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Comprehensive analysis of hepatitis B virus antiviral drug resistance mutations based on a new high-throughput phenotypic assay

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Dedicated to my parents
for all their help and support

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Abstract

Chronic hepatitis B remains a major health problem worldwide. Without treatment, patients chronically infected with hepatitis B virus can progress to liver cirrhosis, hepatic decompensation and hepatocellular carcinoma. Therefore, the goals of therapy are to prevent or reverse these complications and to ultimately improve survival. Over the last decades, treatment of chronic hepatitis B infection with potent antiviral drugs has been well established but is limited by the emergence and selection of resistant HBV variants during long term therapy. Resistance testing, as learnt from HIV therapy, is essential to improving treatment monitoring and optimizing antiviral therapy. However, a time- and cost-effective resistance testing assay to identify genetic changes that cause clinical resistance, confer potential cross-resistance to other therapies and/or affect viral replication has not yet been established.

In this study, a standardized phenotypic assay suitable for high throughput clinical applications was developed based on several key approaches. First, a rapid PCR amplification and cloning strategy of HBV was optimized for screening and genotyping of patients with viral breakthrough/clinical resistance by sequencing. Full length HBV genomes, polymerase or reverse transcriptase sequences, respectively, were amplified from patient material with high sensitivity (viral load limit as low as 10^2 IU/ml) and then cloned into replication competent HBV constructs for all HBV genotypes. Second, a transfection-based method was adapted and scaled down to a standardized high throughput 96-well cell culture format in which treatment with 4 different drugs in 5 serial dilutions each can be conveniently tested in triplicate within 5 days. Third, a new quantification method using a sensitive, selective qPCR to determine HBV replication by measuring HBV progeny in the cell culture supernatant was applied to avoid the conventional, time and labor intensive Southern blot analysis. The selective primers can distinguish HBV rcDNA from input constructs with a selectivity of more than $1:10^{4.5}$, overcoming the biggest hurdle for using qPCR for phenotypic testing. Finally, the biosafety of this assay, especially when dealing with vaccine escape variants, was improved by using fully replication-competent, HBx-minus constructs that produce non-infectious HBV. Overall, the new phenotypic assay proved robust and easy to be embedded into clinical practice.

For a comprehensive analysis of HBV drug resistance a panel of 100 HBV expression plasmids, harboring the dominant resistance mutations individually or in combination, was generated by site-directed mutagenesis or cloning from patient serum. These mutant plasmids enriched the patient sample collection we obtained from clinicians and allowed for analysis of

important or interesting combinations of HBV drug resistance and compensatory mutations. Evaluation of HBV strains with the four major nucleos(t)ide analogues showed a strong correlation between resistance factors measured and previously published data. The comprehensive testing provided a valuable insight into cross resistance profiles of mutation combinations as well as their viral fitness parameters. The whole dataset presented for the first time the big picture of how these mutations interact with each other in a complex context and what the substantial impact of individual variations is for resistance development, especially against highly potent antivirals entecavir and tenofovir.

One of the major goals of this project was to identify and characterize potential novel resistance mutations. Several candidates were found by clinical observations followed by genotypic screening, or by alternative bioinformatics approaches. Remarkably, the phenotypic resistance to adefovir/tenofovir conferred by rtK32R as well as a series of novel rtM204 mutations, especially rtM204K, was clearly demonstrated, as was their association with impaired viral fitness and infectivity. This deep understanding of the different levels of selection pressure during resistance development allowed us to evaluate the clinical significance of these mutations. In this way, the recently reported HBV variants, rtI233V in adefovir resistant patients and rtA194T in tenofovir resistant patients were negated as genotypic resistance mutations, because of the full susceptibility to either drug they showed in phenotypic testing.

Finally, all data obtained in our analysis have been integrated into the geno2pheno (HBV) analysis tool, which is available on a public domain server. With the knowledge generated in this project, this service will be essential to predicting the clinical response to available drugs, improving treatment monitoring, and in the end optimizing antiviral therapy.

1. Introduction

Chronic hepatitis B virus (HBV) infections constitute a major public health problem worldwide. It is estimated that approximately one third of the global population has been exposed to hepatitis B, and of those, more than 240 million individuals harbor chronic liver infections (**Figure 1**). Chronic HBV infection predisposes to severe progressive liver disease, including liver cirrhosis and hepatocellular carcinoma, and about 600,000 people die each year due to the acute or chronic consequences of hepatitis B (WHO estimations in 2012 [1]). HBV is generally transmitted by percutaneous and permucosal exposure to infected blood and other bodily fluids. Modes of transmission are the same as those for the human immunodeficiency virus (HIV), but HBV is 50 to 100 times more infectious. Besides, unlike HIV, the hepatitis B virus can survive outside the body for at least seven days retaining its infectivity. Because of the significant morbidity and mortality associated with HBV infection, improved prevention, screening and surveillance, and treatment strategies are needed.

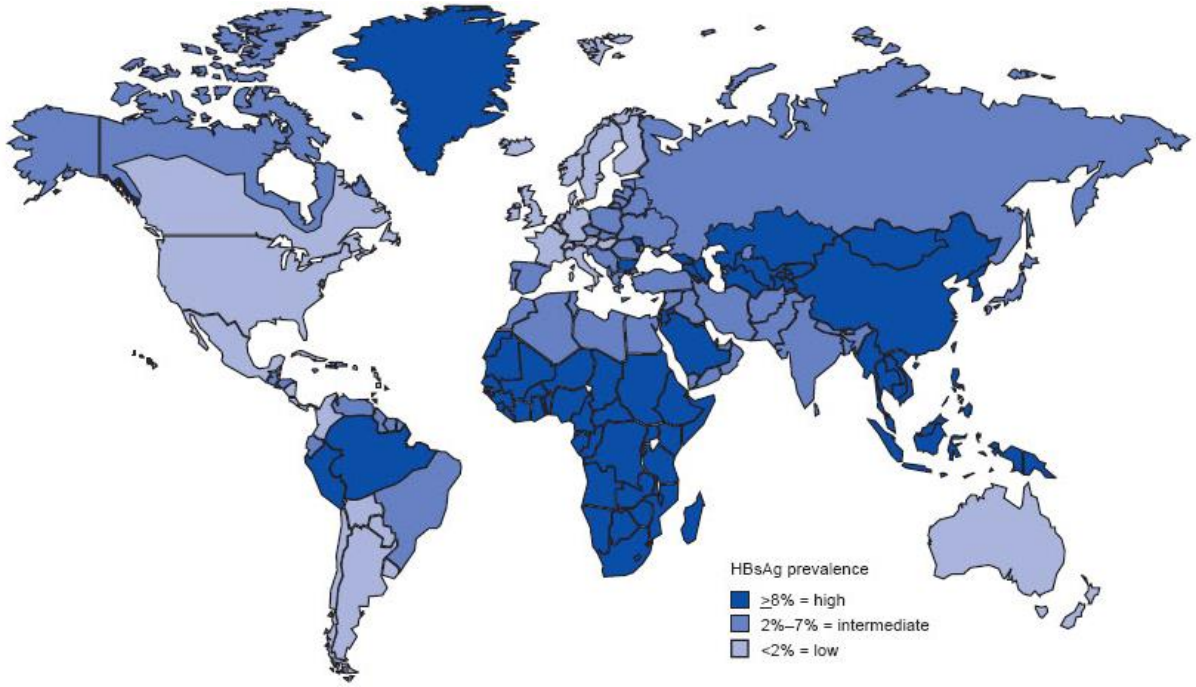


Figure 1: Worldwide prevalence of chronic HBV infection. For Multiple countries, estimates of prevalence of hepatitis B surface Ag (HBsAg), a marker of chronic infection are based on limited data and might not reflect current prevalence in countries that have implemented childhood hepatitis B vaccination. In addition, HBsAg prevalence might vary by subpopulation and locality. Modified after [2].

A better understanding of the HBV life cycle, natural history and pathogenesis studied *in vitro* and *in vivo* has led to the introduction of new oral antiviral therapies with expanded

clinical indications. Nevertheless, chronic HBV infection cannot be completely eliminated or "cured" with current treatments because of the persistence of covalently closed circular HBV DNA in infected hepatocytes that act as a viral reservoir [3]. Indeed, cccDNA may persist in patients with undetectable viremia and even in those with serologic evidence of viral clearance [4]. Therefore, the goal of therapy for HBV infection is to prevent, or reverse, disease progression to cirrhosis, end-stage liver disease, or hepatocellular carcinoma.

Reports of successful antiviral therapy for chronic hepatitis B appeared more than three decades ago, and during the past decade, substantial progress has been made acceleratedly [5]. Along with the progress, however, has come the complexity, in which determining whom, when, and how to treat has become progressively more challenging. Treatment choices should take into account a number of factors that predict response, including clinical circumstances, viral genotype, stage of disease, drug potency, and the likelihood of resistance.

In chronic hepatitis B, where treatment is usually long term and where viral resistance is a significant problem, optimization of treatment to ensure the greatest likelihood of long-term viral suppression for a maximal benefit with a minimal risk of resistance, is particularly of great importance.

1.1 HBV biology, viral life cycle and natural course of HBV infection

Hepatitis B virus is the prototypic member of the hepadnaviridae family, which has a partially double-stranded DNA genome that replicates through an RNA intermediate via reverse transcription. The relatively small 3.2 kb relaxed circular DNA (rcDNA) genome surrounded by an inner nucleocapsid and an outer envelope consists of a full-length minus strand DNA with a terminal redundancy of 8–10 nucleotides and an incomplete plus strand of variable length (**Figure 2**). This incomplete double-stranded genome is organized into only four overlapping but frame-shifted open-reading frames (ORFs) with all recognized coding information on the minus strand. The longest ORF encodes the viral polymerase (Pol); the second ORF referred to as the "envelope" or "surface" (S) ORF encodes the L, M, and S proteins (collectively, hepatitis B surface antigen [HBsAg]); two small ORFs encodes the precore/core proteins (PreC/C) and the X protein, respectively. The Pol ORF contains the whole envelope ORF and partially overlaps the PreC/C and the X ORFs.

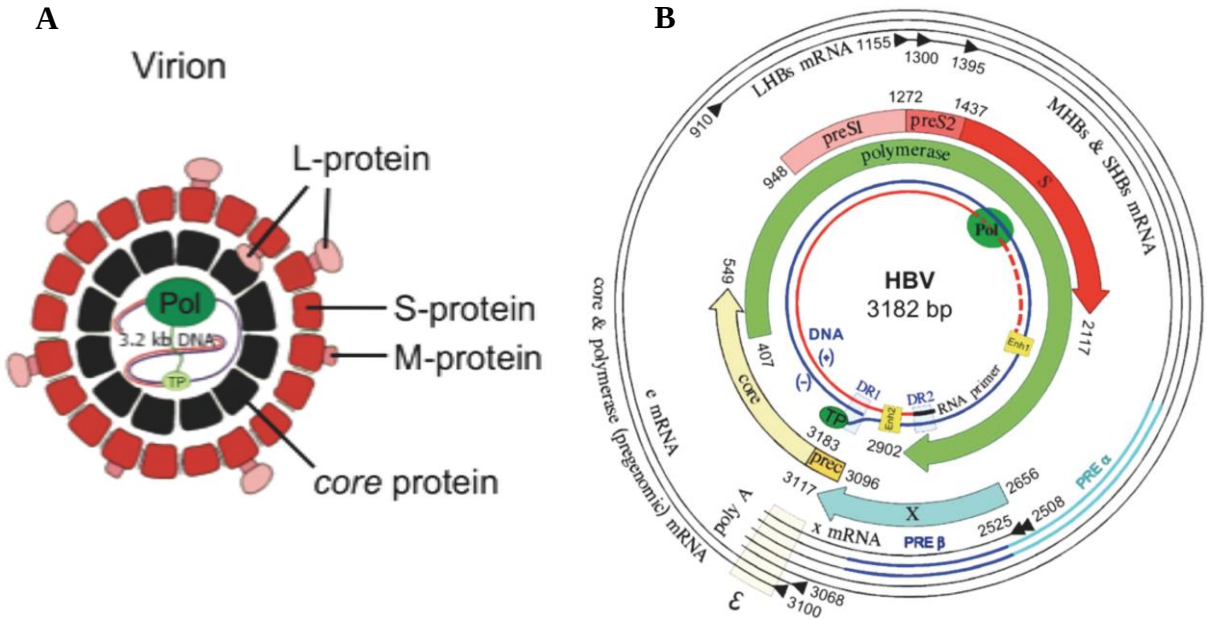


Figure 2: Schematic organization of the HBV virion and genome. (A) The HBV virion consists of an outer envelope containing host-derived lipids and all S gene polypeptides, the large (L), middle (M), and small (S) surface proteins. Within the sphere is an electron-dense inner core or nucleocapsid containing core proteins, a 3.2 kb, circular, partially double stranded viral DNA genome covalently bonded to an endogenous DNA polymerase. **(B)** The inner red and blue circles represent the partially double-stranded HBV genome, which displays the viral rcDNA form, with polymerase (pol) and terminal protein (TP) as green circles, enhancer (Enh1/Enh2) and direct repeat (DR1/DR2) elements as boxes. PreC- (e-), Pre- and subgenomic mRNA transcripts are illustrated in black outer circles. PreC mRNA encodes for the precore protein (HBeAg), the pregenomic mRNA encodes for core protein and polymerase, subgenomic L-, M- and SHBs (large, medium and small hepatitis B surface) mRNAs for the envelope proteins. Triangles indicate transcription starts, the yellow square symbol the encapsidation signal (ε) and blue lined areas the post-translational regulatory element (PRE). The colored arrows inside the scheme depict the four ORFs and the translated viral products: prec/core (HbeAg), core (capsid-protein), polymerase, preS2, preS1 and S (surface proteins L, M and S), and X (X-protein). Modified after [6].

The viral life cycle of HBV (as summarized in **Figure 3**) is relatively well understood despite a lack of robust permissive infection cell culture model. As the first stage of infection, HBV enters the hepatocyte via endocytosis following the binding of the HBV envelope (preS1) to a newly identified receptor NTCP/SLC10A1 [7] and is uncoated in the cytoplasm with the surface proteins shed, releasing the nucleocapsid. The encapsidated viral genome is then transported to the nucleus where the partially double-stranded rcDNA is repaired and converted to covalently closed circular DNA (cccDNA), a stable episomal minichromosome form that serves as the template for viral messenger RNA transcription.

From the cccDNA template/viral minichromosome, two classes of transcripts, greater than genomic length and subgenomic length, are transcribed into four major RNA species: the 3.5, 2.4, 2.1, and 0.7 kb viral RNA transcripts. Both classes contain heterogeneous transcripts

that are of positive orientation, are capped at the 5’ end and are polyadenylated at a common site in the 3’ end. The promoters directing the expression of these four transcripts, respectively, are the enhancer II/basal core promoter (BCP), the large surface antigen (Pre-S1) promoter, the major surface antigen (S) promoter, and the enhancer I/X gene promoter.

These RNAs are then transported to and translated in the cytoplasm to produce the viral proteins, namely hepatitis B core antigen (HBcAg) or nucleocapsid protein, from the 3.5 kb pgRNA; the soluble and secreted HBeAg (from the 3.5 kb precore messenger RNA different from pgRNA); the Pol protein (also from the 3.5 kb pgRNA); the viral envelope proteins L/M/S (from the 2.4 and 2.1 kb RNAs); and the hepatitis B X protein (from the 0.7 kb RNA). In addition to serving as messenger RNA for the nucleocapsid and Pol proteins, the 3.5 kb pgRNA is multifunctional, acting as the template for reverse transcription of the viral genome.

HBV replication takes place through a series of sequential events in the nucleocapsids. First, the viral polymerase begins encapsidation by binding to an encapsidation signal on the pregenomic RNA, which is followed by interactions with core proteins to form nucleocapsids. Reverse transcription of the pregenomic RNA produces the viral genome-negative DNA strand. Through a series of interactions, negative-strand synthesis is completed first, followed by positive-strand synthesis and circularization of the genome. This is followed by assembly of the nucleocapsid with envelope proteins to create mature virions for secretion after budding into the endoplasmic reticulum and passing through the Golgi complex. The replication process is facilitated by several proteins that aid in complex formation and assembly of mature virions.

The unique life cycle and replication strategy of HBV involve two key steps (reviewed in [8]). First, the HBV cccDNA minichromosome that acts as the major transcriptional template for the virus is generated from the rcDNA and stays inherently stable. Second, reverse transcription of the viral pregenomic RNA within the viral nucleocapsid produces progeny HBV rcDNA genomes, completing the cycle.

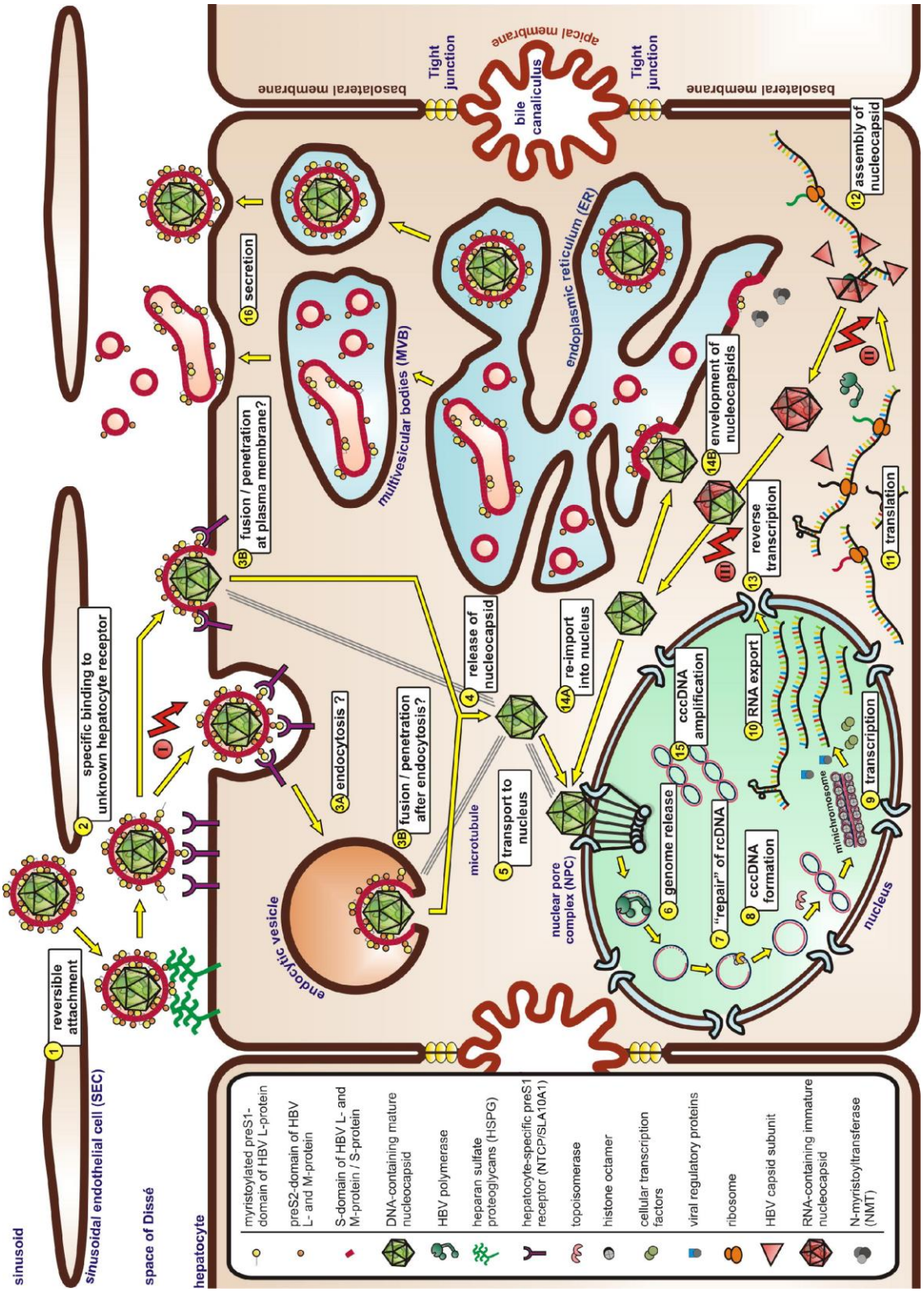


Figure 3: The replication cycle of hepatitis B virus. Once internalized in hepatocytes by endocytosis, the released HBV nucleocapsid is directed towards the nucleus and the HBV genome is translocated into the nucleus. The rcDNA is then converted into cccDNA to initiate HBV RNA transcription and subsequent HBV protein synthesis. The pgRNA is encapsidated and reverse transcribed to result in a new rcDNA molecule. Mature nucleocapsids are then either directed to the secretory pathway for envelopment of new virions for release, or redirected back to the nucleus to amplify the cccDNA pool. Modified from [9].

The first key step, formation and transcription of cccDNA minichromosome, defines successful infection of a hepatocyte. The conversion of rcDNA into cccDNA requires several processes: removal of the Pol protein linked to the 5' end of the minus strand DNA as well as the short terminal redundant sequence in the 5' end of the minus strand; removal of the capped oligoribonucleotide RNA primer linked to the 5' end of the plus strand; completion of the viral plus strand DNA to repair the single-stranded gap region; covalent ligation of DNA extremities for both plus and minus strands. The HBV core protein is a structural component of the viral mini-chromosome and its specific binding results in a reduction of the nucleosomal spacing of the HBV nucleoprotein complexes, while the nuclear hepatitis B X protein binds to the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. New data are emerging regarding the epigenetic control of cccDNA, which might represent another key factor involved in the pathogenesis and natural history of the disease.

The second key step, HBV genomic DNA relication via reverse transcription, is related to but mechanistically distinct from retroviral replication (**Figure 4**). HBV genomic replication is initiated with packaging of the pgRNA and the viral reverse transcriptase into subviral core particles, forming cytoplasmic replication complexes. As the HBV Pol is being translated from the pgRNA molecule, the N-terminal region (terminal protein) binds to a unique RNA stem loop structure, known as epsilon (ε), at the 5' end of the pgRNA. The coordinated binding triggers encapsidation by core particles to from nucleocapsid where reverse transcription occurs. The polymerase bound to epsilon serves as a protein primer for DNA synthesis with epsilon serving as the template for this reaction, in which the first DNA nucleotide is covalently linked to the hydroxyl group of a tyrosine within the terminal protein domain to initiate DNA synthesis (namely the priming of reverse transcription). This Pol-oligonucleotide complex is then translocated to the complementary sequences of a direct repeat (DR1) region located at the 3' end of the pgRNA, from where minus strand DNA synthesis continues until it reaches the 5' end of the pgRNA resulting in a complete minus strand with a short terminal redundancy around 10 nucleotides. While reverse transcription is proceeding, the pgRNA is simultaneously degraded by the C-terminal RNaseH domain of the HBV Pol, except for its capped 5' terminal region including the 5' DR1 region. The capped RNA structure is then translocated to a second DR homology sequence (DR2) on the 5' end of the newly made minus strand DNA, where it acts as a primer for plus strand synthesis using the minus strand as the template, and is extended towards the P bound 5´ end of the minus strand. The short terminal redundancy of the minus strand DNA permits the transfer of the 3' end of the growing short plus strand from the Pol-linked 5' end to the 3' end of the

minus strand, thereby circularizing the genome and allowing continuation of DNA synthesis. Synthesis of the plus strand DNA continues until it reaches approximately 50 to 70% of the length of the minus strand, leaving a single stranded gap of various lengths.

Core particles containing a mixed population of doubled-stranded HBV DNA genomes are transferred to the endoplasmic reticulum, where they are coated with HBsAg. These virions then exit the hepatocyte via budding and vesicular transport, thereby completing the viral life cycle [10].

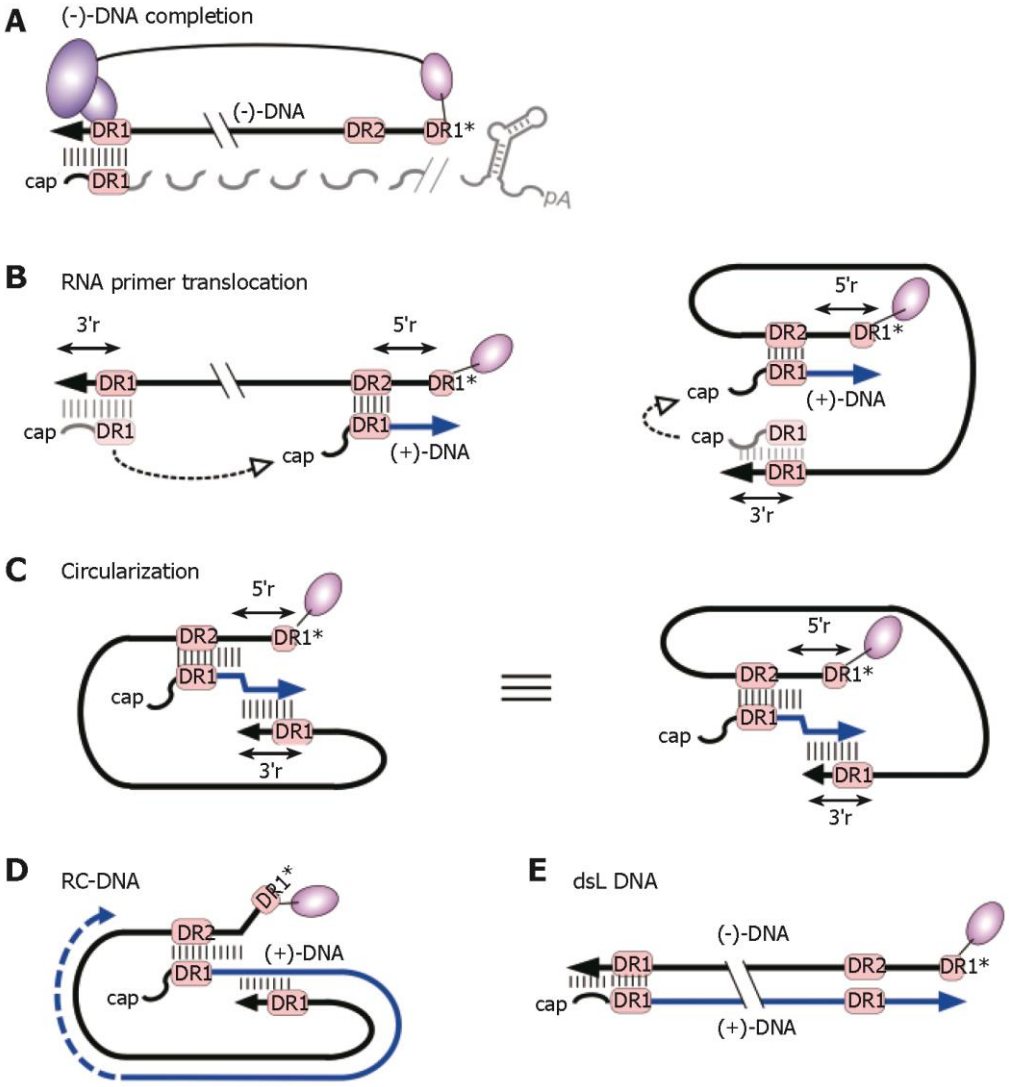


Figure 4: HBV rcDNA formation via reverse transcription. (A) During (-)-DNA synthesis and completion, the DNA primer, still linked to TP, is extended from DR1* to the 5´-end of pgRNA. The pgRNA is simultaneously degraded by the RH domain, except for its capped 5´ terminal region including 5´ DR1. (B) The RNA primer translocates to DR2, and is extended to the 5´-end of (-)- DNA. (C) The growing 3´-end of the (+)-DNA switches to 3´ end (-)-DNA, enabling further elongation and circularization. (D) Elongation on the (-)-DNA template creates rcDNA genome. (E) Double-stranded linear (dsL) DNA synthesis due to failed primer translocation to DR2, called "in situ priming". Modified after [11].

