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To cite this version:

Julien Marlet, Clément Lier, Emmanuelle Roch, Morgan Maugey, Moreau Alain, et al.. Revisiting HBV resistance to entecavir with a phenotypic approach. Antiviral Research, 2020, pp.104869. 10.1016/j.antiviral.2020.104869 . inserm-02908432

HAL Id: inserm-02908432

https://inserm.hal.science/inserm-02908432v1

Submitted on 29 Jul 2020

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Revisiting HBV resistance to entecavir with a phenotypic approach

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Running title: Revisiting HBV resistance to entecavir

Keywords: HBV, entecavir, resistance, phenotypic, polymerase, antiviral

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ABSTRACT

Treatment adaptation after hepatitis B virus (HBV) treatment failure relies on genotypic resistance testing. However, the results of such tests are not always consistent with treatment response. These discrepancies may be due to differences in resistance levels between isolates with the same genotypic resistance testing profiles. We explored this hypothesis by investigating six cases of entecavir treatment failure with an integrative strategy combining genotypic and phenotypic resistance testing, medical record review and therapeutic drug monitoring. Among isolates with genotypic reduced susceptibility to entecavir, one displayed a higher level of resistance to entecavir (mean fold change in entecavir IC_{50} of 1 508 ± 531 vs. 318 ± 53, p=0.008). This isolate harbored a substitution (rt250L) at a position reported to be associated with resistance (rt250V). Reversion to wild-type amino acid at this position partially restored susceptibility to entecavir, confirming that the rt250L mutation was responsible for the high level of resistance to entecavir. One isolate with genotypic resistance to entecavir, harboring the rt173L mutation, displayed a lower level of resistance than the other, harboring the rt202G mutation (mean fold change of 323 ± 124 vs. 6 036 ± 2 100, p=0.20). These results suggest that isolates harboring the rt250L mutations should be considered resistant to entecavir, whereas isolates harboring the rt173L mutations should be considered to display reduced susceptibility to entecavir. An integrative approach to antiviral drug resistance in HBV would provide a more accurate assessment of entecavir treatment failures and help to improve the accuracy of genotypic testing algorithms.

INTRODUCTION

HBV infects 240 million people worldwide and is associated with an increased risk of cirrhosis and hepatocellular carcinoma (1). These complications can be prevented by long-term treatment with nucleos(t)idics analogs, which suppress the replication of HBV DNA (2–4). Entecavir and tenofovir are the two mostly widely used nucleos(t)idics analogs for the first-line treatment of chronic hepatitis B (5–7). They have a much higher genetic barrier to resistance than previous ones (lamivudine and adefovir) (5). In cases of treatment failure, the identification of resistance mutations in the Pol/RT domain is recommended to guide treatment adaptation and minimize the risk of selecting multidrug-resistant strains (5). In research settings, new resistance mutations can be detected *in vitro* with a phenotypic resistance assay which allows the determination the 50% inhibitory concentration (IC_{50}) of a drug for clinical isolates (8).

The lamivudine resistance mutations rt204I/V and the compensatory mutation rt180M, selected in patients with infections refractory to lamivudine, are associated with reduced susceptibility to entecavir *in vitro* (9–17). Patients with lamivudine-refractory infections can nevertheless be treated with entecavir, which has been shown to give good results when used at double the normal dose (1 mg/d) (18–20). Despite this adaptation, entecavir treatment failure can still occur. It is usually associated with selection of additional entecavir resistance mutations (rt184G, rt202I/G or rt250V) (9, 21, 22) but in a subset of patients, these mutations are not detected. In this context, the results of genotypic resistance assays do not always explain treatment failure. The observed discrepancies may be linked to other mutations affecting the Pol/RT domain that are not considered to be resistance mutations in the current guidelines (5). To explore this hypothesis, we retrospectively

analyzed clinical isolates refractory to entecavir, with an integrative strategy combining genotypic and phenotypic assays, together with entecavir drug monitoring.

MATERIALS AND METHODS

Patients and samples

Plasma samples were collected between 2011 and 2018 at Tours University Hospital for routine quantification of HBV viral load (Abbott Real-Time HBV) and genotypic drug resistance testing. Samples were stored at -20°C until further analysis. Patients infected with lamivudine-resistant isolates (rt180M±204V) or entecavir-resistant isolates (rt180M+204V+184G, 202I/G or 250V) and experiencing treatment failure were included in this study. This study was approved by the Tours University ethics committee (no. 2019 055). We retrospectively analyzed the clinical history of each patient. Renal failure, HIV co-infection, cirrhosis and cancer were considered relevant comorbidities. Liver diseases were evaluated with non-invasive markers (transient elastography or biochemical markers) or by liver biopsy. Fibrosis and liver inflammation were classified based on Metavir score. Virological response and treatment failures were defined according to current treatment guidelines (5). We used the online Hep Drug Interactions resource (https://www.hep-druginteractions.org/, University of Liverpool) to check for drug interactions.

