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Original Research Article

Monitoring of hepatitis B virus surface antigen escape mutations and concomitant nucleostide analog resistance mutations in patients with chronic hepatitis B

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ABSTRACT

Background: In hepatitis B virus (HBV), reverse transcriptase (RT) region of the polymerase P gene and surface S gene (HBsAg) are largely overlapped. Mutations in surface S gene may cause escape variants. In the present study, we aimed to study the prevalence and pattern of the typical HBsAg escape mutations and concomitant nucleos(t)ide analogue resistance mutation patterns in patients with chronic hepatitis B (CHB) in Indian population.
Methods: The present observational study was carried out from January 2021 to June 2022 with 156 known cases of CHB infection. Hepatitis B viral load quantitation was done followed by HBV genotyping and drug resistance detection by PCR and sequencing.
Results: Out of 156 cases of CHB, HBsAg escape mutations were found in 50 (32.05%) patients. Genotype D was predominant (90%). Median viral load was 4.43×10^5 copies/ml. Total 128 HBsAg escape mutations of 46 different patterns were observed with overall prevalence of 29.49% (46/156) in CHB infected patients. The most common substitutions were sP127T (16.67%), sA128V (14.74%), sR122K (5.13%), sY134N (3.85%), sK141R (2.56%), sS143L (2.56%) and sT126INST (1.92%). Concomitant RT mutations were detected in 20 (40%) patients. Total 68 (43.59%) RT mutations of 18 different mutation characteristics were found conferring possible or confirmed resistance to nucleos(t)ide analogues.
Conclusions: The emergence of drugs resistant mutants with alteration in 'aa' determinant of the S protein is of some concern. The development of novel nucleos(t)ide analogues with a high barrier to resistance is warranted. National surveillance networks should be set up.

Keywords: Chronic hepatitis B, Surface antigen escape mutations, Nucleos(t)ide analogue resistance, PCR, Sequencing

INTRODUCTION

Hepatitis B virus (HBV) infection is a major cause of hepatic-related morbidity and mortality globally with an estimated 257 million people carrying chronic infection and approximately 887,000 HBV-related deaths per annum by the end of 2015.[1] HBV genome is a small, partially double-stranded circular DNA of approximately 3,300 bases with overlapping open reading frames (ORF).[2] The lack of proof-reading function of HBV RT is characteristic of HBV resulting into high degree of genetic variability which is exacerbated by the high speed of the HBV replication cycle.[3] This has resulted in emergence of HBV genotypes (A-J), based on inter-genotype sequence variation of more than 8%.[2] The distinguished features of HBV genome, particularly the wide spread of overlapping ORFs, have implications on HBV treatment and prevention.[2] High degree of genetic

variability of HBV allows the virus to react to selective pressure by antiviral drugs causing mutations in the RT region which can lead to emergence of virus escape mutants in the adjacent S region with subsequent lack of response to HBV vaccine.[2,4] This is observed particularly in case of inadequate HBV therapy with drugs that have low genetic barrier for resistance with some mutations restoring full replicative capacity and competence effect on HBV.[2] It is now confirmed that HBV transmission can occur in vaccinated persons as evidenced by the persistence of hepatitis B surface antibody (anti-HBs) in these patients.[5] Additionally, HBV with mutations can escape serological detection by currently used enzyme immunoassays resulting into occult HBV infection.[6,7] In India, prevalence HBV was estimated to approximately 4% indicating an intermediate endemicity of the virus with the disease burden of approximately 50 million.[8] However, there is a paucity of data about HBsAg escape mutations and concomitant antiviral drug resistance from population-based clinical investigations in Indian population, which contributes significantly to global HBV infection burden. Thus, the present study was carried out to investigate the patterns of HBV S gene escape mutants and the antiviral drug resistance mutations among chronic HBV infected patients in India.

METHODS

The present cross sectional, observational study was carried out at Metropolis healthcare Ltd, global reference laboratory, Mumbai with 156 known cases of chronic hepatitis B (CHB) infection (defined as the presence of hepatitis B surface antigen [HBsAg] for more than six months) with detectable HBV levels and experience with any nucleos(t)ide analogues and continuing any nucleos(t)ide analogues but with an increase in HBV DNA levels (break-through) from January 2021 to June 2022. Patients with acute hepatitis B infection or CHB infection but treatment naïve or treatment experienced CHB but with sustained virological response (SVR) were excluded. Demographic and clinical data were collected and included information on age, gender, antiviral drug treatment status and viral load. Approval from independent ethics committee [Conscience independent ethics committee; protocol ID MHL/Mol/2021/02] was obtained with a waiver on patient's consent. Whole blood (5 ml) from study subjects was collected in tube containing EDTA (Becton Dickinson vacutainer systems). These samples were stored at 2-8^0 C, transported to the laboratory, and analysed within 24 hours of collection for HBV viral load quantitation, genotyping and drug resistance detection by polymerase chain reaction (PCR) and sequencing.