Hepatitis B virus is a highly evolved pathogen specifically targeting human hepatocyte. As a noncytopathic virus, meaning that under normal circumstances, HBV does not cause degenerative changes during infection and subsequent replication within hepatocytes. Instead, the cellular injury caused by hepatitis B appears to be largely immune mediated [12]. The clinical course and outcome of persistent HBV replication is determined, to a certain extent, by the generation and selection of viral escape mutants. Frequent unsuccessful attempts by the host's immune response to clear wild-type and escape mutants of HBV from infected hepatocytes lead to the necroinflammation and liver damage typically associated with chronic hepatitis B [13].

Two of the major determinants of the outcome of HBV infection are age and immune competence at the time of infection. In adult-acquired HBV, symptomatic acute disease often leads to clearance of the hepatitis B surface antigen (90%-95%), while in neonates and young children, initial HBV infection is usually subclinical and results in high rates (80%-90%) of chronic infection [14].

The typical course of chronic hepatitis B is characterized serologically by the presence of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) for a variable period of time and antibody to hepatitis B core protein (anti-HBc) that persists indefinitely. HBeAg is a reliable marker of HBV replication that correlates with the level of serum HBV DNA. Resolution of infection is characterized by the loss of HBeAg and HBsAg and the development of antibody to HBeAg (anti-HBe) and anti-HBs, along with the continued presence of anti-HBc [15].

The natural history of chronic HBV infection is characterized by an initial period of active replication followed by a late, nonreplicative phase. Three phases of chronic HBV infection have been characterized: immune tolerance, immune clearance (or immune active), and inactive [16]. The immunotolerant phase, which is characterized by the presence of HBeAg and high serum HBV DNA levels ($> 10^5$ copies/mL) but persistently normal alanine aminotransferase (ALT) levels, is present in those infected at birth or in early childhood but is usually absent when infection occurs during adolescence and adulthood. The immunotolerant phase is followed by an immune clearance phase of variable duration. During this period, serum ALT levels are typically elevated, HBV DNA levels are high, and features of chronic hepatitis are present on liver biopsy.

Clearance of HBeAg and development of anti-HBe, so-called HBeAg seroconversion, occurs spontaneously in 50% to 70% of patients after 5 to 10 years and usually heralds a period of inactivity typified by serum HBV DNA less than 10^4 copies/mL, normal ALT

levels, and minimal activity on liver biopsy. However, the disease course after entry into this inactive state varies, and while a subset will remain quiescent and some even clear the HbsAg, a significant proportion will again develop active hepatitis. Further, some patients under immune pressure during the period of HBeAg seroconversion develop viral mutations that do not produce HBeAg but continue to replicate at high levels and cause various degrees of liver damage. This form of chronic HBV infection is referred to as HBeAg-negative chronic hepatitis B. Individuals with chronic HBV infection, whether HBeAg-positive or HBeAg-negative, face an increased risk of developing HCC, especially if they have developed cirrhosis [17].

Given that the virus itself is noncytopathic in immune-competent persons, these stages reflect evolving virus-host interactions, underscoring the importance of the host immunologic response in disease containment, pathogenesis, and progression.

1.2 HBV antiviral drugs and resistance

Our knowledge of the molecular biology of HBV has increased considerably over the past decades, leading to the development of very effective prophylactic vaccines and to the development of therapeutic approaches against HBV [18]. The incidence of new cases of HBV infection has fallen dramatically since the introduction of an effective vaccine in 1982. However, the number of existing chronically infected patients did not drop significantly since the efficient therapy in eradicating the infection is still missing [19].

Currently there are 2 major modalities of approved antiviral therapies for the treatment of chronic HBV infection: the interferons, interferon alfa-2b or peginterferon alfa-2a, and the nucleos(t)ide analogues, including lamivudine, adefovir, entecavir, telbivudine, and tenofovir (**Table 1**). Interferon, given by injection, is used as an antiviral inhibiting viral replication as well as to enhance the host's immune defense system. The treatment is generally finite (ie, given for 48 weeks) because of the associated toxicities, which can be significant. Their use has not been linked to the development of any drug resistance; however, their reduced effectiveness against particular viral genotypes (C and D) and poor tolerability in certain patient populations means that they are not ideal treatment options in many cases [20]. Only 30% of pegylated-IFN-α treated patients achieve a sustained antiviral response [21]. Alternatively, oral nucleos(t)ide agents that specifically block activity of the HBV polymerase/reverse transcriptase, are generally potent and well tolerated. Nevertheless, nucleos(t)ide therapy generally involves a continuous, long-term course of therapy (or even

indefinite suppressive therapy) and, for many patients, a treatment course that is undefined at the start and will depend upon initial response. The main limitation to their use is the selection of and emergence of antiviral resistance during treatment.

Table 1: Approved anti-HBV agents

Drug Class	Name	Activity	Relative Potency	Genetic Barrier to Resistance	Daily Dosage
L-nucleoside analogues	Lamivudine	Pol • Premature chain termination	++	1	100 mg/day
	Telbivudine		+++	1	600 mg/day
Guanosine nucleoside (D-cyclopentane)	Entecavir	Pol • Inhibits priming RT • Inhibits negative strand synthesis • Inhibits positive strand synthesis	++++	3	0.5 mg/day or 1.0 mg/day (with LAM resistance)
Nucleotide (acyclic phosphonates)	Adefovir	Pol • Premature chain termination RT • Inhibits negative strand synthesis	+	1	10 mg/day
	Tenofovir		++++	Unclear	300 mg/day
Interferons	Interferon alfa-2b	• Inhibits viral replication (RNA/Protein synthesis) • Immuno-modulatory effects	++*	/	5 MU/day or 3×10 MU/week for 16-48 weeks
	Peginterferon alfa-2a		+++*	/	180 µg /week for 48 weeks

* Genotype dependent efficacy.

The HBV polymerase is a multifunctional protein that has 4 primary domains: a priming region called terminal protein, a spacer region of unknown function, a catalytic region that functions as a RNA-dependent RNA polymerase/DNA polymerase (also known as reverse transcriptase), and a carboxy terminal region that has ribonuclease H activity. The catalytic region can be subdivided into 7 domains A to G, which are thought to play different roles in

the function of the polymerase. The error-prone HBV rt-polymerase causes a high nucleotide substitution rate coupled with the production of more than 10^{12} virions per day, resulting in the probability of every nucleotide of the HBV genome being substituted many times over each day. A population of viral variants or quasispecies is capable of rapidly responding to endogenous (host immune response) and exogenous selection (antiviral therapy or during viral transmission) pressures. This pool of quasispecies provides HBV with a survival advantage by already having a population of pre-existing escape mutants from the immune response (precore or HBeAg-escape), prophylactic vaccines (vaccine escape), and antiviral therapy (drug resistance).

All currently available nucelos(t)ide analogues essentially target only one site: HBV polymerase/reverse transcriptase. Depending on structure and mechanism of action, they can be grouped into 3 main chemical classes: L-nucleosides, acyclic phosphonate nucleotides, and D-cyclopentane deoxyguanosine analogues (**Figure 5**). The L-nucleoside analogues include lamivudine and telbivudine and block HBV DNA synthesis by being incorporated into the growing DNA chain, causing premature chain termination. Adefovir and tenofovir disoproxil fumarate, which are structurally very similar to one another, are both acyclic phosphonates or nucleotide analogues and function by inhibiting both the reverse transcriptase and DNA polymerase activity, resulting in premature DNA chain termination. The third group, of which entecavir is currently the only approved agent, is characterized by a D-cyclopentane moiety. Entecavir is a deoxyguanosine nucleoside analogue that prevents HBV replication by inhibiting 3 steps: 1) priming of the HBV DNA polymerase, which is a necessary step that precedes DNA synthesis, 2) reverse transcription of the negative strand of HBV DNA from pregenomic RNA, and 3) synthesis of the positive strand of HBV DNA from the negative strand [22, 23]. Understanding the chemical classification of these agents is important because it affects the pathways and patterns of drug resistance and cross resistance, facilitating the selection of the optimal drugs for rescue treatment in the event that resistance does develop [24].

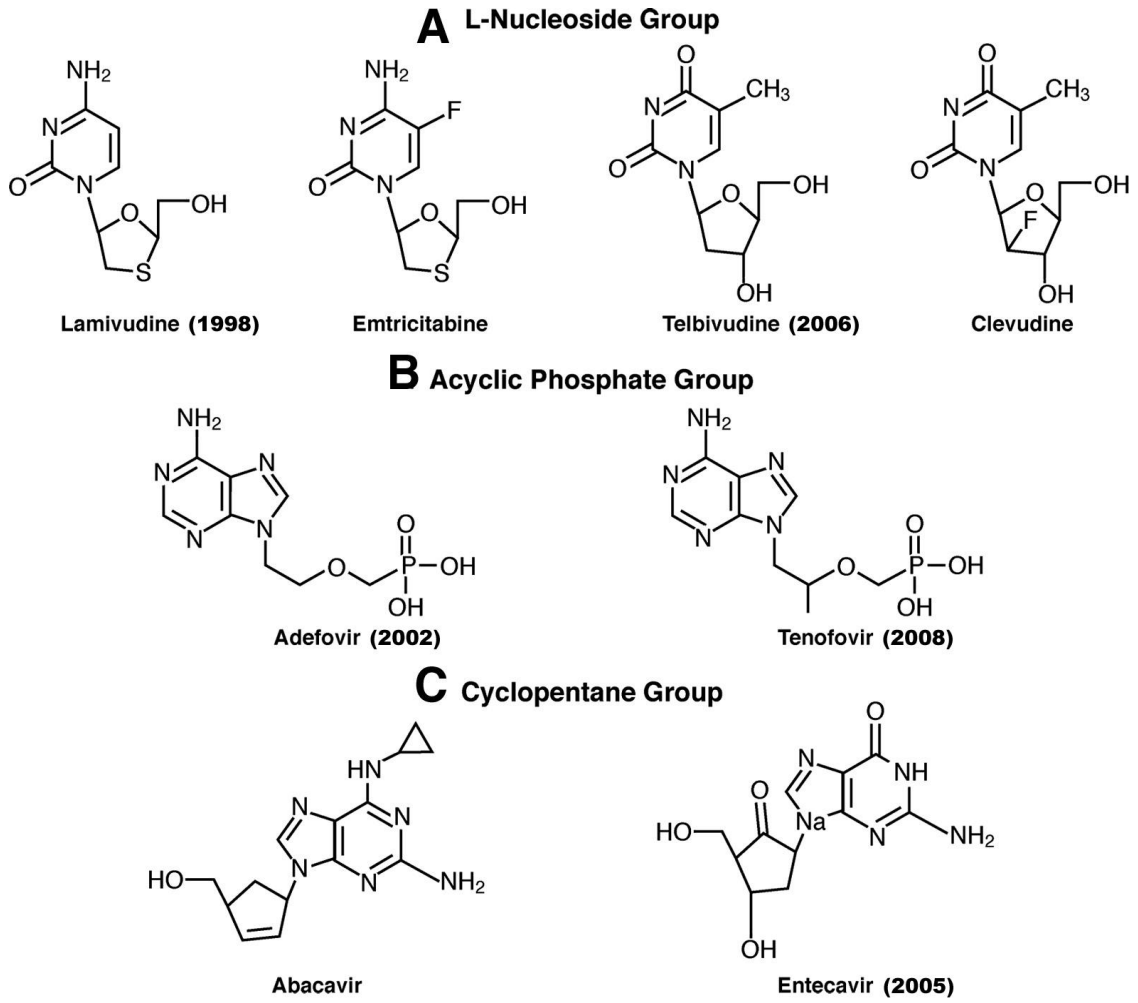


Figure 5: Chemical structures of the 3 main classes of anti-HBV nucleos(t)ide analogues. (A) Lamivudine, emtricitabine, telbivudine, and clevudine are nucleoside analogues with the unnatural β L-configuration, acting as chain terminators. (B) Adeofovir dipivoxil and tenofovir disoproxil fumarate are acyclic nucleotide analogues. They possess a phosphonate group that is equivalent to a phosphate group but cannot be cleaved by the esterases. Both function as chain terminators as well. (C) Entecavir and abacavir are carbocyclic analogues of 2'-deoxyguanosine. The oxygen in the furanose ring is replaced by a vinyl group with entecavir, inhibiting HBV polymerase via several mechanisms including priming, reverse transcription, and DNA elongation. The number in braces indicates the year of FDA approval. Modified after [25].

Given the clinical importance, a standardized nomenclature to describe HBV antiviral response and to define genotypic, phenotypic and clinical resistance to antiviral therapy has been established (**Table 2**). Detailed clarification of terminologies was recently reviewed and summarized [26-28].

Table 2: Nomenclature for antiviral resistance

Term	Definition
Primary nonresponse	Inability of nucleoside/tide analogue treatment to reduce serum HBV DNA by >1 log_{10}IU/ml after the first 12 weeks of treatment
Partial response	Detectable HBV DNA on real-time PCR assay during continuous therapy
Virological breakthrough (viral rebound)	Increase in serum HBV DNA level by >1 log_{10} above nadir on ≥two occasions 1 month apart, while on treatment, after achieving a virological response in a medication-compliant patient
Biochemical breakthrough	Elevation in serum ALT while on treatment, after achieving normalization in a medication-compliant patient
Genotypic resistance	Detection of viral populations bearing amino acid substitutions in the reverse transcriptase region of the HBV genome that have been shown to confer resistance to antiviral drugs in phenotypic assays during antiviral therapy. These mutations are usually detected in patients with virologic breakthrough but they can also be present in patients with persistent viremia and no virologic breakthrough
Phenotypic resistance	Decreased susceptibility of an HBV polymerase to an antiviral treatment *in vitro*
Cross resistance	Decreased susceptibility to more than one antiviral drug conferred by the same amino acid substitution or combination of amino acid substitutions

Adopted from [17, 26, 27].

Antiviral drug resistance is defined as the reduced susceptibility of a virus to the inhibitory effect of a drug, and results from a process of adaptive mutations under the selection pressure of antiviral therapy. This process (**Figure 6**) usually begins with a mutation in the viral genome that becomes selectively amplified by the selective pressure exerted by antiviral therapy. This genotypic resistance leads to phenotypic resistance as resistant variant continue to be selected and amplified with a rise in serum HBV DNA levels. An increase in serum HBV DNA levels > 1 log_{10}copies/mL above the nadir value achieved during antiviral therapy implies emergence of virological breakthrough in a treatment-compliant patient.

Serum HBV DNA levels initially may be relatively low after the emergence of resistance and during virological breakthrough, since these mutations often reduce the replication fitness of the virus. However, the development of compensatory mutations can restore replication fitness, thereby leading to progressive increases in HBV DNA. Virological breakthrough is typically followed by biochemical breakthrough (or clinical resistance), in which alanine aminotransferase (ALT) levels begin to increase above nadir values achieved during therapy. Hepatitis flares and even hepatic decompensation can result. Prompt recognition of viral mutations may allow for modification of antiviral therapy to obviate clinical consequences of resistance that may be poorly tolerated, for instance, in a cirrhotic patient.

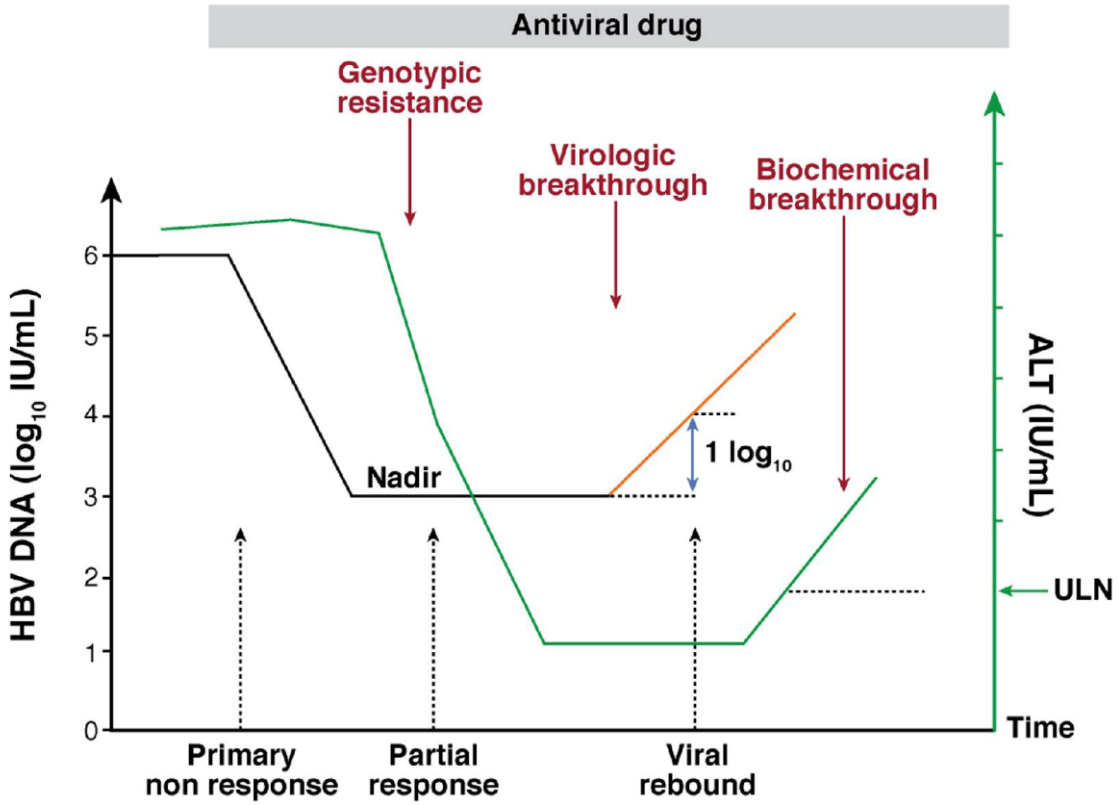


Figure 6: Evolution of viral load and ALT levels during antiviral resistance emergence. After an initial drop in viral load following the initiation of antiviral therapy, virological breakthrough may occur as a consequence of antiviral drug resistance. It corresponds to the rise in serum HBV DNA levels of at least 1-$\log_{10}$ IU/mL compared with the lowest value during therapy (nadir value), in 2 consecutive samples 1 month apart, in patients who have previously responded and have a good treatment compliance. It may be followed by an elevation in serum ALT levels in patients who previously showed transaminases normalization under treatment. It may result in hepatitis flares and in worsening of liver histology. Modified after [26].

The cumulative annual incidences of resistance with different approved antivirals among chronic hepatitis B patients are shown in **Table 3**. Among nucleoside-naïve patients, antiviral

drug resistance had been reported in up to 80% of patients after 5 years of lamivudine [29], 29% after 5 years of adefovir [30, 31], 1.2% after 5 years of entecavir [32], and 8-21% after 2 years of Telbivudine [33]. Resistance rates are substantially higher in patients with prior resistance to lamivudine, with rates of up to 20% after 1 year of adefovir [34] and 51% after 5 years of entecavir [32]. Sequential therapy had also been reported to result in the selection of multi-drug resistant HBV [35]. No tenofovir resistance was noted during 3 years of treatment in the recent trials, although at week 72, the majority of viremic patients were switched to a combination of tenofovir and emtricitabine [36].

Table 3: Cumulative annual incidence of resistance for all five approved agents

Drug and patient population	Resistance at year of therapy expressed as percentage (%)					
	1st	2nd	3rd	4th	5th	6th
Lamivudine	23	46	55	71	80	–
Adefovir	0	3	6	18	29	–
Adefovir (LAM-resistant)	20	–	–	–	–	–
Telbivudine HBeAg-Pos	4.4	21	–	–	–	–
Telbivudine HBeAg-Neg	2.7	8.6	–	–	–	–
Entecavir	0.2	0.5	1.2	1.2	1.2	1.2
Entecavir(LAM-resistant)	6	15	36	46	51	57
Tenofovir	0	0	0	0	–	–

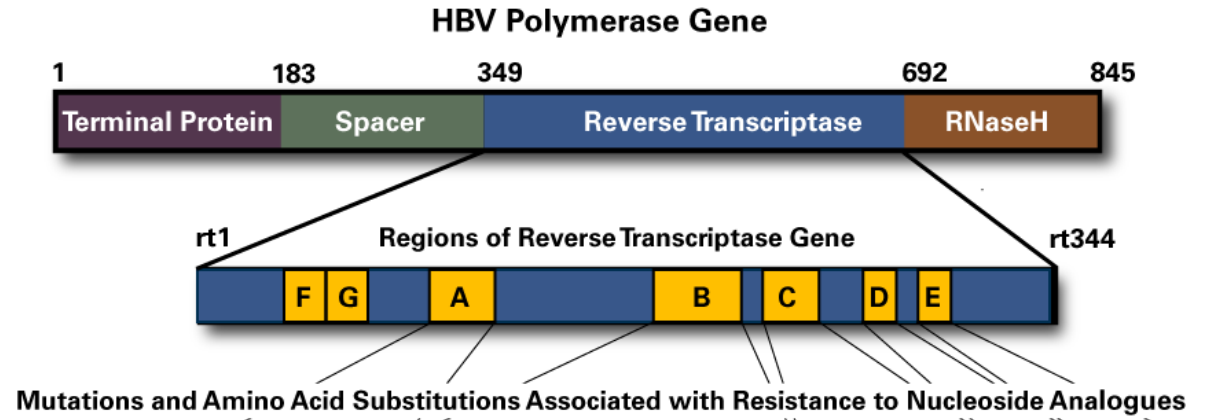
Modified from [26].

Two types of mutations have been identified: primary, which are directly responsible for associated drug resistance, and secondary or compensatory mutations, which occur for the virus to facilitate replication competence because primary resistance mutations may be associated with a reduction in replication fitness. Compensatory mutations are also important because they reduce the deleterious effects to the virus associated with acquisition of primary drug-resistant mutations [37, 38]. The primary and compensatory resistance substitutions associated with treatment failure for chronic hepatitis B are shown in **Figure 7.** To date, changes to eight codons in the HBV polymerase ORF account for primary treatment failure with the currently approved nucleos(t)ide analogues. These resistance-associated mutations selected by a particular nucleos(t)ide analogue confer at least some degree of cross-resistance to other members of its structural group but may also diminish the sensitivity to nucleos(t)ide analogues from a different chemical group [39-42]. Due to the different patterns of resistance

to the five licensed drugs, these substitutions commit subsequent viral evolution to five different pathways (**Table 4**).

- The L-nucleoside pathway (rtM204V/I). In this pathway, LAM and LdT treatment select for rtM204V/I which predisposes to subsequent ETV resistance. Although rtM204I can be found in isolation, M204V are only found with other changes, in particular rtL180M [43]. Compensatory changes have also been found, such as rtL80V/I and rtV173L [44, 45]. They do not confer cross-resistance to ADV or TDF.
- The acyclic phosphonate pathway (rtN236T). ADV and TDF treatment select for and/or consolidate rtN236T [46]. The rtN236T does not significantly affect sensitivity to LAM and ETV.
- Shared pathway (rtA181T/V). In this pathway, treatment with either L-nucleosides or acyclic phosphonates can select rtA181T/V, which occurs in about 40% of cases of ADV failure but less than 5% of cases of LAM failure [47]. ADV and TFV treatment can consolidate rtA181T/V. The rtA181T/V also confer cross-resistance to LdT but do not significantly affect sensitivity to ETV.
- The double pathway (rtA181T/V+rtN236T). In this pathway, treatment with TDF consolidates both of these variants, significantly blunting its antiviral efficacy [46], resulting in persistent viremia [48]. The double mutation also confers cross-resistance to LAM and LdT but does not significantly affect sensitivity to ETV.
- The D-Cyclopentane/ETV naive resistance pathway (rtM204V/I ± rtL180M and one or more substitutions at rtI169, rtT184, rtS202, or rtM250). Three substitutions are required to be selected out for high level resistance on ETV, accounting for the very low resistance rates observed in NA naïve patients [49, 50]. They also confer cross-resistance to LAM and LdT but do not significantly affect sensitivity to ADV and TDF.

The first 3 pathways are associated with only 1 mutation, whereas the fifth pathway requires at least 3 mutations for resistance. This “pathways of evolution approach” facilitates understanding HBV evolution during therapy and can be used to predict patient outcomes and improve our understanding of cross-resistance patterns and profiles [39].



Mutations and Amino Acid Substitutions Associated with Resistance to Nucleoside Analogues

Gene Region	A		B				C		D	E
Baseline Amino Acid Position	80	169	173	180	181	184	202	204	236	250
Baseline Amino Acid	L	I	V	L	A	T	S	M	N	M
Lamivudine Resistance	V/I		L	M	T			V/I/S		
Adefovir Resistance					T/V				T	
Entecavir Resistance		T		M		S/A/I/ L/F/G	G/I	V		V
Telbivudine Resistance					T/V			I		

Figure 7: Mutations in the reverse transcriptase region of HBV polymerase associated with resistance to nucleos(t)ide analogue therapy. The HBV polymerase consists of four domains: terminal protein, spacer, POL/RT and RNaseH. The POL/RT domain itself has seven domains (A, B, C, D, E, F and G), and it is amino acid substitutions in these domains that confer nucleos(t)ide analogue resistance. Mutations designated rtM204I/V/S, in the Tyrosine–methionine–aspartate–aspartate (YMDD) motif in the catalytic or C domain of HBV P ORF, confer resistance to lamivudine and telbivudine. Multiple amino acid substitutions at positions 169, 184, 202 and 250 have been reported to be associated with resistance to entecavir, in addition to a background of substitutions at position 180 and 204. Specific mutation conferring resistance to tenofovir remains to be identified, although adefovir resistance mutations at position 181 and 236 confer reduced susceptibility to tenofovir.