Genotypic drug resistance testing

Genotypic drug resistance testing was performed by amplifying and Sanger sequencing the Pol/RT domain (aa rt1-rt344), as previously described (23). Briefly, HBV DNA was extracted with the DSP Virus kit (Qiagen) on the EZ1 Advanced XL system. Pol/RT domains were amplified as previously described (23), sequenced with BigDye Terminator Mix 1.1 (Applied Biosystems) and analyzed with the CLC

Genomics Workbench software V3 (CLC Bio). Known resistance mutations in the Pol/RT domain and HBs Ag escape mutants were searched for using two online algorithms: Geno2pheno [HBV] v2.0 (Max Planck Institute) and HBV tool v0.8, based on Stanford HIValg-Software (HIV-GRADE) (24, 25). Resistance mutations were interpreted according to the latest EASL guidelines (5).

Therapeutic drug monitoring

Entecavir plasma concentrations were determined in a validated liquid chromatography-tandem mass spectrometry assay (LC-MS/MS). Briefly, 200 µL of plasma samples were thoroughly mixed with 400 µL of 0.1% formic acid in acetonitrile and internal standard solution (20 µL; atazanavir-D5). The mixture was centrifuged at 10 000g for 10 min. The supernatant layer was evaporated off under a stream of nitrogen. The residue was reconstituted in 70 µL of 0.1% formic acid in water, and 15 µL of the resulting solution was injected into the LC-MS/MS system. It was equipped with a positive-mode ion electrospray interface (5500 QTRAP, Sciex, Saint Quentin en Yvelines, France) and an ultra-high-performance liquid chromatograph (Shimadzu, Saint Quentin en Yvelines, France). Data were acquired by multiple reactions monitoring, including m/z 278.1 → m/z 152.1 and m/z 278.1 → m/z 81.1 for entecavir. Chromatographic separation was performed on an Acquity HSS T3® 1.8 µm (2.1 x 100 mm) column (Waters, Saint Quentin en Yvelines, France) maintained at 45°C. The mobile phase consisted of a mixture of solvent A (water, formic acid (99.9/0.1, v/v)) and solvent B (acetonitrile, formic acid (99.9/0.1, v/v)), delivered at a flow rate of 0.5 mL/min with the following linear gradient: a ramp from 5% to 95% of B within 5 min, kept between 5 to 6 min with 95% B. At 6.1 min, the column was put back to 5% of B for a 2 minutes equilibration. The expected residual concentrations ranged from 0.26 to 0.50 ng/mL for patients treated with 0.5

mg entecavir per day and from 0.44 to 0.86 ng/mL for patients treated with 1 mg entecavir per day (26).

Construction of vectors containing Pol/RT from clinical isolates

The plasmid pCI_HBVpg1820 (obtained from Camille Sureau, INTS, Paris, France) contained 1.1 units of HBV genome (genotype D *ayw3*) and allowed the transcription of pregenomic HBV RNA transcription from a CMV promoter (27). This plasmid was digested with *Eco*RI and *Sph*I to remove the HBV Pol/RT domain and generate the pCI_HBV∆RT plasmid. The whole Pol/RT domains (aa 1-344) of clinical isolates were amplified by nested PCR, using primers previously described (28) and Platinum™ PCR Supermix High Fidelity (Invitrogen™). Cycling reactions were performed on a T3000 Biometra thermocycler with the following parameters: 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, 180 s at 68°C and a final extension for 10 min at 68°C. The second round of PCR added *EcoRI* and *SphI* 5' and 3' overhangs to the amplified Pol/RT domain. Cycling reactions were the same as above with a shorter elongation step (120 s). The amplified Pol/RT domains were inserted between the *Eco*RI and *Sph*I restriction sites of pCI_HBV∆RT. After *E.coli* transformation, recombinant clones were analyzed by Sanger sequencing to select one clone harboring the Pol/RT domain from clinical isolates.

Site-directed mutagenesis

The L250M_F (TATTCCCTAAACTTCATGGGTTACATAATTG) and L250M_R (CAATTATGTAACCCATGAAGTTTAGGGAATA) primers were designed to introduce the L250M mutation into the Pol/RT domain of isolate P7. The Pol/RT domain of

isolate P7 containing the L250M mutation was amplified in two fragments (RT_F + L250M_R and RT_R + L250M_F), under the cycling conditions described above, but with a lower annealing temperature (50°C) and a shorter elongation step (60 s). The two PCR products were purified with NucleoFast® 96 PCR clean-up (Macherey-Nagel, Hoerdt, France), fused together by PCR with RT_F and RT_R, and introduced into pCI_HBVΔRT, as described above.

