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A new strategy for studying in vitro the drug susceptibility of clinical isolates of human hepatitis B virus

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Abstract

Background/Aim : Hepatitis B virus resistance to antivirals has become a major clinical problem. Our objective was to develop a new method for the cloning of naturally occurring HBV genomes and a phenotypic assay capable of assessing HBV drug susceptibility and DNA synthesis capacity in vitro.

Methods : Viral DNA was extracted from sera, PCR amplified with newly designed primers, and cloned into vectors that enable, after cell transfection, the initiation of the intracellular HBV replication cycle. Single or multiple clones were used to transfect Huh7 cells. The viral DNA synthesis capacity and drug susceptibility were determined by measuring the level of intracellular DNA intermediate, synthesized in absence or presence of antiviral, using Southern blot analysis.

Results : We have developed, calibrated, then used this phenotypic assay to determine the drug susceptibility of HBV quasi-species isolated throughout the course of therapy from patients selected according to their mutation profile. A multiclonal and longitudinal analysis enabled to measure variation of drugs susceptibility of different viral quasi-species by comparison of IC_{50}/IC_{90s} with standards. The presence of famciclovir, or lamivudine induced mutations in the viral population caused a change in viral DNA synthesis capacity and drug susceptibility *in vitro*, demonstrating the clinical relevance of the assay.

Conclusion : Our phenotypic assay enables the in vitro characterization of the DNA synthesis capacity and drug susceptibility of HBV quasi-species isolated from patients. This assay should allow a better monitoring of patients undergoing antiviral therapy, as well as the screening of novel drugs on emerging resistant strains.

Introduction

Despite the availability of a safe and effective vaccine against hepatitis B, chronic infection with human hepatitis B virus (HBV) remains a major health problem worldwide. The recent development of inhibitors of HBV reverse transcriptase, such as lamivudine (3TC) or adefovir dipivoxil (PMEA), has markedly improved HBV therapy and disease management (1). However the difficulty to eliminate HBV covalently-closed-circular DNA (cccDNA), which is a major determinant responsible for HBV genome persistence in infected cells (2, 3), represents a therapeutic challenge and has required the use of long-term therapy. Unfortunately, the prolonged use of antivirals is associated with the emergence of resistant HBV strains that are responsible for therapeutic failure and progression of liver disease (4-8). Resistance to 3TC and, more recently, to the newly approved anti-HBV nucleotide analogue PMEA, are the consequence of the selection of HBV polymerase gene mutants, i.e. M204V or M204I (4-7) and N236T (9, 10), respectively. Moreover, 3TC resistance mutations can also confer cross-resistance to alternative drugs of the same class, as shown recently for emtricitabine (FTC), a molecule derived from 3TC, and for clevudine (L-FMAU) (11-13). Consequently, the management of drug resistance has become a crucial issue in antiviral therapy against HBV infection.

In the case of HIV therapy, drug resistance testing is now recommended to guide the choice of new drug regimens after the first or multiple treatment failures (14, 15). In addition to genotypic assays (e.g. sequencing), several phenotypic assays have been developed for HIV and are currently used in clinical practice to monitor drug resistance (14, 15). To date, only one phenotypic drug susceptibility assay has been developed for HBV (16). With the current and future development of new anti-HBV molecules, phenotypic drug susceptibility testing could become an important tool for the management of patients infected with resistant HBV isolates and for the evaluation of the effect of new antivirals on circulating clinical HBV isolates.

Until recently, the analysis of the phenotype of naturally occurring or drug induced HBV

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mutants has relied either on PCR-mediated transfer of HBV genome cassettes or on site directed mutagenesis within a well established replication-competent laboratory strain. This allowed for instance to confirm that the M204V/I ± L180M mutants selected *in vivo* were indeed conferring resistance to 3TC *in vitro* (11, 17-19). Moreover, Gunther *et al.* (20) described an original and efficient method for PCR amplification of full-length HBV genomes that was meant to facilitate the analysis of naturally occurring HBV variants. All these methods do not seem appropriate for a standardized phenotypic assay which requires a solid viral genome replication level (i.e. viral DNA synthesis). Here, we describe a new strategy and method for the cloning of HBV genomes isolated from patients into plasmidic vectors and the basis of another phenotypic assay capable of assessing HBV drug susceptibility *in vitro* and evaluate new antivirals on circulating viral strains. To illustrate and validate the assay, the drug susceptibility profile of different viral populations isolated from patients at various times during the course of antiviral treatment is presented.

