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Comparative Analysis of Hepatitis B Virus Genotype A Molecular Evolution in Patients Infected With HBV and in Patients Co-Infected With HBV and HIV

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HIV infection has a significant impact on the natural progression of liver disease caused by infection with hepatitis B virus (HBV), but its role in the molecular evolution of HBV is unknown. It is difficult to study the molecular evolution of HBV longitudinally considering its genomic complexity, which implies the analysis of paired samples. This study aimed to analyze the difference in the evolutionary dynamics of HBV among patients with HIV and uninfected individuals. In this study, 17 patients infected chronically with HBV were recruited, 9 of them were co-infected with HIV. Patients were HBe antigen-positive and infected with HBV genotype A. Paired plasma samples were collected from each patient 3 years apart, and they were compared subsequently to each other. The HBV phylogenetic inference among isolates from patients infected with HBV and co-infected with HBV and HIV tends to cluster separately. Likewise, when comparing the HBV evolutionary rate and genetic distances, values were higher in the former in both preC/C and S genomic regions. Intra-host analyses of HBV isolates revealed high diversity and complexity of quasispecies among patients infected with HBV exhibiting high numbers of viral variants and genetic distance. In summary, after studying the HBV molecular evolution among isolates ascribed to genotype A at inter- and intra-host levels, HBV exhibited low quasispecies complexity and diversity as well as low evolutionary rates in the presence of HIV co-infection, suggesting that the co-infection may have an impact on the HBV molecular evolution most likely from the weakened cellular immune response. ***J. Med. Virol. 9999:1–8, 2012.***

KEY WORDS: HBV; HIV; co-infection; molecular evolution; quasispecies

INTRODUCTION

Approximately 10% of the individuals infected with HIV worldwide are also chronically infected with hepatitis B virus (HBV) and the natural history of HBV infection is modified in these patients. HBV–HIV co-infection increases the risk of developing liver impairments, such as end-stage liver disease and hepatocellular carcinoma [Soriano et al., 2010].

At least eight different HBV genotypes (A to H) have been recognized worldwide by phylogenetic analysis [Kurbanov et al., 2010]. The disease outcome may be influenced by HBV genotypes through different impacts on the development of liver disease [Sumi et al., 2003]. While HBV genotypes D, F, and A are similarly prevalent among patients infected with HBV in Buenos Aires, Argentina [Franca et al., 2004; Pezzano et al., 2011], a marked predominance of genotype A has been reported in individuals co-infected with HBV–HIV [Perez-Olmeda et al., 2003; Lacombe

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et al., 2006; De Maddalena et al., 2007; Quarleri et al., 2007; Trinks et al., 2008; Aizawa et al., 2010].

Despite being a DNA virus, HBV uses RNA as a replicative-intermediate. Its reverse transcriptase lacks proofreading capacity, leading to a population distribution called quasispecies [Chu and Lok, 2002]. The impact of the dynamic changes on the HBV quasispecies heterogeneity from different genomic regions is unknown in HBV–HIV co-infected patients. To date, sequence analyses of the HBV genome in individuals co-infected with HBV–HIV have been limited to direct sequencing of complete genomes [Audsley et al., 2010], and partial genomic regions [Matthews et al., 2006] at a single time-point.

The studies focused on the molecular evolution of HBV involve the understanding of accumulated sequence changes of the genome and the mutation rate observed over a long period. However, it is difficult to determine the magnitude of HBV sequence changes due to the organization of the HBV genome. The nucleotide substitution rate of this virus is determined by both the lack of proofreading activity during retrotranscription and the overlapping open reading frames (ORFs). This denotes that the HBV genomic variation is constrained by amino acid changes over time taking into account that each ORF protein has its own functional and structural constraints [Jazayeri et al., 2010].

