Influence of HIV-associated degree of immune suppression on molecular heterogeneity of hepatitis B virus among HIV co-infected patients

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We have investigated the molecular diversity of Hepatitis B virus (HBV) among the HIV co-infected patients from eastern-India. HBsAg/HBV-DNA positive subjects (n = 73) from 874 HIV-infected patients were analyzed by sequencing followed by genetic diversity quantification. HBV-genotype-D and HBV/sugenotype-D2 were predominant. HBV/D2 isolates from patients with low CD4 count manifested significantly lower non-synonymous substitutions (p < 0.0001) and Shannon entropy (p = 0.0006) in their surface and polymerase gene in comparison to those from moderately increased CD4 count. ART-induced immune-reconstitution therefore might raise non-synonymous immune/therapy escape substitutions among these HBV/D2 isolates. Decreased genetic diversity and increased viral load in the HBV/D2 isolates might facilitate the maintenance of their wild type characteristics in the low CD4 count, leading to its increased prevalence in this group. Interestingly, genetic diversity in HBV/A1, the next common subgenotype, was modified in the opposite manner. Together our results underscore the need for proper HBV molecular monitoring in HIV co-infection.

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Introduction

Introduction of highly active anti-retroviral therapy (HAART) in late 20th century has decreased AIDS-related morbidity and mortality. Thus liver diseases associated with chronic hepatitis have emerged as the second leading cause of death among the HIV-infected patients (Lacombe et al., 2010). According to UNAIDS report (2009), people living with HIV infection is estimated to be 33.3 million in the world (http://www.unaids.org/documents/20101123_GlobalReport_Chap2_em.pdf). Globally, 350–400 million people are chronic Hepatitis B surface antigen (HBsAg) carriers (Datta, 2008). In addition, shared routes of transmission have led 3 million people around this world to be co-infected with HIV and hepatitis B virus (HBV) (Soriano et al., 2010). Several studies have reported that HIV related immune suppression significantly modifies the natural history of HBV infection (Vallet-Pichard and Pol, 2004; Puoti et al., 2006). The presence of HIV is associated with increased chronicity of HBV infection, reduced rate of spontaneous HBsAg and hepatitis B early antigen (HBsAg) seroconversion (Thio, 2009). End-stage liver diseases (ESLDs) such as cirrhosis and hepatocellular carcinoma (HCC) (Lacombe et al., 2006; Salmon-Ceron et al., 2009) and liver related mortality were also found to be increased considerably in the HIV co-infected patients compared to HBV mono-infection (Thio et al., 2002). Moreover, the presence of HIV and consequential immune alterations may act as cofactors in modulating HBV genetic variability as well as its molecular evolution within a HIV co-infected host. However, reports which have addressed this issue are scanty. Recently, HIV co-infection has been reported to be associated with the emergence of specific mutations in the HBV genome (Audsley et al., 2010). HIV driven immune dysfunction also renders low evolutionary rate to HBV (Cassino et al., 2012). Therefore, the role of HIV driven immune dysfunction in the molecular heterogeneity of HBV during coexistence in a host requires further investigation.

Based on > 8% divergence in the complete genome sequence eight HBV genotypes (HBV/A to HBV/H) have been classified and two more genotypes (HBV/I and HBV/J) have been proposed recently (Lin and Kao, 2011). In addition, based on > 4% (but < 8%) divergence in the complete nucleotide sequence within a
particular genotype, subgenotypes have been described. HBV genotypes and subgenotypes vary geographically and differ considerably in the mutational patterns, ethnicity and associated clinical outcomes. In India, HBV/D is predominant, with varying prevalence of HBV/A in different parts of the country (Datta, 2008). In eastern India HBV/C is also circulating in a considerable proportion. Moreover, four subgenotypes were identified within HBV/D (HBV/D1, HBV/D2, HBV/D3 and HBV/D5) and only one subgenotype was found in HBV/A (HBV/A1) and HBV/C (HBV/C1) each in this part of the country.

