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Short Communication

Long-term monitoring drug resistance by ultra-deep pyrosequencing in a chronic hepatitis B virus (HBV)-infected patient exposed to several unsuccessful therapy schemes

M. Sede [a,b,1], D. Ojeda [a,1], L. Cassino [a,b], G. Westergaard [c], M. Vazquez [c], S. Benetti [d], F. Fay [d], H. Tanno [e] J. Quarleri [a,b,*]

[a] Centro Nacional de Referencia para el SIDA, Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
[c] Instituto de Agrobiotecnología Rosario (INDEAR), Santa Fe, Argentina
[d] Centro de Diagnóstico Médico de Alta Complejidad (CIBIC), Rosario, Argentina
[e] Servicio de Gastroenterología y Hepatología del Hospital Provincial de Centenario, Rosario, Argentina

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ABSTRACT

The aim of this study was to analyze the spectrum and dynamics of low-prevalent HBV mutations in the reverse transcriptase (rt) and S antigen by ultra-deep pyrosequencing (UDPS). Samples were obtained from a chronically infected patient who was followed throughout a thirteen-year period. This technology enabled simultaneous analysis of 4084 clonally amplified fragments from the patient allowing detecting low prevalent (<1%) mutations during the follow-up. At baseline, HBV sequences were predominately wild-type. Under sequential HBV monotherapies including lamivudine, adefovir and entecavir, a high frequency of rtM204I mutation was detected initially as unique and then coexisting with rtM204V. Both mutations were statistically associated with rtA200V and rtV207I, respectively. Once the entecavir and tenofovir combined therapy was started, polymerase and consequently envelope gene mutations appeared at several positions at a higher frequency than before, including the entecavir resistance-associated mutation rtT184L.

Hepatitis B virus (HBV) is a circular partially double stranded DNA virus containing a reverse-transcriptase (rt) enzyme. HBV replicates via an RNA intermediate that is responsible for the generation of several related viral variants called quasispecies, favoring the emergence of HBV drug resistance (Locarnini and Zoulim, 2010). HBV resistance to antiviral therapy can lead to HBV treatment failure and progression of liver disease. In general, this is gradually acquired through the selection of pre-existing variants with polymerase resistance-conferring mutations and the accumulation of new amino acid substitutions (Durantel, 2010). As the reading frames of the envelope and polymerase genes overlap, several of such resistance mutations could simultaneously alter the antigenicity of HBsAg inducing an "immune escape" (Chotiyaputta and Lok, 2009; Sheldon and Soriano, 2008; Sloan et al., 2008; Torresi et al., 2002).

The estimated mutation rate of HBV is $>2 \times 10^{-4}$ base substitutions/site/year. This is about 100 times higher than that of other DNA viruses, while about 1000 times lower than that of RNA viruses (Chu and Lok, 2002). The emergence of drug resistance occurs more slowly for HBV than for HIV-1 and HCV, two other viruses that exist as quasispecies (Lai et al., 2003). This fact could be explained by the combination of incomplete inhibition of virus replication by some NRTIs, slow turnover of covalently closed circular DNA in chronically infected hepatocytes and constraints on HBV evolution imposed by its overlapping reading frames, in addition to the host immune response (Soriano et al., 2008).

Direct sequencing and reverse hybridization (line probe assay [LiPA])-based methods are techniques commonly used for detecting HBV drug resistance mutations. Nevertheless, mutations must be present in viral quasispecies with a prevalence $\geqslant$20% to be detected by sequencing, and only known mutations are reported by LiPA. The aim of the present study was to analyze the spectrum and dynamics of low-prevalent HBV mutations at both the reverse transcriptase (rt) and S gene by massively parallel ultradeep pyrosequencing (UDPS) in a patient who did not respond to a 13-year antiviral therapy with different NRTIs. This technology is based on a massively parallel PCR amplification and pyrosequencing

* Corresponding author at: Centro Nacional de Referencia para el SIDA, Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires, Argentina. Tel.: +54 11 4508 3689/3671; fax: +54 11 4508 3705.
E-mail address: quarleri@fmed.uba.ar (J. Quarleri).
[1] These authors contributed equally to this work.