Notably, a specific single amino acid substitution may confer multidrug resistance (MDR). This was shown with the rtA181V/T substitutions, which are responsible not only for decreased susceptibility to the L-nucleosides LAM and LdT but also to the acyclic phosphonates ADV and TDF [47]. This highlights the clinical usefulness of genotypic testing (drug resistance testing) in patients with treatment failure, as has been done for HIV therapy management [51], in order to determine the viral resistance mutation profile and thereby tailor therapy to the major viral circulating strain.

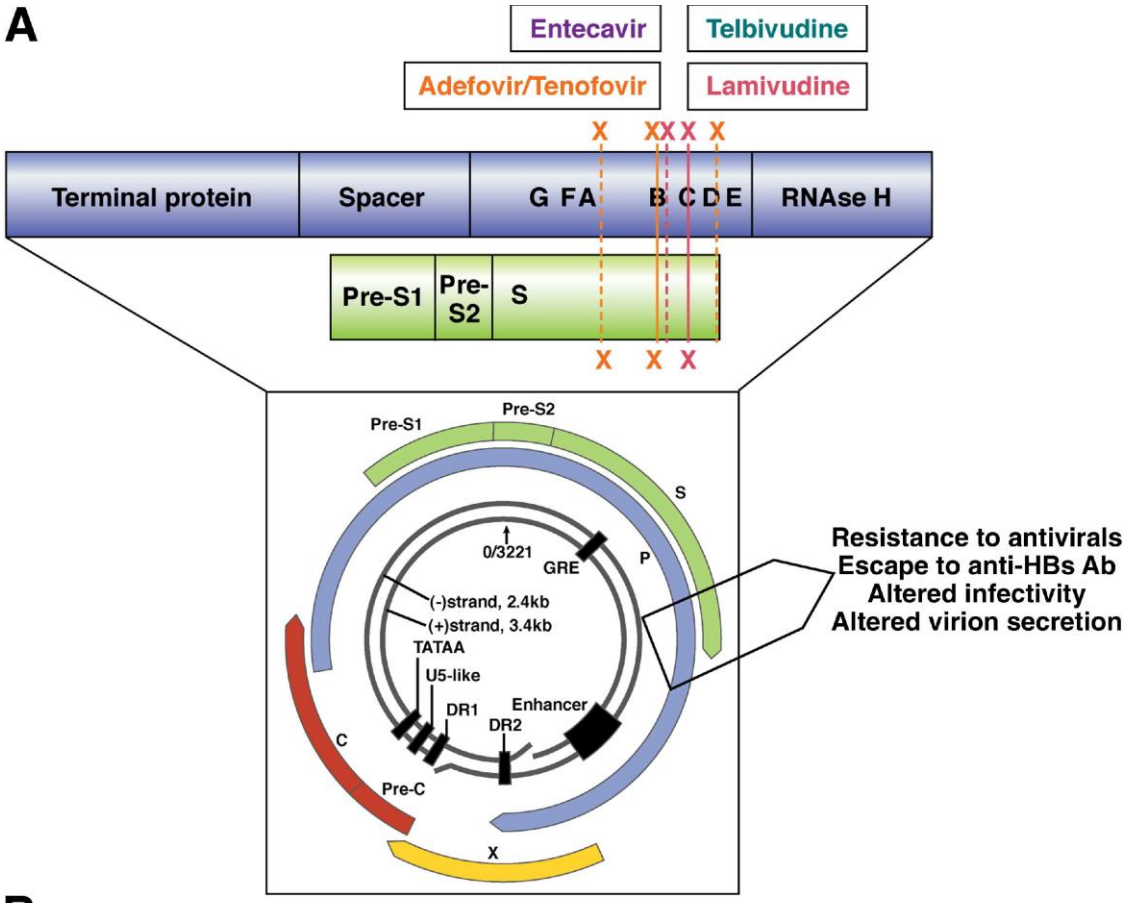
Table 4: Patterns and pathways of drug resistance in the context of cross-resistance

Pathway	Mutation	Level of susceptibility				
		LAM	LdT	ETV	ADV	TDF
	WT	S	S	S	S	S
L-nucleoside (LAM/LdT)	M204I/L180M+M204V	R	R	I	S	S
Acyclic phosphonate (ADV)	N236T	S	S	S	R	I
Shared (LAM/LdT/ADV)	A181T/V	R	R	S	R	I
Double (ADV/TDF)	A181T/V+N236T	R	R	S	R	I/R
D-Cyclopentane (ETV)	*±I196T±V173L±M250V *±T184G±S202I/G	R	R	R	S	S

I, intermediate; R, resistant; S, sensitive; *, L180M+M204V/I. Modified from [52].

The gene that encodes the HBV polymerase overlaps with the gene that encodes the viral envelope, and so mutations in the overlapping reading frame can change both proteins [8]. This raises an important issue that antiviral treatment induced mutation can also have a substantial impact on other aspects of the variant such as HBsAg/virion secretion, infectivity and immune escape [53, 54]. Moreover, the combination of polymerase and surface gene mutations may then result in viruses that exhibit a reduced fitness which may translate into differences of selection kinetics [55]. However, such studies have been hampered by the low infectivity observed with primary human hepatocytes and the HepaRG cell line, the only cellular systems that are available to study the full viral replication cycle, including infection [56, 57].

A



B

Drug Grouping	Resistance Mutations	HBsAg Corresponding Changes
L-Nucleosides (LMV & LdT) (* LdT)	rtL180M	No Change
	rtM204V	sI195M
	rtM204I *	sW196*/S/L
Acyclic Phosphonates (ADV & TFV) (* LdT) (# LMV) *#Also Affected	rtA181T *#	sW172*
	rtA181T *#	sW172L
	rtA181V *	sL173F
	rtN236T	After End of HBs open reading frame
Cyclopenta(e)ne (ETV)	rtI169T	sF161H/L
	rtT184A	No Change
	rtT184C	sL175F + sL176V
	rtT184I	No Change
	rtT184G	sL176V
	rtT184S	sL175F
	rtT184M	sL176*
	rtT184L	sL175F
	rtS202C	No Change / sS193F
	rtS202I	sV194F/S
	rtS202G	No Change / sS193L
	rtM250I	After End of HBs open reading frame
	rtM250V	After End of HBs open reading frame

*STOP CODON

Figure 8: Impact of drug resistance mutations in the viral polymerase gene on the overlapping surface gene. (A) Physical map showing that resistance mutations may result in viral envelope changes leading to altered virion secretion, altered infectivity, and escape to anti-HBs antibodies. **(B)** The main resistance associated substitutions in the viral polymerase and their corresponding changes in the HBsAg. Modified from [26].

1.3 Methods for drug resistance detection and evaluation

Antiviral drug resistance, the major hurdle to the success of chronic hepatitis B therapy with nucleos(t)ide analogues, is conferred by the selection of polymerase gene mutants with reduced susceptibility to nucleos(t)ide analogues. A fundamental issue regarding antiviral resistance is the criteria for defining drug-resistant mutations and the method used to diagnose drug resistance.

Viral load assays

Measurement of serum HBV DNA is used to evaluate disease activity, assess the efficacy of antiviral therapy, and predict treatment outcomes [58]. Studies have demonstrated that persistently high levels of serum HBV DNA are associated with an increased risk of cirrhosis, HCC and liver related mortality, while reduction in HBV DNA typically precedes other beneficial events to halt the progression [59]. These findings have led to an increased reliance on the HBV DNA level as an indication for hepatitis B treatment. On the other hand, Measurement of viral load is indispensable for monitoring antiviral response as well as confirming the presence of drug-resistant virus because nearly all instances of resistance to nucleos(t)ide analogue are initially identified by a sustained rise in viral load that occurs despite continuing antiviral therapy [28]. It is important to use a sensitive HBV DNA assay to monitor for drug resistance, and the same assay should be used over time for consistency. In most treatment guidelines, a ≥ 1.0 $\log_{10}$ IU/mL increase in HBV DNA while on treatment is considered to indicate emergence of drug-resistant virus. The sensitive HBVDNA assays that are currently in use allow detection of rising viral loads because of drug-resistant virus even when the emergence of the drug-resistant HBV population is slow [60]. Because factors other than drug resistance (for example, poor patient compliance and/or pharmacogenomic factors) can affect viral load [61, 62], it cannot be automatically assumed that rising loads are indicative of drug resistance because drug-resistant HBV can only be confirmed by genotyping and/or phenotyping.

To identify potential genotypic resistance, the HBV polymerase sequence isolated from the patient during virologic breakthrough should be compared to either the sequence isolated from a pre-therapy sample [63] or the consensus published sequences(s) of the same genotype when pre-therapy samples are not available [64]. Genotyping relies on either DNA sequencing or hybridization.

Genotypic analysis I – DNA sequencing based methods

Sequencing-based methods include standard population-based polymerase chain reaction (PCR), cloning of PCR products, and restriction fragment-length polymorphism analyses. Direct PCR sequencing is commercially available and is the most commonly used method for detecting the presence of resistance mutations in clinical trials, as this strategy allows for all mutations to be identified, including primary mutations, potential compensatory mutations, and previously unidentified mutations. A major limitation of this technique is its lack of sensitivity in detecting minor populations of resistant virus compared with other methods. In general, only viral variants present at a prevalence ≥ 20% among the total HBV quasispecies pool are detectable using this technique. Cloning can overcome this problem, but analysis of large numbers of clones is required. Viral mutants that constitute as little as 5% of the total population can be detected by restriction fragment-length polymorphism (RFLP) analyses, but separate sets of endonuclease reactions must be designed specifically for each (and known) mutant of interest [65, 66]. These methods are labor intensive, require highly skilled personnel, and are not suitable for high-throughput screening. With the exception of the TRUGENE genotyping test developed by Visible Genetics (Siemens Healthcare Diagnostics), no PCR-based DNA assays have been commercialized or approved by regulatory bodies and remain as "in house" or "home brew" assays lacking standardization.

As a promising trend, next generation sequencing (NGS) has led to a profound revolution in genome research as well as in clinical diagnosis practice. More specifically, in the study of viral quasispecies, NGS can overcome the limitations of classic clonal Sanger sequencing by direct parallel clonal sequencing of mixed samples, resulting in more than 10^3 reads per base (termed as ultra-deep) and has been successfully applied in HIV, HCV and HBV drug resistance analysis [67-73]. The main technical limitation of NGS is that the maximum lengths of individual sequencing reads are shorter than those obtainable with conventional chain termination sequencing methods but this issue is getting continuously improved overtime [74-76]. Ultra-deep sequencing is currently the fastest and probably most sensitive (0.1%) method available for detecting small subpopulations of resistant virus and is likely to become the method of choice in the near future, particularly if the associated instrumentation and running costs become more affordable.

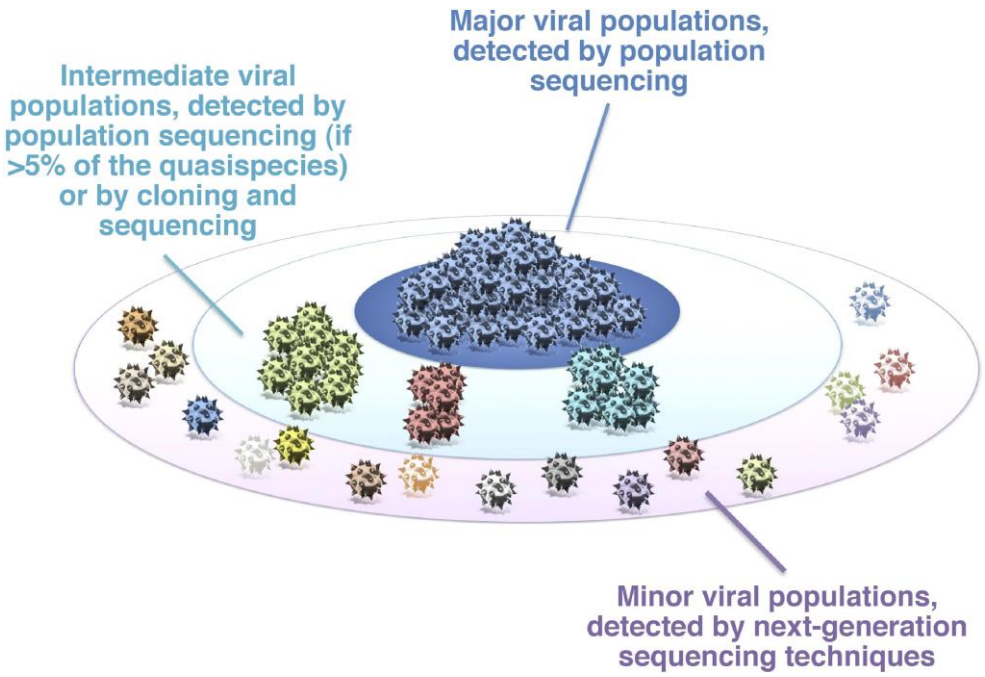


Figure 9: Quasispecies distribution of HBV genomes and methods to detect major (>10% of the viral quasispecies), intermediate, and minor (<1% of the viral quasispecies) viral populations. Modified from [60].

Genotypic analysis II – Hybridization based methods

Hybridization-based genotyping methods, which can detect single nucleotide mismatches, provide an alternative strategy and are also being widely used. The commercially available reverse hybridization assay LiPA DR (Innogenetics, Belgium) uses a series of short membrane-bound oligonucleotide probes to discriminate between wild-type sequences and those of known drug resistant mutants by detecting single nucleotide mismatches in PCR-amplified HBV DNA [77, 78]. Line-probe assays are able to detect antiviral-resistant variants at a prevalence as low as 5% of the total HBV quasispecies pool [79]. Other hybridization-based methods include: (1) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) based restriction fragment mass polymorphism (RFMP) which can detect mutants that comprise ≥ 1% of the viral quasispecies [80-82]; (2) DNA chip technologies using oligonucleotide microarrays with improved sensitivity as well as ability to detect "new" mutants, which are relatively easy to perform for the simultaneous detection of a multitude of unique mutations as well as recognized polymorphisms [83-85]; (3) real time PCR formats with specific probes including allele specific PCRs [86, 87].

The major limitation of all hybridization-based methods is that specific probes must be created for each mutation of interest, and multiple probes may be required to detect a single nucleotide change because of HBV genotypic variability. Besides, natural sequence

variability as well as sequence context and secondary structures in regions of interest may reduce their discriminatory power and specificity.

The more sensitive assays can detect HBV DNA encoding the resistance mutations that represent 5%-10% of the entire HBV quasispecies enabling earlier identification of patients with genotypic resistance mutations prior to or at the time of virologic breakthrough. However, the utility of ultra-sensitive assays that can detect mutants comprising ≤ 1% of the viral population in predicting drug resistance remains to be determined.

In vitro phenotypic testing

In vitro phenotypic assay remains the "gold standard" to confirm genotypic antiviral resistance based on the determination of changes to the effective concentration of the drug required to inhibit 50% of the target (IC_{50}) relative to the "wild-type" reference HBV. Several approaches have been developed for analyzing the *in vitro* drug susceptibility of resistant variants identified *in vivo* in patients. These approaches include viral polymerase enzymatic assays, cell lines permanently expressing HBV resistant mutants, and cell culture models in which the viral genome of resistant mutants is transferred for the analysis of viral replication and drug susceptibility.

Antiviral susceptibility testing involves assaying the activity of the polymerase enzyme or of HBV replication. As mutations conferring resistance to nucelos(t)ide analogues are located in the viral polymerase gene, several enzymatic assays have developed to study their effect in vitro in cell-free assays for viral polymerase activity. The main models to study HBV polymerase activity are based on its expression in insect cells using a baculovirus vector, and on the study of its activity in purified viral nucleocapsids [88, 89]. A surrogate model for HBV polymerase studies has been the use of a cell free assay for the expression of the duck HBV (DHBV) polymerase in a reticulocyte lysate system [90]. Overall, the cross-resistance data obtained in the cell-free polymerase assays were generally consistent with those obtained in tissue culture experiments; however, some discrepancies were observed in the magnitude of the inhibitory effects of the drugs on viral DNA synthesis in the cell-free system and in tissue culture [91, 92].

Meanwhile, several tissue culture models have been developed to study HBV drug resistance, either to understand the mechanism of antiviral drug resistance or to provide cross-resistance data. These assays have also provided important data on viral fitness which is an important determinant in the process of resistant mutant selection in the patient. The HBV

replication, i.e. synthesis of HBV DNA intermediate, is possible in hepatoma cell lines provided that they are transfected with the genetic information necessary and sufficient to enable the synthesis of pre-genomic RNA. Therefore, these assays are based on transient transfection with mutant HBV clones or on the construction of cell lines that permanently express HBV-resistant mutants, while the mutant genomes can be generated either by site-directed mutagenesis or by cloning of the naturally occurring variants (**Table 5**).

Table 5: Cell culture assays for the study of HBV drug resistance

Principle	Advantages	Limitations
Site-directed mutagenesis	Rapid / direct demonstration of the resistance phenotype	Not suited for complex profiles of mutations
Baculovirus vector-mediated transduction	High transduction efficiency; high replication levels	Production of recombinant vector for each mutant
Cell lines that permanently express HBV mutants	High reproducibility; high productivity	Establishment of new cell lines for each new mutant
Whole HBV genome amplification and transfection	Considers variability across the whole HBV genome	Not a stable source of genetic material; low replication levels
Whole HBV genome cloning and transfection	Variability across the whole HBV genome; stable source	Requirement for a cloning step
Exchange of HBV genome fragments	High PCR sensitivity; high cloning efficiency	Chimeric HBV genomes; incomplete variability

Modified from [93].

The study of HBV resistance was initially based on the study of HBV replication in the presence of the antiviral drug following transfection of HBV clones harboring the suspected resistance mutations that had been introduced in the HBV genome either by site-directed mutagenesis or by exchange of viral genome fragments [94, 95]. Then several other alternative methods were developed, relying either on vector-free or on vector-mediated phenotypic assays.

In an attempt to develop a vector-free approach, an original and efficient method for the polymerase chain reaction (PCR) amplification of full-length HBV genomes was developed to facilitate the analysis of naturally occurring HBV variants [96]. PCR amplicons are cut by a special restriction enzyme, whose recognition site is included within primers, to render their extremities competent for circular closing by host enzyme after transfection. Once introduced

in cells, these circularized amplicons serve as covalently closed circular-like DNA templates for the initiation of the intracellular HBV replication. However, depending on the nature of the HBV genome analyzed (e.g., particular mutants or genotypes), the level of intracellular DNA synthesis obtained with this approach can be rather low [97], requiring a sensitive method of detection of viral replication in transfected cells, such as real-time PCR, in view of clinical use.

Until recently, the analysis of the phenotype of naturally occurring or drug-selected HBV mutants has relied on either PCR-mediated transfer of HBV genome cassettes or site-directed mutagenesis within plasmids carrying 1.1 to 2 genome units of well-established replication-competent laboratory strains. These plasmids contain the HBV genetic information necessary and sufficient to initiate the HBV intracellular replication cycle after transfection into cells. The synthesis of HBV pre-genomic RNA can be driven by either the HBV promoter for vectors containing 1.3 to 2 genome units or a strong mammalian promoter for vectors containing 1.1 genome units [98, 99]. The cloning based approach, in comparison to the vector-free assays, allows the study of viral replication upon transfection of one clone or a mixture of clones into hepatoma cells (**Figure 10**). Vector-free and vector-based replication assays are complementary approaches to determine the phenotype of clinical HBV strains.

Another approach to characterize viral drug resistance in tissue culture is the use of cell lines permanently expressing the mutants, to allow a more reproducible measure of drug susceptibility [100]. The advantage of using stable cell lines for phenotype testing is the ability to perform cross-resistance testing in a consistent environment [101]. However, one of the problems of these permanent cell lines is the need to design and produce new cell lines when new resistant mutants are identified. The integration site of HBV DNA within the cellular chromosome may affect HBV replication and cellular function; therefore, these cell lines cannot be used to determine the relative replication efficiency of HBV encoding the antiviral resistance mutations.

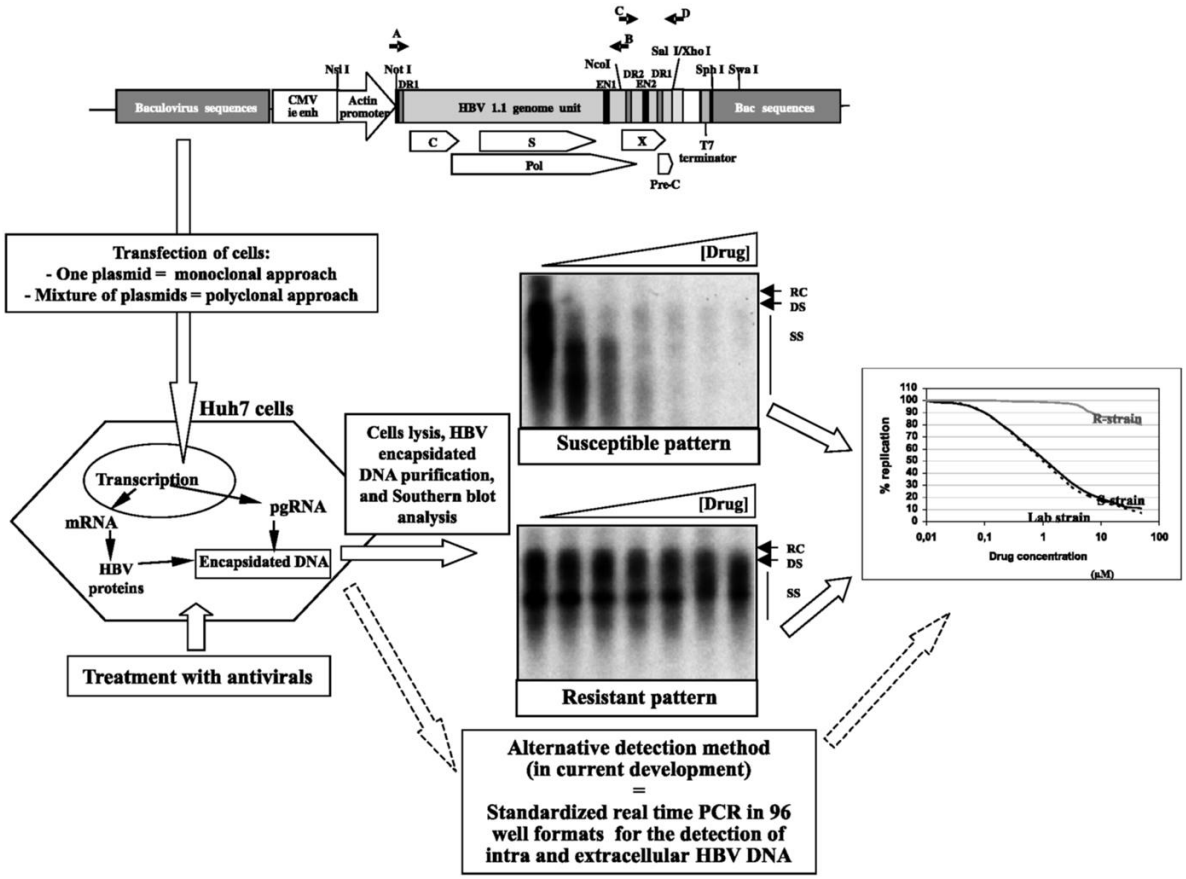


Figure 10: Structure of plasmid used and overall approach for a phenotypic study (drug susceptibility testing) of HBV clinical isolates described in a previous study [99]. A map of the vector pTriEX-HBV is presented at the top. The vector contains 1.1 HBV genome units corresponding to the length of pgRNA. The pTriEx-HBV is transfected into hepatoma cells, in which they trigger HBV genome replication. Transfected cells are treated with drugs for 5 days before viral replicative intermediates are purified and subjected to Southern blot analysis. The IC_{50} and IC_{90} value can be determined by phosphorimager analysis. Modified from [102].

In addition, an *in vitro* assay based on the transduction of HepG2 cells by recombinant baculoviruses harboring replication-competent HBV genomes were developed with several advantages over previously existing cellular models [103, 104]. However, the production of recombinant vector for each mutant can be tedious.

In vivo studies of antiviral drug resistant mutants can be performed with DHBV or WHV in the duck and woodchuck model, respectively [105]; however, the pattern of resistance associated substitutions in the rt-polymerase can differ between species [90, 106]. The study of human HBV resistant mutants is limited to chimpanzee and humanized SCID mouse models, but limited data are available [107, 108]. New convenient small animal models are still in great need [109].

1.4 Clinical aspects: prevention and management of resistance

The development of antiviral resistance is generally associated with worse clinical outcomes as the effectiveness and the benefit of therapy was negated as a consequence of treatment failure [110, 111]. Because HBV uses error-prone reverse transcription to copy its genome, resistant variants emerge frequently even in treatment-naïve patients and are readily to be selected out [112-114]. A major challenge in HBV management is how to most effectively utilize the available oral nucleos(t)ide analogues to achieve long-term durable antiviral suppression while avoiding the selection of viral variants resistant to one or more agents. The spread of drug-resistant HBV variants can be reduced by avoiding unnecessary drug use, choosing drugs and combinations more carefully, and continually monitoring or carrying out targeted surveillance for drug resistance. Because drug-resistant mutant HBV populations are established and expand through replication, antiviral therapy, once initiated, should aim to suppress viral replication as completely and rapidly as possible. In general, the development of resistance depends on a number of variables: pre-treatment HBV DNA levels, rapidity of viral suppression (often correlated with potency of agent), degree of genetic barrier to resistance of agent (threshold probability the virus will mutate under selective pressure from the drug), duration of treatment, and prior exposure to oral antiviral therapy. The lower risk of resistance to TDF and ETV because of their high potency and high genetic barrier (compared with lamivudine, telbivudine, and adefovir) supports their use as first-line therapy whenever possible [52]. Whether initiating treatment with combination therapy will achieve the goal of minimizing the development of antiviral resistance is currently being investigated [115]. Because all available antiviral agents have the same target of action, the utility of this approach would have to be proven in clinical trials because resistance has been reported in a study of de novo combination therapy [116]. In addition, reinforcement of compliance with a prescribed regimen is of paramount importance to preventing resistance since poor adherence substantially reduces viral suppression and therefore increases resistance rates especially for drugs with low barrier to resistance [117].

Monitoring the antiviral response is crucial for the early detection of virological breakthrough, thus permitting early intervention with the prospect of better outcomes. In cases of resistance in compliant patients, an appropriate rescue therapy should be initiated and should have the most effective antiviral effect and minimal risk for selection of multidrug resistance (MDR). It is also important to minimize sequential monotherapy and to avoid use of agents with similar cross-resistance profiles [118]. Therefore, adding a second drug that is not in the same cross-resistance group as the first is the recommended therapeutic approach

[16, 17]. However, although there is a strong virologic rationale for an add-on strategy with a complementary drug to prevent the emergence of MDR strains and raise the barrier to resistance, there is a current trend to recommend a switch to a complementary drug with a high barrier to resistance based on relatively short-term clinical observation [119]. This critical point will need a precise evaluation by long-term clinical and molecular virology studies.