The epidemic spread of HIV has made India the third largest population with this infection in the world next only to South Africa and Nigeria (http://www.unaids.org/en/dataanalysis/monitoring/countryprogress/progressreports/2010countries/india_2010_country_progress_report_en.pdf). In addition; India is in the intermediate endemicity zone of HBV infection (HBsAg prevalence between 2% and 8%) harboring the second largest pool of chronic HBV infection after China (Datta, 2008). HBV mono-infection has been extensively studied in India (Banerjee et al., 2006a,b; Datta et al., 2008; Gandhe et al., 2003; Kumar et al., 2005; Thakur et al., 2002; Vivekanandan et al., 2008), however information regarding HIV–HBV co-infection is lacking. Though a few reports have addressed seroprevalence and associated risk groups of HIV–HBV co-infection in India (Gupta and Singh, 2006; Saravanan et al., 2007), molecular characterization of HBV in HIV-infected patients has not been done.

Therefore in this study, we aimed to characterize HBV genetic heterogeneity among HIV co-infected patients from eastern India and its possible association with the degree of immune suppression. The HBV surface gene (S) containing ‘a’ determinant region and a partial reverse transcriptase (RT) domain of the polymerase gene were analyzed from HBsAg positive HIV-infected patients.

Results

HBV prevalence and HBV genotypes distribution

Among 874 HIV-infected patients (622 males, 252 females; mean age 36.59 ± 8.64; mean CD4 count 276.71 ± 226.38 cells/mm$^3$) HBsAg was detected in 145 (16.59%) patients, which included both anti-retroviral therapy (ART) naïve as well as ART treated subjects. Of these HIV–HBV co-infected patients, 73 (50.34%) samples (67 males, 6 females; mean age 34.65 ± 6.92; mean CD4 count 262.15 ± 191.32 cells/mm$^3$) could be amplified for HBV DNA from the surface gene region. These 73 HIV-infected patients, positive for both HBsAg and HBV DNA, were analyzed in this study. These patients visited the ART clinic of Calcutta School of Tropical Medicine, Kolkata from different districts of West Bengal throughout the study period of almost two years (Fig. 1).

Three HBV genotypes [HBV/A (21/73; 28.77%), HBV/C (3/73; 4.11%) and HBV/D (49/73; 67.12%)] were detected in these HIV co-infected patients by RFLP. When this genotype distribution pattern was compared with that of the HBV mono-infected patients (n=200) coming to our laboratory, significantly higher prevalence of HBV/D (P=0.03) and considerably lower frequency of HBV/C (P=0.001) were observed (Fig. 2A). However, HBV/A distribution did not vary among the HIV co-infected and the HBV mono-infected groups (28.77% vs 28.5%). The partial S gene could be sequenced from 71 isolates for further analysis, 2 samples were insufficient and could not be sequenced.

Subgenotypes of HBV: high prevalence of HBV/D2 and associated substitutions

In the present study, HBV/A (HBV/A1) and HBV/C (HBV/C1) isolates clustered into single subgenotype, whereas HBV/D was distributed among four subgenotypes in the phylogenetic tree [HBV/D1 (6/47; 12.77%), HBV/D2 (32/47; 68.08%), HBV/D3 (4/47; 8.51%) and HBV/D5 (5/47; 10.64%)] (Fig. 3A) similar to HBV mono-infection (Fig. 2B). Interestingly, of these HBsAg/HBV-DNA positive HIV-infected patients from eastern India, HBV/D2 solely contributed to 45.07% among all subgenotypes (32/71) and 68.08% among the HBV/D subgenotypes. Among the HBV mono-infected samples, only 68 HBsAg positive HBV/D isolates that could be subgenotyped were compared with the co-infected samples. HBV/D2 isolates circulated in highly significant (P<0.001) proportion among the...
HIV co-infected patients than the patients with HBV mono-infection (Fig. 2B).

Demographic, biochemical and virological parameters of the HBV/D2 isolates were further investigated in comparison to the non-HBV/D2 isolates (Table 1). The major HBV subgenotype among these non-HBV/D2 isolates was HBV/A1 (21/39; 53.85%). In addition, HBV/C1 (3/39; 7.69%), HBV/D1 (6/39; 15.38%), HBV/D3 (4/39; 10.26%), HBV/D5 (5/39; 12.82%) were also found. In both the groups, males were predominately infected (>90%), sexual contact bear the highest risk of acquiring HBV infection. However, HIV RNA load as well as serum HBV DNA load were comparable between HBV/D2 and non-HBV/D2 group (Table 1).