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Materials and Methods

Plasma samples. Plasma samples, obtained from well-documented chronically HBV-infected individuals, were stored at -20°C until analysis. A reference serum, ACCURUN® 325 HBV DNA positive control manufactured by BBI Diagnostics, was used for the setting up of some experiments.

Antiviral drugs. Lamivudine (3TC), emtricitabine (FTC), and clevudine (L-FMAU) were obtained from Triangle Pharmaceuticals. Adefovir dipivoxil (bis-pom PMEA), and tenofovir disoproxil (bis-poc PMPA) were obtained from Gilead Sciences. Beta-L-FD4C was a generous gift from Dr Y.C. Cheng (Yale University).

Sample preparation and PCR amplifications. Viral DNA was extracted from 200 μl of serum (control or patient serum) using three different commercialized kits (Master pure™ Complete DNA and RNA purification kit [Epicentre], High pure PCR template preparation kit [Roche], and QIAamp™ UltraSens virus kit [Qiagen]). The final volume of elution or resuspension was 60 μl and 5 to 10 μl were used for subsequent polymerase-chain-reaction (PCR) amplifications. All PCRs were performed using a “hot start” procedure with the Expand high fidelity PCR system (Roche) according to manufacturer’s instruction. Three sets of primers were used. Details of primers used are given in Table 1, while their location in the HBV genome is indicated in Figure 1A. Full length HBV (3.2 kb) was amplified according to the method described by Gunther *et al.* (20, 21), with the sense primer P1 containing *HindIII/SapI* sites, and the antisense primer P2 containing *SacI/SapI* sites. A long PCR product of approximately 2800 bp was amplified by 40 cycles (hot-start; 94°C for 40 s, 55°C for 1 min, 68°C for 3 min \pm 2 min after each 10 cycle) with a forward primer A containing a *NotI* site and a reverse primer B containing a *NcoI* site. A short PCR product of approximately 665 bp was amplified by 40 cycles (hot-start; 94°C for 30 s, 50°C for 30 s, 72°C for 45 s \pm 5 s after each cycle starting from cycle 11) with a forward primer C containing a *NcoI* site and a reverse primer D containing a *SalI* site.

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Cloning of more-than-full-length HBV genome. For our cloning strategy, the pTriEX-1.1

vector (Novagen), was transformed into pTriEX-mod by insertion of a unique *NotI* site at position 1717, which is just upstream of the vertebrate transcription start (position 1727) of the vector (Fig. 2). PCR products generated with primers A-B and C-D were digested by *NotI/NcoI* and *NcoI/SalI*, respectively, and cloned in one or two steps using standard procedures (22), between *NotI/XhoI* sites of pTriEX-Mod to derive a vector containing a 1.1x unit length HBV genome. The exact cloning of 1.1x unit length HBV genome is necessary and sufficient to enable the synthesis of full length HBV pre-genomic (pg)RNA required to trigger the intracellular HBV cycle (2, 23). To facilitate the screening of correctly assembled clones, a PCR reaction was set up with primers E and F. The PCR is performed directly with heat-lysed bacterial colonies. An amplicon of approximately 420 nt is expected when the construct is correctly assembled.

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Drug susceptibility assay. Transfection of plasmids (single clones or mixture of clones) into Huh7 cells was performed using Fugene-6 reagent (Roche). Briefly, Huh7 cells were seeded at a density of 7×10^4 cells/cm² and transfected 24 hours later with 150 ng of plasmid per cm². Sixty hours post-transfection, treatment with various concentrations of drugs was started. This treatment was renewed every day for 5 days. Intracellular HBV DNA was then purified, and subjected to Southern blot analysis as described previously (11, 17, 24). The inhibitory concentration 50 (IC₅₀) or IC₉₀ were determined by phosphorimager analysis and compared to standard, i.e. isogenic wild type strain or wild type quasi-species.

