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Cross sectional study on the molecular detection of Hepatitis B virus in patients with chronic liver disease in Guntur, India revealed mutations in Genotype A of occult HBV

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ABSTRACT

Introduction: The present study was aimed at the molecular detection of HBV infection among patients with chronic liver disease (CLD) and to study the mutation spectrum S-gene region in the occult HBV strains.

Materials and Methods: An observational study conducted on 200 clinically diagnosed CLD cases enrolled from the Gastroenterology unit. Two hundred serum samples were screened with Fibroscan and for HBsAg, HBCT and estimated liver enzymes. Samples positive for either HBsAg or Anti HBc total were further evaluated for other HBV markers and presence of HBV DNA. The S region was amplified in occult CHB cases and sequenced.

Results: 70.5% (141) of the patients with CLD were suffering with Chronic Hepatitis B virus (CHB). The HBV DNA was detected in 19.14% patients. Four cases were in occult phase. Two samples of them could be amplified. The isolates belong to the genotype A, and revealed certain mutations leading to novel amino acids.

Conclusions: Mutations in the S gene sequences of HBV virus from the Occult HBV infection (OBI) patients would lead to immune escape or cause occult infection. In the Genotype A at two positions, amino acids that are likely to cause occult infection are observed apart from mutations at certain positions leading to novel amino acids.

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1. Introduction

Hepatitis B is a potentially fatal infection caused by the Hepatitis B virus (HBV), and despite the availability of an effective vaccination, it remains a significant worldwide health problem. According to World Health Organisation estimates, 296 million individuals are infected with chronic hepatitis B in 2019. Many patients with acute infections resolve or move silently to chronic infection, which frequently goes unreported until the virus causes considerable liver damage. Chronic liver disease, such as cirrhosis, liver failure, or liver cancer, affects majority of CHB patients and is associated with considerable morbidity and mortality.[1] The stealthy progression to chronic hepatitis

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is known as 'occult hepatitis,' and in this scenario, molecular tests are positive but conventional testing for Hepatitis B surface antigen is negative.[2]

Hepatitis B surface antigen (HBsAg) has a highly conformational hydrophilic immunodominant region "a" determinant that is targeted by antibodies in serological testing. The HBsAg produces anti-HBs antibodies that are mostly directed to the central portion of the HBsAg known as the "a" determinant. Mutations impact the "a" determinant, resulting in false-negative HBsAg in commercial assays as well as evasion of vaccine-induced immunity, creating a diagnostic quandary.[3] The HBsAg is detectable 6-10 weeks after virus introduction using ELISA, immunochromatographic assays, which is the mainstay in the diagnosis of Hepatitis B infection. After 4-6 months of HBV infection resolution, anti-HBs levels rise as HBsAg decreases. Anti-HBc (anti Hepatitis B Core antigen), which is detectable during both the acute and chronic stages (IgM anti-HBc and IgG anti-HBc, respectively), is used in the diagnosis of HBV in addition to HBsAg. Another marker is the HBeAg (Hepatitis B envelope antigen), whose presence is invariably detectable by commercial immunoassays during acute HBV infection six to twelve weeks after HBV exposure and is associated with high viral transmissibility, infectivity and active viral replication. HBeAg clearance is accompanied by concurrent seroconversion to anti-HBe. An important point to keep in mind is that an occasional negative HbsAg test cannot rule out an active HBV infection. Pre-S/S gene mutations can be seen in the context of CHB infection.[4] Reactivation of the disease in chronic HBV carriers, inability to detect HBsAg by immunoassays, and evasion of the established protective anti-HBs response are all linked to mutations in the S gene. Patients with 'occult' HBV infection are those who test negative for HBsAg yet test positive for HBV DNA, whether or not they have HBV antibodies. The majority of isolates had a mutation that changes aspartate to alanine at residue 144 (sD144A) or glycine to arginine at codon 145 of HBsAg (sG145R).[4]

In the current study, the prevalence of HBV infections were estimated from the clinical cases and the S-gene was molecularly characterised in order to determine the prevalence of HBV infection among chronic hepatitis patients and to explore the mutation spectrum in occult HBV strains.