Table 4 shows the cross-resistance data for the most frequent resistant HBV variants and the major pathways for resistance development. Treatment adaptation should be performed accordingly and is summarized as follows [119]:

- LAM resistance: add TDF (add ADV if TDF not available); a switch to TDF is also advised by some guidelines; however, a switch to ADV is not recommended due to a high rate of resistance and its low potency.
- ADV resistance: it is recommended to switch to TDF if available and add a second drug without cross resistance. If there is no history of LAM prior usage, switching to ETV is also effective for ADV resistance. If genotypic resistance testing is carried out and the rtN236T substitution is present, consider adding LAV, ETV, or LdT to the TDF or switch to TDF plus FTC in a single pill (Truvada); again, if there is no history of previous LAM therapy one could consider switching to ETV. If an rtA181V/T substitution is present, alone or in combination with rtN236T, it is recommended to add-on ETV to the ADV therapy, or to switch to TDF plus ETV if available, as before, if there is no history of prior LAM use, consider switching to ETV.
- LdT resistance: it is recommended to add TDF (or ADV if TDF is not available); a switch to TDF has also been considered in some guidelines; however a switch to ADV is not recommended due to a high rate of resistance and the low potency of ADV.
- ETV resistance: it is recommended to add TDF. Switch to TDF or TDF plus FTC is also advised by some guidelines
- TDF resistance: primary resistance to TDF has not been confirmed so far. It is recommended that genotyping and/or phenotyping be done by a reference-type laboratory to determine the cross-resistance profile. Entecavir, telbivudine, lamivudine, or emtricitabine could be added but would depend on the resistance profile determined genotypically.

1.5 Future and new directions

Very few new drugs are being developed for treatment of chronic hepatitis B; therefore, it is important to continue research into mechanisms of pathogenesis and resistance and to identify new therapeutic targets. Small molecule inhibitors that are directed against multiple HBV targets or steps in HBV life cycle such as viral entry [120], cccDNA formation/maintenance [121], viral pre-genome encapsidation and capsid formation [122, 123] should improve viral clearance and prevent resistance. Therapeutics might also be developed to modulate the innate response of infected hepatocytes [124] and the adaptive immune response [125]. Induction of sustained immunologic control of HBV infection could allow for timed cessation of nucleos(t)ide analogue administration. Clinical or preclinical trials are still ongoing.

Ideally, treatment for CHB should begin at diagnosis; this is not feasible because of limitations of drugs. Clinical trials and concurrent improvements in diagnostic technology ensure that treatment options and expert opinion on patient management will continue to evolve. Many laboratories are genotyping and phenotyping HBV mutants to delineate patterns of resistance and cross-resistance. These data will improve the design of new therapeutic strategies and maximize the benefits of anti-viral agents. Resistance testing methodologies vary, and, although few direct comparisons have been made, in vitro phenotype testing (if possible, in combination with genotype testing) seems superior to conventional genotyping or virtual phenotyping. This is especially true for analysis of HBV isolated from patients who have already been treated with several drugs, in whom multiple mutations are more likely to have become fixed in the genetic archive. Drug resistance testing of HBV isolates is currently performed only in a few specialized “reference-type” laboratories, but it should become routine as more sensitive, reliable, high-throughput, and accurate methods are developed, along with clinically useful algorithms for interpretation. In particular, standardization of tests and definition of resistance/susceptibility thresholds or “cut-offs” that can be used to correlate laboratory results with clinical observations and outcomes are urgently needed.

Aim of this PhD thesis

This study dealing with one of the most important infectious diseases in the world, shall help to improve treatment monitoring and at the end to optimize antiviral therapy. It aimed initially at developing an optimized and standardized phenotypic assay for the analysis of viral fitness with and without drug treatment in high throughput, especially suitable for clinical application. This time- and cost-effective assay should be further validated by evaluating HBV strains with known resistance mutations and determining their resistance factor against the four major nucleos(t)ide analogues in comparison to the published results from the conventional assays. The second aim of the PhD thesis was a comprehensive and systematic analysis of HBV antiviral resistance mutations, by generating and evaluating an extensive panel of carefully selected mutations and their combinations using the established assay. By this means, cross-resistance profiles together with viral fitness parameters should be integrated to reveal the complexity of mutation patterns during resistance development. Moreover, novel resistance mutations should be identified by genotypic screening and phenotypic validation from resistant patient samples, or by bioinformatics approaches. Finally, the complete set of phenotypic evaluation and fitness data generated should be correlated to genotypic analysis to allow a valid prediction from genotypic analyses easier to perform in a standard laboratory.

2. Material and Methods

2.1 Materials

2.1.1 Clinical samples

Serum samples used for genotypic and phenotypic analysis were obtained from chronic hepatitis B patients enrolled in the cohort of the HOPE project, with clinical resistance or partial response to defined treatment. Serum HBV DNA for all samples was measured using the COBAS TaqMan assay.

2.1.2 Chemicals

Adefovir (PMEA)	SeqChem Sequoia, Pangbourne, UK
Agarose	Invitrogen, Karlsruhe, D
Bacillol Plus	Roth GmbH & Co, Karlsruhe, D
Collagen	Serva, Heidelberg, D
Dimethylsulfoxide	Sigma-Aldrich Chemie GmbH, Steinheim, D
Di-thiothreitol	Sigma-Aldrich Chemie GmbH, Steinheim, D
EDTA	Roth GmbH & Co, Karlsruhe
Ethanol	Merck, Darmstadt, D
Ethidium bromid solution (10 mg/ml)	Invitrogen, Karlsruhe, D
Entecavir	SeqChem Ltd., Pangbourne, UK
Glycerol	Roth GmbH & Co, Karlsruhe, D
Glycine	Roth GmbH & Co, Karlsruhe, D
Guanidine hydrochloride	Roth GmbH & Co, Karlsruhe, D
HBSS (+Ca, +Mg)	Invitrogen, Karlsruhe, D
HCl 37% (w/w)	Roth GmbH & Co, Karlsruhe, D
Hoechst 33342, trihydrochloride, trihydrate	Sigma-Aldrich Chemie GmbH, Steinheim, D
Hybond-P PVDF membrane	GE Healthcare, Freiburg, D
Isopropanol	Roth GmbH & Co, Karlsruhe, D
Lamivudine (3TC)	SeqChem Ltd., Pangbourne, UK
Methanol	Roth GmbH & Co, Karlsruhe, D

Nonidet P40	Sigma-Aldrich Chemie GmbH, Steinheim, D
PBS	Invitrogen, Karlsruhe, D
Poly-L-lysine solution	Sigma-Aldrich Chemie GmbH, Steinheim, D
Potassium chloride	Sigma-Aldrich Chemie GmbH, Steinheim, D
Protogel Ultra Pure 30%,	
RNA loading buffer	Sigma-Aldrich Chemie GmbH, Steinheim, D
Safeseal tips	Biozym, Oldendorf, D
Skim milk powder	Merck, Darmstadt, D
Sodium chloride	Roth GmbH & Co, Karlsruhe, D
Sodium deoxycholate	Roth GmbH & Co, Karlsruhe, D
Sodium dodecylsulfate	Roth GmbH & Co, Karlsruhe, D
Tenofovir (PMPA)	SeqChem Ltd., Pangbourne, UK
Tris	Roth, Karlsruhe, D
Triton-X- 100	Sigma-Aldrich Chemie GmbH, Steinheim, D
Trizol	Gibco/Invitrogen, New York, USA
Trypsin- EDTA	Gibco/Invitrogen, New York, USA
Tween 20	Roth, Karlsruhe, D
Uridine	Sigma-Aldrich, Steinheim, D
X-ray films Kodak Biomax MS	Becton Dickinson, Heidelberg, D

2.1.3 Kits

BioSprint 96 One-For-All Vet Kit	Qiagen, Hilden, D
Cell Proliferation Kit II (XTT)	Roche, Mannheim, D
Dual-Luciferase® Reporter Assay System	Promega, Mannheim, D
Fugene 6 Transfection Reagent	Roche, Mannheim, D
Fugene HD Transfection Reagent	Roche, Mannheim, D
GeneJET Plasmid Miniprep Kit	Fermentas, Waltham, USA
LightCycler 480 SYBR Green I Master	Roche, Mannheim, D
Phusion HotStart II High-Fidelity PCR Kit	Fermentas, Waltham, USA
QIAamp MinElute Virus Spin Kit	Qiagen, Hilden, D
QIAamp UltraSens Virus Kit	Qiagen, Hilden, D
QIAGEN Plasmid Plus Midi Kit	Qiagen, Hilden, D
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden, D
QIAquick Gel Extraction Kit	Qiagen, Hilden, D

QIAquick PCR Purification Kit	Qiagen, Hilden, D
Rapid DNA Dephos & Ligation Kit	Roche, Mannheim, D
Rediprime II Random Prime Labelling	Amersham, Buckinghamshire, UK
SEAP Reporter Gene Assay Kit	Roche, Mannheim, D
Zero Blunt TOPO PCR Cloning Kit	Invitrogen, Karlsruhe, D
TRUGENE HBV Genotyping Assay	Invitrogen, Karlsruhe, D

2.1.4 Cell lines

HepaRG - human hepatic progenitor cell line that retains many characteristics of primary human hepatocytes [126]

HepaRG-TR-X - HepaRG derived cell line expressing HBx in a tetracycline inducible manner [127]

HepG2 H1.3 - stably HBV producing hepatoma cell line, containing one copy of a 1.3-fold overlength HBV genome, genotype D, which establishes HBV cccDNA as additional transcription template [128]

HepG2.2.15 - stably HBV producing hepatoma cell line, containing four copies of HBV genome of two head-to-tail dimers, genotype D [129]

HuH7 - human hepatoma cell line [130]

2.1.5 Cell culture consumables and additives

Cell culture flasks/dishes/well plates	TPP, Trasadingen, CH
Centrifuge vials (15/50ml)	Falcon, BD GmbH, Heidelberg, D
Collagenase	Worthington Biochemical, Lakewood, USA
Cryotubes	Corning Inc., USA
DMEM (High Glucose)	Invitrogen, Karlsruhe, D
DMSO	Merck, Darmstadt, D
FCS	Invitrogen, Karlsruhe, D
Freezing Container	Nalgene, Wiesbaden, D
Gentamicin	Ratiopharm, Ulm, D
HBSS	Invitrogen, Karlsruhe, D
HEPES	Invitrogen, Karlsruhe, D
Hydrocortisone	Sanofi Aventis, Paris, FR
Insulin	Pfizer, New York, USA

L-Glutamine (100X)	Invitrogen, Karlsruhe, D
Non-essential amino acids (100X)	Invitrogen, Karlsruhe, D
Penicillin/Streptomycin (100X)	Invitrogen, Karlsruhe, D
Sodium pyruvate (100X)	Invitrogen, Karlsruhe, D
Trypsine-EDTA (10X)	Invitrogen, Karlsruhe, D
Williams Medium E	Invitrogen, Karlsruhe, D

2.1.6 Cell culture media

Cell freezing medium	DMEM, 20% FCS, 10% DMSO
HepaRG growth medium	WME, 10% FCS, P/S, L-Glu, INS, HC, GM
HepaRG differentiation medium	WME, 10% FCS, P/S, L-Glu, INS, HC, GM, 1.8% DMSO
HepG2.2.15/HepG2 H1.3 growth medium	DMEM, 10% FCS, L-Glu, P/S, NEAA, SP
HepG2.2.15 virus production medium	WME, 5% FCS, L-Glu, P/S, HC, 1% DMSO
HuH7 growth medium	DMEM, 10% FCS, L-Glu, P/S, NEAA, SP
HuH7 wash medium	DMEM, 2% FCS, P/S
HuH7 seeding medium	DMEM, 5% FCS, 1% DMSO, 25mM HEPES, L-Glu, NEAA, P/S, SP

2.1.7 Oligodeoxynucloetides

Primers for PCR-Cloning were designed using Vector NTI software from Invitrogen and the online program Primer3 (http://primer3.sourceforge.net/).

Primer ID	Sequence	Comment
HBV-3103-fw	5'-CCGGAAAGCTTATGCTCTTCTTTTTCACCTCTGCCTAATCATC-3'	HBV whole genome amplification
HBV-3107-rev	5'-CCGGAGAGCTCATGCTCTTCAAAAAGTTGCATGGTGCTGGTG-3'	
HBV-398-fw	5'-GACCACCAAATGCCCCTATC-3'	HBV polymerase gene amplification
HBV-2862-rev	5'-ACACGGTCCGGCAGATGAGA-3'	
HBV-1266-fw	5'-CAGGCCATGCAGTGGAATTC-3'	HBV RT region amplification
HBV-2527-rev	5'-GGTTCCACGCATGCGCTGAT-3'	
PCH200-fw	5'-GTCGACGCACCATGCAACTTTTTGAAGAGCTCTTCTTTTTCACCTCTGCCTAATCA-3'	pCH-9/200 construction
PCH200-rev	5'-GAATTTGATCAGATCAATTCGAGC-3'	

Primers for site-directed mutagenesis were designed using two web-based programs: QuikChange Primer Design from Agilent (http://www.genomics.agilent.com), and PrimerX (http://www.bioinformatics.org/primerx/).

Primer ID	Sequence	Comment
HBV-a1506g-fw	5'-GGGTTTTTCTTGTTGACAGGAATCCTCACAATACCGC-3'	rtK32R
HBV-a1506g-rev	5'-GCGGTATTGTGAGGATTCCTGTCAACAAGAAAAACCC-3'	
HBV-a1659t-fw	5'-CTGGTTATCGCTGGTTGTGTCTGCGGCG-3'	rtD83V
HBV-a1659t-rev	5'-CGCCGCAGACACAACCAGCGATAACCAG-3'	
HBV-a2021g/ t2022c-fw	5'-GTTTGGCTTTCAGTTATGCGGATGATGTGGTATTGGG-3'	rtM204A
HBV- a2021g/ t2022c-rev	5'-CCCAATACCACATCATCCGCATAACTGAAAGCCAAAC-3'	
HBV-t2022a-fw	5'-GTTTGGCTTTCAGTTATAAGGATGATGTGGTATTGGG-3'	rtM204K
HBV-t2022a-rev	5'-CCCAATACCACATCATCCTTATAACTGAAAGCCAAAC-3'	
HBV-a2021c-fw	5'-CTGTTTGGCTTTCAGTTATCTGGATGATGTGGTATTGGG 3'	rtM204L
HBV-a2021c-rev	5'-CCCAATACCACATCATCCAGATAACTGAAAGCCAAACAG 3'	
HBV-a2021c/ t2022a-fw	5'-CTGTTTGGCTTTCAGTTATCAGGATGATGTGGTATTGGG-3'	rtM204Q
HBV- a2021c/ t2022a-rev	5'-CCCAATACCACATCATCCTGATAACTGAAAGCCAAACAG-3'	
HBV-t2022g/ g2023t-fw	5'-GTTTGGCTTTCAGTTATAGTGATGATGTGGTATTGGG-3'	rtM204S
HBV-t2022g/ g2023t-rev	5'-CCCAATACCACATCATCACTATAACTGAAAGCCAAAC-3'	
HBV-t2022c-fw	5'-GTTTGGCTTTCAGTTATACGGATGATGTGGTATTGGG-3'	rtM204T
HBV-t2022c-rev	5'-CCCAATACCACATCATCCGTATAACTGAAAGCCAAAC-3'	
HBV-c2914t-fw	5'-CCGTGAACGCCCACTAAATATTGCCCAAGG-3'	HBx C-terminal truncation (1-86)
HBV-c2914t-fw	5'-CCGTGAACGCCCACTAAATATTGCCCAAGG-3'	

Primers for quantitative real-time PCR were designed using LightCycler Probe Design Software 2.0 from Roche.

Primer ID	Sequence	Comment
HBV-3053-fw	5'-TACTAGGAGGCTGTAGGCATA-3'	rcDNA (genotype D) selective qPCR
HBV-134-rev	5'-GGAGACTCTAAGGCTTCCC-3'	
pSelect-gA-fw	5'-CTGTAGGCACAAATTGGTC-3'	rcDNA (genotype A) selective qPCR
pSelect-gA-rev	5'-ATACAGAGCTGAGGCG-3'	
pSelect-gC-fw	5'-CTAGGAGGCTGTAGGCATA-3'	rcDNA (genotype C) selective qPCR
pSelect-gC-rev	5'-TAGAGAGCAGAGGCGG-3'	
HBV-1745-fw	5'-GGAGGGATACATAGAGGTTCCTTGA-3'	HBV total DNA (all genotypes)
HBV-1844-rev	5'-GTTGCCCGTTTGTCCTCTAATTC-3'	
HBV-2251-fw	5'-GCCTATTGATTGGAAAGTATGT-3'	HBV cccDNA (all genotypes)
HBV-108-rev	5'-AGCTGAGGCGGTATCTA-3'	

All oligodeoxynucleotides were synthesized by Invitrogen.

2.1.8 Enzymes

DNase I (recombinant, RNase-free)	Roche, Mannheim, D
Proteinase K	Roth, Karlsruhe, D
Restriction Endonucleases	Fermentas, Leon-Rot, D
RNaseA (10mg/ml)	Roche, Mannheim, D
T4 DNA Ligase	Fermentas, Leon-Rot, D

2.1.9 Plasmids and bacterial strains

pCH-9/3091: encodes a replication competent HBV 1.1-fold overlength genome (genotype D, subtype ayw1; genBank accession number J02203/V01460) under control of the CMV promoter in a pBR322 based plasmid backbone (**Figure 11**). All the plasmids for phenotypic testing in this thesis are its derivatives obtained either through site-directed mutagenesis or by fragment exchange.

pCH-HBV1.1-CPLX$^-$ (pCH-HBV1.1-all Del): derived from pCH-9/3091, with all HBV genes knocked out by introducing a premature stop codon into each ORF. In core ORF T18G and A19T (C), in polymerase ORF T447A (P), in preS1 ORF T1003A, in preS2 ORF G1279A, in S-ORF C1453A (collectively marked as L), and in X-ORF C2677T (X) were mutated.

pRV-HBV1.5-A: contains a HBV overlength genome (genotype A, subtype adw2; genbank accession number X02763) in a pBluescript KS(+) based plasmid backbone.

pTriEx-HBV1.1-C: contains a HBV 1.1-fold overlength genome (genotype C, subtype ayw; genbank accession number AY148342) in a pTriEx based plasmid backbone.

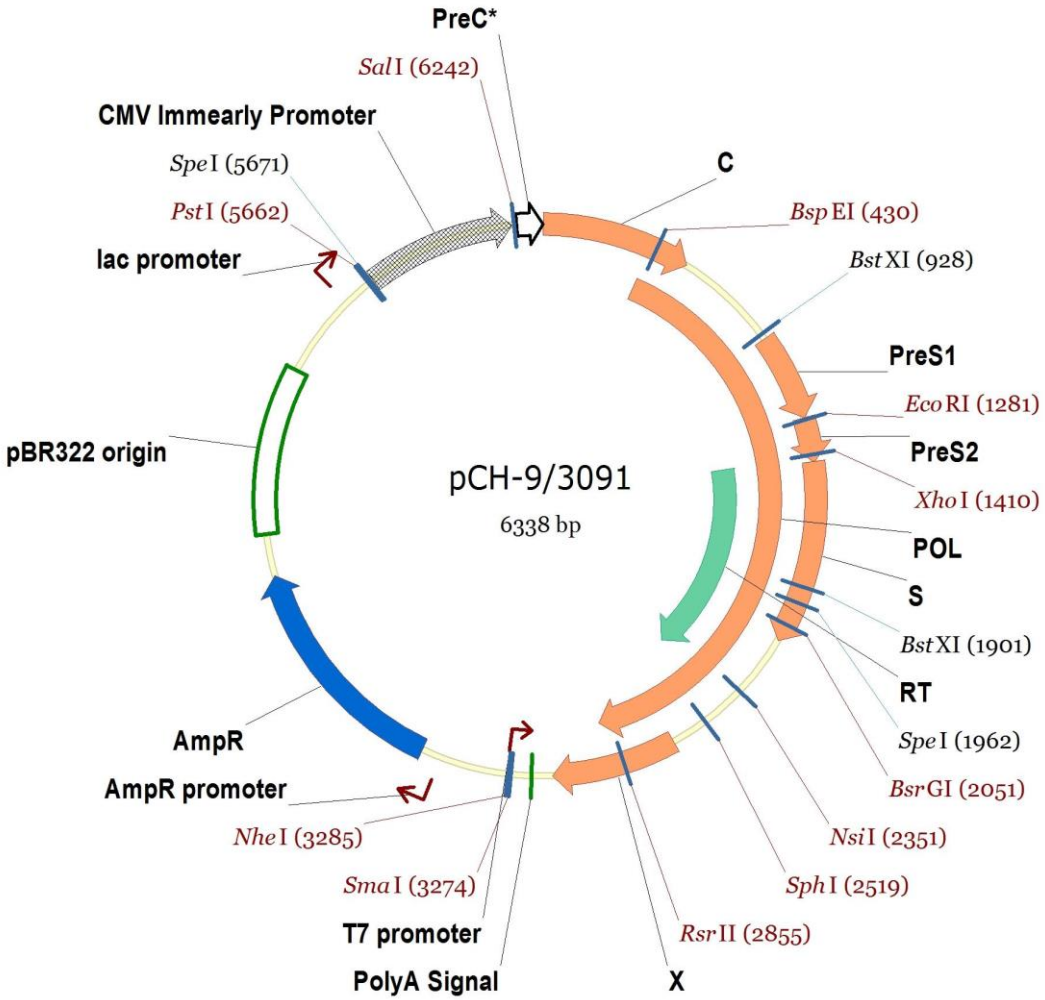


Figure 11: Schematic illustration of the pCH-9/3091 plasmid. This construct contains a terminally redundant HBV genome (SalI/NheI; HBV nucleotides 3091 to 3182-1 to 3182/1 to 84, numbering from the core initiation codon) functionally mimicking the circular DNA genome from which the pregenomic HBV RNA is transcribed. Synthesis of the pregenomic RNA is driven by a CMV-IE enhancer/promoter element (PstI/SalI), whereas subgenomic RNAs, which encode the preS/S and the S envelope proteins and the X protein, are produced from native internal promoters. The important restriction enzyme sites for cloning, recombination and construction of new derivatives are shown with numbering.

All the HBV overlength containing Plasmids were amplified in and purified from the *E. coli* strain Stbl3 (Invitrogen; Genotype: F- *mcr*B *mrr hs*dS20 (r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14*gal*K2 *lac*Y1 *pro*A2 *rps*L20 *(Str^R) xyl-5* λ^- *leu mtl-1*r). This strain is designed for cloning unstable inserts such as direct repeats found in lentiviral/HBV expression vectors. The other plasmids were purified from the TOP10 *E. coli* strain (Invitrogen).

2.1.10 Instruments

Automated immunoassay analyzer AxSYM	Abbott, Wiesbaden, D
Biosafety cabinet	Heraeus, Hanau, D
Centrifuges:	
Beckmann Avanti	Beckmann, Krefeld, D
Beckmann TL-100 ultracentrifuge	Beckmann, Krefeld, D
Eppendorf 5417C/R	Eppendorf, Hamburg, D
Sigma 4K15	Sigma, Osterode am Harz, D
CO_2 Incubator	Heraeus, Hanau, D
DNA/RNA electrophoresis systems	Peqlab, Erlangen, D
Electronic multichannel pipetter	Thermo, Dreieich, D
Electrophoresis gel imaging system	Peqlab, Erlangen, D
ELISA reader	Tecan, Männedorf, CH
Fluorescence microscope IX81	Olympus, Hamburg, D
Fuchs-Rosenthal Hemocytometer	Roth, Karlsruhe, D
Heating block Thermomixer comfort	Eppendorf, Hamburg, D
Incubator	Heraeus, Hanau, D
LightCycler 480 II	Roche, Mannheim, D
Microplate reader Infinite 200 PRO	Tecan, Männedorf, CH
Pipets (0.5-10µl; 10-100µl; 100-1000µl)	Eppendorf, Hamburg, D
Pipetus	Hirschmann, Eberstadt, D
Robotic platform Freedom EVO 100	Tecan, Männedorf, CH
Spectrophotometer NanoVue	GE Healthcare, Freiburg, D
Thermal microplate sealer	4titude, Berlin, D
Thermocycler T3000	Biometra, Göttingen, D
Tube luminometer	Berthold, Bad Wildbad, CH
Waterbath	GFL, Burgwede, D

2.2 Methods

2.2.1 Molecular biology methods

The basic methods in molecular biology, which were applied according to "Molecular Cloning: A Laboratory Manual (Third Edition)" and/or following the kit instructions, were not described here in detail. Competent cell preparation and transformation; DNA/RNA gel electrophoresis; restriction enzyme digestion and fragment extraction/purification; fragment dephosphorylation and ligation; Mini/Midi/Maxi- plasmid preparation; TOPO cloning; polymerase chain reaction;

2.2.1.1 Site-directed mutagenesis

The in vitro site-directed mutagenesis protocol was adapted from the QuikChange (Agilent) strategy to make point mutations. The basic procedure utilizes a supercoiled double-stranded DNA plasmid with a target site and two synthetic oligonucleotide primers, both containing the desired mutation. The primers, each complementary to opposite strands of the plasmid, are extended during temperature cycling by Phusion Hot Start II High-Fidelity Polymerase, without primer displacement. Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks which can be transformed and purified from *E. coli.*

The mutagenic primers were designed using 2 online programs (QuikChange Primer Design and PrimerX) based on the following considerations:

A. Annealing to the same sequence on opposite plasmid strands.

B. Between 25 and 40 bases in length, with melting temperature (Tm) of ≥78°C.

C. With the desired mutation in the middle; 10–15 bases of correct sequence on each side.

D. Terminating in one or more C or G bases with a minimum GC content of 40%.

Prepare the 50 µl PCR reaction(s) as indicated below:

- 5 µl 10× Phusion HF buffer
- 1 µl plasmid DNA template (10-20 ng)
- 1.5 µl forward/reverse primer (20 µM each, Cartrige/HPLC purified)
- 1.5 µl dNTP mix (10 mM each)
- 1 µl Phusion Hot Start II High-Fidelity polymerase (2 U)
- 40 µl PCR grade H_2O

Cycling Parameters for Mutagenic PCR

Step	Cycles	Program	Temperature (°C)	Hold Time
1	1	Initial denaturation	98	30 s
2	20	Denaturation Annealing/Extention	98 72	10 s 30 s/kb
3	1	Final annealing Cooling	72 4	8 min ~

Add 1 µl of the Dpn I restriction enzyme (10 U/µl) to digest the parental plasmid at 37°C for 1 hour. Follow the nomal protocol for transformation and mini plasmid preparation. Analyze 2-5 mutated plasmid clones by sequencing for validation. Do fragment exchange with parental plasmid backbone to exclude any undesired mutations outside the target region induced by possible PCR error.