HBV mutants emerging from genomic changes as a result of overlapping ORFs may exhibit different antigenicity and fitness, leading to a different natural history of the infection in comparison to wild type HBV. Certain mutations in the polymerase gene are associated with resistance to nucleos(t)ide analogues. The S gene is completely overlapped by the polymerase gene. As a consequence, mutations in the polymerase gene may produce changes in the S gene leading to reduced HBsAg antigenicity gene after long-term therapy [Jazayeri et al., 2010].

The presentation of peptides derived from HBV proteins to host cytotoxic T-lymphocytes plays a crucial role in eliminating the infection. The immune selection largely contributes to the evolution of HBV chronic infection by generating positively selected escape mutants. Most T-cell epitopes derive from HBV structural (core and surface) rather than non-structural (polymerase) proteins [Wang et al., 2010].

Patients who have cleared the HBe antigen show a higher number of nucleotide substitutions than those who are HBe positive [Osiowy et al., 2006], likely as a function of increasing immune pressure during the immune clearance phase of infection [Simmonds, 2001]. Thus, HBeAg-positive and negative populations present different evolutionary behaviors which should be studied in a separate analysis. Among HBV–HIV co-infected patients, the HBe-positive profile predominates [Puoti et al., 2002].

The main goal of the study was to analyze the impact of HIV coexistence on HBV genotype A (GtA) molecular evolution based on a 3-year longitudinal study of the S and preC/C ORFs in HBe positive patients, at both inter- and intra-host levels.

PATIENTS AND METHODS

Study Population

This study included 17 individuals chronically infected with HBV who expressed HBe antigen (8 infected with HBV and 9 co-infected with HBV and HIV). No epidemiological link was observed among patients. Paired plasma samples were collected 3 years apart from each patient. Demographic characteristics of patients were recorded, as well as risk group, virological parameters (plasma viral load and S-gene based genotype), immune status (CD4+ T cell counts), lamivudine (LMV)-therapy and its resistance-associated mutations (by pol gene analysis), and HIV viral load.

All patients signed a written consent to participate in this study. This document was then evaluated and approved by a local ethics committee according to the ethical guidelines of the 1975 Declaration of Helsinki.

Among the study population, seven patients co-infected with HBV and HIV co-infected and five infected with HBV were treated with LMV and exhibited resistance-associated mutations.

The mean ± SD CD4+ T cell counts among patients co-infected with HBV and HIV and patients infected with HBV was significantly different ($P = 0.0016$; 237.77 ± 468.87 cells/ml, and 952.09 ± 187.76 cells/ml, respectively).

The time of known chronic infection with HBV, and HIV—only for indivuduals with co-infection—was obtained from clinical records according the first positive serological result and no differences were found ($P > 0.05$; T-test) (Table I).

Cloning and Sequencing

DNA was extracted from 200 μl of plasma using a commercial kit (Qiagen AG, Basel, Switzerland) according to the manufacturer's instructions and subjected to nested PCR amplification, particularly for HBV basal core promoter, preC/C (positions 1631–2000) and S (positions 203–787) genomic regions according to the protocols described previously [Chan et al., 1999; Quarleri et al., 2007].

PCR products were cloned into pGEM-T Easy (Promega[Q5]) according to the manufacturer's instructions. Twenty clones were obtained from each PCR product.

PreC/C and S gene partial nucleotide sequences were obtained by direct PCR and clone-derived sequencing.

The GenBank accession numbers of the sequences reported in this study are HQ589358–HQ589838 and HQ603688–HQ603727 for those obtained by previous cloning and, JN800448–JN800475 and JN809651–JN809678 for those obtained by direct sequencing.