Viral load quantitation

The COBAS Ampliprep/COBAS Taqman (CAP-CTM) HBV DNA test, v2.0 (Roche diagnostics GmbH, Mannheim, Germany) was used for in vitro nucleic acid amplification test for the quantitation of HBV DNA. CAP-CTM is based on fully automated sample preparation (nucleic acid extraction and purification) followed by PCR amplification and detection based on a dual-labelled hybridization probe targeting the pre core and core regions associated with an HBV DNA automated extraction based on the affinity of DNA for silica gel-covered magnetic beads. The COBAS HBV test and CAP/CTM v2.0 test utilizes three external controls (a high titre positive, low titre positive, and negative control) and an internal control (QS), which is a non-competitive armored quantitation standard for viral load quantification. HBV DNA was extracted from plasma sample through automated lysis with chaotropic agents and proteinase K, DNA capture by use of glass particles and purification. After DNA elution at high temperature (80°C), a robotic arm loaded nucleic acids in microvials containing the PCR master mix prepared for each sample by the same robotic arm. Real-time PCR test was performed by the COBAS TaqMan with amplification of two targets HBV DNA and the internal QS. Titer results were reported in international units per mililitre (IU/ml) with lower detection limit of 20 IU/ml.

HBV genotyping by PCR and sequencing

The Qia symphony DSP virus/pathogen kit (Qiagen GmbH, Germany) was used in combination with QIA symphony SP for automated isolation and purification of nucleic acids. Extracted DNA was amplified for HBV DNA polymerase gene, followed by a nested PCR. The PCR amplified products were confirmed by a 2% agarose gel electrophoresis (Figure 1). DNA sequencing of PCR product (729 bp) with forward primer (SprA2F) was then done on ABI 3500XL genetic analyzer (ThermoFisher scientific, Massachusetts, USA). The sequence was analysed for HBV genotyping by BioEdit sequence analysis software using national centre for biotechnology (NCBI) viral genotyping tool.[9]

HBV drug resistance by PCR and sequencing

The QIA symphony DSP virus/pathogen kit (Qiagen GmbH, Germany) was used in combination with QIA symphony SP for automated isolation and purification of nucleic acids. Extracted DNA was amplified for HBV DNA polymerase gene, followed by a nested PCR. The PCR amplified products were confirmed by a 2% agarose gel electrophoresis. DNA sequencing of PCR product (729 bp) with forward primer (SprA2F) was then done on the ABI 3500XL Genetic analyzer (ThermoFisher Scientific, Massachusetts, USA). The sequence was then analysed for drug resistance mutations responsible for resistance to lamivudine (LAM), telbivudine (LdT), adefovir (ADV), tenofovir (TDF) and entecavir (ETV) by BioEdit sequence analysis software.

Statistical analysis

The data was collected, compiled, and analyzed using EPI info (version 7.2). The qualitative variables were

expressed in terms of percentages. Quantitative variables were both categorized and expressed in terms of percentages/ in terms of mean, median and SD. The difference between two proportions was analyzed using chi-square or Fisher exact test. To test difference between two means, student t test was applied. All analysis was two tailed and the significance level set at 0.05.

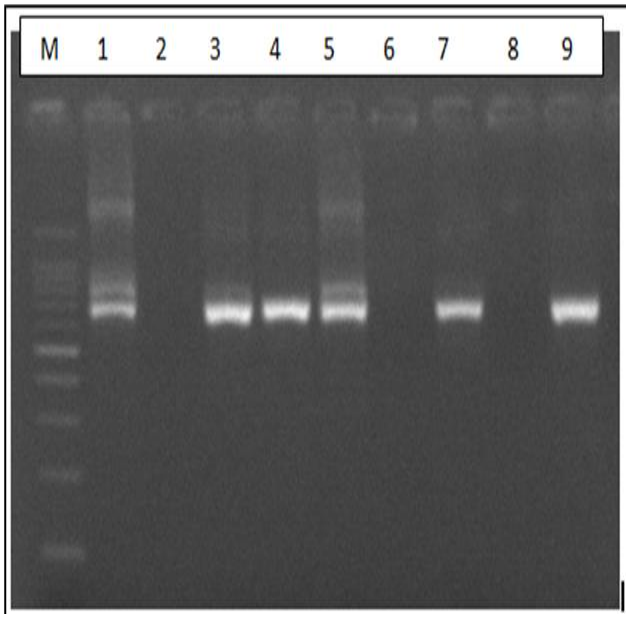

Figure 1: Agarose gel electrophoresis of the nested PCR used to detect HBV DNA.