2.2.1.2 DNA extraction from serum of HBV infected patients

Refer to the instructions of QIAamp UltraSens Virus/QIAamp MinElute Virus Kit. Use preferentially the QIAamp UltraSens kit with a volume of serum from 500 µl-1 ml, diluted consistently in PBS (if necessary). If the serum volume is less than 200 µl, then QIAamp MinElute is recommended. Add a negative serum or PBS as negative control for the extraction (optional). Elute or resuspend the DNA in 60µl (2×30 µl) super pure water (pH 7.0-8.5) or 10 mM Tris-HCl buffer (pH 8.0-8.5) provided. Use 5-10 µl of this eluate for subsequent PCR.

2.2.1.3 Preparative PCR for cloning HBV genome or subgenome

All PCR analyses were performed using Phusion Hot Start II High-Fidelity DNA Polymerase with both low error rate (6-fold lower than Pfu polymerase) and high processivity (10-fold higher than Pfu polymerase) according to the manufacturer's instruction.

Three sets of primers were used to amplify HBV whole genome (WG), polymerase gene (POL) and reverse trascriptase domain (RT), respectively. Calculate primer Tm to determine the annealing temperature for PCR with the modified Breslauer's thermodynamics method (http://www.finnzymes.com/tm_determination.html).

Prepare the 50 µl PCR reaction(s) as indicated below (always multiply the PCR mix (a bit more) without DNA template first, spread 40-45 µl of it in each reaction tube and add the DNA template at last before running the PCR).

- 5 µl 10× Phusion HF buffer
- 5 µl – 10 µl DNA template
- 1.5 µl forward/reverse primer (20 µM each)
- 1.5 µl dNTP mix (10 mM each)
- 1 µl Phusion Hot Start II High-Fidelity polymerase (2 U)
- x µl PCR grade H_2O (make up to 50 µl)

Sequence and position of primers used for HBV genome amplification

Name	Sequence and restriction site	Position	Amplified Region
pWG-fw	5´-CCGGA*AAGCTT*AT*GCTCTTC*TTTTTCACCTCTGCCTAATCATC-3´ (*HindIII*, *SapI*)	3103-3215	Whole Genome
pWG-rev	5´-CCGGA*GAGCTC*AT*GCTCTTC*AAAAAGTTGCATGGTGCTGGTG-3´ (*SacI*, *SapI*)	3107-3086	
pPOL-fw	5´-CCTATCCTATCAACACT*TCCGGA*-3´/ 5´-GACCACCAAATGCCCCTATC-3´ (*BspEI*)	412-434/398-417	POL Gene
pPOL-rev	5´-ACA*CGGTCCG*GCAGATGAGA-3' (*RsrII*)	2862-2843	
pRT-fw	5´-CAGGCCATGCAGTG*GAATTC*-3´ (*EcoRI*)	1266-1285	RT Region
pRT-rev	5´-GGTTCCAC*GCATGC*GCTGAT-3 (*SphI*)	2527-2508	

PCR setting		WG		POL		RT	
	Cycles	Temp	Time	Temp	Time	Temp	Time
Initial Denaturation	1	98°C	30 sec	98°C	30 sec	98°C	30 sec
Denaturation		98°C	10 sec	98°C	10 sec	98°C	10 sec
Annealing	10	65°C	20 sec	65°C	20 sec	65°C	20 sec
Extension		72°C	1.5 min	72°C	60 sec	72°C	25 sec
Denaturation		98°C	10 sec	98°C	10 sec	98°C	10 sec
Annealing	10	65°C	20 sec	65°C	20 sec	65°C	20 sec
Extension		72°C	2 min	72°C	90 sec	72°C	25 sec
Denaturation		98°C	10 sec	98°C	10 sec	98°C	10 sec
Annealing	10	65°C	20 sec	65°C	20 sec	65°C	20 sec
Extension		72°C	3 min	72°C	120 sec	72°C	25 sec
Denaturation		98°C	10 sec	98°C	10 sec	98°C	10 sec
Annealing	10	65°C	20 sec	65°C	20 sec	65°C	20 sec
Extension		72°C	4 min	72°C	180 sec	72°C	25 sec
Final Extension	1	72°C	10 min	72°C	10min	72°C	10min
Holding		16°C	~	16°C	~	16°C	~

The PCR products were checked on a 1% agarose gel for their correct sizes and quantities. Load 5 µl out of the 50 µl from the PCR reaction. If there is a band at the correct size, purify the rest 45 µl with the QIAquick kit from Qiagen and elute in 40 µl Qsp water. Do multiple PCR reactions to get sufficient DNA amount for cloning, if necessary. Consider using UV-Free Gel Purification, S.N.A.P. Kit from Invitrogen for example, without ethidium bromide to avoid damage to long DNA fragment or PCR products by UV light.

In case of the serum sample with viral load below 10^5 genome copies/ml, subgenomic fragment (POL gene or RT region) PCR is recommended as alternatives to whole genome PCR. Nested PCR for POL/RT amplification can be applied to increase the sensitivity.

2.2.1.4 Quantification of nucleic acids

To determine the concentration of a DNA (or RNA) preparation, the absorption at a wave length of λ = 260 nm (A_{260}) was measured using a spectrophotometer (NanoVue). Absorption at 260 nm of 1 corresponds to a concentration of 50 µg/ml for dsDNA (40 µg/ml for RNA). The ratio of the absorption at 260 nm and 280 nm allows estimating the purity of the DNA (or RNA) and a ratio of 1.8 for DNA or 2.0 for RNA ensures high-quality samples. The absorption should be normalized to the corresponding elution buffer or ddH_2O.

2.2.1.5 Vector construction for competent replication of clinical HBV isolates

The basis vector pCH-9/3091 encodes a replication competent 1.1× unit overlength HBV genome driven by CMV promoter and the vector pCH-9/200 was derived from pCH-9/3091. The 1.1× unit-length HBV genome of pCH-9/3091 was excised using the restriction enzymes SalI/NheI and replaced with a short recombinant HBV fragment amplified from pCH-9/3091 with primers PCH200-fw and PCH200-rev. The upstream primer PCH200-fw contains 17 base-pair (nucleotides 3091–3107) and 21 base-pair (nucleotides 3103–3123) regions of the HBV genome linked by two SapI sites. The downstream primer PCH200-rev binds vector sequences and allowed amplification of a 160 base-pair HBV DNA fragment that includes the polyadenylation site at the 3' end of the HBV genome. A SapI site present in the parent vector backbone was removed by site-directed mutagenesis. Thus, the final pCH-9/200 contains a CMV promoter upstream of a short, recombinant HBV sequence that allows the in-frame insertion of a full-length HBV genome following SapI digestion.

Generation of replication competent clinical HBV isolates using pCH-9/200 was adapted as formerly described. PCR by the methods modified from Gunther et al. yields linear

genome-length HBV DNA that lacks 5' and 3' sequences necessary for HBV expression. These full-length HBV genomic DNA can be digested directly with SapI and inserted into SapI-digested pCH-9/200 to produce a replication competent construct comprised of 95% patient-derived sequence. Alternatively, the amplified HBV genomes were subsequently blunt-end cloned into pCR-Blunt II-TOPO Vector using Zero Blunt® TOPO® PCR Cloning Kit, and then released by SapI digestion for efficient subcloning into pCH-9/200.

In case of insufficient amount of full-length HBV genomic DNA for cloning, mostly due to low viral load or poor DNA quality, subgenomic fragment (POL or RT) were amplified directly or with a nested PCR stratedy (using 1:10 - 1:100 diluted full-length HBV PCR product as template). The amplicons were digested (using BspEI/RsrII for POL or EcoRI/SphI for RT) and exchanged into pCH-9/3091 (or pCH-9/3091-X*) with the same digestion in replacement of the wild-type fragments.

2.2.1.6 Quantitative real-time PCR

The real-time qPCR was performed on LightCycler 480 II system using LightCycler 480 SYBR Green I Master reagent, according to the manufacturer's instructions. The general PCR setting and condition were listed as below:

Reaction mix:

- 10 µl 2× master mix
- 1 µl forward/reverse primer (20 µM each)
- 2-8 µl DNA template
- x µl PCR grade water (up to a total volume of 20 µl)

To confirm a specific real-time PCR product, 5 µl of reaction obtained was submitted to agarose gel electrophoresis, followed by optional DNA sequencing analysis. The downstream analysis including absolute and relative quantification, high-resolution melting curve analysis, and genotyping was carried out with LightCycler 480 Software Suite (Version 1.5).

Real-Time PCR program

Program:	Denaturation			Type:	Melting Curves	Cycles:	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2°Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95	300	20	0	0	0	None

Program:	Amplification			Type:	Quantification	Cycles:	45
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2°Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95	15	20	0	0	0	None
2	60	15	20	0	0	0	None
3	72	20	20	0	0	0	Single

Program:	Melting Curve			Type:	Melting Curves	Cycles:	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2°Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95	0	20	0	0	0	None
2	65	15	20	0	0	0	None
3	95	0	0.1	0	0	0	Continuous

Program:	Cooling			Type:	None	Cycles:	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2°Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	40	30	20	0	0	0	None

2.2.1.7 Secreted alkaline phosphatase (SEAP) reporter gene assay

The SEAP reporter system was used as an internal control and normalization reference for transfection efficiency. SEAP is secreted into cell culture supernatant and therefore offers many advantages over intracellular reporters. It allows determining reporter activity without disturbing the cells especially preparing the cell lysates.

The cells were co-transfected with target plasmid and pSEAP control vector in a ratio between 4:1 and 10:1. The culture supernatants were collected 24-72h post transfection in a 96-well plate and 50 µl of each was used for the SEAP reporter assay. The downstream analysis was performed according to the standard protocol from Roche SEAP Reporter Gene Assay Kit with the measurement read at 405 nm on a Tacan ELISA reader.

2.2.2 Cell biology methods

All experiments with cultured mammalian cells were performed in the biosafety level 2 (BSL-2) laminar flow cabinets. All procedures were carried out under sterile conditions using sterile solutions and equipments. Cell cultures were regularly screened by PCR to ensure the absence

of *Mycoplasma sp.* and disposable plastic pipets and gloves were used to avoid any contamination of the cells with microbes. All cells were cultivated in a humidified incubator at 37°C containing 5% CO_2.

2.2.2.1 Cultivation and passaging of mammalian cell lines

All cells were cultured in cell culture flasks and incubated at 37°C and 5% CO2. The human hepatoma cell lines HuH7/HepG2/HepG2 H1.3/HepG2.2.15 were grown in DMEM growth medium and passaged at a ratio of no less than 1:6 when confluent. In addition, HepG2 H1.3 and HepG2.2.15 cells should be maintained in virus production medium for better viral productivity. HepaRG cells were cultured in WME growth medium, and for infection, differentiated in WME differentiation medium for 4 weeks. Growth medium were exchanged every two days for all cells except HepaRG cells (once per week is sufficient). After reaching confluence the cells were twice rinsed with PBS and detached from the culture flask by incubation with 1× Trypsin/EDTA solution for 5-10 min. Cells were resuspended in an appropriate volume of culture medium and splited into new flask/plate for further usage/ applications.

2.2.2.2 Cell counting

Cultured cells were detached from (**2.2.2.1**) and resuspended in 10 ml culture medium. An aliquot was diluted 1:10 in culture medium and transferred to a Fuchs-Rosenthal hemocytometer for counting. The number of cells per ml was determined according to the following equation:

$$\text{Cells/ml} = \text{the average count per square} \times \text{dilution factor} \times 10^4$$

2.2.2.3 Cell cryoconservation

Confluent cell monolayers were detached from the plate as described in (**2.2.2.1**), transferred to a 15 ml Falcon tube and cells were pelleted by centrifugation at 21°C and 1000 rpm for 10 min (Sigma 4K15 centrifuge). Cells were resuspended in freshly prepared cryo-medium (culture medium with 20% FCS and 10 % DMSO). Aliquots of 1 ml were transferred to cryoconservation tubes, placed at -80°C in the freezing container overnight and then transferred to a liquid nitrogen tank for long-term storage.

2.2.2.4 Transient transfection of mammalian cells

Two transfection reagents, Fugene HD and Fugene 6, were chose to introduce foreign DNA into mammalian cells depending on the specific applications. Both are non-liposomal multicomponent formulations designed to transfect plasmid DNA into a wide variety of cell lines with high efficiency and low toxicity. The protocol does not require removal of serum or culture medium and does not require washing or changing of medium after introducing the transfection reagent/DNA complex. In brief, cells were seeded one day before transfection and cultured until reaching the optimal condition (90% confluence for Fugene HD and 60% confluence for Fugene 6) at the time of transfection. Plasmid DNA was diluted with serum free medium to 2 µg per 100 µl (0.02 µg/µl). The transfection reagent was pipetted directly into the medium at a 3:1 reagent:DNA ratio (6 µl reagent per 100 µl total volume) and mixed immediately. The transfection reagent/DNA mixture was incubated for 15 minutes at room temperature and then added to the cells (8-12 µl/cm^2 or 3-5µl/10^4 cells) in a drop-wise manner with well mixing. 24-72 h post transfection, subsequent assays can be performed accordingly.

2.2.2.5 XTT Cytotoxicity assays

Viability of HuH7 cells under different antiviral treatemts with different concentrations was tested in triplicate using XTT-Cell Proliferation Kit II in a 96-well culture format. 25µl XTT labeling mixture was added to each well and incubated for 2 hrs. The cell viability assay determining cleavage of tetrazolium salts (XTT) to formazan by the "succinate-tetrazolium reductase" system. This system belongs to the respiratory chain of mitochondria, and is only active in metabolically active cells. The assay is based on the cleavage of the tetrazolium salt XTT in the presence of an electron-coupling reagent, producing a soluble formazan salt. This conversion only occurs in viable cells. Cells grown in a 96-well tissue culture plate are incubated with the XTT labeling mixture for approximately 2-20 hours. After this incubation period, the formazan dye formed is quantified using a scanning multi-well spectrophotometer. The absorbance revealed directly correlates to the cell number.

2.2.3 Virology Methods

2.2.3.1 *In vitro* replication capacity testing

To measure the ability of HBV mutants to replicate *in vitro*, transient transfection into HuH7 cells was performed as the standard assay. 12-well plates were seeded with 1.5×10^5 HuH7 cells/well, and on the following day (12-18 hrs) after medium change, the cells were transfected at approximately 90% conflurency with 1 µg plasmid DNA plus 3 µl Fugene HD transfection reagent according to the manufacturer's instructions. 12-18 hrs post-transfection, cells were washed with PBS, then fed with fresh seeding medium and incubated for 72 hrs. The supernatants were centrifuged at 800× g for 5 min to remove the dead cells and collected for HBV DNA extraction and HBsAg assay. The extracted HBV DNA was qPCR quantified and normalized to HBsAg expression level as transfection efficiency control. The mutant's replication capacity was expressed as a percentage with 100% being the replication capacity of wild-type virus.

2.2.3.2 Isolation of intracellular HBV DNA replication intermediates

500 µl of lysis buffer (50 mM pH8.0 Tris-HCl, 1 mM EDTA, 1% NP40) was added to each well of a 12-well plate for 1 minute till the monolayer detached from the plate. The cell lysate was well mixed, transferred to an eppendorf tube and incubated on ice for 10 minutes to have a complete lyse. After centrifugation at 10000 rpm for 1 min, the supernatant was transferred to a new tube containing 5 µl of 1 M Mg-acetate and mixed with 10 µl DNase I/RNase A solution (10 mg/ml each), then incubated for at least 1 hour at 37°C. 12.5 µl of 0.5 M EDTA, 10 µl of 5 M NaCl, 11.5 µl of 20% SDS and 7.5 µl of Proteinase K at 20 mg/ml were added and mixed in the tubes at 42°C overnight. After mixing well with 500 µl of phenol:chloroform:isoamyl mix (25:24:1) for 5 minutes at room temperature followed by spining for 10 minutes at 10000 rpm, the aqueous phase was transferred into a new eppendorf tube with 50 µl of 3 M sodium acetate pH5.2 and 1 µl tRNA (10mg/ml), and mixed with 750 µl of isopropanol followed by spinning at maximum speed at 4°C for 45 minutes. At the end of the spin the pellet was washed with 500 µl of 70% ethanol and air-dried. The pellet was resuspended in 30 µl 10 mM pH8.0 Tris-HCl solution containing 1× DNA loading buffer.

2.2.3.3 Production of wild type and variant HBV

For the production of genotype A and D wtHBV, two cell lines, HepG2.2.15 and HepG2 H1.3 were used. These two HepG2-derived cell lines contain respectively either four dimeric HBV genomes (genotype A) or one copy of a 1.3-fold overlength HBV genome (genotype D) stably integrated into the cellular genome. Therefore, both cell lines permanently produce HBV, which can be harvested and purified from cell culture supernatants. For virus preparation, the cells were cultivated in complete DMEM growth medium until 100% confluent. Then, the medium was exchanged to WME virus production medium. For production of rHBV and variant HBV, HuH7 cells were transfected with pCH-9/3091 plasmid derivative/s containing specific mutations or isolates in the HBV genome. Transfection was performed in 10-cm cell culture dishes at 90% cell confluence using 8 µg plasmid DNA plus 24 µl Fugene HD transfection reagent, as described before. 16-18 h post transfection, cells were washed with DMEM wash medium and fed with DMEM seeding medium (containing 5% FCS and 1% DMSO). Every two days, the virus-containing medium was collected and cell debris was removed by centrifugation at 1000 rpm for 10 min. The supernatant was transferred to centrifugal filter devices (Centricon Plus-70, Biomax 100, Millipore Corp., Billerica). The first centrifugation was performed at 3500 × g for 1 h at 4°C, to capture the virus particles in a filter. Due to the exclusion limit of 100 kDa, serum proteins flow through, while proteins larger than 100 kDa are retained by the filter. In a second, invert centrifugation step with the filter system turned upside down, was performed at 2600 × g for 10 min, to elute the viruses. The virus concentrates were supplemented with 10% glycerol and stored at -80°C. The titer of the produced HBV was determined as enveloped DNA-containing viral particles by dot blot analysis following a CsCl density-gradient as outlined below, or alternatively, by real time PCR quantification.

2.2.3.4 Caesium chloride density gradient

In the context of a virus production, different types of particles can be obtained in the preparation: nacked DNA, unenveloped DNA-containing capsids and enveloped virions. Their different densities enable to separate them in a density gradient. DNA is centrifuged down to the bottom, because of the very small size; DNA-containing capsids sediment at a density of 1.3 g/l, while intact virions can be found in the 1.22 g/l fraction. The CsCl density gradient ultra-centrifugation was performed with the SW-60 swing bucket rotor. In SW-60 polyallomer vials 500 µl of CsCl solutions with the following densities were carefully layered

one upon the other: 1.4 g/l, 1.3 g/l and 1.15 g/l. On top of the CsCl solutions 500 µl of a 20% sucrose solution was layered, and the sample was applied. The vials were filled up with PBS and tarred on micro scales. Ultra-centrifugation was performed at 55000 rpm at 20°C for 4 h to overnight. The first 12 density fractions from the bottom were collected with a Dot Blot Fraction recovery system. Each fraction contained a volume of approximately 175 µl. The fractions were subjected to quantitative dot blot analysis.

2.2.3.5 Dot blot

To quantitatively and qualitatively analyze the produced wtHBV or rHBV, a DNA dot blot was performed. The DNA fractions, obtained by CsCl density centrifugation, were dotted to a nylon membrane in a dot blot aperture. A HBV plasmid standard ranging from 8 to 1000 pg was added. Samples and standard were washed with 200 µl PBS once, before the membrane was transferred to a 3 mm Whatman paper soaked with denaturation solution. Subsequently the membrane was transferred to a 3 mm Whatman paper soaked with neutralisation solution. Then the DNA was cross-linked to the membrane in a UV oven at 125 mJ/cm^2. The membrane was hybridised with a ^{32}P-labelled HBV DNA probe at 65°C overnight. At the next day, the membrane was washed 3-fold 20 min each at 65°C with wash solution. After wrapping the radioactive membrane in seran wrap, the DNA was exposed to a phosphor-screen and quantified with a phosphoimager.

2.2.3.6 HBV infection of HepaRG cells

Infection of differentiated HepaRG cells with HBV was performed in differentiation medium in presence of DMSO before, during and after infection. The preparation of differentiated HepaRG cells was in 12-well plate format and lasted for 4 weeks. For the first 2 weeks, the cells were maintained in standard growth medium with medium exchange 2 times per week. Then the cells were maintained in differentiation medium for another 2 weeks with medium exchange 2 times per week as well until ready. For infection, a viral dose of 200 MOI (or viral genome equivalent per cell) was diluted with differentiation medium to a final volume 500 µl/well containing additional 5% PEG, and then applied to the cells. The incubation for infection lasted for over-night and the cells were washed with PBS for 3 times and then fed with fresh differentiation medium. 8-10 days after infection, HBV markers such as HBsAg, HBeAg and rcDNA in the supernatant and intercellular cccDNA were measured respectively to characterize the infection.

2.2.4 Antiviral resistance testing: genotypic and phenotypic methods

2.2.4.1 Sequencing analysis for genotypic testing

Two different sequencing strategies, PCR direct sequencing and clone sequencing were adopted to analyze the viral quasispecies isolated from patients and identify potential genotypic resistance.

PCR direct sequencing

PCR amplification of the HBV whole RT region from patient serum was performed as indicated. The PCR product with the right size (≈ 1.2 kb) was purified after gel electrophoresis using QIAquick Gel Extraction Kit. 10 µl of the eluate at a concentration of 10-30 ng/µl was submitted for direct bidirectional sequencing using the same PCR primers.

Clone sequencing

PCR amplification of HBV whole genome, polymerase gene or reverse transcriptase region, respectively, followed by restriction digestion and ligation into HBV backbone vectors, generated HBV replication competent recombinants containing different length of patient-derived sequence. Depending on direct sequencing results from the same patient, 10-50 transformed clones were randomly picked for plasmid preparation using GeneJET Plasmid Miniprep Kit. 20 µl of each prepared plasmid (30-50 ng/µl) was submitted for sequencing. For cloned full genome or polymerase, pRT-rev primer was used first to identify the interesting clones with desired mutations within the RT region. Then the selected clones were further full-length sequenced using the optimized primer sets as indicated.

Sequence analysis and mutation annotation

The direct sequencing trace files were analyzed using QSVAnalyser software to determine the possible quasispecies with proportion estimation. The derived DNA sequences were then submitted to the webserver (http://hbv.bioinf.mpi-inf.mpg.de) based on a Gene2Pheno algorithm for mutation annotation. The HBV subgenotype and possible mutations in the RT region as well as in the overlapping S gene can be indicated from the input sequence in comparison to wild-type reference sequences or sequence consensus.

2.2.4.2 Preparation of drug solutions

The four antiviral drugs (Lamivudine, Adefovir, Entecavir and Tenofovir) used in phenotypic testing were synthesized chemically and provided as pure substances (purity > 98%) in powder form by SeqChem Sequoia. The stock solutions were prepared by dissolving drug powders in PBS to the proper concentrations as indicated below and filtering through a 0.20 µm microfilter.

Drug	Molecular weight	Stock concentration (mM)	Solvent
Lamivudine	229.3	20	PBS
Adefovir	273.2	5	PBS
Entecavir	295.3	5	PBS
Tenofovir	287.2	5	PBS

For preparation of the working solutions for drug treatment, stock solutions were diluted in reseeding medium in succession. Serial dilutions were performed to obtain 5 different series with 2× final concentrations in treatment.

Drug	Dilution series (µM, 2×)	Final concentration (µM, 1×)
Lamivudine	0.02/0.2/2/20/200	0.01/0.1/1/10/100
Entecavir	0.001/0.01/0.1/1/10	0.0005/0.005/0.05/0.5/5
Adefovir	2/4/8/16/32	1/2/4/8/16
Tenofovir	1/2/4/8/16	0.5/1/2/4/8

Both stock solutions and working solutions were frozen in aliquots at -20°C. The freshly prepared working solution/ medium can be stored for at least 2 months.

2.2.4.3 Drug susceptibility assay for phenotypic testing

96-well testing format

HuH7 cells were plated into 10 cm^2 dishes at 2.5×10^6 cells/dish. Approximately 24 h post plating, cells were transfected at > 90% conflurency with 8 µg plasmid DNA plus 24 µl FuGENE HD transfection reagent (ratio 1:3). The following day, transfected cells were

separated by trypsinization and washed twice by centrifugation at 300 g for 3 min and resuspension with washing medium. Finally the cells were resuspended in reseeding medium (containing 5% FCS and 1% DMSO) and seeded into a 96-well plate at a concentration of 1.5×10^4 cells/well in a volume of 110 µl. After a brief centrifugation of the plate followed by incubation at 37°C for 1-2 h, the cells were fed with 110 µl/well prepared reseeding medium containing the 5 serial dilutions of each drug for treatment in triplicates (same reseeding medium without drugs as non-treatment control). 72 h after treatment, the supernatants were collected and centrifuged at 500 g for 5 min to remove the dead cells and transferred to a new plate for immediate DNA extraction or short-term storage at -20°C.

Automatic DNA extraction

For each complete phenotypic testing with 4 drugs, a total of 64 supernatants were obtained and submitted for HBV DNA extraction. A protocol for high throughput DNA extraction using BioSprint 96 One-For-All Vet kit was optimized on Tecan's robotic platform Freedom EVO 100, with a comparable performance to the standard spin column based method.

Selective qPCR for HBV rcDNA quantification

The extracted DNA was then subjected to real-time qPCR for HBV DNA quantification in the supernatants, using the selective primers to distinguish HBV rcDNA from input plasmid. Each PCR well contained the following: 10 µl LightCycler 480 SYBR Green I Master, 1 µl selective forward primer (20 µM), 1 µl selective reverse primer (20 µM) and 8 µl extracted HBV DNA in a final volume of 20 µl. The qPCR was conducted on LightCycler 480 II with a sequence of conditions as: denaturation at 95°C for 5 min, followed by 40 amplification cycles of 95°C for 15, 60°C for 15 s and 72°C for 20 s, ended with a standard melting curve procedure. HBV DNA copy numbers were calculated based on a serial dilution of calibrated HBV DNA standard. The average values from 3 replicate wells at each drug concentration were used to calculate the 50% inhibitory concentration (IC_{50}) based on dose response equations.