TABLE I. Demographic, Biochemical, Virological, and Risk Data of Patients Included in the Study

ID	Gender, age (year)	Risk	ALT[a]	HBV VL (log)[b]	3TC therapy[c]	CD4 cells/ml[d]	3TC resistance mutations[d]	HIV VL (log)[d]	Time[Q3] of infection[e] (HBV–HIV)
P2	M, 41	HTSX	N	7.3	>30	108|108	wt|wt	NA|4.8	3 4
P7	F, 32	HTSX	N	>7.6	>30	253|308	wt|L180M/M204V	<1.7|<1.7	4 4
P9	M, 48	HMX	N	>7.6	>30	1000|650	V173L/L180M/M204I/V/S|V173L/L180M/M204I/V/S	<1.7|<1.7	4 4
P12	M, 47	HMX	1-2X	3.5	>30	108|252	wt|L180M/M204I/V/S	<1.7|<1.7	3 5
P13	M, 28	HTSX	N	>7.6	>30	1384|1073	L180M/M204I/V/S|V173L/L180M/M204I/V/S	<1.7|<1.7	3 5
P16	M, 27	IDU	N	>7.6	>30	130|315	wt|L180M/M204I/V/S	5.1|<1.7	4 4
P19	M, 28	IDU	1-2X	6.7	28	564|761	wt|L180M/M204I/V/S	<1.7|<1.7	4 5
P20	M, 30	HTSX	N	>7.6	>30	26|69	wt|wt	<1.7|<1.7	4 6
P21	M, 31	HTSX	N	>7.6	>60	312|338	L180M/M204I/V/S|L180M/M204I/V/S	<1.7|<1.7	4 6
M1	F, 49	HTSX	N	7.1	0	838	wt		NA NC
M2	M, 31	HTSX	N	6.8	0	670	wt		3 NC
M3	F, 43	HTSX	4X	>7.6	18	1240	L80V/V173L/L180M/M204V|L80V/V173L/L180M/M204V		4 NC
M4	M, 20	HMX	N	7.1	16	907	L80V/V173L/L180M/M204V|L80V/V173L/L180M/M204V		4 NC
M5	F, 28	IDU	N	>7.6	22	NA	V173L/L180M/M204I|V173L/L180M/M204V		5 NC
M6	M, 47	HTSX	1-2X	>7.6	0	1130	M204V		3 NC
M7	M, 48	IDU	1-2X	4.8	16	1007	L180M/M204V|L180M/M204V		4 NC
M8	M, 35	HMX	N	>7.6	24	987	L80V/V173L/L180M/M204V|L80V/V173L/L180M/M204V		5 NC

NA, not available; NC, do not correspond.
[a]Times of the upper limit of normal (N).
[b]At baseline, expressed as log of copies/ml using AMPLICOR HBV MONITOR Test v2.0 (Roche Molecular Diagnostics[Q4]).
[c]Duration in months.
[d]Vertical bars separate baseline and last sample values.
[e]Expressed in years and estimated since serological diagnosis for each virus.

Phylogenetic Analysis of Sequences

Nucleotide sequences introduced in this study and sequences obtained from GenBank database were aligned with the ClustalX v1.83 program [Thompson et al., 1997] and edited using the Bioedit v7.0.9.0 program [Hall, 1999]. A Maximum Likelihood (ML) phylogenetic tree was obtained using the PhyML software v3.0 [Guindon et al., 2010] with the substitution model that best fit the data, among those ranked by the ModelTest software v3.06 [Posada and Crandall, 1998] according to Akaike Information Criterion (AIC).

Phylogenetic grouping robustness was evaluated by bootstrap analysis using ML (1,000 replicates) with the PhyML software.

Molecular Evolutionary Rate Analysis

In order to estimate substitution rates for each group, Bayesian coalescent analyses were performed on preC/C and S direct sequences from patients infected with HBV and patients co-infected with HBV and HIV, at first and last time-points. The analyses were carried out with the BEAST v1.6.0 software package [Drummond and Rambaut, 2007] using the uncorrelated lognormal (UCLN) molecular clock model [Drummond and Rambaut, 2007] and a constant population size as the demographic model in order to avoid overparametrization. Convergence was assessed by effective sample size (ESS) values higher than 200 and 10% of the sampling was discarded as burn-in. Uncertainty in parameter estimates was evaluated in 95% highest posterior density (HPD95%) interval.

