core region from valine to phenylalanine results in low or no synthesis of HBeAg[13], and this mutation was present in the sequence and was the probable reason that the patient was serologically negative for HBeAg and anti-HBeAg antibodies positive.

The sequence reported in the study showed fewer number of mutations compared to the mutations reported from south[16], north[9] and east[10] India, probably because the virus is not under high immunological and antiviral pressure owing to low circulation in the area. Further studies with larger sample size and full genome next generation sequencing will help shed more light on not only on these aspects but will also help understand if there are any quasispecies complex as described from African country[22] in genotype A specific to this region.

This study was based on single sequence and this was a major limitation of the study; however, the study reported maiden detection and detailed full genome characterization of HBV genotype A1 from central

India and a full genome analysis of HBV genotype A1. Continuous molecular surveillance is required to keep a track of emerging genotypes and the mutations occurring in a region which will help public health programme and treating clinicians.

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