2. Materials and Methods

2.1. Ethics

A prospective observational cross-sectional study was conducted from 2018 to 2020 in the study area. A total of 200 clinically diagnosed Chronic liver disease cases from the Gastroenterology unit of the Hospital were included in the study. The study has been approved by the Institutional Ethical Committee. After obtaining informed consent relevant demographic, clinical data was recorded from the participants.

2.2. Sample collection

Approximately 10 ml of blood was collected. The Serum was separated and aliquoted in 1 ml into 3-4 screw cap tubes, stored one aliquot at -20^{0}C and remaining at -80^{0}C until testing.

2.3. Sample screening

Fibroscan Transient elastography (Echosens, Paris, France) was performed for all the patients to measure the liver stiffness and the extent of fibrosis in kilo Pascal's (kPa), to assess the liver pathogenesis. Fibroscan results are staged F0 to F4.[5] The serum samples were screened for HBsAg and HBc Total antibodies (HBCT) using Enzyme Linked Fluorescent Assay (ELFA) (Minividas, Biomerieux, 30315-HBS, 30314-HBCT). Simultaneously all serum samples were tested for liver parameters namely bilirubin, Aspartate Aminotransferase, Alanine aminotransferase, Alkaline Phosphatase as per manufacturers instruction (Beckman coulter AU 480 wet chemistry analyser). The serum samples that were positive for either HBsAg or Anti HBc total were further evaluated for HbeAg, Anti Hbe, Anti HBc IgM, Anti HBs antibodies using ELFA and CLIA (Chemiluminescent immunoassay) methods and also were subjected to HBV DNA testing. The viral DNA was extracted by the spin-column based method (Qiagen DNA extraction kit, AIAge, Germantown, MD 20874) and the HBV DNA was eluted in 40 μl. The elutes were stored at -20^{0}C until further testing.

2.4. Statistical methods

Chi-square test is performed to test the significance among proportions.

2.5. Molecular testing

The HBV DNA was detected using Altostar® HBV PCR Kit 1.5 (Altona diagnostics, Germany), which is a real-time PCR based method, as per manufacturer's instructions. The HBV DNA Positive and HBsAg negative samples were then amplified using conventional PCR for the region spanning the PreS1/PreS2/S. The primers were designed using the Hepatitis B virus isolate FR_BV_248 (Accession No MZ097809). The Forward primer is 5'- GTGGGTCACCATATTCTTGG -3' and the reverse primer sequence is 5'- GAGAGTAACCCCATCTCTTTG-3' resulting in 1239 bp product. The amplicon was then sequenced using sanger method of sequencing at Barcode Biosciences, Bangaluru. The sequences were aligned using MEGA 6 program and the mutations pertaining to immune

escape and Occult HBV infection (OBI) that were reported in the literature[6]. Cluster analysis is performed using A to H genotypes of HBV to identify the genotypes.

3. Results

3.1. Prevalence of HBV infections

Among the 200 samples, 101 (50.5%), 111 (55.5%) were detected positive for HBsAg using rapid Immuno chromatography test (ICT) and by Immuno fluorescent assay (IFA; Vidas Biomerieux) methods respectively and the remaining are negative for HBsAg. A total of 131 (65.5%) samples were positive for HBc Total (HBCT) of which 30 (15%) cases were negative for HbsAg. Majority of the cases of Chronic Liver Disease are male patients (71.5%) and 28.5% are female patients. Among these hepatitis B infected males were 49.5% and females were 21%. Out of 200 CLD cases, 56 (28%) were in the age group of 21-30 years, followed by 47 (23.5%) cases in 31-40 years, 44 (22%) cases in 41-50 years, 28 (14%) cases in 51-60 years, 18 (9%) of cases were above 60 years. Only 07 (3.5%) cases were in the age group of 11-20 years and no case was in less than 10 years. The age distribution among the CHB was 30.5% (43) in 21 to 30 years, 21.3% (30) in 31 – 40 years, 21.9% (31) in 41-50 years and 14% (20 cases) in 51-60 years. Majority of cases (73.75%) were observed to be in the range between 21 and 50 years (Figure 1). The mean age of the CHB patients was 40.08 years.