Phenotypic drug resistance and replication capacity testing

Huh7 cells (29) were maintained in DMEM medium (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (Thermo Fisher Scientific), at 37°C, under an atmosphere containing 5% CO_2. On day 0, Huh7 cells were used to seed a 96-well plate at a density of 10^4 cells per well. The cells were then transfected with 100 ng/well pCI_HBV1.1x in the presence of Fugene 6 (Promega), according to the manufacturer's protocol. Entecavir monohydrate (Sigma Aldrich) was added to the cells at six different final concentrations ranging from 2.5 to 50 000 nM. DMSO was adjusted to a final concentration of 0.05% in all wells. All drug concentrations were tested in triplicate. All steps from this point until cell lysis were performed in a level 3 biosafety laboratory. On day 2, the cell culture medium was removed and replaced with fresh medium and drugs. On day 4, the medium was removed, and the cells were washed twice with PBS. Cellular and viral membranes were lysed with 1% IGEPAL® CA-630 (Sigma Aldrich) and one freeze-thaw cycle. The resulting lysate was treated with 1 U of RQ1 DNase (Promega) for 3 h at 37°C, to eliminate any residual plasmid DNA. Intracapsid viral DNA was extracted with 30 µL of Quickextract (Epibio), according to the manufacturer's protocol. HBV and plasmid DNA levels in this extract were quantified by duplex real-time PCR, using primers described elsewhere (28), on a LightCycler 480 II apparatus (Roche) using TaqMan

Universal PCR Mastermix II without UNG (Thermo Fisher Scientific). Cycling reactions were performed with the following parameters: 10 min at 95°C, followed by 45 cycles of 15 s at 95°C, 30 s at 60°C, 30 s at 72°C. Inhibitory concentrations 50% (IC_{50}) were determined using GraphPad Prism v5.0 by least square regression. Fold change in resistance corresponded to entecavir IC_{50} of a given isolate, relative to entecavir IC_{50} of the wild-type HBV genotype D *ayw3* strain (pCI_HBVpg1820) tested in parallel. Entecavir ICs_{50} and fold change in resistance were determined in three independent experiments and compared in Mann-Whitney tests. Replication capacity relative to the same wild-type strain as before was determined by the quantification of HBV DNA replication in six different wells after four days of culture without antiviral drugs. Results were represented as the means and standard errors.

Data availability

All sequences were deposited in the GenBank database (Accession numbers: MN562223-MN562231).

RESULTS

Genotypic drug resistance testing

During the study period, 110 genotypic drug resistance assays were performed on HBV clinical isolates, including nine isolates harboring mutations conferring reduced susceptibility or resistance to entecavir (Table 1). Among these, six isolates were detected in the context of entecavir treatment failure (P3B, P4, P5, P6, P7 and P8) and three in other contexts of treatment failure (P1, P2 and P3A) (Table 1). Isolates P3A and P3B were from the same patient (P3). This patient experienced lamivudine treatment failure (P3A), followed four years later by a partial viral response to entecavir (P3B). Treatment history and HBV viral loads for the six patients with

infections refractory to entecavir are presented in *Figure 1*. No relevant interactions between HBV treatment and other concomitantly used drugs were detected for these patients. Impaired immune responses in patients P5 and P3, both of whom were co-infected with HIV, may have contributed to treatment failure. No other clinical factors potentially associated with resistance were detected in the other patients.

Genotypic drug resistance testing detected mutations associated with resistance to entecavir and treatment failure in patients P3B (rt204V+180M+173L) and P8 (rt204V+180M+202G) (Table 1 & *Figure 2*) (5). By contrast, isolates P4, P5, P6 and P7 harbored only mutations associated with reduced susceptibility to entecavir (rt204I/V±180M) that could not account for entecavir treatment failure (Table 1 & Figure 2) (5). Isolate P7 harbored one additional mutation (rt250L) located at the same position as another substitution associated with resistance (rt250V) (5). Phenotypic resistance testing was performed on isolate P7 Pol/RT, to determine the role of the rt250L mutation in entecavir treatment failure.

A retrospective analysis of plasma entecavir concentrations revealed that these concentrations were lower than anticipated (0.44-0.86 ng/mL), suggesting poor treatment compliance or inappropriate dosage, for patients P6 (0.32 ng/mL) and P8 (0.29 ng/mL). By contrast, patients P7 and P3B had entecavir concentrations in the expected range (>0.86 ng/mL). Samples P4 and P5 were no longer available for entecavir plasma concentration measurement.

None of these isolates harbored mutations conferring cross-resistance tenofovir (rt181T/V, 236T). As predicted by this genotypic testing, rescue therapy with tenofovir (with or without entecavir) lead to viral response (P4, P5, P6, P7 and P8) or partial viral response (P3) in entecavir refractory patients.