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Results

Extraction of viral DNA from sera and PCR performance. The comparison of three extraction kits showed that viral DNA purification using the QIAamp™ UltraSens virus kit was the most efficient, as a PCR product was visible in an ethidium bromide-stained gel when as low as 5 copies of genome, corresponding to a theoretical serum titer of 300 genomes/ml, were used as initial template (Fig. 1C). To establish and compare the performance of our PCR amplifications to the PCR assay developed by Gunther *et al.*, PCRs were carried out with the different pairs of primers (P1-P2, A-B, and C-D) using dilutions (1/10th) of DNA purified from control serum. All PCRs were performed using the Expand high fidelity PCR system and PCR conditions described previously (20, 21), or above. PCRs performed with primer pairs A-B and C-D were 2-5 and 10-15 times less effective, respectively, than that done using P1-P2 (Fig. 1D), indicating a slightly lower performance of our PCRs. The result with primers C-D was expected as the PCR product spans the gap in the circular HBV DNA contained in virions. Generally, for serum with very low viremia (i.e. <10⁴ genomes/ml), a nested PCR using a P1-P2 PCR product as template was necessary to obtain clonable amount of viral DNA.

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Cloning of HBV genomes in the pTriEx vector: feasibility and performance. A vector in which expression of the HBV pgRNA is controlled by a heterologous promoter such as the cytomegalovirus (CMV) promoter, has proven useful for the analysis of post-transcriptional steps in the viral life cycle (11, 17, 25, 26). To date the construction of such a vector was not extensible to all HBV genomes isolated from patients, mainly because of the difficulty to fuse the +1 of pgRNA transcription with +1 of mammalian promoter transcription. To address this problem, a *NotI* site was introduced into the pTriEx-1.1 plasmid just upstream of the +1 of transcription of a chicken β -actin promoter (Fig. 2A). No modification of the promoter activity was observed for the new plasmid, i.e. pTriEX-Mod, as measured by reporter gene expression (data not shown). A primer A, hybridizing at the +1 of transcription of the pgRNA and containing a *NotI* tail, was designed (Fig. 1B) and used in combination with the reverse

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primer B, which hybridizes at the ATG of HBV X gene and contains a unique *NcoI* site, to generate a 2.8 kb amplicon covering around 85% of the HBV genome. When this fragment was cloned into pTriEx-Mod at the *NotI* site, the +1 of transcription of the HBV pgRNA and the +1 of transcription of the β -actin promoter were fused. The PCR products obtained with the two primer pairs A-B and C-D were digested with *NotI/NcoI* and *NcoI/SalI* and simultaneously cloned into pTriEx-Mod (using an approximate 2:2:1 molar excess of insert PCR products to vector) to produce pTriEx-HBV vectors (Fig. 2B). Alternatively, cloning could be obtained in two steps, by inserting first the 0.65kb C-D amplicons and then the large A-B amplicon. This fusion of promoters enabled the production, in transfected-hepatoma cell, of HBV pgRNA at a steady level regardless of the information contained in the 1.1x unit length of genome cloned. On average between 50 and 75% of the clones analyzed presented the expected restriction pattern, and between 40% and 75% of these clones were replication-competent and led to the formation of HBV DNA replicative intermediates after transfection into Huh7 cells.

Comparison of the synthesis of intracellular replicative intermediates observed in Huh7 hepatoma cells after transfection with pTriEx-HBV with other previously reported methods.

The genome of a well defined HBV genotype D (serotype ayw) was cloned i) into the pTriEX-Mod vector, ii) as head-to-tail tandem into a pUC vector, or iii) according to Gunther's method into a pUC vector (HBV genomes with *SapI* sticky ends). The HBV genome cloned by Gunther's method was first released by *SapI* digestion before the cells were transfected. The three types of construction were used to transfect Huh7 cells with the

same amount of cloned HBV genome. A plasmid called pCMV-HBV containing a 1.1x unit length HBV genotype D genome (a kind gift of Dr Seeger) under the control of the strong

CMV promoter, was used as control. As expected, due to presence of the strong chicken β -actin promoter, the pTriEx-HBV vector gave rise, along with pCMV-HBV, to the highest level of intracellular replicative intermediates (Fig. 2D), while the level of intracellular replicative intermediates obtained after transfection with the head to tail HBV dimer was very

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low. This suggests the superiority of pTriEx-HBV vector to analyze HBV post transcriptional events.