3.2. Liver enzymes during HBV infections

Elevated liver enzymes were noted in 48.5% (n=97) of which 54.6% were positive for hepatitis B. Among those with normal enzyme levels (51.5%), 88 (85.4%) were cases of Chronic Hepatitis B. Fibroscan results of CLD cases showed 82 (41%) in F0, F1 with no or minimal scarring, 28 (14%) in F2 with moderate fibrosis, 16 (8%) in F3 with severe fibrosis and 68 cases (34%) in F4 with cirrhosis or advanced fibrosis. Among the hepatitis B cases 48.9% (69) were in F0/F1 stage, 17.7% (25) in F2 stage, 7.09% (10) in F3 stage, 23.4% (33) in F4 stage. More number hepatitis B cases were seen in F0-F1 stage.

3.3. Serological markers

Of the 200 cases, HBsAg could be detected in 111 (55.5%) while 131 (65.5%) were positive for HBCT(Anti HbcTotal). Among 141 CHB patients 101 were positive for both HBCT, HBsAg whereas 10 patients were negative for HBCT but HBsAg positive (total= 111). 30 patients with CHB were positive for HBCT but negative for HbsAg. Among 141 CHB patients, 25.5% (n=36) were positive for anti HBs antibodies while 74.5% were negative. Anti HBs antibodies were detected in 14 of the HBsAg positive cases and not detected in other 97 cases. HBe antigen was positive in 4.3% (6), anti HBe antibodies were detected in 95% (n=134) and HBc IgM antibodies were present in 11.34% (n=16) of CHB cases (n=141).

3.4. HBV DNA detection

Out of 141 CHB patients HBV DNA was detected in 19.14% (27) by Realtime PCR assay. Out of 27 DNA positive samples 4 samples were of occult CHB with HBsAg negative but viral DNA detectable by Realtime PCR assay. These four samples were subjected to conventional PCR using primers spanning for S gene, only two samples could be amplified using conventional PCR. Both the samples were sequenced and the sequences are identical. Phylogenetic analysis of S gene sequences of known genotypes and the sequences were found to be grouped together with genotype A (Figure 2). The sequence in the present analysis vary at 7 positions, in comparison with the genotype A (Accession No. LC533934) (Table 1), of which 6 were synonymous and variation at 7^{th} position i.e 534 is altering the amino acid from G to A, and likely to affect protein function.

Majority of the amino acid positions in the S gene in the present study are wild type amino acid with respect to immune escape.[6] However, the present study noted 13 novel mutations that neither belong to wild type or mutant type (Table 2). Similarly, among the amino acids that can cause occult HBV infection, in two positions, mutant amino acids are observed, and in 14 positions, novel amino acids are noticed (Table 3).

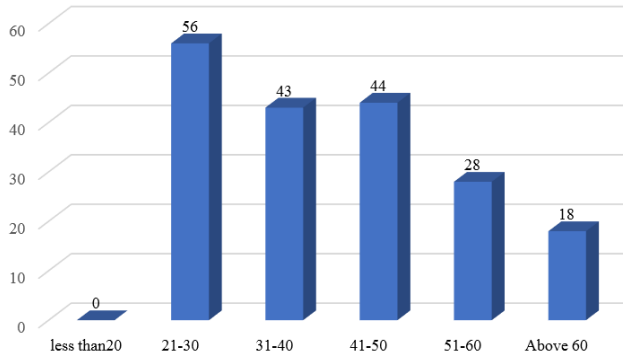

Figure 1: Age-wise distribution of the chronic liver disease cases

4. Discussion

The rapid ICT method is a routine method of screening of HBsAg in majority of the laboratories as compared to IFA. Both the methods showed minor difference and the differences are not statistically significant (P<0.01). Only 10 cases were false negative by the ICT method. The false negative results based on rapid test are attributed to inadequate coating of the antigen, different nature of antigen

Table 1: Variations in the sequences

Nucleotide position	12	87	297	438	497	522	534
LC533934	T	A	C	T	G	T	A
CLD72	C	T	T	C	C	C	C
CLD101	C	T	T	C	C	C	C