PreC/C and S Gene-Based Analysis of HBV Heterogeneity

For an inter-host analysis, the genetic distance among the different HBV isolates from patients infected with HBV and patients co-infected with HBVand HIV was estimated based on preC/C and S direct sequences at the first and last time-points. The mean genetic distance (d) was calculated at nucleotide level using the Tamura-Nei model and a Gamma distribution (four categories) for substitution rates at sites (with alpha values estimated by the ModelTest software), implemented in the MEGA 4.0 software [Tamura et al., 2007].

The intra-host analysis included the comparison of the genetic distance or diversity (d) and complexity among quasispecies from both study groups, patients infected with HBV and patients co-infected with HBV and HIV, at the first and last time-points of the 3-year period. Clone sequences of preC/C and S regions from each patient were used in this analysis. HBV viral intra-host genetic distances were analyzed using the same methodology used for inter-host distances. HBV viral complexity was measured by calculating the normalized Shannon entropy (Sn) at

nucleotide level. The Sn was calculated according toVolkenstein [1994].

Selection Analyses

Selection analyses were carried out using the HyPhy program [Pond et al., 2005] which allowed for comparing the number of synonymous and non-synonymous substitution rates at each site.

In order to compare the degree of the positive selection in HBV nucleotide sequences, the preC/C and S ORFs alignments (and their overlapping genes X and pol, respectively) were analyzed using the Fixed Effects Likelihood (FEL) method [Kosakovsky Pond and Frost, 2005] at two different levels: (a) intra-host: clone-derived sequences were studied longitudinally, and (b) inter-patient: the selection pressure was compared among clone sequences from patients infected with HBV and patients co-infected with HBV and HIV at first time-point. The FEL method enabled the detection of nonneutral selection, based on the maximum likelihood approach. Only sites identified by FEL with a significant P-value ($P < 0.05$) were included in the final result.

Statistical Analysis

Differences between data groups were analyzed by independent t-test.

The Spearman's correlation test was used to evaluate potential correlations among changes over time in CD4-T cell counts and the number of resistance mutations with genetic diversity and complexity in the S and preC/C genomic regions.

All P values were 2-tailed; $P < 0.05$ was considered statistically significant. All the analyses were performed by the SPSS 13 software (Demo version).

RESULTS

The ML phylogenetic tree for S-preC/C concatenated sequences confirmed that all the isolates reported in this study belonged to subgenotype A2. Nucleotide sequences from this study are intermingled with previous Argentinean isolates. However, those samples from patients infected with HBV grouped with very low support (Fig. 1). Since most patients had either past or current LMV treatment (14 out of