Phenotypic resistance assays

Phenotypic resistance assays were performed on these nine clinical isolates of HBV, to quantify the level of resistance (*Figure 3*A and Table 2). The mean entecavir IC_{50} and the mean fold change in resistance differed significantly (p=0.0005) between: i) wild-type isolate (47 ± 12 nM, mean fold change of 1.00 ± 0.12), ii) isolates P1, P2, P3A, P4, P5 and P6 harboring rt204I/V±180M mutations associated with reduced susceptibility to entecavir (16 160 ± 3 148 nM, mean fold change of 318 ± 53) and iii) isolate P8 harboring the 202G mutation associated with high-level resistance to entecavir (229 466 ± 79 796 nM, mean fold change of 6 036 ± 2 099) (*Figure 3*A). Interestingly, phenotypic resistance differed from genotypic predictions for two (P7 and P3B) of six isolates associated with entecavir treatment failure (*Figure 3*A).

Isolate P7, harboring the rt204V+180M+250L mutations, displayed a higher level of resistance to entecavir than other isolates harboring only the rt204I/V±180M resistance mutations (75 303 ± 19 651 nM vs. 16 160 ± 3 148 nM, mean fold change of 1 508 ± 531 vs. 318 ± 53, p=0.008, *Figure 3*A). Reversion to wild-type amino acid at position rt250 by site-directed mutagenesis (P7 del 250L) decreased resistance (mean fold change of 1 508 ± 531 vs. 394 ± 171, p=0.10) to a level similar to that for other isolates harboring only the rt204I/V±180M resistance mutations (*Figure 3*A). The 250L mutation was, therefore, responsible for the high level of resistance to entecavir.

Isolate P3B, harboring the rt204V+180M+173L resistance mutations, displayed a lower level of resistance to entecavir than isolate P8 harboring the rt204V+180M+202G entecavir resistance mutations (mean fold change of 323 ± 124

vs. 6 036 ± 2 099, *p*=0.20), although not significantly so. Isolate P3B displayed a level of resistance similar to that of other isolates harboring only the rt204I/V±180M resistance mutations (mean fold change of 323 ± 124 vs. 318 ± 53, *p*=0.79). Thus, in HBV clinical isolates harboring the rt204V+180M mutations, the rt202G mutation was associated with resistance to entecavir, whereas the rt173L mutation was not.

The last three isolates associated with entecavir treatment failure (P4, P5 and P6) had low levels of resistance (mean of 351 ± 60, *Figure 3*A), consistent with the genotypic assays results.

Replication capacities

Replication capacities relative to the wild-type strain (HBV D *ayw3*) were determined for these nine HBV clinical isolates and for the P7 del 250L mutant (*Figure 3*B). The values obtained were plotted against the fold change in entecavir IC_{50} for each isolate, to visualize the cost in replication fitness (*Figure 4*).

Replication capacities were reduced for isolate P7, and to a lower extent for isolates P4 and P5 (*Figure 3* & *Figure 4*, *p*≤0.01). Reversion to wild-type amino acid at position rt250 in isolate P7 (P7 del 250L) restored a normal level of replication capacity (21% ± 3% vs. 98 ± 9%, *p*=0.002), similar to that for other isolates with rt204I/V±180M mutations. This confirmed that the rt250L mutation was responsible for both the increase in resistance and the decrease in replication capacity (*Figure 3* & *Figure 4*). Isolate P5 lacked the rt180M compensatory mutation, which could explain its reduced replication capacity. Isolate P4 replication capacity was also reduced but was not different from that of isolate P2, harboring the same resistance

mutations (64% ± 5% vs 80% ± 10%, *p*=0.32). As such, the reduced replication capacity of isolate P4 might not be biologically significant.

An analysis of sequential samples P3A (rt204I+180M) and P3B (rt204V+180M+173L) revealed a moderate, although not significant, increase in replication capacity (93 ± 4% vs 124 ± 13%, *p*=0.07) with no increase in resistance (mean fold change of 300 ± 231 vs. 323 ± 124, *p*=0.80). These observations are consistent with the selection of isolate P3B and the rt204V+173L mutations in a context of partial viral response to entecavir.

DISCUSSION

In this work, we explored six entecavir-refractory HBV isolates through a combined genotypic and phenotypic approach. As others (9, 11–16, 22, 30, 31), we used a phenotypic resistance assay based on Pol/RT recombinant viruses to determine the IC_{50} of entecavir for clinical isolates. These recombinant viruses harbored the same Pol/RT domain as detected by genotypic drug resistance testing (i.e. major variant) as this was the most relevant approach in the context of treatment failure. We used qPCR for HBV DNA quantification (13–15, 28) rather than southern blotting, the technique generally used until recently (9, 11, 12, 14–16, 22, 30, 31). This qPCR technique could not distinguish between cccDNA, pgRNA and rcDNA, but it did allow the precise and sensitive measurement of HBV DNA levels.