Lanes: M molecular weight marker (100bp DNA Ladder); 1-Positive control; 2-Negative control; 3, 4, 5, 7, 9- positive for HBV DNA; 6, 8-negative for HBV DNA.

Demographics of study population

Out of 156 cases of CHB, HBsAg escape mutations were found in 50 (32.05%) patients. Mean age of the patients was 40.2 years. Out of these 50 patients, 36 (72%) were male and 16 (32%) were female patients. Median viral load was 4.43×10^5 copies/ml (5.64 Log10 copies/ml) with IQR $0.62\text{-}3.67\times10^5$ copies/ml. Genotype D was predominant, found in 45 (90%) patients followed by genotype A in 3 (06%) and genotype C in 02 (04%) patients as shown in Table 1.

Table 1: Demographic characteristics of the study population.

Characteristics	N (%)
Study population	156
HBsAg escape mutations detected	50 (32.05)
Male	36 (72)
Female	16 (32)
Age (Years ± SD)	
Mean age	56.55±8.44
Male	53.67±7.18
Female	44.12±8.22
Genotypes, n (%)	
Genotype A	3 (06)
Genotype C	02 (04)
Genotype D	45 (90)
Viral load, copies/ml	
Mean viral load	4.91×10^5
Genotype A	1.13×10^5
Genotype C	0.11×10^5
Genotype D	4.38×10^5

RESULTS

HBsAg escape mutations

Total 128 HBsAg escape mutations of 46 different patterns were observed. The overall prevalence of HBsAg mutants was 29.49% (46/156) in CHB infected patients. The most common substitutions were sP127T (16.67%), sA128V (14.74%), sR122K (5.13%), sY134N (3.85%), sK141R (2.56%), sS143L (2.56%) and sT126INST (1.92%). Typical HBsAg escape amino acid substitutions of the study patients were evaluated and categorized into hepatitis B immunoglobulin (HBIg) selected escape, vaccine escape, hepatitis B diagnosis-escape and immune-selected amino acid substitutions. Rates for HBIg selected escape, vaccine escape, hepatitis B diagnosis-escape and immune-selected amino acid substitutions-7.05%, 13.46%, 14.74% and 45.51% (Table 2). No sign of critical G145R mutation was probed in any of cases. Furthermore, some other mutations of unknown significance such as sC121/124/147CGRS, sC147R, sL109d, sL109M, sL109I, sL109FILMV, sP142HLPR, sS132F, sT131APST were also noted.

Table 2: Typical HBsAg amino acids substitutions of the study population.

HBsAg amino acid substitution category	Mutation pattern	Freq. of mutation (%)	References number
HBIg escape	sD144E, sP120APST, sQ129R, sT123A, sY134H/N	11 (07.05)	2, 4, 10, 16, 19, 22, 25
Vaccine escape	sD144E, sI126T, sL109R, sP120APST, sS143L, sQ129H, sT126I	21 (13.46)	2, 4, 16, 22, 25
Diagnosis-escape	sM133K, sP120APST, sR122G/K/T, sS143L, sT126INST, sT131I, sP127A/L	23 (14.74)	2, 4, 16, 22, 25
Immune-selected amino acid substitution	sA128V, sC137R, sC139S, sD144E, sG119R, sG130R, sK141E/N/R, sP120T, sP127T, sS132F, sS143L, sT131I, sY134F	71 (45.51)	4, 16, 17, 19, 22, 26, 27

Table 3: RT mutations pattern of the study population.