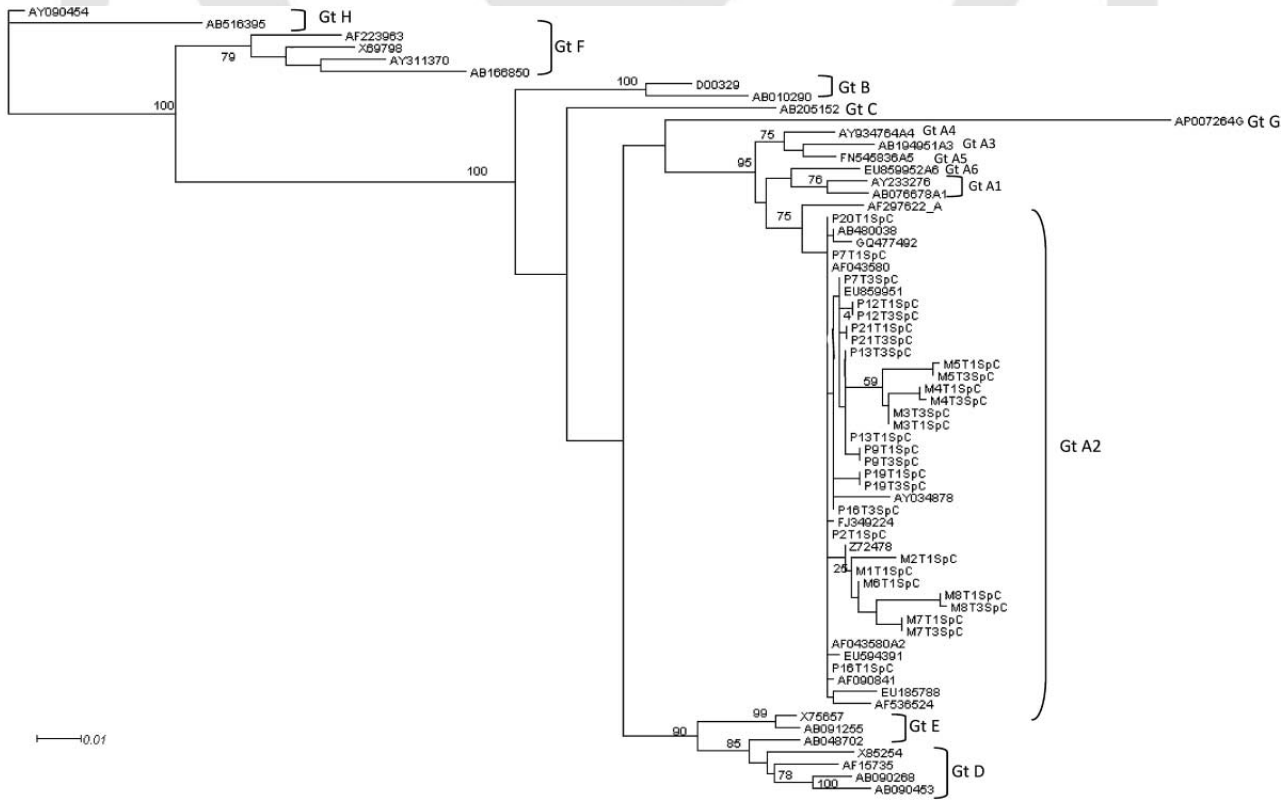

Fig. 1. Maximum-likelihood analysis of concatenated S and preC-C nucleotide sequences (named as "SpC") from 8 HBV-monoinfected patients (named as "M") and 9 HIV-HBV co-infected patients (named as "P") obtained by direct sequencing. Each HBV isolate is also referenced to the sampling time (T1 or T3). The GenBank accession numbers of S nucleotide sequences are JN800448–JN800475 while those for the preC nucleotide sequences are JN809651–JN809678. Reference sequences (genotypes A to H) were retrieved from GenBank and are indicated by their accession number; those Argentinean HBV isolates previously characterized are indicated in brackets with an asterisk. The phylogenetic tree topology was evaluated by bootstrap analysis (1,000 replicates) and values are shown at each node. The ruler shows the branch length for a pairwise distance equal to 0.1.

TABLE II. Molecular Evolutionary Rates of HBV Isolates From Mono- and Co-Infected Patients

HBV genomic region (mono/co)	Rate (substitution site^{-1} year^{-1})	Lower HPD	Upper HPD
preC (mono)	3.11×10^{-4}	1.92×10^{-7}	1.03×10^{-3}
preC (co)	1.63×10^{-4}	4.48×10^{-9}	5.10×10^{-4}
S (mono)	4.50×10^{-4}	6.64×10^{-8}	1.32×10^{-3}
S (co)	7.86×10^{-5}	4.57×10^{-8}	2.48×10^{-4}

17), clustering by treatment in order to determine plausible differences due to LMV treatment was not achieved.