We demonstrate here that a phenotypic approach can detect resistance in some isolates predicted to display reduced susceptibility by genotypic resistance testing. One isolate (P7) harboring the rt250L mutation was more resistant to entecavir than other isolates lacking this mutation (mean fold change of 1 508 ± 531 vs. 318 ± 53, *p*=0.008). Reversion to wild-type amino acid at position rt250 in isolate P7 by site-

directed mutagenesis restored reduced susceptibility to entecavir (394 ± 171, *p*=0.10), confirming that the rt250L mutation conferred high level resistance to entecavir. The rt250V and 250L mutations affect the same position in the protein; both were known to confer a high level of resistance to entecavir *in vitro* but they were associated with different treatment outcomes *in vivo* (22, 32). The rt250V mutation was enough to result in viral breakthrough in lamivudine-resistant isolates (rt204V+180M) (5, 22). By contrast, the rt250L mutation was associated with viral breakthrough only in the presence of other entecavir resistance mutations (rt184G, 202G or 250V) (5, 22, 32). As a result, only the rt250V mutation was considered a resistance mutation in algorithms for genotypic resistance testing (5). To our knowledge, this is the first description of viral breakthrough on entecavir treatment associated with the selection of the rt204V+180M+250L resistance mutations without selection of additional entecavir resistance mutations. These findings suggest that rt250L should be considered as an entecavir resistance mutation in future guidelines. Unlike other entecavir resistance mutations, the rt250L mutation was associated with a significant decrease in HBV replication capacity (21 ± 3%), which may have prevented its emergence *in vivo* and may account for the rarity of its description in clinical studies (22).

Isolates harboring the rt204V+180M+173L or 202G resistance mutations are considered to be resistant to entecavir in current treatment guidelines (5). In contrast with the treatment guidelines, only mutation rt202G, but not rt173L, is associated with high level resistance to entecavir. Mutation rt173L is a compensatory mutation associated with restauration of normal replication capacity (5, 9, 16, 33). We confirm these findings here, with the observation of a high level of resistance to entecavir associated with the rt202G mutation (P8) but not with the rt173L mutation (mean fold

change of 6 036 ± 2 099 vs. 323 ± 124, p=0.20). Thus, genotypic predictions may not always be in accordance with phenotypic observations.

This study demonstrates the need for an integrative approach in the context of entecavir treatment failure, particularly in situations in which no resistance is detected in genotypic and/or phenotypic assays. One patient had kidney failure and insufficient entecavir dosing (P6). Another patient was co-infected with HIV and may have impaired immune response (P5). Together with the reduced susceptibility to entecavir, these factors may have led to entecavir treatment failure without the selection of additional resistance mutations.

Using a phenotypic assay, we were able to quantify the level of resistance to entecavir of a series of HBV isolates that were refractory to entecavir treatment. In clinical practice, genotypic drug resistance testing is used as a first-line assay for guiding treatment adaptation. This assay is based on only a subset of known mutations and does not reflect the heterogeneity of susceptibility between clinical isolates. By combining genotypic and phenotypic approaches, we were able to develop a clearer understanding of the reasons for entecavir treatment failure. Our results argue for isolates harboring the rt180M+204V+250L mutations to be considered resistant to entecavir and for those harboring the rt180M+204V+173L mutations to be considered to display reduced susceptibility to entecavir. Further combined phenotypic and genotypic characterizations of HBV clinical isolates may help to improve the accuracy of genotypic testing algorithms and to guide treatment adaptation more effectively in clinical practice.

ACKNOWLEDGMENTS

We thank the Thellie endowment fund and our generous donators for funding this project. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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Table 1: Treatment history and characteristics of the HBV isolates harboring mutations conferring reduced susceptibility or resistance to entecavir

Isolate	Sex	Age (years)	Comorbidities	Metavir score	Treatment history	Treatment at failure	Total treatment duration (years)	Type of treatment failure	HBe Ag[a]	HBV viral load (log IU/mL)[b]	HBV genotype	Pol/RT resistance mutations			Treatment adaptation	Treatment outcome[c]
												L180	M204	Other		
Mutations conferring reduced susceptibility to entecavir																
P1	M	26	-	U	None	Telbivudine	4	Breakthrough	+	5.6	C	M	I	-	Tenofovir	Unknown
P2	M	37	-	A1F1	None	Lamivudine	13	Breakthrough	-	2.5	E	M	V	-	Entecavir [d]	VR
P3A	F	44	HIV	F1	None	Lamivudine	7	Breakthrough	+	7.4	E	M	I	-	Tenofovir	Partial VR
P4	M	52	-	U	Adefovir Entecavir	Entecavir	6	Breakthrough	-	3.0	A	M	V	-	Tenofovir	VR
P5	M	55	HIV Kidney	A1F2/3	Lamivudine Tenofovir	Entecavir [d e]	28	Breakthrough	+	1.4	C	-	I	-	Tenofovir	VR
P6	F	76	Kidney	A1F3	Lamivudine Adefovir	Entecavir [d e]	8	Primary non response	-	3.3	A	M	V	-	Tenofovir + Entecavir [d e]	VR
P7	F	62	-	A2F3	None	Entecavir	4	Breakthrough	-	4.4	A	M	V	250L[f]	Tenofovir + Entecavir	VR
Mutations conferring resistance to entecavir																
P8	M	45	-	F0F1	Lamivudine Adefovir Tenofovir	Entecavir [d]	17	Primary non response	-	2.7	A	M	V	202G	Tenofovir	VR
P3B	F	44	HIV	F1	Lamivudine Tenofovir	Tenofovir + Entecavir [d]	11	Partial VR	+	1.5	E	M	V	173L	Tenofovir + Entecavir [d]	Partial VR