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at a picoliter scale of individual DNA molecules (454 Life Sciences distributed by Roche), allowing the simultaneous analysis of thousands of clonally amplified regions, increasing the probability of detecting minority variants (Shendure and Ji, 2008).

The patient is a 48 year-old woman with chronic active hepatitis B. Her HBV-related parameters and biochemical characteristics have been previously described (Cassino et al., 2011). HBV isolates from this case were ascribed to genotype A2 and showed the presence of A1762T, G1764A, and T1753C mutations at the basal core promoter (BCP) during the entire follow-up. Written informed consent from the patient was obtained.

Non-adherence to different HBV therapy schemes was excluded by the medical practitioner.

HBV genomic analysis was performed using six serum samples. The first sample was collected under the naïve condition, while the other five were obtained on therapy and distributed in two time periods 7 years apart.

The patient exhibited three different HBe/anti-HBe profiles during follow-up. From 1996 to mid 2004, such profile was HBe(−)/anti-HBe(+), then she showed a sero-reversion [HBe(+)/anti-HBe(−)] up to 2007. From 2008 to 2010 both markers were simultaneously detected.

High levels of HBV plasma viral load were observed during follow-up despite antiviral therapy (Table 1).

HBV-DNA was extracted and the HBV polymerase domain, amino acids 1–344 (Stuyver et al., 2001), was amplified by PCR using primers with the sequences (forward from nt 3196 to 3215) 5′-CCTCAGGCCATGCAGTGGAA-3' and (reverse from nt 1265 to 1308) 5′-CCT GCTGCGCGCAAAACAAGCGGCTAG-GAGTTCCGCAGTATGGA-3′.

UDPS was used to sequence the HBV polymerase gene after PCR amplification. Purified amplicons were quantified by Quant-it Picogreen (Invitrogen, Life Technologies, MI). In addition, the Agilent 2100 bioanalyzer (Agilent Life Science, Santa Clara, CA) was used to verify the quality and length of the amplicons. After quality controls, PCR amplicons were pooled in equimolar concentrations and sequenced on the 454 Life Science platform (GS-FLX, Roche Applied Science) according to the Manufacturer's instructions. A total of 4084 partial HBV polymerase gene sequence reads were returned from the UDPS on the six serum samples from the patient; the number of reads per sample ranged between 474 and 917. In order to analyze the data generated by UDPS, the GS Amplicon Variant Analyzer (454 Life Sciences, Roche) was used. To distinguish authentic minority variants from technical artifacts, we used an estimation threshold of 1%. Those variants detected above the threshold by UDPS were unlikely to have resulted from a technical artifact based on a previously reported statistical theoretical calculation (Eriksson et al., 2008).

Table 1 depicts the main nucleotide and amino acid changes observed in the surface antigen (s) and the retrotranscriptase (rt) domain of the viral polymerase.

Three substitutions at the S protein sF134L, sY161F, and sI208T were observed when the patient was treatment-naïve and anti-HBe positive. The last two mutations were synonymous for HBV polymerase; only the rtV142A appeared with an unknown role on HBV drug-resistance. Interestingly, these S and rt mutations disappeared when the patient had been under lamivudine (LMV) therapy for two years. Instead, HBV displayed (i) a (C–T) substitution at nt 730 that affected both the S protein (sL192F) and the polymerase (rtA200V), and (ii) a (G–T) change at nt 743, leading to the appearance of the LMV resistance YIDD motif. Both rt mutations were associated ($p < 0.05$, Chi-squared test) and once LMV was stopped, remained as a minor population. After LMV interruption, monotherapies were sequentially introduced: LMV – as second cycle – (May-01 to Jun-06), adefovir – ADV – (Jul-06 to Nov-07) and, entecavir – ETV – (Dec-07 to May-08). Subsequently,