2.2.5 Bioinformatics and statistics

All the bioinformatics analysis was performed using the data and software publicly available. The statistical analysis was performed using Microsoft Excel and GraphPad Prism. Quantitative data are shown as mean +/- standard deviation. For evaluation of statistical significance, the student *t* test was used. A *p*-value less than 0.05 is considered as significant.

2.2.5.1 HBV Sequence manipulation

HBV full genome sequences of selected representative virus genotypes were retrieved from NCBI. As the viral genomes are circular and the start point of the sequences in databases were different, most of which were the EcoRI restriction enzyme cutting site, a unanimous start point, the start codon ATG of the core ORF, was selected and the all sequences were manually curated to begin at this same location.

Multiple sequence alignment was generated using MUSCLE (multiple sequence comparison by log-expectation) program followed by manual correction. To identify the highly conserved regions in HBV genome for primer design, the software BioEdit was used to scan through the alignment base by base. Using Primer 3, primers were selected within the candidate regions, taking target regions, primer length, sequence specificity and secondary structure, GC content and Tm etc. into consideration.

2.2.5.2 Molecular modeling of HBV polymerase

Building a 3D structure model for a protein usually consists of a number of steps: (1) sequence alignment of target with template; (2) generation of initial homology model; (3) model refinement; and (4) model evaluation.

Sequence alignment

The protein segment from amino acid position 336 to 679 of HBV polymerase is responsible for the RT activity of HBV. A BlastP query was performed towards the entire amino acid sequence database available in the Protein Data Bank (PDB) searching for proper templates. The sequence alignment was generated for the entire RT region (rt1-344) in comparison to the chosen template, using the Needleman-Wunsch algorithm followed by manual refinement.

Homology modeling

Based on the alignment, the backbone coordinates of the residues in HBV RT were generated with Swiss-Pdb Viewer as the initial scaffold. A loop search was carried out using all the structures in the PDB. The less conserved regions, insertions, and side chains were built by manual modeling using the program Modeller and its reference to databases of known main-chain conformations and preferred side-chain rotamers.

Model refinement and assessment

The final model was minimized using the molecular graphics and simulation program X, and the quality of the geometrical parameters of the model was evaluated by PROCHECK.

Mutation analysis and visualization

After energy minimization, the substrates and nucleotide analog inhibitors were then docked into the active sites of the polymerase model using the program ICM-Pro. The desired mutations were introduced into the complex and visualized with Discovery Studio Visualizer.

2.2.6 Scientific research services

2.2.6.1 DNA synthesis

DNA synthesis service was provided by GeneArt to facilitate a panel of site-directed mutagenesis designed in a similar pattern. After sequence analysis, oligonucleotides synthesized in-house, with maximum sequence accuracy, served as the building blocks for assembling long DNA fragments, the master DNA, for individual mutagenesis. The mutated fragments were sequence-verified and then cloned into designated vectors.

2.2.6.2 DNA sequencing

DNA sequencing service was provided by GATC Biotech using Applied Biosystems 3730xl DNA Analyzer in a "NightXpress" fashion. DNA samples and sequencing primers (20µl each, sufficient for 8 reactions) were dispatched in separated tubes with individual barcodes at a concentration of 10-50 ng/µl for PCR fragments, 30-100 ng/µl for plasmids and 10 pmol/µl (10 µM) for primers. The universal sequencing primers were provided by the company. Sample/primer delivery and sequencing reactions were carried out during the night and then sequencing results could be downloaded from the website on the next working day.

3. Results

3.1 Establishment of a new high throughput phenotypic assay

The strategy developed in this study for phenotyping clinical HBV isolates consists of several sequential parts: extraction of viral DNA from sera and PCR amplification; cloning of HBV genome/subgenome into the HBV expression vectors (pCH-9/200 or pCH-9/3091) followed by sequencing analysis; transfection based *in vitro* drug susceptibility test in 96-well cell culture format; automated DNA extraction and qPCR quantification, for IC_{50} and resistance factor determination. **Figure 12** represents schematically the overall workflow.

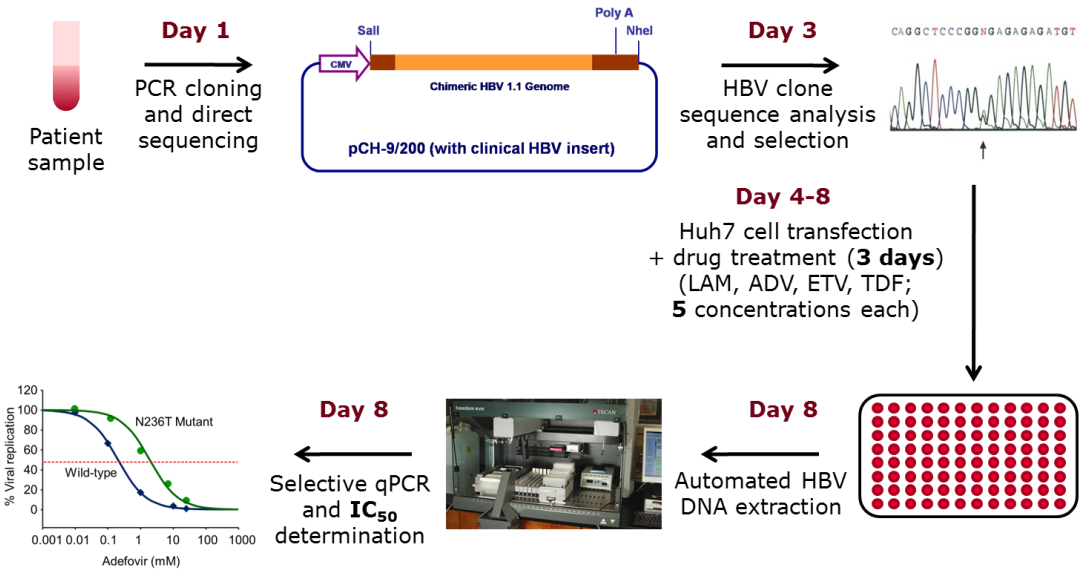


Figure 12: Schematic workflow of phenotypic resistance analysis. HBV full genome sequence, whole polymerase gene or entire reverse transcriptase region was PCR amplified from patient sample and cloned into an HBV replication competent construct forming a chimeric 1.1 fold- overlength HBV genome driven by a CMV promoter. After sequencing analysis, desired clones with interesting mutations were selected and transfected into HuH-7 cell, followed by treatment with 4 drugs in 5 different concentrations for 3 days. These experiments were adapted to a 96-well cell culture format for high throughput analysis. HBV DNA in the supernatants are automatically extracted and quantified by a selective qPCR specifically recognizing the newly synthesized HBV rcDNA. Finally, the IC_{50} values for each drug were calculated and the fold resistance factors were determined by comparing the IC_{50} of each variant to that of the wild-type clone.

3.1.1 Successful PCR amplification of HBV genome and subgenome sequences

The comparison of different viral DNA extraction kits showed the QIAamp UltraSens Virus kit has the highest DNA purification efficiency. For successful PCR amplification of HBV

full genome (≈ 3.2 kb) sufficient to be cloned, the serum titer limit is ≈ 10^3 IU/mL. PCR amplification of HBV subgenomic POL gene (≈ 2.5 kb) and RT region (≈ 1.2 kb), has higher sensitivity, resulting in a serum titer limit as low as 10^2 IU/mL. Moreover, a nested PCR using full genome PCR product as template can further increase the PCR sensitivity of POL gene and RT region, but may also increase the risk of contamination. All PCR analyses were performed using the Phusion Hot Start High-Fidelity PCR system and PCR conditions described above, which can be applied to most of the HBV genotypes with similar efficiency (**Figure 13**).

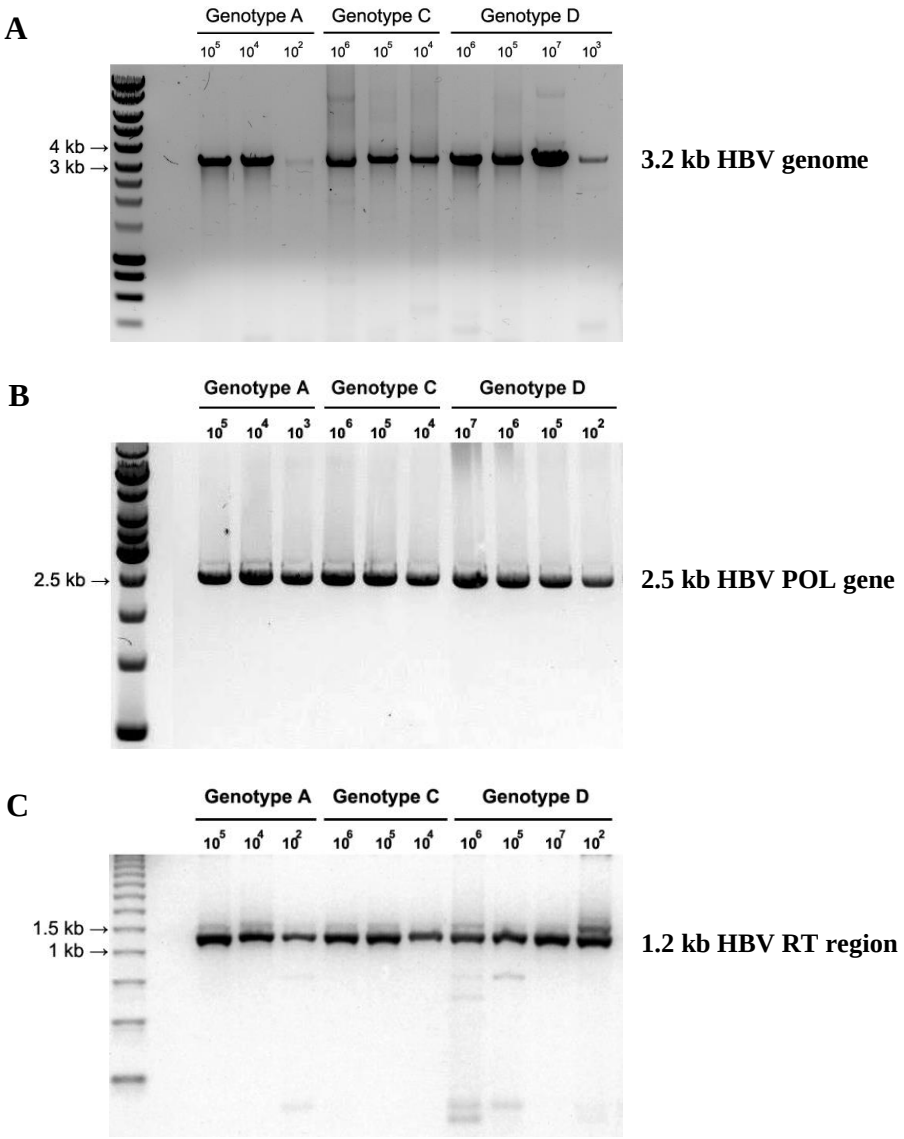


Figure 13: PCR amplification of HBV genome and subgenome from patient serum samples with different viral loads and genotypes. HBV 3.2 kb full genome (**A**), 2.5kb polymerase gene (**B**) and 1.2kb reverse transcriptase region (**C**) were amplified, respectively, from genotype A, C and D serum virus with viral loads between 10^7 IU/ml and 10^2 IU/ml.

3.1.2 Cloning of HBV genome/subgenome into the pCH-9 Vectors

For full HBV genome cloning and expression, a cassette vector pCH-9/200 was constructed that contains the minimum HBV sequence necessary for viral transcription and replication after insertion of a genome-length PCR product (**Figure 14**). Following digestion of pCH-9/200 and full-length HBV genomes with SapI, HBV genomes can be inserted in-frame, resulting in the generation of replication-competent HBV clones that are 95% derived from patient virus. This fusion enabled the efficient production of HBV 3.5 kb pgRNA in transfected hepatoma cells at a constant level independent of HBV genotype or sequence.

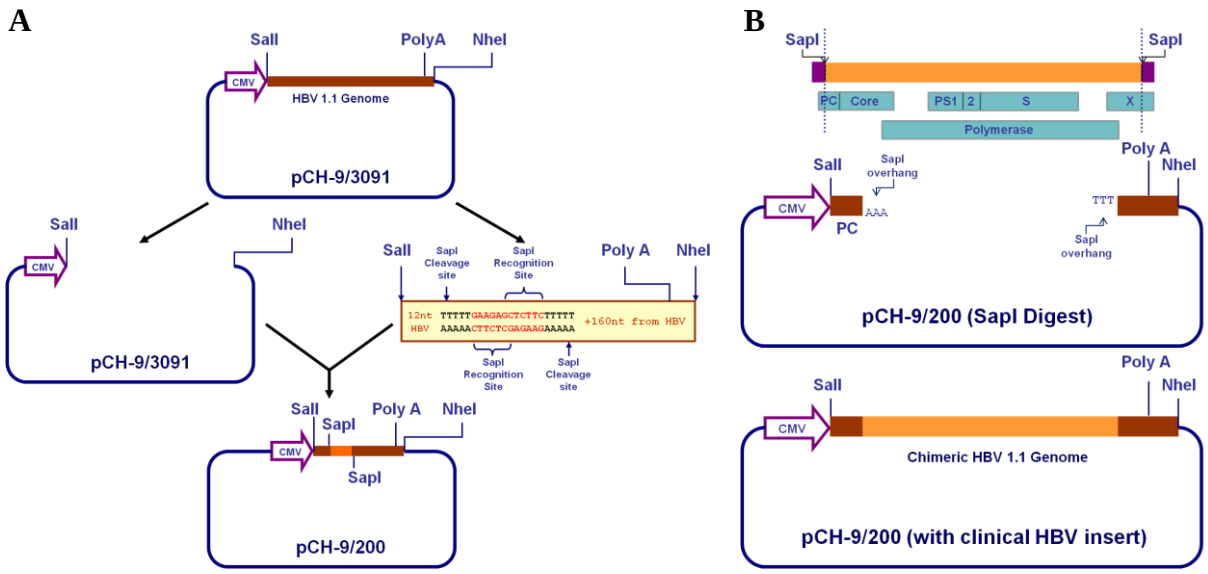


Figure 14: Construction of pCH-9/200 for generation of replication competent clinical HBV isolates. (**A**) The pCH-9/200 was generated by PCR amplification of a small sequence from the 5' end of the HBV genome linked by two SapI sites to a highly conserved 160 base-pair HBV DNA fragment including the polyadenylation site at the 3' end of the HBV genome followed by ligation into the empty vector. (**B**) After SapI digestion, the amplified HBV genome was ligated into SapI-digested pCH-9/200. This allows the in-frame insertion and expression of full-length HBV genome. Modified from [98].

The basis vector pCH-9/3091 was used as another cassette vector for cloning of HBV subgenome and expression of the derived chimeric HBV genome. HBV polymerase sequence aa 8 to aa 818 (out of aa 1-832) was amplified with primers HBV POL-fw and HBV POL-rev which introduced a BspEI or RsrII site into the 5’ or 3’ end of each amplicon, respectively. Similarly, HBV polymerase sequences aa 291 to aa 704 including the entire RT region (RT aa 1–344) was amplified with primers HBV RT-fw and HBV RT-rev which introduced an EcoRI or SphI site into the 5’ or 3’ end of each amplicon, respectively. Restriction digestion of the PCR products and the vector followed by ligation formed the chimeric HBV constructs

consisting of either almost whole polymerase (≈ 97%) or entire RT region in a unified WT genotype D backbone.

The BspEI, RsrII and SphI sites are conserved in all HBV genotypes. The EcoRI site is only conserved in genotype A and B, but comprises just one possible nucleotide change in the other genotypes. Given that the EcoRI-containing primer binding site is highly conserved except this single nucleotide variation, the PCR amplifying HBV RT region and the subsequent cloning confer comparable efficacy among different genotypes. Similarly, most of the amplified POL gene can be correctly inserted into pCH-9/3091 vector. However, HBV genome, especially genotype C, may contain more than one BspEI cutting site inside the POL gene which will result in a truncated form after digestion and insertion, thereby incompetent for viral replication. For those samples, full genome or RT region cloning should be performed instead.

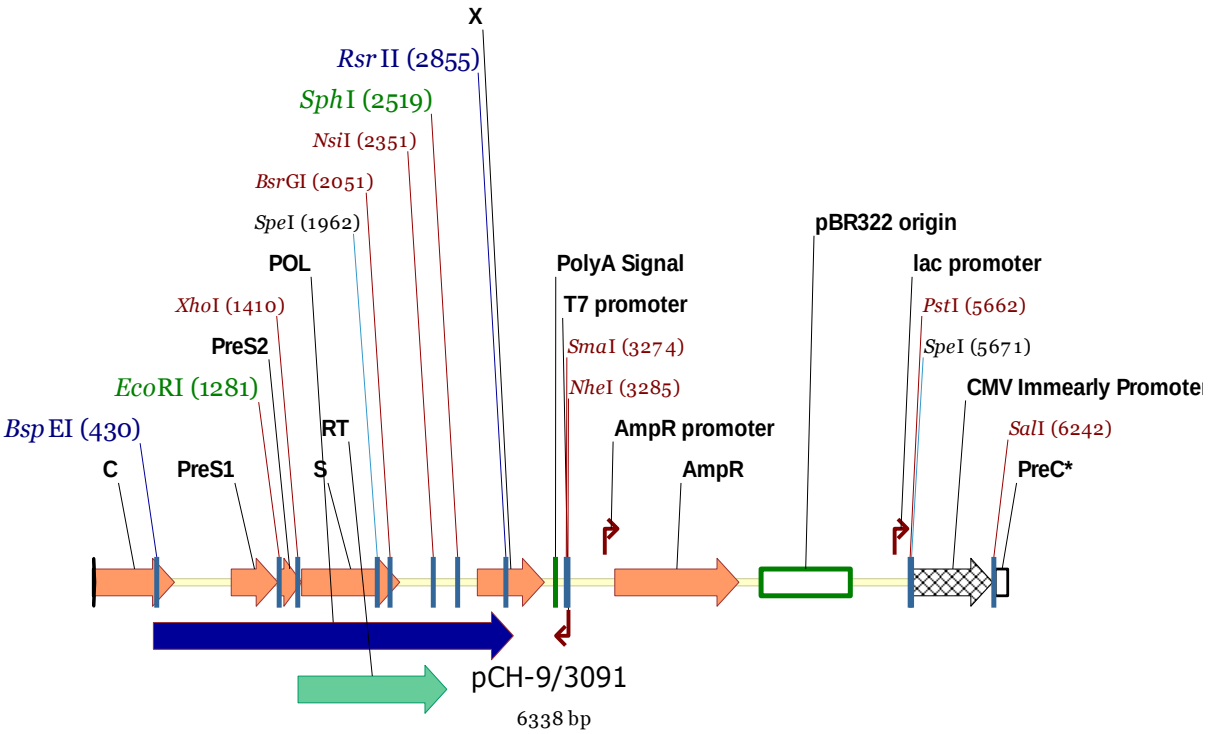


Figure 15: Generation of replication competent chimeric HBV isolates by subgenome sequence exchange using pCH-9/3091. Polymerase gene (POL, blue arrow) or reverse transcriptase region (RT, green arrow) amplified from patient samples contained the indicated restriction sites introduced by PCR primers that bind to highly conserved regions in all HBV genotypes. Replication competent chimeric HBV constructs were generated by replacing the corresponding region in pCH-9/3091 with the amplified HBV subgenome sequences.

3.1.3 Direct and clone sequencing analysis: feasibility and performance

HBV DNA isolated from patient serum was sequenced by two means: direct and clone sequencing. Direct sequencing of HBV RT region was performed after the highly sensitive

PCR amplification before the cloning to determine the viral genotype and to evaluate the mutations in the main viral population. By this, patient material was screened for rare HBV genotype and/or mutation pattern. Multiple clone sequencing was performed after cloning in order to select the representative or desirable mutant clones for phenotyping. Meanwhile, the mutation frequency and distribution among the clones also indicated its proportion within the viral population and the linkage between different mutations.

The signal-to-noise ratio in direct sequencing trace file determined the sensitivity in detecting minor populations of resistant mutants. In general, with our method we could detect particular mutations that represent ≥10% of the total quasispecies pool. Proportion in percentage was estimated by QSVAnalyser (**Figure 16**).

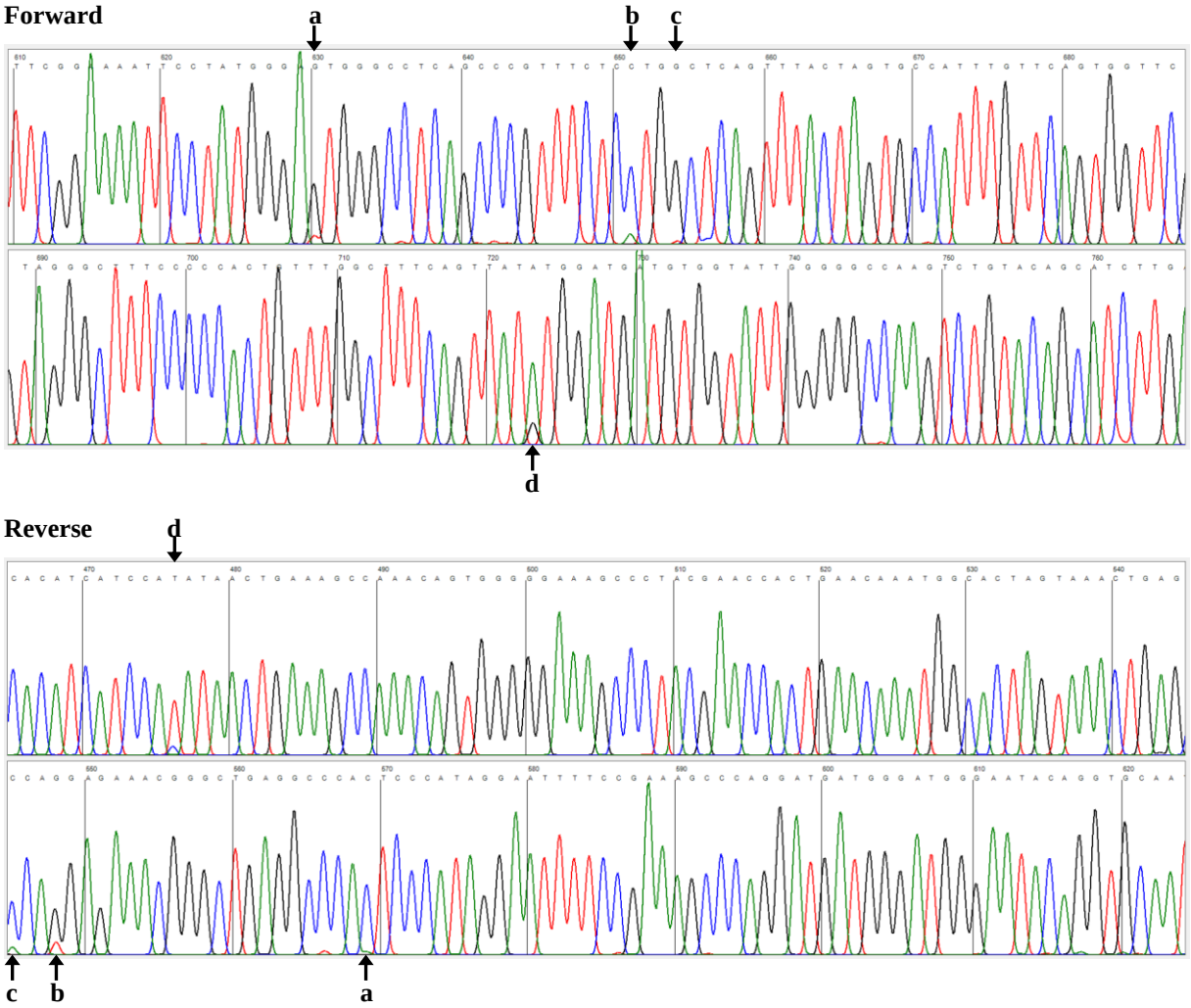


Figure 16: Mutation detection and proportion estimation by sequencing trace file analysis. HBV RT region was PCR amplified from patient serum and then submitted for sequencing. The proportion of detected resistance mutations within the quasispecies was estimated using QSVAnalyser: (**a**) T/G, rtV173L, 10%; (**b**) A/C, rtL180M, 24%; (**c**) T/G, rtA181S, 12%; (**d**) G/A, rtM204V, 22%. Sequencing of both **forward** and **reverse** strands was required for confirmation.

Clone sequencing overcame the low sensitivity of direct sequencing, through intensive multiclonal analysis. The limit of detection was estimated according to the number of sequenced clones and the desired level of confidence (**Table 6**). Accordingly, a number of at least 20-30 clones were required for detection and acquisition of low-frequency mutations or variants (around 10%) with a high probability (confidence interval ≥ 90%).

Table 6: Limit of Variant Detection Depending on the Number of Clones Analyzed

	Clones analyzed, n					
	10	20	30	40	50	100
Detection limit (95% CI)	25.9%	13.9%	9.5%	7.2%	5.8%	3.0%
Detection limit (90% CI)	20.6%	10.9%	7.4%	5.6%	4.5%	2.3%
Detection limit (80% CI)	14.9%	7.7%	5.2%	3.9%	3.1%	1.6%
Detection limit (75% CI)	12.9%	6.7%	4.5%	3.4%	2.7%	1.4%

Note. Based on Bayesian estimation assuming adequate template sampling during PCR amplification. CI, confidence interval.

The information concerning the basic consensus sequence, potential mutations and their possible proportions within the viral population can be obtained from direct sequencing, and helps the downstream cloning, selection and determination. Next, the expression constructs with single cloned HBV genome can be full-length sequenced using a minimal set of well-designed primers suitable for all HBV genotypes, based on a time- and cost-efficient strategy. This allows HBV full length sequencing within 2 days with relatively low costs (only 4 sequencing reactions needed per interesting clone).

3.1.4 Drug susceptibility testing in 96-well format

To adapt the cloning-based drug susceptibility assay to a more convenient, standardized 96-well culture format, HuH-7 cells were transfected with recombinant variant constructs in 10-cm dishes and then reseeded into 96-well plates, treated with 4 different drugs in 5 serial dilutions each in triplicate for 3 days without medium change. The suspension and seeding steps with cell washing in between reduced the remaining input plasmids in supernatant by approximately 1 log and made the cells homogeneous and individualized for distribution in a culture format suitable for carrying out an assay in high throughput format. HBV DNA in the supernatants was automatically extracted by a Tecan robotic platform, where a total of 64 samples produced in a standard testing could be fully processed within 4 hours.