The patients harboring HBV/D2 were younger than those infected with non-HBV/D2 strains (Table 1, P = 0.028) and tended to be more HBeAg positive compared to those with other HBV subgenotypes (P = 0.014). Notably, the mean CD4 count was lower in the HBV/D2 isolates than non-HBV/D2 isolates though not statistically significant (240.38 ± 148.42 vs 318.02 ± 339.32; P = 0.23). (Table 1).

In the surface gene region, various immune escape mutations were found among the HIV–HBV co-infected patients (Table 1). Three HBV/D2 and one HBV/D5 isolates displayed a premature stop codon at amino acid position 69 (C69stop) regardless of HBSAg positive status. Clonal alignment confirmed the presence of C69stop by sequencing 10 clones randomly chosen from each sample.

Further highest prevalence of HBV/D2 among the HIV–HBV co-infected patients was confirmed by phylogenetic analysis of the polymerase RT region sequences generated from 25 subjects (Fig. 3B). Additionally, investigation of polymerase gene region from the 11 ART treated patients included in the study indicated the presence of lamivudine resistant triple mutation (rtV173L + rtL180M + rtM204V) in two HBV/D2 isolates and lamivudine resistant double mutation (rtL180M + rtM204V) in one HBV/D1 subject. Thus, the two HBV/D2 isolates which harbored lamivudine mutation rtL173V; also had vaccine escape mutation E164D in the overlapping S gene (Table 1).

Association of HBV/D2 with CD4 count

Distribution of HBV subgenotypes was evaluated in different CD4 count groups among these HIV co-infected patients. Interestingly, an increased prevalence of HBV/D2 was found with decreasing CD4 count (Fig. 4). Patients with CD4 count < 200 cells/mm³, had highest occurrence of HBV/D2 (40.63%) while least occurrence was observed among those with CD4 count ≥ 500 cells/mm³ (3.13%). Moreover, the frequency of HBV/D2 isolates in varying CD4 count groups showed a nearly significant (Spearman r = −0.812, P = 0.058) negative association. This correlation was absent in non-HBV/D2 isolates (Spearman r = −0.696, P = 0.136) as well as in its major subgenotype, HBV/A1 (Spearman r = −0.706, P = 0.136). A major change in distribution of HBV/D2 was observed around CD4 count 350 cells/mm³. We therefore, tried to analyze nucleotide diversity of HBV/D2 in this region. The sample size between CD4 count < 300 cells/mm³ and ≥ 300 cells/mm³ was comparable (Fig. 4), so we performed the analysis around CD4 count 300 cells/mm³.

Nucleotide diversity of HBV with CD4 count

When nucleotide diversity was determined among HBV/D2, the isolates from patients with CD4 < 300 cells/mm³ showed significantly decreased relative amounts of non-synonymous substitutions (dN) in the surface gene region compared to the HBV/D2 subjects from patients with CD4 ≥ 300 cells/mm³ (median dN 0.004 vs 0.012 respectively, P < 0.0001) without significant difference in relative amounts of synonymous substitutions (dS) (Fig. 5A and B). Moreover, dN/dS was also varied significantly among the HBV/D2 isolates in these two CD4 count groups (Fig. 5C; P = 0.001). In contrast, a different pattern of dS and dN were observed in HBV/A1, the most frequent subgenotype in the non-HBV/D2 group. Interestingly, no non-synonymous substitution could be found in the HBV/A1 isolates from patients with CD4 ≥ 300 cells/mm³. Though negative selection persisted (dN/dS < 1) among HBV/A1 in the two CD4 count groups similar to HBV/D2, a different dN/dS pattern was noticeable in the surface gene region of HBV/A1 isolates (Fig. 5C).

This association of decreased non-synonymous substitutions in the HBSAg region with lower immune pressure was consistent in XYPLOT of cumulative behavior of average synonymous and non-synonymous substitutions of the coding region of polymerase RT domain (Supplementary data 1). In contrast, the HBV/D2 isolates from patients with CD4 ≥ 300 cells/mm³ showed increased non-synonymous substitutions in both the surface as well as polymerase gene region. The difference in non-synonymous substitutions would result in amino acid variation which would be further reflected in HBV viremia.