HBV evolutionary rates were estimated separately based on the S or preC/C nucleotide sequences, and expressed as the number of nucleotide substitutions per site and per year. The values obtained were in consonance with those reported previously [Zhou and Holmes, 2007] (Table II). In both, patients infected with HBV and patients co-infected with HBV and HIV, the HBV substitution rates obtained by the preC/C sequence analysis were lower than those based on the S sequences. When the impact of co-infection of HIV was considered, the HBV estimated rates from patients infected with HBV were five and two times higher in the S and preC/C regions, than those from patients co-infected with HBV and HIV, respectively (Table II).

The genetic distance (d) analysis between HBV isolates was carried out at both inter- and intra-host levels. The former was calculated on S or preC direct sequences and showed higher values (mean ± SE) among patients infected with HBV than those obtained among patients co-infected with HBV–HIV (Table III).

When the intra-host analysis of the genetic distance was performed, no significant differences were found in mean distance among clones calculated from the S gene in patients infected with HBV and patients co-infected with HBV and HIV. However, distance values calculated from preC/C in the former group were higher than those from patients co-infected with HBV and HIV at first ($P = 0.0132$) and last ($P = 0.0149$) time-points.

Similarly, quasispecies complexity (by Shannon entropy analysis, Sn) was analyzed at first and last time-points based on preC/C and S genes sequences. When comparing the data obtained between patients infected with HBV and patients co-infected with HBV and HIV as an average of the values for each patient within the class, the former showed a tendency to achieve higher complexity values in both genetic regions (Table IV).

The longitudinal analysis of viral heterogeneity along the 3-year period revealed that none of the HBV isolates from either group of patients exhibited statistically significant differences in Sn values ($P > 0.05$).

Positive selection occurs when an amino acid alteration confers an adaptation or selective advantage. Inter-patient analyses revealed that two codons on pol, six on S, three on preC/C and five on X were identified as positively selected in patients infected with HBV. However, in patients co-infected with HBV and HIV only three codons on preC/C were estimated to be under positive selection.

At intra-patient level, isolates from patients infected with HBV exhibited one codon on pol, five on S gene, three on X and none on preC/C region under positive selection. However, positive selection was identified only on one codon on S gene in patients co-infected with HBV and HIV (Table V). Nevertheless, these results should be interpreted with caution in the context of the overlapping reading frames.

DISCUSSION

HIV infection modifies the natural history of HBV infection resulting in higher rates of persistence and increasing liver-related morbidity and mortality [Soriano et al., 2010].

HBV A is the most prevalent genotype among patients co-infected with HBV and HIV in Argentina, [Quarleri et al., 2007; Trinks et al., 2008]. A similar scenario has also been described elsewhere [Perez-Olmeda et al., 2003; Lacombe et al., 2006; De Maddalena et al., 2007; Aizawa et al., 2010].

HBV-genotype A infection is associated with a significantly higher cumulative rate of sustained

TABLE III. Inter-Patient Analysis of Genetic Distances (d) of HBV Isolates From Mono- and Co-Infected Patients

	S		preC/C	
	HBV–HIV co-infected patients	HBV monoinfected patients	HBV–HIV co-infected patients	HBV monoinfected patients
First time	0.0069 ± 0.0030[a]	0.018 ± 0.0070	$0.0040 \pm 0{,}0030$[c]	0.010 ± 0.0050
Last time	0.0028 ± 0.0016[b]	0.020 ± 0.0054	0.0040 ± 0.0030	0.002 ± 0.0017

[a]Statistically significant differences in d from S region among HBV monoinfected and HBV–HIV co-infected patients at first time-point, $P < 0.0001$.
[b]Statistically significant differences in d from S region among HBV monoinfected and HBV–HIV co-infected patients at last time-point, $P < 0.0001$.
[c]Statistically significant differences in d from preC/C region among HBV monoinfected and HBV–HIV co-infected patients at first time-point, $P < 0.0001$.