Table 1
Nucleotide (white column) and amino acid (light gray columns) replacements (non-synonymous are bolded) in the reverse transcriptase (rt) domain of polymerase and the surface antigen (S protein) by comparison with the prototype sequence of genotype A2 (GenBank X70185) taken as wild type (wt). Frequency (dark gray columns) of each replacement is depicted according to the sample analyzed which is identified by the date (a), the on-therapy status of the patient at the time of collection (b) and, the HBV viral load as $\times 10^6$ IU/mL (c). Only values with frequency above 1% are shown. The (−) means absence or frequency <1%

HBV Polymerase (rt domain)				**HBV S protein**			**Frequency (%) of substitutions detected by UDPS**					
Nucleotide position: wt/mut	wt	Pos	mut	wt	Pos	mut	a. Jun-98 b. Naïve c. 6	Feb-01 IFN LMV 39.2	May-01 Stop 36.7	Dec-08 ETV+TDV >110	May-09 ETV+TDV 6	Aug-09 ETV+TDV 36
437:T/C	L	102	L	**L**	**94**	**S**	-	-	-	9.5	1.3	9.6
514:C/A	**T**	**128**	**N**	**P**	**120**	**T**	-	-	1.0	-	25.0	9.1
556:T/C	**V**	**142**	**A**	**F**	**134**	**L**	16.2	-	13.8	20.6	9.7	10.5
558:T/A	**S**	**143**	**T**	**F**	**134**	**L**	-	-	-	-	8.8	5.5
558:T/G	**S**	**143**	**A**	**F**	**134**	**L**	-	-	-	9.8	-	-
638:A/T	I	169	I	**Y**	**161**	**F**	29.1	-	11.3	26.3	23.4	33.3
647:A/G	G	172	G	**E**	**164**	**G**	-	-	-	-	1.1	7.8
741:T/A	**L**	**180**	**M**	S	171	S	-	1.2	-	99.0	98.9	92.7
681:A/C 682C/T	**T**	**184**	**L**	**L** L	**175** 176	**F** L	-	-	-	9.7	1.3	5.5
730:C/T	**A**	**200**	**V**	**L**	**192**	**F**	-	71.7	4.5	82.1	87.4	71.2
743:G/T	**M**	**204**	**I**	**W**	**196**	**L**	-	99.1	6.0	82.1	85.7	74.9
741:A/G 743G/T	**M (I)**	**204**	**V**	**I**	**195**	**M**	-	-	-	15.2	12.0	20.1
750:G/A 752G/A	**V**	**207**	**I**	**M** **W**	**198** **199**	**I** **END**	-	-	-	3.7	9.4	9.6
779:T/C	H	216	H	**I**	**208**	**T**	48.9	-	25.5	14.9	13.7	24.2
786:T/G	**S**	**219**	**A**	**S**	**210**	**R**	1.7	-	-	39.7	10.2	16.0
791:G/A	**V**	**224**	**I**	L	215	L	-	-	-	-	13.3	6.4

Abbreviations: mut: mutated; Pos: amino acid position (following each gene number); IFN: interferon; LMV: lamivudine; ETV + TDV: Entecavir plus tenofovir; stop: therapy interruption.

tenofovir (TDV) was added to ETV therapy, and this combination continues to the end of the follow-up period.

Interestingly, when the patient was on the combined therapy scheme, the frequency of variations at S and polymerase was higher than the previously observed under monotherapy. Still, it did not appear to be detrimental for viral fitness, considering the persistent high level of viral load. After three years under the same antiviral therapy, the HBV viral load levels reveal persistent high replication (Nov-2009: $>110 \times 10^6$ IU/mL; Mar-2010: 4.3×10^6 IU/mL; Jul-2010: 4.9×10^6 IU/mL; Sep-2010: 2.4×10^5 IU/mL; May-2011: 1.6×10^5 IU/mL). Likewise, the simultaneous presence of HBe/anti-HBe was related to an extensive hepatocyte damage and severe immune-mediated liver injury and subsequent dysfunction, as well as reactivation of HBV replication (Wang et al., 2011).