Amino acid variation in HBV with CD4 count

Amino acid variations were measured by Shannon entropy which reflected non-synonymous substitutions harbored in the genome. In the surface gene region, a significant difference in median entropy was observed between the HBV/D2 isolates from the two CD4 count groups (0.199 in CD4 < 300 cells/mm³ and 0.305 in CD4 ≥ 300 cells/mm³, P = 0.0006) (Fig. 5D). Among all the four groups analyzed, the HBV/D2 isolates from patients with CD4 < 300 cells/mm³ showed the least median Shannon entropy. But no significant difference was found in median entropy between the non-HBV/D2 isolates (P = 0.106) including HBV/A1.
Increased viral replication of HBV/D2 in lower CD4 count

A significant difference was observed in HBV DNA load of the HBV/D2 isolates in the two CD4 count groups ($P=0.03$) but no such difference in HBV viremia was found among the non-HBV/D2 subjects in these CD4 count groups ($P=0.40$) (Fig. 6). Notably, the major non-HBV/D2 subgenotype, HBV/A1 isolates, also demonstrated contrasting viremia in the two CD4 count groups compared to the HBV/D2 isolates. Moreover, HBeAg positivity was increased in the HBV/D2 subjects with low CD4 count (89.47% in < CD4 300 cells/mm$^3$ vs 75.0% in CD4 ≥ 300 cells/mm$^3$) (data not shown).

Discussion

The present study sheds light on the genetic heterogeneity of hepatitis B virus under the influence of HIV induced immune modulation among the HIV–HBV co-infected patients from eastern India. To the best of our knowledge, this is the first study from...
India on genetic diversity of HBV in HIV co-infection. In the present study, we found that the prevalence of HBV/D2, the major HBV subgenotype among these co-infected patients, was inversely associated with lowering of CD4 count. Molecular heterogeneity analysis of HBV/D2 revealed that the degree of immune suppression could influence nucleotide diversity and amino acid variation among them. The consequence was reflected in the replication of HBV/D2 isolates. In contrast, HIV driven immune dysfunction could modulate genetic diversity in a reverse manner among the isolates of HBV/A1, the next frequent subgenotype after HBV/D2.

The HBV genotypes/subgenotypes found among these co-infected patients are consistent with our previous data on HBV mono-infection (Banerjee et al., 2007; Chandra et al., 2009), but the proportion differs between the HIV–HBV co-infected and the HBV mono-infected patients of eastern India. This is in contrast to a recent report from Mexico that showed HBV/G predominated in the HIV co-infected patients, while HBV/H was common in the HBV mono-infected patients (Mata Marí et al., 2012). In our study, HBV/D was the predominant genotype in the HIV co-infected patients from eastern India. Among the subgenotypes of HBV/D, HBV/D2 circulated in significantly higher proportion and thus became the major HBV subgenotype. Notably, these patients came from different parts of the state of West Bengal in eastern India (Fig. 1); throughout the study period of almost two years. Additionally, HBV/C1 was found rarely among these co-infected patients. Being a recent introduction to this region (Vivekanandan et al., 2004; Banerjee et al., 2006a), HBV/C possibly have not been spread among the HIV–HBV co-infected patients in this region.

In the present study, we observe an overall male predominance in the study subjects and an association of younger age with the HBV/D2 isolates which are consistent with our previous HBV mono-infection study (Chandra et al., 2009). In addition, the HIV co-infected patients with HBV/D2 had elevated ALT level (63.90 ± 71.64 IU/L) which is contradictory with the report by Chandra et al. (2009) (36.81 ± 19.53 IU/L). This might be due to the presence of HIV infection which renders hepatocytes more prone to liver injury (Babu et al., 2009).

The key factor which induces the development of mutations in HBV genome is the immune pressure of a host. HIV infects the immune cells, primarily targeting the CD4+ T cells which are eventually destroyed during the course of infection. Depletion in the number of CD4+ T cells might be considered as a measure of degree of immune suppression. Therefore, HIV and its associated immune suppression might influence the molecular heterogeneity of the co-infecting HBV. An association between functional immune status and HBV serological outcome in the HIV–HBV co-infected adults was demonstrated by Landrum et al. (2010). In the present study, the mean CD4 count was found to be lower in the HBV/D2 subjects compared to non-HBV/D2 subjects, though dysfunction could modulate genetic diversity in a reverse manner among the isolates of HBV/A1, the next frequent subgenotype after HBV/D2.