VR, virological response; U, unknown; Kidney, chronic kidney failure; [a] Architect HBe Ag assay; [b] Abbott HBV Real Time assay; [d] Double-dose ETV was used for isolates with reduced susceptibility; [e] ETV dose was adapted according to creatinine clearance for patients with renal failure; [f] Unlike other mutations at this position, these mutations are not considered to be entecavir resistance mutations

Table 2: Fold change in entecavir 50% inhibitory concentrations (ETV ICs_{50}) and replication capacities of HBV isolates harboring Pol/RT mutations conferring reduced susceptibility or resistance to entecavir

Isolate	Pol/RT resistance mutations			Phenotypic drug resistance testing		
	L180	M204	Other*	ETV IC_{50} (nM)	Fold change	Replication capacity (%)**
WT	-	-	-	47 ± 12	1.00 ± 0.12	100 ± 11
P1	M	I	-	15 736 ± 8 912	275 ± 109	87 ± 8
P2	M	V	-	13 317 ± 6 409	282 ± 172	80 ± 10
P3A	M	I	-	12 643 ± 7 034	300 ± 231	93 ± 4
P4	M	V	-	13 377 ± 6 045	296 ± 105	64 ± 5
P5	-	I	-	20 676 ± 9 577	461 ± 94	63 ± 5
P6	M	V	-	21 212 ± 13 036	296 ± 117	90 ± 16
P7	M	V	M250L	75 303 ± 19 651	1 508 ± 531	21 ± 3
P7 del 250L	M	V	-	2 990 ± 1 298	394 ± 171	98 ± 9
P8	M	V	**S202G**	229 466 ± 79 796	6 036 ± 2 099	121 ± 8
P3B	M	V	**V173L**	8 225 ± 6 230	323 ± 124	124 ± 13

* Mutations associated with resistance to entecavir and treatment failure (in bold, mutations included in current treatment guidelines *(5, 32)*); **, relative to wild-type strain HBV D *ayw3*