Table 2: Status of known immune escape mutations in amino acids[6]

	CLD72	CLD101	Mutation Type
Y134H/N	F	F	N
S143L	T	T	N
L108V/I	P	P	N
T87I	L	L	N
I84T	L	L	N
A91T	L	L	N
T68V	I	I	N
S78N	R	R	N
V60A	H	H	N
T31I/T	S	S	N
P34N/T	S	S	N
G31E	S	S	N
Q2K/B	E	E	N
E164D, T116N, P120S/E, I/T126A/N/I/S, Q129H/R, M133L/T, K141E, P142S, D144A/E, G145R/A, M103I, L109I, T118K, P120A, S171F, C48G, V96A, L175S, G185E, V190A, T140I, G130N, F134I			W

Foot Note: The mutation type 'N' indicates novel and 'W' indicates wild type.

Table 3: Status of known OBI mutations in amino acids[6]

	CLD72	CLD101	Mutation Type
I126S/T	**T**	**T**	M
S114T	T	T	M
R122P	K	K	N
S143L	T	T	N
I126S/A	T	T	N
S78N	R	R	N
L108I	P	P	N
A90V	C	C	N
Q118L	T	T	N
T87S	L	L	N
A90T	C	C	N
KL45F	S	S	N
P47T	V	V	N
N56T	T	T	N
G73E	R	R	N
P94S	L	L	N
Y100S, Q101R, P105R, T115N/A, T116N, P120L, T123N/A, P127H/L, Q129P/R, M133T, S167L, R169H, G145R/A, C124Y, D144A, G119R, C124Y, C139R, I110L, S117N, P127S/T, M133T			W

Foot Note: The mutation type 'N' indicates novel and 'W' indicates wild type.

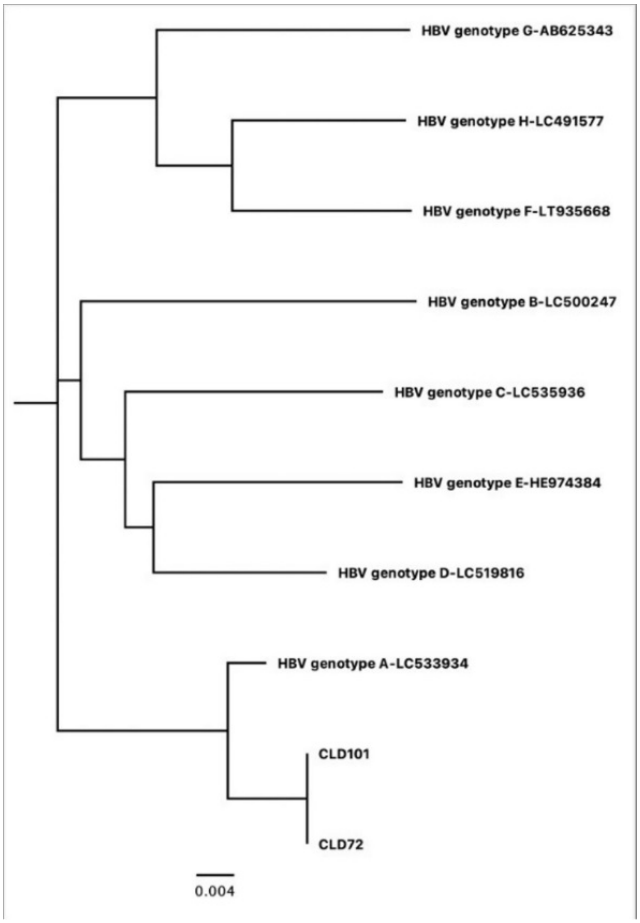

Figure 2: Phylogenetic position of the isolates with respect to different known genotypes of the HBV

used and genetic heterogeneity of the virus prevalent in that area.[7,8]

The cases which were negative for HBsAg but positive for HBCT (n=30) could be either that the infection resolved in these HBsAg negative patients or the patients are in occult phase. All together there is high prevalence of hepatitis B infection among chronic liver disease cases (n=141, 70.5%). A multicentric study from India identified that HCV was the commonest cause of liver disease in the northern region with prevalence of 44.9%, and HBV was predominant with 47.9% and 40·6% in eastern and southern regions, respectively.[9]