Table 1
Demographic, biochemical and virological characteristics of study subjects (N=71).

<table>
<thead>
<tr>
<th></th>
<th>HBV/D2</th>
<th>Non-HBV/D2 (A1, C1, D1, D3 and D5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. (%)</td>
<td>32 (45.07)</td>
<td>39 (54.93)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>29 (90.63)</td>
<td>36 (92.31)</td>
</tr>
<tr>
<td>Age (mean ± SD)*</td>
<td>32.54 ± 6.88</td>
<td>36.41 ± 7.32</td>
</tr>
<tr>
<td>Risk factors (%)</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>Sexual contact (%)</td>
<td>15 (57.69)</td>
<td>22 (70.97)</td>
</tr>
<tr>
<td>Blood transfusion/surgical operation (%)</td>
<td>6 (23.08)</td>
<td>4 (12.90)</td>
</tr>
<tr>
<td>Multiple exposures (%)</td>
<td>5 (19.23)</td>
<td>5 (16.13)</td>
</tr>
<tr>
<td>ALT, IU/L (mean ± SD)</td>
<td>63.90 ± 71.64</td>
<td>62.08 ± 27.11</td>
</tr>
<tr>
<td>CD4 count, cells/mm³ (mean ± SD)</td>
<td>240.38 ± 148.42</td>
<td>318.02 ± 339.32</td>
</tr>
<tr>
<td>HIV RNA load, log copies/ml (mean ± SD)</td>
<td>4.44 ± 1.17</td>
<td>4.36 ± 1.13</td>
</tr>
<tr>
<td>HBsAg positive (%)b</td>
<td>25/30 (83.33)</td>
<td>25/37 (67.57)</td>
</tr>
<tr>
<td>HBV DNA load, log copies/ml (mean ± SD)</td>
<td>5.3 ± 1.23</td>
<td>5.15 ± 1.48</td>
</tr>
<tr>
<td>Amino acid substitutions in surface gene region including MHR (frequency)</td>
<td></td>
<td></td>
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<tr>
<td>Immune escape mutations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A128V(30),Y134N/H (1/1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M133T/I(A1, C1, D1, D3 and D5)</td>
<td>M133T/C1, 1,</td>
<td></td>
</tr>
<tr>
<td>Q101H(C1), Y134N(D1, 1), Y134H(D3, 1), Y129(R/D1, 1)</td>
<td></td>
<td></td>
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<tr>
<td>Vaccine escape mutations</td>
<td></td>
<td></td>
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<tr>
<td>E164D(2)</td>
<td></td>
<td></td>
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<tr>
<td>Premature stop codon</td>
<td>C69stop(3)</td>
<td>C69stop (D3, 1)</td>
</tr>
<tr>
<td>No. of patients on ART Lamivudine resistant mutations (frequency)</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>rTL73V ± rtL180M + rtM204V</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>rTL180M + rtM204V</td>
<td>–</td>
<td>1</td>
</tr>
</tbody>
</table>

* P=0.028.  
** P=0.014.

Fig. 4. Distribution of HBV/D2 isolates in varying CD4 count groups. Sample distribution between HBV/D2 and non-HBV/D2 in CD4 < 300 cells/mm³ and ≥ 300 cells/mm³ is shown in the table. * CD4 count was not available for one sample.
the difference is not statistically significant (Table 1). In addition, a trend of HBV/D2 clustering in the immunosuppressive phase was also evident from a nearly significant negative association ($P = 0.058$) between its frequency and corresponding CD4 count. This observation suggested that the predominance of HBV/D2 could be related to the HIV driven degree of immunosuppression.

The deciding parameter of selection in a particular environment, $dN/dS$, showed an overall predominance of negative selection on HBV among these HIV co-infected patients ($dN/dS < 1$). This supports a recent finding that identified negative selection on HBV in the HIV co-infected patients from Caucasian ethnicity (Audsley et al., 2010). However, it did not address whether the genetic diversity of HBV varied with different CD4 counts. In a recent report by Cassino et al. (2012) have suggested that as a consequence of lower CD4 cell count, HBV subjects from HIV co-infected patients had low quasispecies diversity as well as evolutionary rate when compared to that from HBV mono-infected patients. Interestingly in our study, we observed that the molecular heterogeneity of HBV is modulated with the degree of immune suppression and varied in different HBV subgenotypes.