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Description, calibration and performance of the drug susceptibility assay. Huh7 cells were transfected with clones or a mixture of clones (up to 20) of pTriEx-HBV obtained by the cloning of HBV genomes purified from serum. When possible, the multiclonal approach was preferred to monoclonal approach, as a mixture of clones more closely resembles the natural quasi-species. Both replication-competent and replication-deficient clones could be mixed and used for transfection. The analysis of intracellular viral DNA replicative intermediates, synthesized in the presence or absence of antivirals, enabled to determine the drugs' IC₅₀ and IC₉₀ for each clinical isolate (see principle on Fig. 2B). Experiments were performed at least in duplicate. Inhibitory concentration 50 and IC₉₀ were compared to those obtained with standard, i.e. isogenic wild type strain or with wild type, e.g. pre-therapeutic, quasi-species. Many anti-HBV drug already available or in development were evaluated in transient cellular system using isogenic wild type strains, mostly of genotype D. To calibrate the assay, the IC_{50s} and IC_{90s} of various drugs available in our laboratory were determined using different wild type quasi-species (genotypes A, C, D, and G) cloned in pTriEx rather than the isogenic wt strain. No significant differences in drug susceptibility were observed *in vitro* between natural wt quasi-species and wt isogenic strains (data not shown). As example, IC_{50s} and IC_{90s} obtained with these drugs in Huh7 cells for a genotype D are presented in Table 2. These data were subsequently used as standard for all phenotypic assays performed using unknown samples.

To test the sensitivity of the assay, incremental mixtures of plasmids containing either wild type derived HBV genomes or 3TC-resistant derived HBV genomes were used to transfect cells. Treatment with 3TC was performed to analyze the variation of drug susceptibility associated with the different mixture of genomes. The results demonstrate that the assay readily distinguished the variation in 3TC-susceptibility of mixtures that contained 25, 50, 75, or 90% resistant virus from the samples with 100% drug sensitive or 100% drug-resistant

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HBV genomes (Fig. 3; panels A and B). The assay was not sensitive enough to distinguish mixtures containing 10% of 3TC-resistant virus and 90% of wt from a pure wt population.

We also analyzed the amount of viral DNA replicative intermediates obtained with mixtures of plasmids containing different ratios of wild type derived HBV genomes or 3TC-resistant derived HBV genomes transfected in Huh7 cells. The results showed a gradual decrease of the level of viral DNA synthesis concomitantly with the increased proportion of mutant genomes in the mixture (Fig. 3C).

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Longitudinal studies of the variation of drug susceptibility of viral population *in vivo*. To validate and exemplify the utility of our phenotypic assay, two patients who underwent drug treatment and developed resistance were selected. Serum samples taken through the course of the therapy were extracted and HBV genomes cloned in pTriEx-Mod. Mixtures of 10 to 20 replication-competent pTriEX-HBV clones for each time point studied were transfected in Huh7 cells and treatment with antivirals was performed as described above.

(i) Patient 1. The profile of patient 1 corresponded to a typical pattern of 3TC-acquired resistance by selection of the double mutation L180M/M204V. Only two samples, pre-therapeutic and post-breakthrough, were used for the phenotypic analysis, as a proof of concept. For the pre-therapeutic HBV genome population, the IC₅₀ and IC₉₀ of 3TC were determined to be at approx 100 nM and 2μM, respectively (Fig. 4A). For the viral population corresponding to breakthrough under 3TC treatment, the IC₅₀ was higher than 50 μM, which represents a decreased susceptibility of more than 500 fold, while an IC₉₀ could not be achieved (Fig. 4A). For both viral populations, the susceptibility to other drugs tested, including PMEA and PMPA, remained unchanged (Fig. 4B).

(ii) Patient 2. The profile of patient 2 was more complex, as he was first treated with famciclovir for 12 months (01/97 until 12/97), switched to 3TC for 45 months (01/98 until 10/01), then started on bitherapy 3TC/PMEA from November 2001. The genotypic analysis (direct sequencing) of circulating HBV revealed the sequential appearance of L180M (between July and December 1997) during famciclovir therapy and L180M+M204V

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mutations (between April and July 1998) during 3TC administration, in the polymerase gene.