Male preponderance was noted among Chronic liver disease patients as well as those infected with Hepatitis B ($P<0.05$). Several other studies also noted similar finding that chronic hepatitis B infection was more prevalent among males.[10–12] Exposure to risk factors like alcohol, sexual behaviour is relatively high in males is attributed for these differences. Age has a significant effect and most susceptible age group is 21-50yrs. Low prevalence of chronic hepatitis B infection (3.5%) was noted in the subjects below 20yrs. This observation could be due to successful implementation of national HBV immunization program in India since 2010, increased awareness, availability and use of disposable devices for invasive procedure. In a systematic review and meta-analysis[13,14] a significant decline in HBsAg prevalence was observed in the more recent birth years.

Liver function tests alone are not good markers for predicting CHB. If only liver enzymes levels are monitored to initiate antiviral therapy, a considerable number of patients will probably lose the opportunity to receive therapeutic interventions. It was demonstrated that the liver function markers exhibited a marked variation among patients infected with HBV and majority of the CHB patients were processing with mild liver fibrosis.[15–17] In contrast to HCV infection, CHB infection, a threshold exists with approximately $10^3$to 10^4IU/ml HBV DNA, below which liver injury is negligible or absent.[18] Hence, assessment of fibrosis using non-invasive fibroscan will help in staging the disease and choice of treatment which is also crucial for prognosis, and surveillance.

Analysis of the clinical stages among CHB, in the present study, 5 cases (3.5%) were in Immunotolerant phase, 9 (6.38%) in immune active phase, 65 (46.1%) in immune inactive phase and 4 cases in OBI phase. In OBI phase, HBeAg negative CHB is common and hence in the clinical practice, it has become more cognizant to detect HBV-DNA for the diagnosis.[19] In addition, the OBI patients in CHB will have detectable anti HBc with or without anti HBs antibodies.[20] In this study, the OBI patients were identified with HBsAg negative, HBeAg negative, anti HBe positive, with or without HBc IgM and detectable DNA. The presence of HBV-DNA in the serum or plasma can be utilized to determine prognosis and direct HBV treatment as it indicates an active HBV infection. Hence the patients with chronic liver illness should undergo a HBV DNA testing to identify OBI infection who otherwise may be missed if tested for HBsAg alone. The four OBI samples were subjected to conventional PCR using primers spanning the S- gene of the virus but only two samples could be amplified. In chromic HBV patients, after the loss of HBeAg in the serum, the HBV levels become low and not detectable by conventional PCR.[21] It has been established that the mutations in the S- gene sequences of HBV virus from the OBI patients would lead to immune escape.[6] In the present study, both the sequences didn't show known immune escape amino acid variants but revealed certain novel mutations. It would be interesting to establish functional role of these novel mutations in those positions in the Genotype A. In addition, at two positions, amino acids that are likely to cause occult infection are observed apart from mutations at certain positions leading to novel amino acids (Table 3). Further, identification of patients infected with the mutations in S gene would be useful in preventing them

from blood donation, in order to prevent from transmission of these variants.

5. Conclusions

The present study showed 70.5% of HBV infection in patients suffering from CLD. Based on S-gene sequence, the HBV genotypes in the present study belong to Genotype A. Mutations in OBI patients in the S gene would lead to immune escape and occult infection. Screening and eliminating such mutations would help in avoiding such patients from blood transfusion, thereby preventing spread of the immune escape mutant HBV variants in the population.

6. Limitations of the study

The study was based in a tertiary care facility rather than based on a population-based sampling, where more feasibility and known expertise in liver disease care available. Further functional studies with large sample size would help in characterizing the role of novel mutant AA in immune escape or occult infections.

7. Ethics Approval

The study was approved by the institutional ethics committee vide GMC/IEC/079/2017 dated 14.06.2017 of the Guntur Medical College, Guntur, India.

8. Source of Funding

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9. Conflict of Interest

None.

10. Acknowledgement

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