Mutation pattern	Mutation characteristics	Nucleos(t)ide analogue (n)					Total
		LAM	ADV	ETV	LdT	TDF	
Primary resistance mutation	rtA181AI/AT	7	7	ND	7	ND	21
	rtL180LM/M/MT	4	ND	ND	3	1	8
	rtM204I/L/LV/MV/V	9	ND	9	8	1	27
	rtN236R/T	ND	1	ND	ND	ND	1
	rtM204I/L/LV/MV/V ± rtL180LM/M/MT ± A181AI/AT	5	ND	1	4	1	11
Partial resistance mutation	rtA194A/T	ND	ND	ND	ND	3	3
	rtT184IST/S	ND	ND	2	ND	ND	2
	rtM250I/M	ND	ND	5	ND	ND	5
	rtS202G	ND	ND	1	ND	ND	1

LAM-Lamivudine, LdT-Telbivudine, TDF-Tenofovir, ADF-Adefovir, ETV-Entecavir, ND-Not detected.

RT mutations

Concomitant lamivudine (LAM), telbivudine (LdT), adefovir (ADV), tenofovir (TDF) and entecavir (ETV) mutations were detected in 20 (40%) patients. Total 68 (43.59%) RT mutations of 18 different mutation characteristics were found conferring possible or confirmed resistance to nucleos(t)ide analogues. Primary resistance mutations observed were rtA181AT/IT (n=21), rtM204I/L/LV/MV/V (n=27), rtL180LM/M/MT (n=8), and rtN236R/T (n=1). Partial resistance mutations observed were rtT184IST/S (n=2), rtA194AT (n=3), rtM250I/IM/MV (n=5) and rtS202G (n=1). All these mutations rates and commutations are presented in Table 3.

DISCUSSION

An HBV mutant is a variant arising as a result of specific selection, conferring a specific phenotype.[10] In the case of vaccine-escape mutants, selection pressure is conferred by either endogenous factors such as the host immune system that leads to mutations in the S-ORF or exogenous antiviral therapy leading to mutations in the overlapped P-ORF region.[10] Although, these HBV mutants remain stable over time, they can be transmitted horizontally as well as vertically.[10] It was proposed by Carman et al that some variants from the HBV pool having similar replication potentials are selected by humoral and cellular immune response; which are known as immune escape mutants.[11] Therefore, the resulting mutant viruses are able to escape from host's humoral and cellular immune responses because of mutated S-genes, thus reducing diagnostic and immune prophylaxis efficacy.[11] This underlines the importance of detection of S-mutants in chronic HBV infected patients to identify patients requiring appropriate preventive therapy and closer strict follow-up for early detection of hepatocellular carcinoma (HCC). It is well known that primary and compensatory RT mutations reduce anti-HBs antibody binding (vaccine associated) by altering the second loop of the immunodominant region of HBsAg ('aa' determinant) thus causing epidemiological and diagnostic issues.[12] However, recent worrisome evidence is related to P-mutants inducing vaccine-escape S gene mutants due to treatment induced mutations.[10] The overlapping nature of S and P-ORFs in the HBV genome may cause every single mutation to influence one or more functions of the corresponding nucleotide sequence. Hence, immune escape mutations in S-ORF may be translated as mutations in the P-ORF that eventually may result in resistance to the antiviral therapy. Likewise, mutations occurring in P-ORF during antiviral therapy may be translated as mutations in the S-ORF that may eventually produce an S-immune escape mutant.[10] The circulation and subsequent transmission of S-escape mutants resistant to the nucleos(t)ide analogues currently used as anti-HBV therapy and vice versa, pose a significant risk since these mutants may potentially infect both treatment naïve and immunized individuals and negatively affect the efficacy of both the antiviral treatment and the vaccination programs.[13,14] This highlights the importance of continuous monitoring and surveillance.

In the present study of 156 chronic HBV infected patients, ≥1 HBsAg escape mutations were observed in 50 (32.05%) patients. A total of 46 nucleotide substitution patterns were identified in S gene. We evaluated hepatitis B Ig escape mutations, vaccine escape mutations, hepatitis B diagnosis-escape and immunoselected amino acid substitutions and the mutation rates were detected as 7.05%, 13.46%, 14.74% and 45.51% respectively (Table 2). It was reported by Avellon and Echevarria that amino acid substitutions in HBV variants were responsible of 12.5% failure in disease diagnosis, 6.6% invalid vaccination, and 9.2% escape from immunoglobulin therapy.[15] In a study by Ozguler and Sayan, hepatitis B Ig escape mutations, vaccine escape mutations, hepatitis B diagnosis-escape and immunoselected amino acid substitutions rates were detected as 9.6%, 6.9%, 5.2%, and 11.9% respectively.[16] Kandpal et al observed the prevalence of 16.09 % (14/87) escape mutations in the genotyping studied group in India.[17] Colagrossi et al reported immune-associated escape mutations in 22.1% of patients with rising temporal-trend in Europe.[4] Prevalence of 18.9% was reported in Jordan.[2] In the Korean population of HBV