For automated DNA extraction, 4 different magnetic bead-based purification kits were compared together with the spin column-based kit as a "gold standard". Based on the same platform and extraction protocol, the BioSprint 96 One-For-All Vet Kit from Qiagen showed the best performance: highest extraction efficiency and lowest running variability.

3.1.5 HBV selective qPCR: distinguishing rcDNA from input plasmid

Drug susceptibility testing in 96-well format scales up the throughput but requires a more sensitive quantification method, which was still missing so far. Unambiguous rcDNA detection in the presence of excess HBV plasmid DNA is not technically trivial, not even by PCR approaches.

We developed a sensitive real-time qPCR method that selectively amplifies HBV rcDNA (a 264 bp small fragment located near the 5' of positive strand across the nick) but not the input HBV plasmid. The principle is based on the intervention of backbone sequence in the plasmid between primer binding sites combined with a short PCR elongation period (**Figure 17A**). This method enabled facile and higher throughput quantification of replicated HBV DNA without the need for arduous Southern blot to separate the replicated DNA from the input. By using series diluted plasmids from 10^9 to 10^2 copies as template, the selective qPCR showed a $\geq$ 4.5 log reduction on input constructs (selectivity $\approx 1{:}10^{4.5}$, **Figure 17B**). In our standard phenotypic testing, background input plasmid quantification routinely had levels > 100-fold less than that of HBV DNA untreated positive control. This confirmed a nearly complete subtraction of input plasmid, thereby minimizing the potential overestimation of newly produced virus.

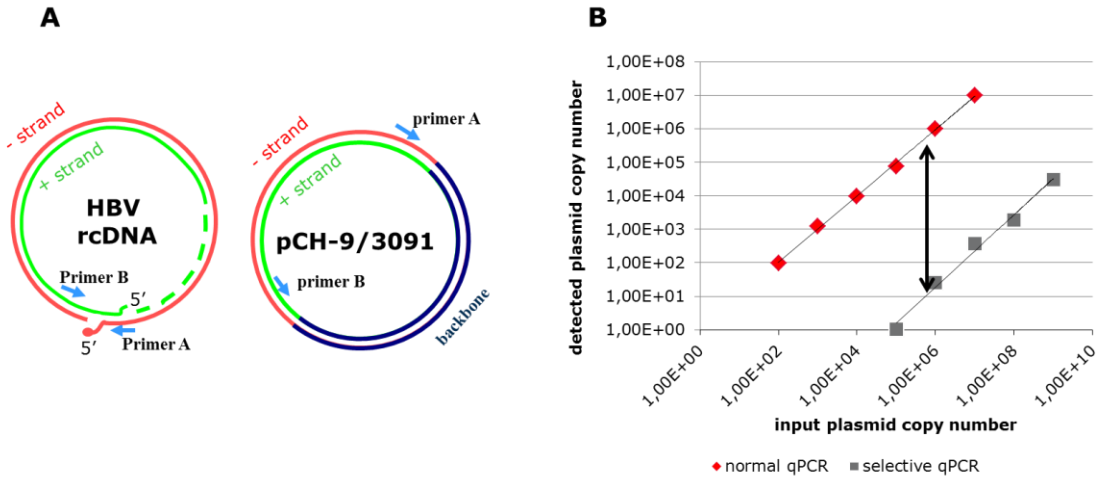


Figure 17: Principle and efficiency of the selective qPCR. (A) Primers were designed to span the nick region of the HBV rcDNA genome as well as the redundancy region of the overlength HBV genome in the plasmid. The amplicon on rcDNA is about 264 bp, while on input plasmid it is around 3.5 kb. (B) Series diluted HBV plasmid samples from 10^9 to 10^2 copies were used as PCR template with normal HBV DNA primers or selective primers. The selectivity is calculated as the ratio of quantification by selective and normal qPCR.

This quantitative PCR was able to measure down to 5 copies of HBV rcDNA, corresponding to a theoretical supernatant titer of 1000 genomes/ml. Notably, the amplified region with the selective primers is unique for HBV rcDNA instead of other intracellular HBV DNA forms such as single-stranded replicative intermediates and double-stranded linear DNA. This allows a more accurate and specific quantification of rcDNA when intracellular total HBV DNA is applied.

3.1.6 Resistance analysis: IC_{50} and resistance factor determination

The analysis of extracellular HBV rcDNA, synthesized in the presence or absence of antivirals with different concentrations in triplicate, enabled the determination of the IC_{50} of the drugs for each variant. This IC_{50} was compared to those obtained with our standard, wild-type laboratory strain, to determine the corresponding resistance factor, a direct representation of its *in vitro* susceptibility to each drug.

To assess the assay sensitivity and variability, especially for low level resistance as typically associated with adefovir resistance mutations *in vitro*, HBV variants with rtN236T mutation, introduced by site-directed mutagenesis or by cloning of two patient derived polymerase genes, were tested at least in duplicate. As shown in **Figure 18**, the IC_{50} value of the point mutated variant rtN236T for adefovir had a 2.3-fold increase over that of wild type,

consistent with previous reports. This result also proved that the assay was able to detect resistance with ≥2-fold changes.

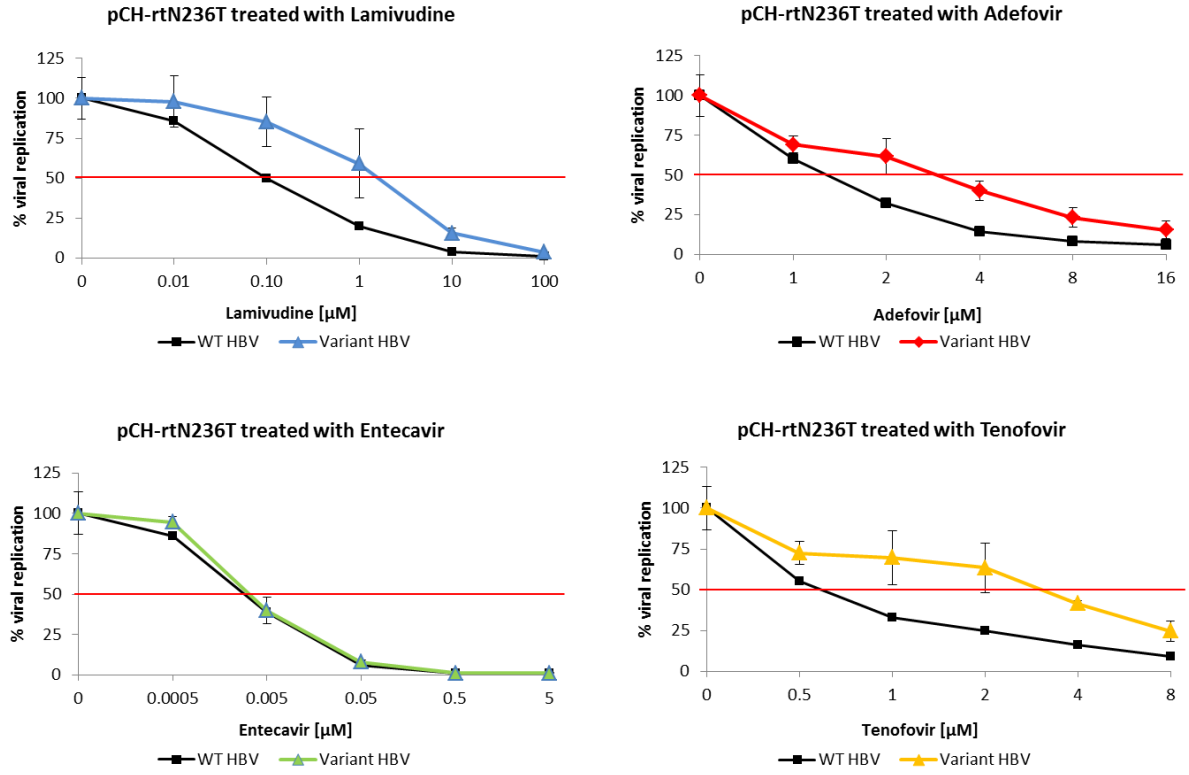


Figure 18: *In vitro* susceptibility of rtN236T to four drugs. HuH-7 cells were transfected with wild-type (WT) or rtN236T HBV plasmid and treated with lamivudine, adefovir, entecavir or tenofovir in different concentrations as indicated. The percentage of HBV DNA production is based on the HBV DNA concentration values as measured by selective qPCR in comparison with the level of HBV DNA in the absence of drug (untreated control). Dose–inhibition curves for the two HBV strains were used to estimate the IC_{50} values for each drug.

Phenotypic results, including IC_{50} and resistance factor values of each recombinant for each drug, were summarized in **Table 7**. Two HBV isolates from different patients with the same rtN236T mutation showed similar IC_{50} values close to those of the rtN236T point mutant for almost all 4 drugs. Of note was the rtN236T variant cloned from the second patient (Pt2-rtN236T), showing a higher IC_{50} value against adefovir in comparison with the other 2 rtN236T variants. Sequencing analysis revealed that the RT region of Pt2-rtN236T contained no extra known resistance mutations but a genotype-specific natural polymorphism, rtL217R, which may account for a lower response to adefovir as previously reported.

All fold resistance factors obtained correlated well with the clinical observations and were consistent with previously published results. No significant variation was found between repeated independent tests, indicating a low interassay variability and good reproducibility of the assay.

Table 7: *In vitro* drug susceptibilities of tested rtN236T recombinants.

Recombinant	LAM		ETV		ADV		TDF	
	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
pCH-WT	0.1	1	0.003	1	1.3	1	0.6	1
pCH-rtN236T	1.2	12	0.003	1.1	3.0	2.3	3.1	5.2
Pt1-rtN236T	1.0	10	0.002	0.8	4.2	3.0	3.5	6.1
Pt2-rtN236T	1.5	15	0.003	1.0	6.2	4.8	3.8	6.4

Pt, patient; RF, resistance factor; IC_{50} in µM.

3.1.7 The influence of HBV backbone genotype on resistance analysis

Amplification and cloning of full length HBV can often be technically challenging, limiting its use especially on samples of low serum HBV DNA levels, impractical in large scale clinical applications. HBV subgenome cloning strategy provided a more convenient alternative to phenotype clinical isolates by cloning into a wild-type laboratory backbone.

In total three wild-type laboratory strains of different genotypes (A, C, D) were tested as backbone in this study. The HBV expression vector pCH-9/3091 contains the genotype D laboratory stain J02203 organized as described before. Two more HBV expression vectors (pCH-HBV1.1-A / pCH-HBV1.1-C) containing genotype A and C laboratory stains, respectively, were constructed in the same manner as for pCH-9/3091. These three wild-type strains conferred very similar drug susceptibility phenotypes but different replication capacities (**Table 8**). Genotype D replicated best, followed by genotype C, while genotype A replicated 20-fold less efficiently, which correlated well with *in vivo* observations.

Table 8: IC_{50} and replication capacity of wild-type strains with different genotypes

Genotype	LAM (IC_{50})	ETV (IC_{50})	ADV (IC_{50})	TDF (IC_{50})	RC
D	0.1	0.003	1.3	0.6	100%
A	0.1	0.003	1.3	0.8	5%
C	0.1	0.002	1.3	1.0	95%

RC, replication capacity in percentage compared to wild type; IC_{50} in µM; all genotypes in the same construct.

By cloning the polymerase gene or RT region from other genotypes into the replication competent construct with genotype D HBV backbone, intergenotypic chimeric HBV genomes were created. To evaluate whether the diverse genetic background can influence the replication capacity as well as the resistance phenotype conferred by cloned POL gene/RT

region, intergenotypic chimeric HBV recombinants were tested for drug susceptibility and compared with the intragenotypic chimeric HBV recombinants containing same insert (**Table 9**). No significant difference in resistance factor was observed between intra- and intergenotypic chimeric variants harboring the same insert/mutation. Moreover, the intergenotypic chimeric recombinants using genotype D laboratory strain as backbone replicated the same, if not better, as the intragenotypic ones. These results clearly supported the idea of using the universal genotype D backbone for cloning and phenotyping variants from all genotypes, fitting in a standardized pipeline.

Table 9: Resistance phenotypes of intra- and intergenotypic chimeric variants

Genotype		Mutation	Resistance Factor				RC
Backbone	Insert		LAM	ETV	ADV	TDF	
A	A	AV	25	<1	3.2	2.6	9%
D	A		25	<1	3.6	2.3	13%
A	A	VL+LM+MV	>1000	5	1.0	4.2	3%
D	A		>1000	6	1.0	4.8	4%
C	C	NV	2	<1	1.4	4.5	16%
D	C		5	<1	1.4	4.2	14%
C	C	LI+LM+MI	>1000	2	<1	1.5	41%
D	C		>1000	3	<1	1.0	34%

RC: replication capacity in percentage compared to wild-type genotype D; AV, rtA181V; VL+LM+MV, rtV173L+rtL180M+rtM204V; NV, rtN236V; LI+LM+MI, rtL80I+rtL180M+ rtM204I.

3.1.8 Phenotypic assay in an HBx-deficient manner: toward a better biosafety

Vaccine escape mutations can be present in drug resistant patients or even be selected during antiviral treatment. This certainly raises an inconvenient biosafety issue of the phenotypic assay which should not be neglected when dealing with those variants. To solve this issue, a new strategy using an HBx-deficient HBV vector for phenotyping was proposed and tested.

Two HBx premature stop codons (xQ8* and xQ87*) were introduced into pCH-9/3091 by site-directed mutagenesis after the SphI and RsrII restriction sites, without affecting the overlapping polymerase. This allowed the introduction of an HBx deficiency in the chimeric HBV after insertion of any cloned RT region or POL gene. *In vitro* phenotypic results showed that HBx-deficiency in wild-type or variant HBV had no significant impact on either

resistance phenotype or replication capacity in transfected HuH-7 cells (**Table 10**), but as expected, led to non-productive infection in HepaRG cells (**Figure 19**).

Table 10: IC_{50} and replication capacity of variants with or without HBx

Recombinant	LAM (IC_{50})	ETV (IC_{50})	ADV (IC_{50})	TDF (IC_{50})	RC
pCH-WT	0.1	0.003	1.3	0.6	100%
pCH-X^-	0.1	0.003	1.5	0.8	85%
pCH-rtK32R	1.0	0.0005	4.5	6.5	47%
pCH-rtK32R-X^-	1.2	0.0005	5.0	7.5	41%

X^-, HBx minus; RC, replication capacity in percentage compared to wild type; IC_{50} in µM.

The inability of HBx-deficient HBV to replicate viral DNA and to produce viral antigens after infection demonstrated a strong attenuation of the virus, and therefore, provided a feasible option to improve the phenotypic assay for a better biosafety.

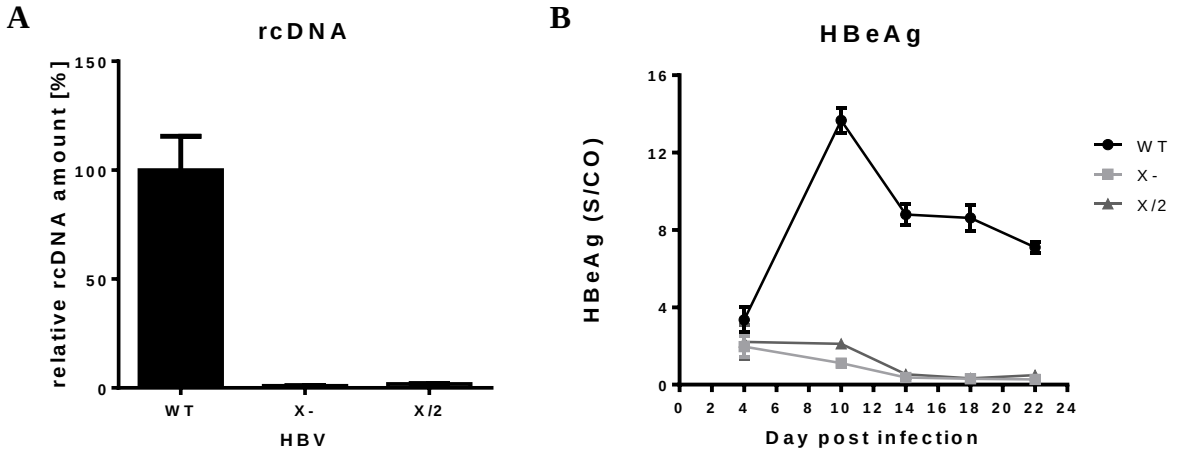


Figure 19: HBx deficiency led to non-productive HBV infection in HepaRG cells. Wild-type (WT), HBx-minus (X-) and HBx truncated (X/2) virus were produced by transfecting HuH-7 cells with WT, HBx double mutated (xQ8*+xQ87*) or HBx single mutated (xQ87*) HBV plasmid, respectively, and purified by heparin column chromatography. HepaRG cells were infected with each virus at an MOI of 100 vp/cell and analyzed for HBV rcDNA and HBeAg production in the supernatants.

3.2 Comprehensive analysis of HBV resistance mutations

The genetic changes that confer resistance to the approved nucleos(t)ide analogues used for chronic hepatitis B treatment have been described extensively in the literature. A comprehensive evaluation of these resistance mutations together with their combinations using a unified and standardized assay shall provide comparable and compatible results of

resistance phenotype, cross resistance profile as well as important viral fitness parameters for an integrated analysis.

3.2.1 A representative panel of HBV expression plasmids with known mutations

In total, thirteen known dominant resistance mutations and two recently identified resistance-associated mutations from controversial findings were initially included in the mutation list (**Table 11**). Since the resistance mutations in HBV RT may also affect the overlapping S gene, the corresponding HBsAg mutations were also indicated. In particular, when the same RT mutation had different possibilities on HBsAg due to alterative codon options, the most closed substitution was chosen to avoid the possible negative effect. In addition, the optimal codon was chosen, if applicable, for better expression. Finally, all amino acid or nucleotide changes can actually be found in nature.

Table11: HBV antiviral resistance substitutions and corresponding changes

RT Mutation	HBsAg Mutation	POL Codon	S Codon	Genome SDM
rtL80I	-	TTA→ATA	GGT→GGA	T1649A
rtI169T	sF161L	ATT→ACT	TTC→CTC	T1917C
rtV173L	sE164D	GTG→CTG	GAG→GAC	G1928C
rtL180M	-	CTG→ATG	TCC→TCA	C1949A
rtA181T	sW172L	GCT→ACT	TGG→TTA	G1951T+G1952A
rtA181V	sL173F	GCT→GTT	CTC→TTC	C1953T
rtT184G	sL176V	ACT→GGT	CTA→GTA	A1961G+C1962G
rtA194T	-	GCT→ACT	GGG→GGA	G1991A
rtS202I	sV194F	AGT→ATT	GTT→TTT	G2016T
rtS202G	-	AGT→GGT	TCA→TCG	A2015G
rtM204V	sI195M	ATG→GTG	ATA→ATG	A2021G
rtM204I	sW196L	ATG→ATT	TGG→TTG	G2023T
rtI233V	-	ATA→GTA	GTA→GTG	A2108G
rtN236T	-	AAC→ACC	GGT→GGA	A2118C
rtM250V	-	ATG→GTG	TTC→CTC	A2159G

SDM, site-directed mutagenesis; the number indicates the mutated position in HBV genome.

A panel of 65 HBV expression plasmids harboring the selected mutations individually or in combination was generated by DNA synthesis or site-directed mutagenesis, and by fragment exchange between constructs (**Table 12**). These variant plasmids enriched the patient sample collection we obtained from clinicians and allowed for analysis of important or interesting combinations of resistance and compensatory mutations that could hardly be isolated from patient material. In particular, complex mutation combinations with respect to entecavir resistance were generated to analyze how mutation interactions determine the resistance phenotype, modulate the fitness cost, and affect the treatment outcome. Combinations of mutations within different resistance pathways were also created to evaluate the possible selection of multi-drug resistant variants during combination therapies. Besides, the possible resistance mutations in different contexts were generated for comparative confirmation.

3.2.2 Phenotypic analysis of resistant HBV panel

Antiviral susceptibility (IC_{50}) to lamivudine, entecavir, adefovir and tenofovir, respectively, were determined for all variant clones. Because of the highly sensitive qPCR quantification, we were able to accurately measure the IC_{50} of clones that replicated and released very low levels of extracellular HBV DNA (even below 1% of wild type control) without a significant bias. Additionally, antiviral susceptibilities for recombinant clones containing polymerase genes from clinical isolates were tested and compared in parallel. The phenotypic results were summarized in **Table 12.**

As consistent with previous studies, all variants containing typical YMDD mutations (rtL180M+rtM204V or rtM204VI) in the panel conferred high-level resistance to lamivudine (RF >1000). Primary resistance mutations rtA181T/V and rtN236T conferred merely low-level resistance to adefovir *in vitro* with RFs <3, but meanwhile, a moderate-level resistance to lamivudine (RF ≥10). Surprisingly, rtM204V individually, but not in combination with rtL180M, showed a significantly reduced susceptibility to adefovir and tenofovir (RFs of 6.1 and 10). When combined with another adefovir resistance mutation, for example, rtM204V+rtN236T, its resistance could be increased to an even higher level. However, without rtL180M, single rtM204V showed a strongly reduced RF (from >1000 to <50) to lamivudine and a minor RF of 3 to entecavir.

Each of the tested variants at rtI169/rtT184/rtS202/rtM250 in combination with rtL180M+rtM204V or rtM204I conferred a significantly reduced susceptibility (RF>100) to entecavir. Additional mutations at these positions could further increase resistance (e.g.,

addition of the mutation rtI169T to rtL180M+rtS202I+rtM204V increased resistance 10-fold, to RF>1000), but could also countervail it (e.g., the addition of rtT184G of rtV173L+rtL180M+rtS202G+ rtM204V (RF~120) decreased resistance (RF~30)), or even abolish it (e.g., addition of rtM250V to rtL180M+rtT184G+rtM204V (RF100 reduced to RF<5). Notably, the resistance phenotype of the rtL180M+rtS202I+rtM204V+rtM250V combination could not be determined due to its completely eliminated replication (< 0.05% of WT). Besides, most of the entecavir resistance associated combinations also showed slightly reduced susceptibility to adefovir and tenofovir *in vitro*.

The recently identified mutations associated with adefovir resistance (rtI233V) or tenofovir resistance (rtA194T) were tested individually or in combinations with other mutations (rtL180M+rtM204V) as mentioned in the original studies. However, none of the phenotypic tests on these variants could confirm the resistance conferred by these two mutations to adefovir or tenofovir. Besides, rtI233V didn't further increase the resistance of rtA181T/V to adefovir or tenofovir in combination.

Table 12: IC_{50} and resistance factors determined for the resistant HBV panel

ID	Mutation	LAM		ETD		ADV		TDF	
		IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
MUC-000	WT	0.1	1.0	0.003	1.0	1.3	1.0	0.6	1.0
MUC-001	rtL80I	0.1	1.0	0.003	1.0	1.3	1.0	1.0	1.7
MUC-002	rtI169T	0.2	1.8	0.001	0.3	1.1	0.9	0.6	1.0
MUC-003	rtV173L	0.5	5.0	0.005	1.8	1.3	1.0	1.0	1.7
MUC-004	rtL180M	4.5	45	0.004	1.3	1.7	1.3	0.6	1.0
MUC-005	rtA181V	2.0	20	0.004	1.3	2.6	2.0	1.7	2.8
MUC-006	rtA181T	2.5	25	0.002	0.8	3.0	2.3	1.4	2.3
MUC-007	rtT184G	0.05	0.5	0.001	0.3	1.3	1.0	0.5	0.8
MUC-008	rtA194T	0.8	8.0	0.005	1.7	1.3	1.0	0.7	1.2
MUC-009	rtS202I	0.3	3.0	0.001	0.3	2.2	1.8	1.5	2.4
MUC-010	rtS202G	0.2	2.0	0.002	0.8	2.0	1.6	1.3	2.1
MUC-011	rtM204V	4.5	45	0.005	1.6	6.5	5.0	6.0	10
MUC-012	rtM204I	>1000	>1000	0.1	30	2.5	1.9	1.1	1.8
MUC-013	rtI233V	0.2	2.0	0.002	0.8	1.5	1.1	1.1	1.8
MUC-014	rtN236T	1.2	12	0.003	1.0	3.2	2.5	3.0	5.0
MUC-015	rtM250V	0.2	2.0	0.002	0.8	0.5	0.4	0.3	0.5
MUC-016	rtL80I + rtM204I	>100	>1000	0.03	10	1.3	1.0	1.1	1.9

Table 12: IC_{50} and resistance factors determined for the resistant HBV panel (continued)

ID	Mutation	LAM		ETD		ADV		TDF	
		IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
MUC-017	rtV173L + rtM204I	>100	>1000	0.1	30	2.9	2.2	3.5	5.8
MUC-018	rtL180M + rtM204I	>100	>1000	0.05	16	2.5	1.9	1.8	3.0
MUC-019	rtL80I + rtM204V	8.0	80	0.005	1.6	2.6	2.0	3.8	6.3
MUC-020	rtL180M + rtM204V	>100	>1000	0.04	13	1.8	1.4	3.0	5.0
MUC-021	rtV173L + rtL180M + rtM204V	>100	>1000	0.05	17	2.0	1.5	2.7	4.5
MUC-022	rtI169T + rtM204V	>100	>1000	0.05	17	10	7.7	8.0	13
MUC-023	rtI169T + rtV173L + rtL180M + rtM204V	>100	>1000	0.2	66	2.0	1.5	0.4	0.7
MUC-024	rtL180M + rtM204V + rtM250V	>100	>1000	0.2	66	1.3	1.0	1.3	2.1
MUC-025	rtV173L + rtL180M + rtM204V + rtM250V	>100	>1000	0.1	33	1.4	1.1	2.0	3.3
MUC-026	rtI169T + rtL180M + rtM204V + rtM250V	>100	>1000	0.6	200	1.3	1.0	0.9	1.5
MUC-027	rtI169T + rtV173L + rtL180M + rtM204V + rtM250V	>100	>1000	0.4	130	1.5	1.2	0.9	1.5
MUC-028	rtI169T + rtV173L + rtL180M + rtT184G + rtM204V	>100	>1000	0.3	100	1.7	1.3	2.6	4.3
MUC-029	rtT184G + rtM204V	>100	>1000	0.15	5.0	8.5	6.5	4.0	6.7
MUC-030	rtL180M + rtS202I + rtM204V	>100	>1000	0.4	133	4.0	3.1	8.0	14
MUC-031	rtL180M + rtT184G + rtS202I + rtM204V	>100	>1000	0.5	166	4.0	3.1	2.0	3.3
MUC-032	rtV173L + rtL180M + rtS202G + rtM204V	>100	>1000	0.5	166	3.0	2.3	4.0	6.7
MUC-033	rtV173L + rtL180M + rtT184G + rtS202G + rtM204V	>100	>1000	0.1	33	3.0	2.3	2.6	4.4