TABLE IV. Intra-Host Analysis of Complexity (Sn, Shannon Entropy) and Diversity (d, Genetic Distance) of HBV Isolates From Mono- and Co-infected Patients

	HBV–HIV co-infected patients		HBV monoinfected patients	
	First time	Last time	First time	Last time
Sn				
S	0.45 ± 0.16	0.47 ± 0.19	0.66 ± 0.21[a]	0.64 ± 0.15
preC/C	0.72 ± 0.18	0.73 ± 0.14	0.82 ± 0.12[b]	0.9 ± 0.81[c]
d				
S	0.0022 ± 0.0015	0.0045 ± 0.0041	0.0032 ± 0.0010	0.0026 ± 0.0010
preC/C	0.0045 ± 0.0024	0.0037 ± 0.0011	0.0084 ± 0.0030[d]	0.0082 ± 0.0026[e]

[a]Statistically significant differences in Sn from S region among HBV monoinfected and HBV–HIV co-infected patients at first time-point, $P = 0.041$.
[b]Statistically significant differences in Sn from preC/C region among HBV monoinfected and HBV–HIV co-infected patients at first time-point, $P = 0.059$.
[c]Statistically significant differences in Sn from preC/C region among HBV monoinfected and HBV–HIV co-infected patients at last time-point, $P = 0.018$.
[d]Statistically significant differences in d from preC/C region among HBV monoinfected and HBV–HIV co-infected patients at first time-point, $P = 0.0132$.
[e]Statistically significant differences in d from preC/C region among HBV monoinfected and HBV–HIV co-infected patients at last time-point, $P = 0.0149$.

biochemical remission, HBV DNA clearance, and HBsAg clearance than the HBV-genotype D infection in patients infected chronically with HBV [McMahon, 2009].

This comparative study analyzed the genetic and evolutionary characteristics of HBV-genotype A in patients infected with HBV and in patients co-infected with HBV and HIV, along a 3-year period, with precedents of similar times of infection with HBV.

The HBV virus from patients infected with HBV showed some unique characteristics. In the phylogenetic tree, all the sequences were intermingled, but tended to cluster together in these patients, although not statistically significant (Fig. 1). However, other evolutionary characteristics reinforced the hypothesis of a different behavior since they also exhibited higher genetic distances and rates of evolution other than HBV in patients co-infected with HBV and HIV. The Bayesian coalescent analysis that evaluated the dynamics and evolutionary rates of these populations showed that the evolutionary rates of patients infected with HBV were two and five times higher than those observed in patients co-infected with HBV and HIV, from the preC/C and S regions respectively. Such observation could be influenced by the selection and the small size of the study population, being inconvenient to draw any definitive conclusion.

The plausible outlook of independent HBV evolution in both groups of infected subjects—with and without coexistence of HIV—led us to investigate the

TABLE V. Positively Selected Sites for Inter- and Intra-Patient Selection Analyses*.

	Inter-patient analysis		Intra-patient analysis				
ORF[a]	HBV monoinfected	HBV–HIV co-infected	M3	M4	M5	M8	P19
S	42 (0.015)	117 (0.026)	94 (0.024)		94 (0.009)		144 (0.047)
	71 (<0.001)	124 (0.026)				71 (<0.001)	
	94 (<0.001)	142 (0.026)					
	118 (0.037)				118 (0.005)		
	122 (0.011)			122 (0.027)			
	171 (0.005)						
P	500/rt152 (0.002)	—		475/rt122 (0.009)	—	—	—
	521/rt173 (0.043)						
preC/C	6 (0.047)	—	—	—	—	—	n.a.
	23 (0.036)						
	60 (0.014)						
X	126 (0.026)	—	—	—	—	92 (0.031)	n.a.
	132 (0.035)					105 (0.014)	
	136 (0.027)						
	137 (0.037)		137 (0.022)				
	154 (0.022)						

n.a., Not available.
[a]Numerations correspond to the small S gene or to the complete ORFs in the other genes (for P gene, numeration according to the RT domain is also provided). Sequence AF090839 was used as reference.
*P-values form REL analyses associated with each positively selected site are shown in parentheses.