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The double mutations (L180M+M204V) remained detectable until the last point tested (November 2001). Six samples were selected throughout the course of the therapy to perform phenotypic analysis *in vitro* and follow drug susceptibility of the viral population (Fig. 5A). Treatment with 3TC, FTC, PMEA, and PMPA were performed. At the end of the treatment, encapsidated HBV DNA was purified and subjected to Southern blot analysis (Fig. 5B and 5C). The change in 3TC susceptibility for the six viral populations was clearly demonstrated, as measured by the gradual variation of the IC₅₀. This change in *in vitro* 3TC-susceptibility was associated with the sequential apparition of L180M and L180M/M204V mutations in the polymerase gene, as determined by both direct sequencing of the circulating viral quasispecies (Fig. 5A), sequencing of either mixed clones (Fig. 5D) or individual clones (10 randomly-chosen clones shown for Pey1, Pey2, Pey4 and Pey6 on Fig5E) used for the phenotypic analysis.

Therefore the phenotypic analysis correlated closely with the *in vivo* genotypic and clinical data. For the six viral populations, the susceptibility to FTC paralleled that of 3TC, as expected (11, 27), while the susceptibility to the other drugs tested, including PMEA, remained unchanged (data not shown), and was consistent with the initial virological response observed *in vivo* when adéfovir dipivoxil was added to 3TC in the treatment of this patient in November 2002.

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Discussion

The novel cloning technique described in this report enables the construction of molecular clones, containing a 1.1 HBV genome-unit derived from clinical isolates, which allow the study of viral replication upon transfection of eukaryotic cells. When transfected into Huh7 cells, these clones trigger the expression of pre-genomic HBV RNA under the control of a heterologous promoter, and therefore initiate the viral replication cycle including synthesis of intracellular viral DNA replicative intermediates. Therefore, such vectors allow the analysis of post transcriptional steps of the viral life cycle, including nucleocapsid formation and HBV-polymerase-mediated reverse transcription. pTriEx-HBV vectors are obtained by a one-step insertion of two PCR products that cover the 1.1 genome-unit, which corresponds to the length of the HBV pgRNA (2, 23). Alternatively, the cloning can be done in two steps, with first the insertion of the C-D amplicon followed by the insertion of the large A-B fragment. The latter strategy was used for instance for the cloning of some HBV genomes isolated from sera with very low viral load. In average, between 40 and 75 % of the correctly assembled clones were found to be competent for viral DNA synthesis in transfected cells. This result is consistent with previous observations (20, 21). The lack of genome replication competence of a clone has two potential origins: i) the insertion of artificial mutations by PCR polymerase as described previously (20, 21), ii) the cloning of a naturally occurring replication-deficient genome, most likely derived from the spontaneous error rate of the HBV polymerase itself (28). As the frequency of mutations introduced by the high fidelity polymerase used in the PCR is likely to be constant, our results suggest that the amount of viable genomes in the circulating viral quasi-species is highly variable from patient to patient, but also during the course of the disease.

The main interest of our drug susceptibility assay is to determine the phenotypic status of the viral population derived from a patient in response to a given antiviral. The method described here has several advantages over previously reported methods, which include i) sub-cloning of PCR amplified HBV genome fragment into, or site directed mutagenesis of, a plasmid

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containing a well established replication-competent laboratory strain (11, 17-19), and ii) the method developed by Gunther *et al.* (20). For instance, methods based on the exchange of a cassette or site directed mutagenesis do not take into account the HBV genome variability existing in other parts of the genome. Moreover, the exchange of a cassette can create non-natural chimeric genomes, especially when the genotypes of the HBV to be studied, is different from that installed in the receiver plasmid. By contrast, our method enables the cloning of the whole HBV genome isolated from a given sample, and appears theoretically more appropriate to study complex situations, i.e. multiple mutations dispatched all over the genome (e.g. precore + polymerase mutant, as described in Chen *et al.* (29)) or genotypically undefined phenotypes. As compared to the strategy developed by Gunther *et al.* (20), the vector we have designed presents several advantages for phenotypic analysis. Firstly, it can be directly transfected into cells without any time-consuming and expensive *SapI* digestion. Moreover, the pTriEx-HBV vector does not require intracellular repair as in the case of the *SapI* digested vector, and is readily utilized by the host-cell transcription machinery for the synthesis of HBV mRNAs, including pgRNA. Secondly, the initial synthesis of pgRNA is determined by the mammalian promoter of pTriEx and is identical from one vector to another irrespective of the HBV genome cloned. This ensures a constantly high level of pgRNA synthesis and consequently a higher level of viral DNA synthesis, although the latter depend on nucleocapsid assembly and viral polymerase activity. This is particularly important in the context of a phenotypic assay which requires high level of HBV DNA synthesis to facilitate the determination of drug susceptibility by standard procedures. Altogether, transfection of Huh7 cells with a standard amount of pTriEx-HBV is a reproducible and efficient method to analyze the phenotypic status of HBV viral populations.