Among the HBV/D2 isolates, genetic diversity was varied significantly in the patients with CD4 $\geq 300$ cells/mm$^3$ when compared to the patients with CD4 $< 300$ cells/mm$^3$. The median $dN$ (both in the surface gene region containing B cell epitope as well as in the polymerase gene RT region) was significantly lower in the later in comparison to the former. As a result, amino acid variation was less frequently observed among the HBV/D2 subjects isolated from patients with lower CD4 count group and they showed reduced Shannon entropy. Thus decreased genetic diversity renders increased replication capacity to the virus resulting in higher HBV viral load among them. Additionally, greater HBeAg positivity was also found in the HBV/D2 infected patients with CD4 $< 300$ cells/mm$^3$ compared to the patients with higher CD4 count. Taken together, it can be assumed that low CD4 count may help the HBV/D2 isolates to retain their wild type characteristics. Thus, the gradual decrease in CD4 count in the course of HIV co-infection may provide increased viral fitness to the HBV/D2 isolates, which might lead to increased association of HBV/D2 with lower CD4 count. Furthermore, in an earlier HIV–HBV co-infected study (Audsley et al., 2010), it was established that HBeAg positive status is correlated with lower mutation frequency which supports our results.
It is noteworthy that the co-infected patients harboring HBV/D2 exhibited significantly higher HBeAg positivity when compared to other HBV subgenotypes. This possibly indicates that HBV/D2 from the co-infected patients is more infectious than other HBV subgenotypes. Due to lack of data on liver histology or other non-invasive parameters that detect pathogenicity we are unable to assess whether HBV/D2 is more pathogenic than the other subgenotypes.

All of the treated patients in our study received lamivudine as the only drug effective against HBV in their ART regimen. Two HBV/D2 isolates among the 11 exposed to lamivudine therapy (mean CD4 count 451.5 cells/mm^3), showed the presence of lamivudine resistant polymerase gene mutation (rtV173L+rtL180M+rtM204V) as well as corresponding vaccine-immune escape mutation (E164D) in the overlapping surface gene region. Widespread use of antiretrovirals, specially the regular use of lamivudine as a part of ART regimen in India might predispose the emergence of polymerase mutants that result in the development of ‘vaccine-escape’ mutants in the overlapping surface gene region. Thus, an increased CD4 count which renders higher dN in the HBV/D2 isolates suggests a possible emergence of higher rate of HBV mutants to evade immune pressure. The mutations in S gene can significantly modify hepatitis B surface antigen synthesis and reduce the binding efficiency to antibodies. As a result, these mutants might cause even the vaccinated individuals vulnerable to HBV infection, indicating its importance from public health perspectives also. Thus, this study underscores the importance for molecular monitoring of HBV in these patients to study the incidence of such mutations, their effect on HBV-related liver disease in the HIV co-infection, as well as their transmission potential. Furthermore, for hepatitis B treatment options in these patients more potent drugs, that have higher transmission potential. Furthermore, for hepatitis B treatment options in these patients more potent drugs, that have higher genetic barrier against resistance development, must be seriously considered.

Mutations in the polymerase gene may produce changes in the overlapping surface gene region of HBV. Furthermore to evade the host immune response, virus may develop immune escape mutants. A128V, reported to be an immune escape mutation, was found in most HBV/D2 isolates (30/32) in HIV co-infected patients (Lazarevic et al., 2010). However, Tallo et al. (2008) mentioned this substitution as a subgenotype specific substitution and thus supported our findings. In addition, several other immune escape mutations (M131I/T, Q101H, Y134N/H and Q129R) were also present but in very low frequency (Table 1). Interestingly, in the upstream of ‘a’ determinant region, C69stop was found in three HBV/D2 isolates and one HBV/D5 isolate from HIV co-infected patients who were positive for HBsAg, HBeAg and had higher viral load (> 10^6 copies/ml). Recently a study has shown the presence of this unusual stop codon in Iranian patients with cirrhosis who were HBsAg positive with higher viremia (Vejzjalali et al., 2009). Therefore, these patients might be at a risk of developing ESDs.