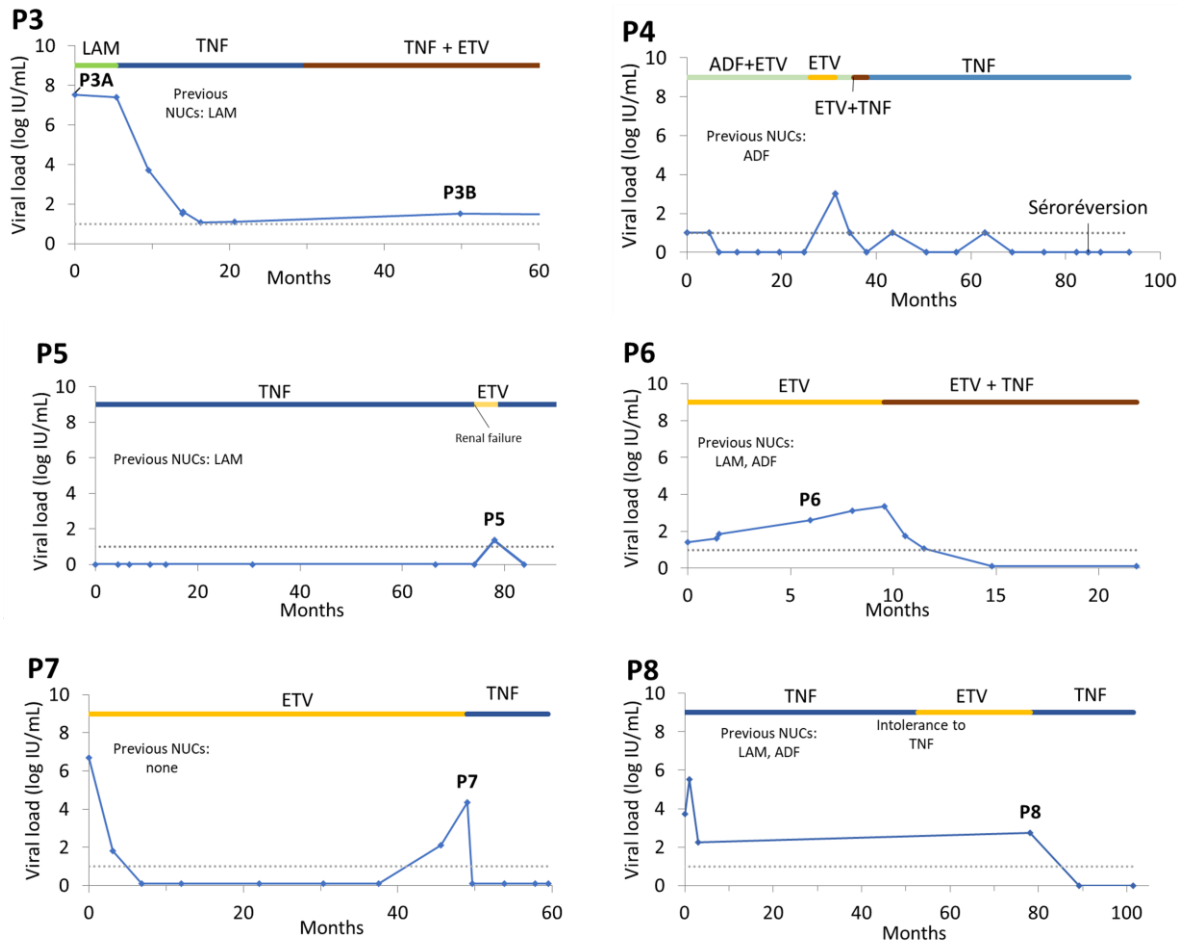

Figure 1: Biological and clinical follow-up of patients experiencing entecavir treatment failure

The clinical isolates included in this study are identified with bold characters. NUCs, Nucleos(t)idics analogs; LAM, lamivudine; ETV, entecavir; TNF, tenofovir disoproxil fumarate; ADF, adefovir. HBV viral loads were quantified with the Abbott HBV Real Time assay. Its lower limit of detection (10 IU/mL) is represented with a dashed line.

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                    140       150       160       170       180       190       200
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Consensus A     NLHDSCSRQL YVSLMLLYKT YGWKLHLYSH PIVLGFRKIP MGVGLSPFLL AQFTSAICSV VRRAFPHCLA
P4              .......... .......... ..R....... ..I....... .........M .......... ..........
P6              .......... .......... .......... .......... .........M .......... ..........
P7              .......... .......... .......... .......... .........M .......... ..........
P7 del 250L     .......... .......... .......... .......... .........M .......... ..........
P8              .......... .......... ..R....... .......... .........M ...I...... ..........
Consensus C     DLHDSCSRNL YVSLLLLYKT FGRKLHLYSH PIILGFRKIP MGVGLSPFLL AQFTSAICSV VRRAFPHCLA
P1              .......... .......... .......... .......... .........M .......... ..........
P5              .......... .......... .......... .......... .......... .......... ..........
Consensus E     NLHDSCSRNL YVSLMLLFKT FGRKLHLYSH PIIMGFRKIP MGVGLSPFLL AQFTSAICSV VRRAFPHCLA
P2              S......... .G........ .......... .......... .........M .......... ..........
P3A             .......... .......... .......... .......... .........M .......... ..........
P3B             .......... .......... .......... .......... ..T......M .......... ..........

                    210       220       230       240       250
                ....|....| ....|....| ....|....| ....|....| ....|....|
Consensus A     FSYMDDVVLG AKSVQHLESL YTAVTNFLLS LGIHLNPNKT KRWGYSLNFM
P4              ...V...... ......R.A. .......... .......... ..........
P6              ...V...... ......R... .......... .......... ..........
P7              ...V...... ..T...R... .......... .......... .........L
P7 del 250L     ...V...... ..T...R... .......... .......... ..........
P8              .G.V..I... ......R.A. .......... .......... ..........
Consensus C     FSYMDDVVLG AKSVQHLESL FTSITNFLLS LGIHLNPNKT KRWGYSLNFM
P1              ...I...... .......... .......... .......... ..........
P5              ...I...... .......... .......... .......... ..........
Consensus E     FSYMDDVVLG AKSVQHLESL YTSVTNFLLS LGIHLNPNKT KRWGYSLNFM
P2              ...V...... .......... .......... .......... ..........
P3A             ...I...... .......... .......... .......T.. ..........
P3B             ...V...... .......... .......... .......T.. ..........
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Figure 2: Pol/RT domain sequences of HBV clinical isolates harboring mutations conferring reduced susceptibility or resistance to entecavir. Amino acids 130 to 250 of Pol/RT domains are represented, including all known positions associated with resistance. Mutations associated with reduced susceptibility or resistance to entecavir are shown in gray and black, respectively. Amino acids identical to the consensus sequence for the genotype concerned are indicated with a dot (26,27). The conserved YMDD motif is framed in red. Two Pol/RT mutations (rt131S and 142G framed in black in isolate P2) were associated with concomitant HBs Ag escape mutants in the overlapping S gene (s123A and s134V, not shown).