Recently another method to determine the drug susceptibility of clinical HBV isolates has been reported ((16). Both methods are very convergent, but present few differences. Amongst similarities, we can note i) the cloning of 1.1x unit length HBV genome, ii) the use of a strong promoter to drive the expression of pgRNA, iii) the use of southern blot analysis to determine

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IC₅₀/IC₉₀. The main difference relies in the fact that Yang and colleagues used a vector which contains already the 0.1x unit HBV redundancy, and therefore needed to clone only 1x unit length genome, generated by one PCR amplification. One disadvantage of this approach is that the sequence of the redundancy is not acquired from the clinical isolate, thus leading to potential mosaic genomes. With our method one cannot exclude the possibility of creating mosaic genomes at the 3' end during cloning. However, this would not affect the polymerase gene in which most of the mutations conferring resistance have been described so far. To obtain data on chimeric genome formation, we have sequenced 38 individual clones at both extremities (data not shown). At the nucleotidic level, less than 1% differences were obtained with the analysis of 38 pairs of PCR amplicons (i.e. analysis of 5' and 3'). Notably, 20 out of 38 pairs of PCRs were strictly identical (100% homology). These results suggest a high degree of conservation in the redundant region within a given viral population in sequential samples.

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Historically, phenotypic assays have first been developed for monitoring drug susceptibility of HIV viral populations. Some of them are currently used in clinical practice to monitor drug resistance (14, 15). The phenotypic assay we have developed for HBV is based on the cloning genome and the use of replication competent vectors in a cell-transfection based assay. As we use Southern blot analysis to measure the impact of drug treatment on the synthesis of intracellular HBV DNA, three to four weeks were necessary to perform a phenotypic assay on a given sample. To shorten the time of completion for an assay, a real time PCR method is being developed to quantify intracellular HBV DNA synthesis and virion release. This should simplify and facilitate phenotypic studies towards an easier clinical use.

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As compared to the HIV phenotypic assay, the development and clinical use of the HBV phenotypic assay is still at an early stage. However, we demonstrate in this study the relevance of this type of assay for longitudinal monitoring of the phenotypic status of the viral population isolated from clinical samples. Indeed, in the case of one patient (patient #2) who acquired sequentially famciclovir and 3TC resistance, we have shown that the result of

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phenotypic analysis were consistent with those of the detailed genotypic analysis performed on the same clinical samples (Fig. 5). Furthermore, in another study we applied the same phenotypic assay to study the PMEAResistance associated with the N236T mutation and a viral breakthrough in a liver transplanted patient (9).

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The assay described in this study has proven useful to perform multiclonal analysis and to determine the sensitivity of viral quasi-species to antivirals. Indeed, this assay allowed to monitor the kinetics of emergence of drug resistant mutants in longitudinal studies. With the development of new anti-HBV molecules, this phenotypic drug susceptibility assay could become an important tool for the management of patient infected with resistant HBV isolates and for the evaluation of new antivirals on clinical isolates circulating in the population.

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Table

Table 1 . Sequence and position of primers used for HBV genome amplification.