In contrast to HBV/D2, among the non-HBV/D2 isolates genetic diversity and viral load were modified in a different manner with the degree of immune suppression. The non-HBV/D2 isolates included HBV/A1 as the major subgenotype (53.85%). In the HBV/A1 isolates, median dN was increased in the patients with lower CD4 count (< 300 cells/mm^3) but surprisingly no non-synonymous substitutions were found in the HBV/A1 isolates with higher CD4 count i.e. CD4 ≥ 300 cells/mm^3. Differences in the molecular heterogeneity as well as the clinical outcome between these two HBV genotypes (HBV/D and HBV/A) has been addressed in several previous studies (Datta et al., 2008; Weinberger et al., 2000; Zehender et al., 2008). A possible explanation for this contrasting substitution pattern with varying immune suppression between HBV/A1 and HBV/D2 could be the difference in host immune response against these two subgenotypes. Actually, these two subgenotypes demonstrated differential expression of genes related to innate and adaptive immune response. In our preliminary study, Cathelicidin antimicrobial peptide (CAMP) which is reported to target enveloped virus (Hancock and Diamond, 2000), was found to be upregulated significantly in HBV/A1 infection with CD4 < 300 cells/mm^3 compared to HBV/A1 infection with CD4 ≥ 300 cells/mm^3 (Supplementary data 2). It seems likely that in order to evade the effect of upregulated CAMP gene, non-synonymous substitutions might have been introduced in HBV/A1 genome. In contrast, CAMP gene is downregulated significantly in HBV/D2 isolates with lower CD4 count where decreased non-synonymous substitutions were observed. This variation between HBV/A1 and HBV/D2 was noted in the surface gene, which is the major target of neutralizing antibody for immune-clearance; however study from the whole genome might help in better understanding of this phenomenon.

In conclusion, HBV/genotype-D and HBV/subgenotype-D2 predominantly circulated among the HIV co-infected patients in eastern India. The notable findings of this study include a trend of negative association of frequency of the HBV/D2 isolates with CD4 counts and modification of HBV molecular heterogeneity by HIV driven immune dysfunction particularly the degree of immune suppression. Being associated with decreased genetic variability and increased viremia in low CD4 count, selection of HBV/D2 seems to be favored in isolates with low CD4 count. Increased non-synonymous substitutions with moderate increase in CD4 count also suggests that ART induced immune reconstitution might lead to the development of vaccine/immune escape and lamivudine resistant mutations among them. In contrast to HBV/D2, interestingly in HBV/A1 genetic variability is modified differently in presence of HIV. Further work is needed to confirm the role of HIV on HBV evolution by prospective studies. Taken together, our study emphasizes the necessity for molecular monitoring of HBV and use of potent antivirals that reduce the risk of HBV mutations development, in the setting of HIV co-infection.

**Material and methods**

**Study participants**

A total of 874 HIV-infected patients coming to the ART clinic of Calcutta School of Tropical Medicine, Kolkata were referred to ICMR Virus Unit, Kolkata for testing HBV markers during March 2010–December 2011. They were initially screened for HBsAg status followed by tested for detection of HBV DNA among HBsAg positive patients. Among them 73 HIV-infected patients were positive for both HBsAg and HBV DNA and they were included in the study. HCV positive patients were excluded from the study. Among the study subjects, 11 patients were exposed to antiretroviral therapy (ART including Lamivudine) with median duration of two years. After obtaining informed consent, blood samples were collected from the patients. Plasma was separated on the same day of collection and was stored at −80°C in aliquots until further analysis. Ethical clearance was obtained from the Ethics committee of the Institution.

**Serological assays**

HBsAg and HBeAg were tested by commercial EIA kits (Dia- sorin, S.P.A, Saluggia, Italy) according to manufacturer’s instructions. Anti-HIV and anti-HCV were screened using kits from
General Biologicals, Taiwan and Hepanostika, Boxtel, The Netherlands respectively.

DNA isolation, HBV DNA detection and genotyping

DNA was extracted from 200 µl of plasma using the QIAamp DNA Blood MiniKit (QIAGen, Hilden, Germany). HBV DNA was detected by nested-PCR amplification from the surface gene region as reported by Datta et al. (2006). Hemi-nested-PCR followed by restriction fragment length polymorphism (RFLP) was done to determine HBV genotypes as described previously (Biswas et al., 2009). For avoiding false positive results guidelines by Kwok and Higuchi (1989) were strictly followed.