contribution of quasispecies to the molecular evolutionary behavior of HBV. Two different factors, such as the high HBV replication capacity and the lack of proofreading activity by the HBV polymerase, contribute to the development of quasispecies complexity and diversity during infection. The HBV quasispecies composition appears to be different when HIV is present, as previously reported [Cassino et al., 2009; Audsley et al., 2010]. The present study demonstrates that the intra-host analyses of HBV isolates revealed high complexity and diversity among patients infected with HBV exhibiting higher numbers of viral variants and genetic distance. Because the number of clones analyzed is low, minor variants may be undetected, leaving out useful information [Gretch et al., 1996].

These observations suggest that there is a consequence of higher immune pressure in patients infected with HBV, since the immune pressure appeared to be weaker and CD4-T cell counts were significantly lower in patients co-infected with HBV and HIV. This rationale is supported by previous findings concluding that the magnitude, frequency, and quality of the HBV-specific T cell responses decreased with lower CD4-T cell counts in patients co-infected with HBV and HIV [Chan et al., 1999]. In both groups of patients, the detection of a response correlated significantly with CD4-T cell counts. In fact, this putative higher selection pressure was found in patients infected with HBV since more codon sites were positively selected among them than in patients co-infected with HBV and HIV. Moreover, although an important number of HBV mutations were detected in several epitopes, the overall frequency of mutations under selection pressure was low. Even so, the frequency of mutations under positive selection was lower among patients co-infected with HBV and HIV. This is consistent with the weaker HBV-specific T-cell response observed in HIV-HBV co-infection [Audsley et al., 2010].

It is important to point out that all the patients expressed HBeAg, and as previously reported, the positive HBeAg status was associated with a decreased mutation frequency regardless of the presence of HIV. The lower evolution rate in HBeAg positive patients may be a consequence of reduced immune pressure [Lim et al., 2007].

Conversely, the behavior of HBV quasispecies did not seem to be modified during the follow-up of each patient, regardless the coexistence of HIV or the presence of antiviral treatment. This behavior might reflect an adaptation of the virus to environmental conditions, such as therapy or dissimilar immune pressure for both antibody and T-cell response [Vignuzzi et al., 2006; Westover and Hughes, 2007].

In summary, after studying the HBV molecular evolution among isolates ascribed to genotype A at inter- and intra-host levels, HBV exhibited low quasispecies complexity and diversity as well as low evolutionary rates in the presence of HIV co-infection, suggesting that the co-infection may have an impact on the HBV molecular evolution most likely from the weakened cellular immune response. Further research is needed to determine the effect of this phenomenon on disease progression.

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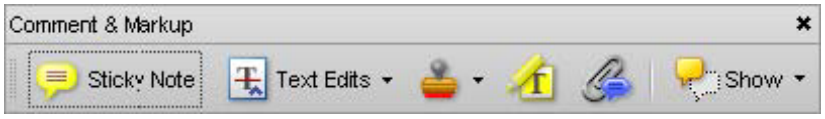

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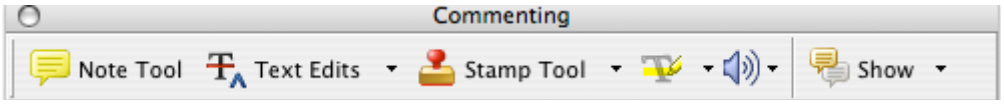

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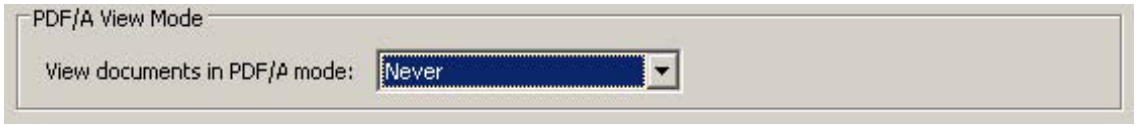

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