carriers prevalence of HBsAg escape mutants was reported close to 33%.[18] Overall variable prevalence of 8.3% and 17.9% of typical HBsAg escape mutations were noted in studies on Turkish population.[16,19] In a recent study by Araujo et al, a comprehensive analysis of clinically significant HBV mutations in relation to genotype, sub genotype and geographic region was done. No mutants were detected in central Asia. HCC associated mutants were reported predominantly in Southern Asia (41%), followed by North America (37%) and Eastern Asia (36.5%) and the lowest rates were observed in Middle Africa, Western Europe, and Australia and New Zealand (14.6-19.6%). Immune escape mutants were detected often in North America (16.3%), Eastern Asia (12.3%) and South-eastern Asia (12%) and less frequently in Middle Africa (0), Eastern Africa (1.4%) and the Caribbean (1.6%) by Araujo et al. [20]

The typical HBsAg escape mutations detected in the present study have been shown in many investigations to be associated with diagnostic problems in HBsAg assays and vaccine or HBIg therapy escape (Table 2). Globally, G145R which is known to cause an immune-escape during vaccination, is the most frequent and stable amino acid change in HBsAg. It is reported to be associated with HBV genotype D. [21] Although in the present study, genotype D was predominant, no existence of G145R mutation was found, instead we found sG145A/E in two patients (1.28%). Similar findings were reported by Ababneh et al and Al-Qudari et al. [2,21] In Turkey, 1.2% of sG145R mutations were found by Sayan et al.[19] Ozguler et al defined the frequency of sG145 mutation as 1.8%.[16] However, in American blood donors, sG145R was detected as high as 22%.[16] Significance of sR122 as a serologic determinant for HBV subtype, with subsequent effect on the antigenicity and immunogenicity of the virus had been established in previous study reports.[2,22] It was reported that these sR/K122 mutations, providing either d to y or the opposite change, were observed frequently in association with a mutation at position 144 (sD144A/E).[23] The importance of this pattern was shown by Martel et al, using site-directed mutagenesis. It was determined by Martel et al that point mutation at position 122 or 144 could markedly reduce recognition while their combination completely abolished recognition of the modified HBsAg mutant variant.[24] In the present study, combination of sD144E and sR122K was found in only one patient. It's therefore important to carry out a comprehensive study with a greater number of HBV patients in India, especially those escaping from standard diagnostic and vaccination measures, which will help in monitoring the potential existence of such amino acid substitution. Insufficient data are available for interpretation, and the clinical effects of some of the detected HBsAg escape mutations (i.e., sC121/124/147CGRS, sC147R, sL109d, sL109M, sL109I, sL109FILMV, sP142HLPR, sS132F, sT131APST) in the present study are not clear**.** Although, obvious significance of these mutations could not be established, potential existence of mutations affecting the antigenic properties of HBsAg in the circulating viral strains cannot be ruled out.

In the present study, analysis of RT drug resistance showed a 43.59% (n=68) frequency of resistance-related substitutions. In the literature, varied LAM resistance from 23-80% was reported in chronically infected HBV individuals, after 1 and 5 year(s) of monotherapy.[16] In the present study, LAM resistance was found to be 8.97% (14/156). Nucleos(t)ide analogues therapies have been successful in sustained viral suppression however, long-term use of treatment with a low barrier to resistance leads to resistant HBV mutant's emergence. Therefore, it's recommended to not use such agents (LAM, LdT and ADV) as first-line therapy.[20] Nevertheless, management of nucleos(t)ide analogues failure remains a crucial issue, particularly in low-resource countries where ETV and TDF are not readily available. In thepresent study, the overall rates of mutations inducing resistance to LAM (8.97%) and LdT (8.33%) were higher than those for other nucleos(t)ide analogs ADV (5.13%) and TDF (2.56%). The frequency of rtM204 mutation was found at 17.30% (n=27). Pattern of rtM204I alone was observed predominantly (5.77% [n=9]), followed by rtM204V + rtL180M mutations (5.13% [n=8]) which were present as co-mutations. In a similar study by Lee et al, rtM204I/V (50.2%) mutation was most frequently detected mutation, followed by rtL180M (39.2%).[28] Ozguler et al observed 16.5% were rtM204I/V mutations followed by rtL180M mutation (10.5%).[16] Ababneh et al reported rtL180M and rtM204V as predominant mutations in Jordan.[2] In the present study, frequency of rtA181 mutations was observed to be 40% and rtA181AT alone was observed predominantly [36% (n=18)]. Other identified mutations were rtT184IST/S and rtN236R/T associated with ETV and ADF resistance respectively. In the seven patients with LAM resistance mutations, partial resistance to ETV was detected which is likely related to selection of HBV entecavir mutants in those patients. This finding is in line with recent findings by Ababneh et al and Geipel et al.[2,29] rtM204I/V mutations were accompanied by compensatory mutations in other domains such as rtT184IST/S, rtA194AT, rtM250IM, rtS202G and rtL180LM. Similar findings were reported by Ababneh et al and Caligiuri et al.[2,30] In the previous studies, it was demonstrated that mutations in the HBsAg selected by LAM therapy developed more frequently in HBV genotype A when compared with genotype D.[31] In the present study, genotype D was predominant (90%); therefore, it was not possible to make a comparison of mutations related to the different HBV genotypes.