At this time, the three S protein changes (sF134L, sY161F, and sI208T) previously detected when the patient was treatment-naïve, reappeared and remained during follow-up. The rtM204I and rtA200V also reappeared accompanied by another well-known LMV resistant mutation rtL180M at high frequency (>90%) along the follow-up. The latter had been previously detected at very low frequency (1.2%) seven years before, but it was not found by clonal analysis (Cassino et al., 2011). Another substitution at the rt204 codon (nt 741 A–G), leading to rtM204V (which also causes the sI95M), emerged at lower frequency. Concomitantly, two G–A mutations (nt 750 and 752) emerged, affecting the S protein at sM198I and sW199 as well. The rtM204V and rtV207I appeared as non-independent changes ($p < 0.05$, Chi-squared test) during follow-up. The rtA200V and rtV207I mutations are associated with NRTI resistance (Margeridon-Thermet et al., 2009; Rhee et al., 2010). Few studies have reported the HBV infection of a single individual exhibiting the concomitant detection of rtM204I/V mutations (Pallier et al., 2006) that were not detected when clonal analysis was carried out (Cassino et al., 2011); their relative abundance could be influenced by the HBV genotype and the replication environment, including the therapy switch from LMV to ETV plus TDV (Bottecchia et al., 2008; Leemans et al., 2008).

The changes (A–C and C–T) at nt 681 and 682 promoted the rtT184L ETV resistance associated mutation that also affected the S protein (sL175F), and remained at low frequency during the ETV-TDV therapy. Another two rare substitutions (nt 437 and 786) were detected affecting the S protein -sL94S, sS210R-, but only the latter had an impact on the polymerase (rtS219A). At the last two sampling times, three new nucleotide changes were detected. The first one (C–A at nt 514) affected both the S protein (sP120T) and the polymerase (rtT128N). The mutations sP120T and sF134L affected the major hydrophilic region (MHR) of HBsAg and the sP120T also reduced the antigenicity to a comparatively lesser extent than sG145R (Tian et al., 2007; Torresi et al., 2002). Here, when the sP120T emerged, the viral load decreased since both the HBsAg secretion and replication of LMV-resistant variants were impaired. However, when the frequency of sP120T decreased, the viral load was restored, probably due to the presence of 1762/1764 dually mutated BCP (Amini-Bavil-Olyaee et al., 2010). Although having found other S protein mutations outside the "a" determinant occurring in combinations (sY161F, and sI208T), the influence of each and other single mutations (sL94S, sE164G) on HBsAg antigenicity assessed by binding of these variants to the "a" determinant-specific monoclonal antibody as well as HLA typing and measurement of cytotoxic T lymphocyte activity, are needed to evaluate their functional significance.

Despite the high-sensitivity of UDPS analysis on the diversity of quasispecies, the mutations conferring primary resistance (or reduced sensitivity) to ADV/TDV (rtA181V/T, rtN236T) (Locarnini and Zoulim, 2010) were not detected at any sampling time. This scenario was previously described among patients with ongoing HBV viremia who did not have demonstrable drug associated mutations to these drugs (Schirmer et al., 2011). In this regard, it is important to consider that among heavily pre-treated patients, as in our case, the TDV activity against HBV appears diminished in comparison to naïve patients (Lam et al., 2011) and sequential NRTI monotherapy after the occurrence of resistant mutations with LMV and ADV may result in an increased rate of multidrug-resistant HBV by sequential selection of mutations conferring resistance to both the initial and subsequent therapy (Petersen et al., 2012). The spectrum of viral mutants detected by UDPS reflects the level of genetic heterogeneity of the HBV population and the rapid emergence of resistance to antiviral drugs with high genetic barrier based on the previous selected resistance to other NRTIs.

To our knowledge, this is the first study to report the long-term dynamics of drug-resistant HBV and its altered S antigenicity longitudinally analyzed by UDPS. Monitoring the accumulation of drug resistant HBV mutants by UDPS during antiviral therapy appears to be useful in the clinical management of patients with chronic hepatitis B. Understanding the circumstances leading to the appearance of such HBV strains may help to guide future therapies.

Disclaimers

The authors do not have any commercial or other association that might pose a conflict of interest.

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