Name	Sequence and restriction sites	Position ^a	Genotypes amplified
P1	5'-CCGGA AA AGCITATGCTCTTCTTTTTCACCTCTGCCTAATCATC-3' <i>HindIII</i> <i>SapI</i>	1821-1843 forward	All genotypes
P2	5'-CCGGAGAGCTCATGCTCTTCAAAAAGTTGCATGGTGCTGGTG-3' <i>SacI</i> <i>SapI</i>	1825-1804 reverse	All genotypes
A	5'-TGCGCACCGCGGCCGCAACTTTTTCACCTCTGCC-3' <i>NotI</i>	1816-1835 forward	All genotypes
B1^b	5'-GGCAGCACASCTTAGCAGCCATGG-3' <i>NcoI</i>	1395-1372 reverse	Genotypes A, B, D, E and G
B2^b	5'-GGCAGCACASCCGAGCAGCCATGG-3' <i>NcoI</i>	1395-1372 reverse	Genotypes C and F
C1^c	5'-ACMTCSTTTCCATGGCTGCTAGG-3' <i>NcoI</i>	1360-1385 forward	Genotypes A, B, D, E and G
C2^c	5'-ACMTCSTTTCCATGGCTGCTCGG-3' <i>NcoI</i>	1360-1385 forward	Genotypes C and F
D	5'-CTAAGG GT CGACGATACAGAGCWGAGGCGG-3' <i>SalI</i>	2027-1998 reverse	All genotypes
E	5'-TAAACAATGCATGAACCTTTACCCCGTTGC-3' <i>NsiI</i>	1124-1154 forward	All genotypes
F	5'-GGGAGWCCCGTAAAGAGAGGTGCG-3'	1549-1525 reverse	All genotypes

a) Nomenclature is according to Galibert *et al.* (30)

b) Primers B1 and B2 are mixed to perform PCR when the HBV genotypes is unknown.

c) Primers C1 and C2 are mixed to perform PCR when the HBV genotypes is unknown.

Supprimé : (29)

Table 2. Inhibitory concentration 50 and 90 of various drugs, as determined in Huh7 cells

transfected with wild-type HBV quasi-species cloned into pTriEx vectors.

Drug	Inhibitory Concentration (μ M)	
	IC ₅₀	IC ₉₀
3TC	0.1 + 0.1	7.5 + 3.2
FTC	0.15 + 0.1	15 + 5.4
PMEA	10 + 4.6	100 + 6.4
PMPA	20 + 2.5	100 + 10
DXG	50 + 7.7	> 100
DAPD	60 + 6.2	>>100
L-FMAU	7.5 + 2.5	100 + 10
β-L-FD4C	0.07 + 0.02	1 + 0.1

Figure legends

Figure 1. Design of the PCR assay to amplify HBV genomes : positions of primers and comparison of PCR-sensitivity according to primer pairs used. (A) A map of the open circular HBV genome with its open reading frames is shown. The different primers used in this study are indicated. Primers P1 and P2 were described by Gunther *et al.* (20). (B) Sequence of the forward primer A used for PCR amplification and primer binding site at the triple-stranded region of the HBV genome are indicated. The primer P1 is also shown. (C) Viral HBV DNA was extracted from a reference serum using three different kits commercially available. Serial dilutions ($1/10^{\text{th}}$) of this DNA were used to set up the PCR with primers P1 and P2. PCR-products resulting from 40 cycles of amplification were run on a 1% agarose gel and stained with ethidium bromide. The amount of genome equivalent used to set up the PCR reaction, determined after real time quantification of the DNA stock solution, is indicated on the top of the gel. The names of the companies commercializing the kits used in this experiment are indicated underneath the gel. (D) Serial dilutions ($1/10^{\text{th}}$) of HBV DNA were used to set up PCR with different primer pairs (P1-P2, A-B, and C-D; as indicated on the bottom of the gel with the corresponding size of amplicon). PCR-products resulting from 40 cycles of amplification were run into a 1% (P1-P2 and A-B) or 2% (C-D) agarose gel and stained with ethidium bromide. The amount of genome equivalents used to set up the PCR is indicated on the top of the gel.

Supprimé : (19)

Figure 2. Schematic representation of the vectors constructed in the study and principles of the phenotypic assay. (A) The maps of pTriEX-1.1 and derived plasmids are shown as well as the detailed sequence of the basic core promoter (BCP) of the chicken β -actin gene. The modification of the BCP by insertion of a *NotI* site and the inclusion of the blasticidin cassette are also shown. (B) A map of the vector pTriEX-HBV is presented on the top of the figure. The vector contains 1.1 HBV genome units corresponding to the length of pgRNA. The primers A-B and C-D used to generate the two products which have been cloned to obtain the vector are also indicated. Vectors pTriEx-HBV are transfected into cells, in which

they trigger HBV genome replication. (C) Transfected-cells are treated with drugs for 5 days before viral replicative intermediates are purified and subjected to Southern blot analysis. The IC_{50s} and IC_{90s} can be determined by phosphorimager analysis. (D) Comparison of the amount of intracellular replicative HBV DNA intermediates produced after the transfection of Huh7 cells with the same HBV genome cloned in different vectors. An autoradiography resulting from Southern blot analysis of intracellular HBV DNA performed 4 days post transfection, is presented. Lanes pCMV, pTriEx, pDimer and SapI (in duplicate) correspond to DNA extracted from viral core particles derived from Huh7 cells that were transfected with pCMV-HBV, pTriEx-HBV, pHBVdimer, or SapI-linearized pUC-HBV plasmids respectively (see text for details). A molecular marker was run on the right hand side of the gel and radiolabeled for visualization after X ray exposure.