Amplification of RT region of polymerase gene

Reverse transcriptase (RT) region of HBV polymerase (pol) gene was amplified using Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, CA,USA) by nested polymerase chain reaction (PCR). Six microlitre of the extracted DNA was added in 30 µl reaction mix of the first round PCR using primers HB1F [5’-AAGCTCTGCTAGATCCCAGAGT-3’] and HB6R [5’-AACAGACCAATTTAGCTA-3’] (antisense 1803–1874)] and 4 µl from the first round PCR product was amplified using primers NIV3 [5’-GACTCTGGTGACTTCTTC-3’] and HS4R [5’-CATACTTTCAAATCTAGGG-3’] (antisense 989–970)]. The nucleotide sequence numbering was according to the suggested reference strain sequence E03188 [Genbank accession number DQ315783] (India). The thermal cycling profile included incubation for 10 min at 95°C. 45cycles at 95°C for 50 s, 55°C for 45 s, and 72°C for 45 s, followed by a final extension step at 72°C for 7 min. Second round PCR followed the same profile for 40 cycles.

Sequence analysis of surface gene/polymerase gene region and determination of subgenotypes

Amplified surface gene region for 115 amino acids including ‘a’ determinant region and 190 amino acids of polymerase RT region were sequenced directly using Prism Big Dye kit in ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA,USA) and analyzed using Bioedit version 7.0.9.0 (Hall, 1999). HBV genotypes/subgenotypes and sequence substitutions were analyzed by aligning study sequences with Genbank retrieved sequences. Phylogenetic analysis was performed in Mega 4, using the neighbour-joining method (Tamura et al., 2007). Phylogenetic groups were evaluated by the bootstrap test with 1000 replicates.

The nucleotide sequence data reported in this article have been submitted to the DDBJ/EMBL/GenBank. Accession numbers for the HBV partial surface gene sequences: JQ349405–JQ349475. Accession numbers for the HBV partial polymerase gene sequences: JQ349118–JQ349146 and JX244153-JX244171.

Genetic diversity calculation

Genetic diversity was determined from nucleotide diversity followed by amino acid variation in HBV surface gene and polymerase gene region. Nucleotide diversity was evaluated by the number of synonymous substitutions per synonymous site (dS) and the number of non-synonymous substitutions per non-synonymous site (dN). These relative amounts were estimated by the method of Nei and Gojobori using sequence analysis tool—SNAP available at the Los Alamos National Laboratory website (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entopy.html) (Korber, 2000). The ratio of dN and dS (dN/dS) was calculated to estimate selection (Negative selection if dN/dS < 1 and Positive selection if dN/dS > 1). The cumulative behavior of average synonymous and non-synonymous substitutions of a coding region was automatically generated by SNAP as XYPLOT. The Shannon entropy per site was determined from the deduced amino acid sequences of amplified gene region using the Entropy program on this Los Alamos National Laboratories website (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy.html) as an indicator of amino acid variation.

Ligation, cloning of PCR products

PCR products of the surface gene region were cloned using InsTA clone PCR cloning kit, (MBI Fermentas, Vilnius, Lithuania). DNA from transformed colonies were amplified and sequenced as mentioned above.

Quantification of HBV DNA and HIV RNA

HBV DNA was quantified by TaqMan based real time PCR assay in Applied Biosystems SDS 7000 (Foster City, CA, USA) with lower detection limit of 10^{2} copies/ml (Chandra et al., 2007). Quantification of HIV RNA was measured by COBAS TaqMan HIV–1 test in COBAS Tagman 48 analyzer using human plasma. Lower detection limit was 47 copies/ml.

Statistical analysis

Statistical calculations representing the continuous data were expressed as the mean value ± standard deviation (SD). Mann–Whitney U test, unpaired t test were performed for comparisons of continuous variables between the groups using the Graphpad Prism (version 4.0.3). Categorical variables were analyzed using the chi-square test or Fisher exact test, as appropriate. Correlation was analyzed by Spearman’s rank test. All P-values were 2-tailed and P-value < 0.05 was considered to be significant.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2012.11.003.

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