Sayan et al had suggested a possibility of circulation of HBV encoding surface mutations with concomitant nucleos(t)ide analogues associated resistance variants specifically for the populations with the highest prevalence rates. Their findings also suggested the possibility of pre-existing typical HBsAg escape and nucleos(t)ide analogues resistance mutations.[19] HBV encoding LAM-resistant mutations had been reported in a

cohort of dialysis patients with occult HBV by Besisik et al.[32] On the other hand, Ozaslan et al reported a high secondary mutation rate for the HBV surface gene in family members of HBV patients.[33] Based on a large-scale clinical investigation, Huang BX et al had reported a finding supporting the influence of HBV immune escape associated mutations on drug resistance.[21] They found that the sA159V mutation might increase the fitness of LAM/ETV resistant mutants by decreasing the HBsAg levels and increasing the viral replication capacity.[34] In the present study, concomitant HBV surface escape variants and nucleos(t)ide analogues resistant variants were observed in 12.82% (n=20) patients. Our analysis of the resistance mutation-positive patients clearly showed that the frequency of immune escape-associated mutations is significantly higher in resistance mutation-positive patients than in resistance mutation-negative patients (p=0.0023).

In this study we focused on the typical HBsAg escape mutations via DNA sequencing of the polymerase gene region. By this method, it is possible to monitor nucleos(t)ide analogues resistance mutations concomitantly in the same sequencing. Drug resistance mutations in the HBV polymerase gene may not always result in a direct impact on the nature of HBsAg and its function, and vice versa. However, HBV S escape variants are viable and pathogenic and may infect properly vaccinated people. Thus, "nucleos(t)ide analogues-resistant, possible vaccine escape mutants" have the potential to infect both naïve and immunized people, negatively affecting the efficacy of both the antiviral treatment and the vaccination programs.[14] This issue should be considered by the recent therapeutic strategies aimed at achieving HBV-cure. A number of limitations of this study need to be considered. Of note, our study was based on patient-derived viral strains rather than artificially generated strains, thus providing convincing evidence. Nevertheless, in vitro experimental data may not always fully reflect in vivo processes. Because of limited number of unique sequences from patients with genotype A (n=3) and genotype C (n=2), it was not possible to make a comparison of mutations related to the different HBV genotypes. This study is limited by the fact that we could not do long term follow up of patients to correlate concomitant HBsAg escape variants with drug resistance mutations in relation to clinical outcome. More prospective multicentric studies with larger age matched study population are needed to establish this correlation.

CONCLUSION

We investigated HBV genotype distribution, patterns of HBV S gene escape mutants and antiviral drug resistance mutations among chronic HBV infected patients in India. HBV genotype D was found to be predominant. Total 128 HBsAg escape mutations of 46 different patterns were observed. Total 68 (43.59%) RT mutations of 18 different mutation characteristics were found conferring possible or confirmed resistance to nucleos(t)ide analogues. Concomitant drug resistant RT mutations were detected in 20 (40%) patients. Present study has provided important data on the sequence diversity of HBsAg in chronic HBV strains circulating in India. The emergence of drugs resistant mutants with alteration in 'aa' determinant of the S protein is of some concern. However, it would be worthwhile to see if mutations related to HBsAg escape occur more frequently in other HBV genotypes. The development of novel nucleos(t)ide analogues with a high barrier to resistance is essential. National surveillance networks need to be set up to monitor the epidemiological dynamics and public health impact of HBsAg escape mutants.

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