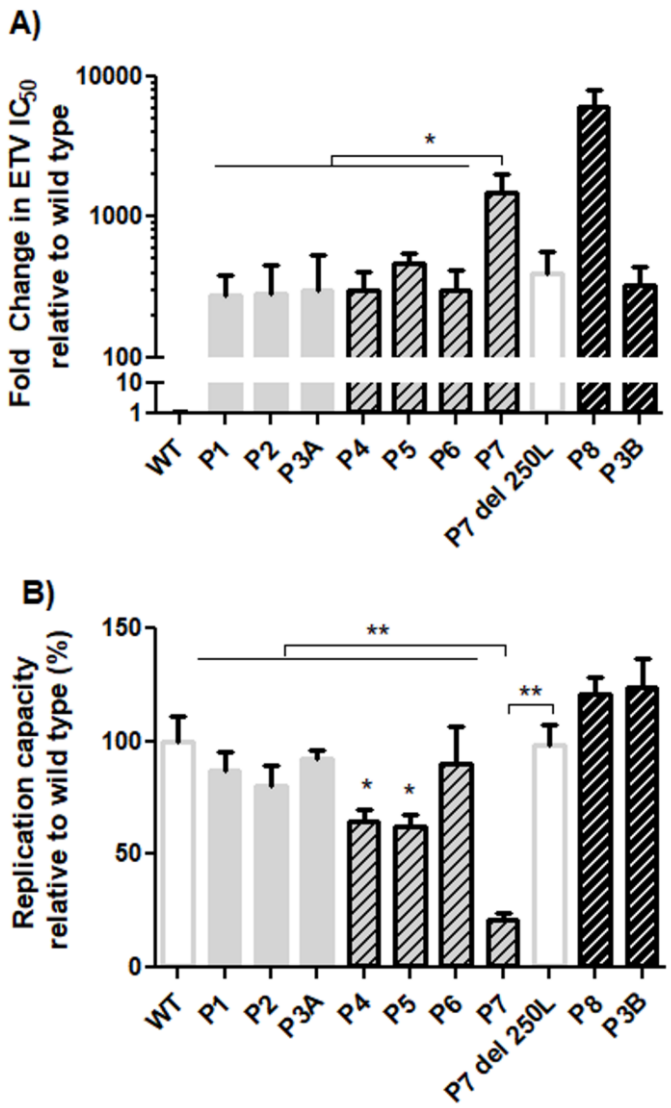

Figure 3: Phenotypic analysis of HBV isolates associated with entecavir treatment failure (hatched bars) or with lamivudine or telbivudine treatment failure (gray solid-color bars).

Laboratory strains, including wild-type HBV D *ayw3* (WT) are shown as white bars. Isolates harboring the rt204I/V±180M resistance mutations associated with reduced susceptibility to entecavir are shown in light gray. Isolates harboring the resistance mutations rt204V+180M+202G (P8) or rt204V+180M+173L (P3B) associated with resistance to entecavir are shown in black. The values shown are the mean and standard error of the mean for three independent experiments. A) Phenotypic

resistance assays performed with a gradient of entecavir concentrations from 0.4 to 4.7 log nM. B) Measurement of replication capacities without entecavir. *, p-value≤0.01; **, p-value<0.005.

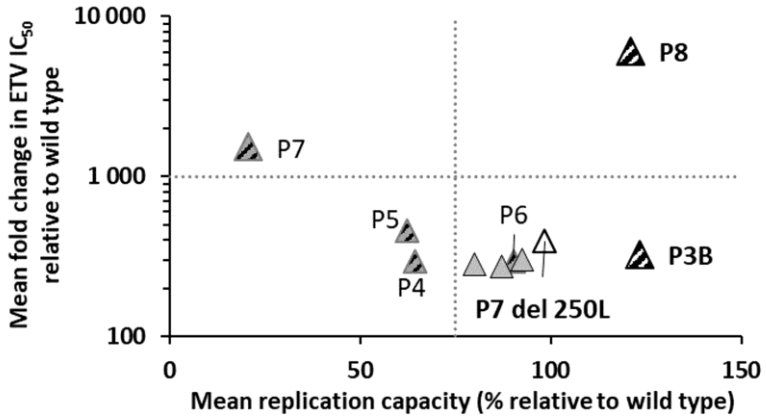

Figure 4: Mean fold change in entecavir IC_{50}, and replication capacity of clinical HBV isolates associated with entecavir treatment failure (hatched triangles), lamivudine or telbivudine treatment failure (solid gray triangles) and laboratory mutant P7 del 250L (white triangle). Isolates harboring the rt204I/V±180M resistance mutations predicted to be associated with reduced susceptibility to entecavir are shown in light gray. Isolates harboring the rt204V+180M+173L or 202G mutations predicted to be resistant to entecavir are shown in black. A vertical dotted and dashed line separates off the isolates with a replication capacity significantly lower than that of the wild-type strain HBV D *ayw3* (p<0.01). The horizontal dotted and dashed line separates the isolates with reduced susceptibility from those displaying resistance (p<0.005).