Figure 3. Sensitivity of the phenotypic assay to detect drug resistant mutants within a mixed viral population. (A) Various mixtures of pTriEx-HBV clones containing either wild type HBV genomes or 3TC-resistant HBV genomes (containing the L180M and M204V mutations) were used for transfection of Huh7 cells. Cells were left untreated or treated for 5 days, starting 60 hours post-transfection, with various concentrations of lamivudine, i.e. from 1.56 to 25 μ M. A Southern blot analysis of intracellular replicative HBV DNA intermediates was performed. The composition of the mixture used is indicated on the bottom of the autoradiography. (B) Phosphorimager analysis of blots was performed and the results presented on the graph. The 3TC-susceptibility curves of the different mixtures are shown. The percentage of inhibition of viral replication is drawn as a function of the amount of lamivudine used (logarithmic scale). (C) The replication capacity of the same mixture was analysed in absence of drug treatment using the approach described above. A quantification of the amount of intracellular HBV DNA was performed by phosphorimager analysis. Histograms showing the percentage of replication of each mixture as compared to control (100% with pure wt) are presented.

Figure 4. Results of the phenotypic assay for a typical patient (patient#1) with

lamivudine resistance. (A) Autoradiograms showing the *in vitro* 3TC-susceptibility profile of two viral populations. Two mixed population of pTriEx-HBV vectors obtained from the cloning of HBV genomes from pre-therapeutic (top panel) and breakthrough (bottom panel) samples were used for the transfection of Huh7 cells. Cells were treated for 5 days, starting 60 hours post-transfection, with increasing concentrations of lamivudine (shown on the top of each autoradiogram). Southern blot analysis of intracellular replicative HBV DNA intermediates was performed. The IC₅₀ and IC₉₀, determined by quantitative analysis, are indicated within the frames. (B) Graphs showing the *in vitro* susceptibility of three viral populations, including the pre-therapeutic (open square and dashed line) and breakthrough (cross and semi-dashed line) of patient#1, and a standard wt population (plain circle and plain line), to 3TC, PMEA, and PMPA. The percentage of viral replication relative to HBV replication without drug is represented as a function of the concentration of drugs (logarithmic scale).

Figure 5. Phenotypic assay for patient#2. (A) Diagram indicating the variation of viral load throughout the course of therapy with famciclovir, lamivudine, and PMEA. The genotypic status of the viral population is indicated on the top of the figure, while the samples used for the phenotypic analysis are indicated at the bottom (Pey-1 to Pey-6). Six pTriEx-HBV multiclonal obtained by cloning of HBV genome from the different samples, were used to transfect Huh7 cells. Cells were treated for 5 days, starting 60 hours post-transfection, with increasing concentration of lamivudine. Southern blot analysis of intracellular replicative HBV DNA intermediates was performed. (B) Phosphorimager analysis of the blots was performed and the results are presented on the graph. The 3TC-susceptibility curves of the different viral population (Pey-1 to Pey-6) are shown. The percentage of inhibition of viral replication is drawn as a function of the concentrations of lamivudine (logarithmic scale). (C) Autoradiograms of Southern blots are presented for each viral population (Pey-1 to Pey-6). (D) Protein sequence alignment of each viral population (Pey-1 to Pey-6), as represented in the mixture of clones used for the phenotypic analysis. Positions where mutations can occur

as characterized in previous studies (L180, M204, and N236), are indicated (both colour/bold marked and position). (E) Amino acid sequence alignment of 10 individual clones used for the phenotypic analysis for different samples (Pey-1, Pey-2, Pey-4, Pey-6). Positions where mutations can occur as characterized in previous studies (L180, M204, and N236), are indicated (both colour/bold marked and position).

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