Table 12: IC_{50} and resistance factors determined for the resistant HBV panel (continued)

ID	Mutation	LAM		ETD		ADV		TDF	
		IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
MUC-034	rtV173L + rtL180M + rtT184G + rtM204V + rtM250V	>100	>1000	0.02	6.6	1.3	1.0	1.5	2.5
MUC-035	rtV173L + rtL180M + rtS202G + rtM204V + rtM250V	>100	>1000	0.2	66	2.1	1.6	2.5	4.1
MUC-036	rtL180M + rtA181V	20	200	0.004	1.3	3.3	2.5	2.8	4.7
MUC-037	rtL180M + rtN236T	10	100	0.003	1.0	18	14	7.2	12
MUC-038	rtA181V + rtN236T	5.0	50	0.003	1.0	5.8	4.5	3.9	6.5
MUC-039	rtL180M + rtA181V + rtN236T	>100	>1000	0.02	6.6	15	12	>16	>26
MUC-040	rtA181T + rtN236T	10	100	0.004	1.3	4.5	3.5	3.6	6.0
MUC-041	rtV173L + rtN236T	1.0	10	0.004	1.3	4.0	3.1	7.8	13
MUC-042	rtV173L + rtL180M + rtA181V + rtN236T	85	850	0.003	1.0	4.0	3.1	3.0	5.0
MUC-043	rtV173L + rtL180M + rtA181V + rtM204V + rtN236T	>100	>1000	0.002	6.7	2.6	2.0	3.0	5.0
MUC-044	rtV173L + rtL180M + rtA181V + rtM204V	>100	>1000	0.05	17	2.6	2.0	3.0	5.0
MUC-045	rtV173L + rtL180M + rtA194T + rtM204V	>100	>1000	0.03	10	2.0	1.5	2.4	4.0
MUC-046	rtL180M + rtM204V + rtI233V	>100	>1000	0.03	10	2.1	1.6	1.8	3.0
MUC-047	rtA181V + rtI233V	1.5	15	0.004	1.3	4.0	3.1	3.0	5.0
MUC-048	rtA181T + rtI233V	1.2	12	0.003	1.0	3.2	2.4	3.0	5.0
MUC-049	rtL180M + rtA181V + rtI233V	18	180	0.01	3.3	2.7	2.1	2.2	3.6
MUC-050	rtV173L + rtM204I + rtM250V	100	1000	0.02	6.7	1.2	0.9	1.3	2.1

Table 12: IC_{50} and resistance factors determined for the resistant HBV panel (continued)

ID	RT Mutation	LAM		ETD		ADV		TDF	
		IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
MUC-051	rtI169T + rtL180M + rtA181V + rtM204V	>100	>1000	0.05	17	1.3	1.0	2.0	3.3
MUC-052	rtI169T + rtM204V + rtN236T	>100	>1000	0.04	13	42	32	16	27
MUC-053	rtI169T + rtM204I	>100	>1000	0.5	167	5.5	4.2	5.5	9.0
MUC-054	rtI169T + rtA181T	8.0	80	0.004	1.3	3.2	2.5	3.2	5.3
MUC-055	rtI169T + rtN236T	1.1	11	0.005	1.7	6.8	5.2	3.0	5.0
MUC-056	rtL180M + rtA181V + rtM204V + rtM250V	>100	>1000	0.05	17	1.7	1.3	2.1	3.5
MUC-057	rtI169T + rtV173L + rtL180M + rtM204I	>100	>1000	0.15	50	1.5	1.2	3.0	5.0
MUC-058	rtI169T + rtL180M + rtM204V	>100	>1000	0.5	167	2.5	1.9	2.0	3.3
MUC-059	rtL180M + rtT184G + rtM204V	>100	>1000	0.3	100	2.1	1.6	0.3	0.5
MUC-060	rtI169T + rtL180M + rtS202I + rtM204V	>100	>1000	>5	>1000	1.8	1.4	2.8	4.7
MUC-061	rtI169T + rtL180M + rtS202G + rtM204V	>100	>1000	>5	>1000	1.6	1.2	2.9	4.8
MUC-062	rtM204I + rtM250V	>100	>1000	0.05	17	1.3	1.0	1.9	3.2
MUC-063	rtL180M + rtT184G + rtM204V + rtM250V	>100	>1000	0.015	5.0	1.1	0.8	1.9	3.2
MUC-064	rtL180M + rtS202I + rtM204V + rtM250V	–	–	–	–	–	–	–	–

IC_{50} in µM; RF, resistance factor; –, not detectable.

3.2.3 HBsAg secretion and replication capacity of HBV variants

Because of the overlap between the viral polymerase and envelope genes, the rtI169T, rtV173L, rtA181T, rtA181V, rtT184G, rtS202I, rtM204V, and rtM204I polymerase substitutions were associated with sF161L, sE164D, sW172L, sL173F, sL176V, sV194F, sI195M, and sW196L mutations in the S domain of the three envelope proteins, respectively, in the panel. All other mutations caused no corresponding amino acid changes. HBsAg secretion in cell supernatants was analyzed by automated chemiluminescent microparticle immunoassay. The results showed that HBsAg of all single variants could be secreted at a similar level to the wild type (**Figure 20**). Nevertheless, the multiple variants, especially those with three or more HBsAg mutations, had slightly decreased HBsAg levels, probably due to the reduced binding affinity to the mutated HBsAg of the antibody used in the assay as previously reported.

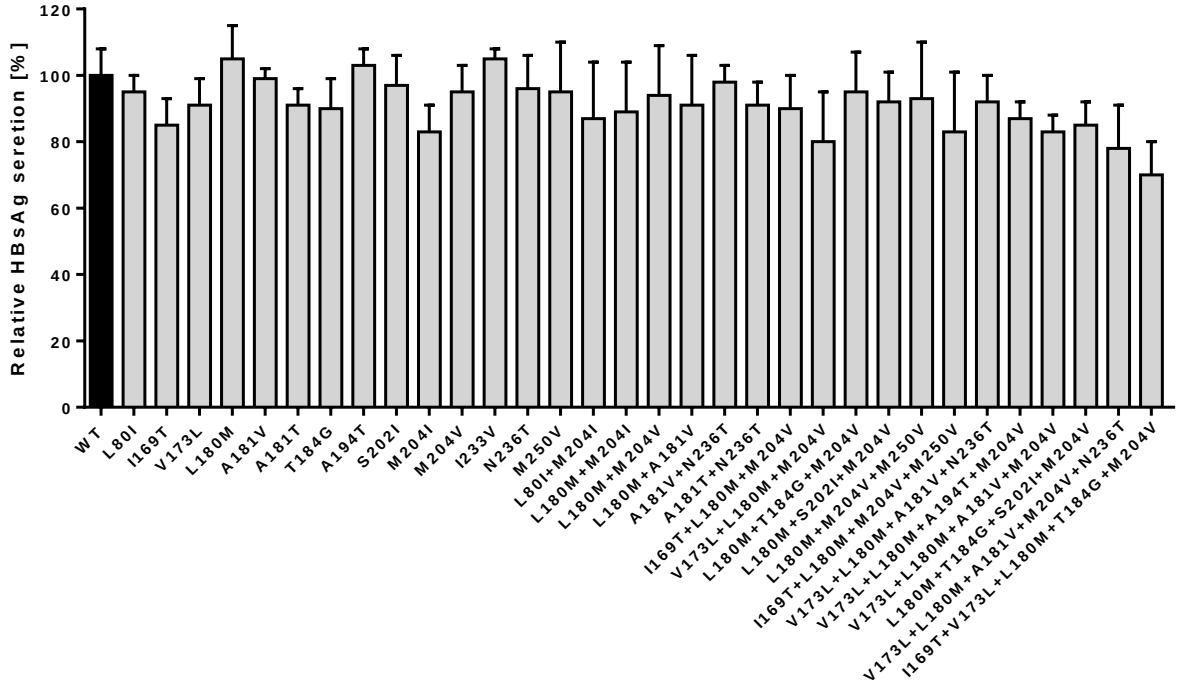


Figure 20: HBsAg secretion of selected variants in the panel. Each variant plasmid was transfected into Huh-7 cells and the secreted HBsAg in the supernatants were quantified using AxSYM HBsAg immunoassay and compared to the wild type level.

The level of replicated HBV DNA associated with each variant selected from the panel was assessed by normalized quantification of HBV DNA in the supernatant after transfection. Only extracellular instead of intracellular assessment was made since changes in the overlapping HBsAg residues induced by HBV polymerase mutations in the panel did not

affect the HBsAg/virion secretion. Most of the variants showed impaired replication albeit to different extents relative to the wild type (**Figure 21**). Among them, single variants harboring primary resistance mutations such as rtA181V, rtM204V/I, and rtN236T, had moderate to large reductions on replication capacity. Secondary mutations including rtL80I, rtV173L, and rtL180M also conferred decreased replication capacity with mild to moderate reductions, but were able to partially restore the replication fitness of the primary resistance mutations in proper combination. Variants with entecavir resistance associated mutations including rtI169T rtT184G, rtS202I/G, and rtM250V, showed varied levels of replication. Variant with rtT184G showed no significant reduction of replication capacity while those with rtI169T, rtS202G, and rtM250V were associated with mild to moderate reductions. Notably, rtS202I resulted in a severe impairment in any context, which could not be compensated by rtT184G in the way reported previously, and even completely diminished the replication in combination with rtM250V. Secondary mutation rtV173L could enhance the replication of most entecavir resistance associated variants or, more generally, variants with mutations rtL180M+ rtM204V/I. Interestingly, rtA181T as a primary resistance mutation also yielded a highly replication competent variant and could even increase the replication capacity of other primary resistance mutations like rtN236T in combination.

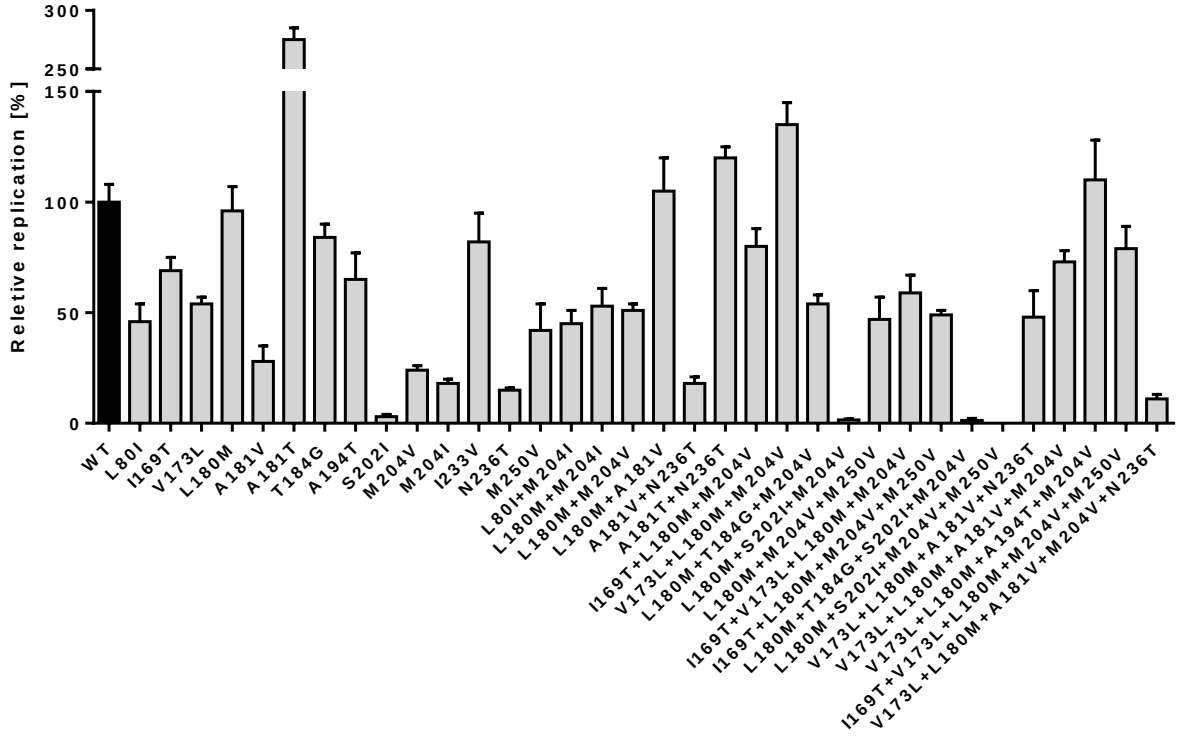


Figure 21: Replication capacity of selected variants in the panel. Relative replication capacity of selected variants was determined after normalized transfection of replication competent plasmids into HuH-7 cells.

3.3 Identification of new mutations potentially resistant to adefovir and tenofovir

Adefovir and tenofovir are acyclic nucleotide analogues with similar structure differing only by a methyl group. Adefovir resistance associated mutations including rtA181T/V and rtN236T conferred low-level cross resistance to tenofovir *in vitro* as confirmed by phenotypic testing. Meanwhile, the rtA194T mutation, reportedly associated with tenofovir resistance as recently identified in tenofovir treated HIV/HBV-coinfected patients, did not confer *in vitro* resistance to tenofovir either as a single mutation or in a lamivudine-resistant viral background (rtL180M+ rtM204V). Given that tenofovir has replaced adefovir as a first line and/or rescue therapy option being used more and more widely, resistance mutations associated specifically with tenofovir therapy are therefore of continuing interest and remain to be identified.

3.3.1 rtK32R presumably corresponding to HIV K65R with tenofovir resistance

According to a recent computational 3D model of HBV reverse transcriptase, the residue rtK32 of HBV RT was properly aligned and matched with K65 of HIV-1 RT (**Figure 22**). Previous studies have reported that the K65R mutation in HIV-1 RT confers intermediate resistance against a number of NRTIs including tenofovir, adefovir, and lamivudine. Therefore, rtK32R could be expected to play a similar role to that by K65R in HIV-1 RT for binding of similar analogues and the development of resistance to inhibitors. This hypothesis was tested by mutation studies of this residue.

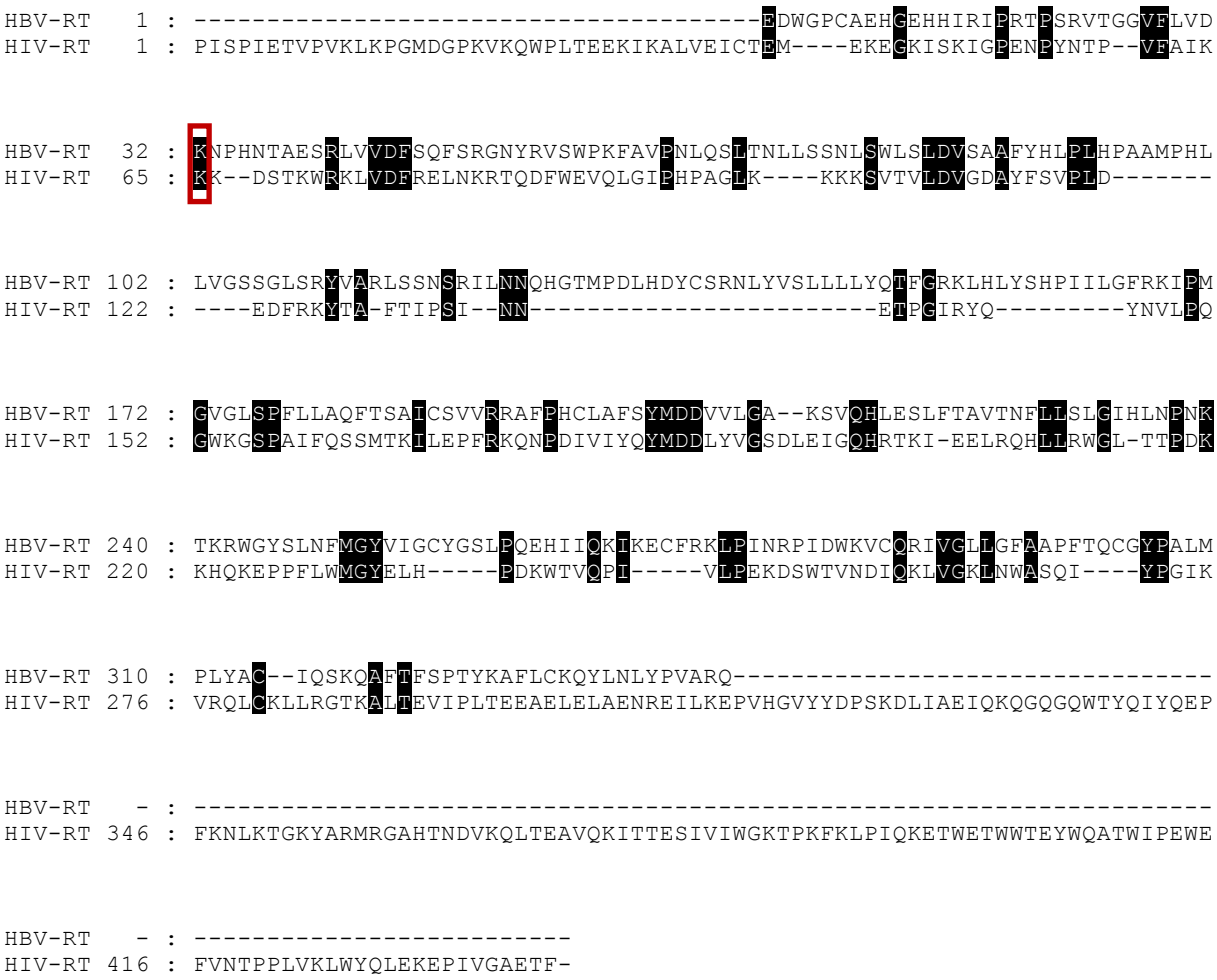


Figure 22: Sequence alignment of HBV RT and HIV-1 RT. The conserved residues between HBV and HIV RT were highlighted in black. The conserved rtK32 corresponding to HIV K65 was indicated in red block.

We first introduced the mutation rtK32R into pCH-9/3091 by site-directed mutagenesis and further generated two more constructs containing mutation combinations, rtK32R+rtA181T and rtK32R+rtN236T, by fragment exchange with existing construct containing rtA181T or rtN236T. In addition, rtK32R were also introduced into pCH-HBV1.1-A construct with a genotype A HBV background for comparison.

Drug susceptibility test of these variants showed that single rtK32R mutation conferred 10-fold, 3.5-fold and 11-fold reduced susceptibility to lamivudine, adefovir and tenofovir, respectively (**Figure 23**). Similar resistance folds were obtained from the genotype A rtK32R variant. This resistance profile was nicely consistent with that of HIV K65R which has been well characterized *in vitro* and *in vivo*. Interestingly, rtK32R increased the sensitivity to entecavir by 6-fold.

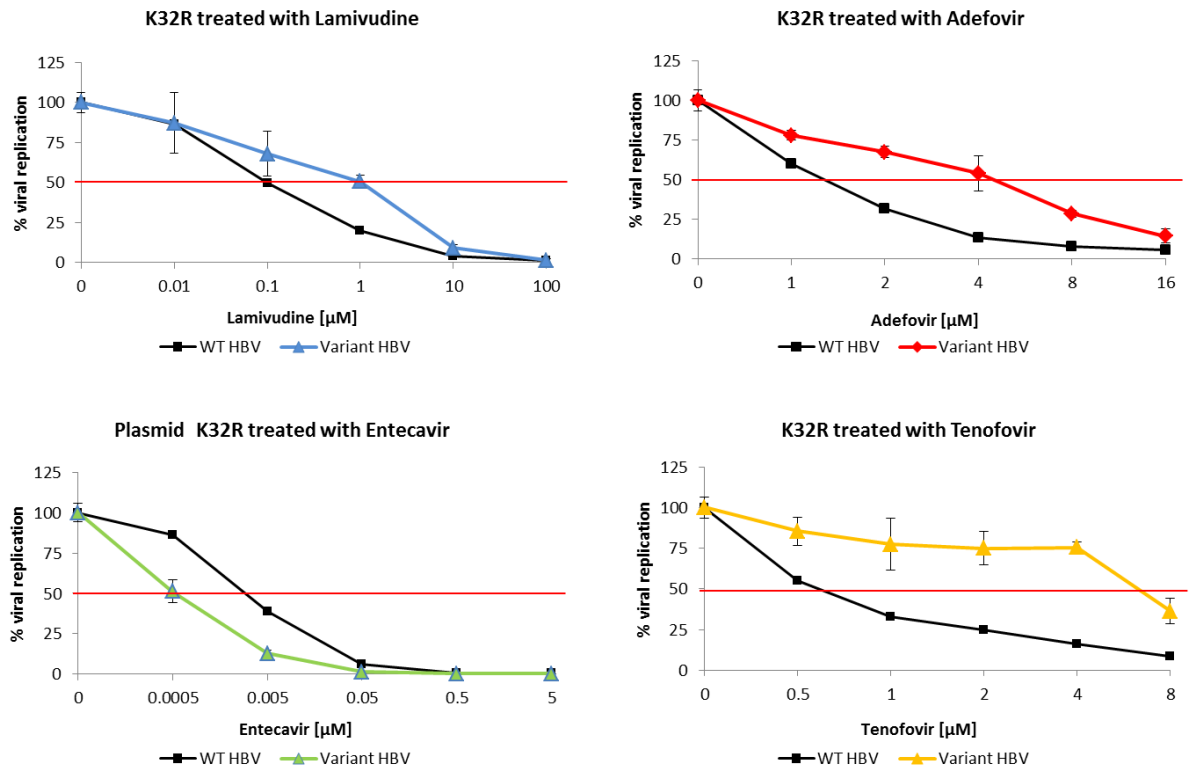


Figure 23: *In vitro* susceptibility of rtK32R to four drugs. HuH-7 cells were transfected with wild-type (WT) or rtK32R HBV expressing construct and treated with lamivudine, adefovir, entecavir or tenofovir in different concentrations as indicated. The percentage of HBV DNA production is based on the HBV DNA concentration values as measured by selective qPCR in comparison with the level of HBV DNA in the absence of drug. Dose–inhibition curves for the two HBV strains were used to estimate the IC_{50} values for each drug.

The rtA181T and rtN236T also conferred reduced susceptibility to lamivudine, adefovir and tenofovir in different levels as determined above. By combination, double mutation rtK32R+rtN236T further reduced the susceptibility to adefovir and tenofovir to ever higher levels (36- and 40-fold). However, the combination of rtK32R with rtA181T did not induce the same effect (**Table 13**).

Table 13: Resistance factor of different rtK32R variants

Mutation	Resistance Factor			
	LAM	ETV	ADV	TDF
WT	1.0	1.0	1.0	1.0
rtK32R	10	0.16	3.5	11
rtK32R(gA)	12	0.25	4.0	8.5
rtK32R+rtA181T	35	0.33	2.2	9.0
rtK32R+rtN236T	25	0.20	36	40

gA, genotype A background; IC_{50} in µM.

For further characterization of rtK32R, its impacts on viral replication capacity and infectivity were evaluated *in vitro*. HBV real-time PCR quantification after normalized transient transfection in HuH-7 cells showed that rtK32R conferred a decreased replication capacity of 47% in comparison to the wild-type level. The combination with rtN236T even reduced the replication capacity down to approximately 4%, while rtA181T partially restored the replication capacity of rtK32R up to 81% in combination (**Figure 24**).

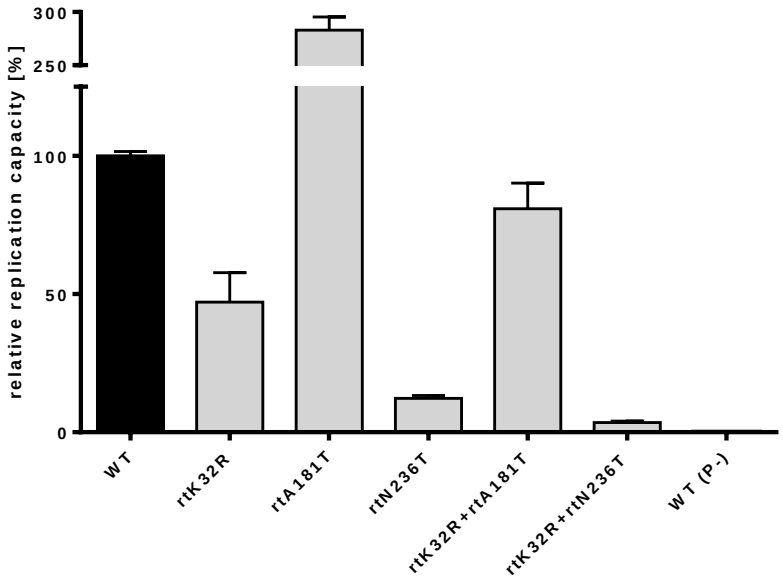


Figure 24: Replication capacity of different rtK32R variants. Relative replication capacity of rtK32R, rtA181T and rtN236T single and combination variants compared to wild-type vilevel was determined after normalized transfection of replication competent plasmids into HuH-7 cells. HBV plasmid WT (P^-) containing a polymerase knockout was used as a non-replicating control.

The introduction of rtK32R in the HBV polymerase simultaneously induced a sR24G mutation in the overlapping S gene. Infection of HepaRG cells using purified recombinant HBV showed that rtK32R/sR24G conferred decreased infectivity in comparison to wild type. The efficiency of viral entry, determined by measuring the established cccDNA after infection, was reduced to 25% compared to wild type, while viral replication and virion secretion measured by intracellular or extracellular rcDNA qPCR was further reduced to 18% and 12%, respectively (**Figure 25**). These results demonstrated a strong impact of the simultaneous sR24G mutation on viral fitness during infection in addition to the replication loss resulting from rtK32R.

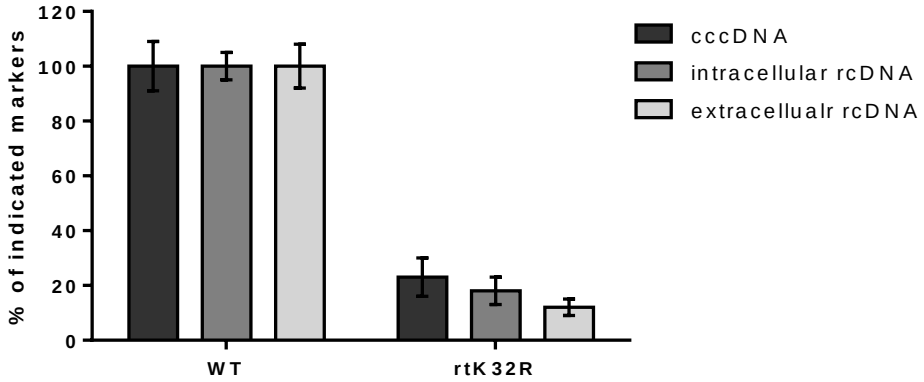


Figure 25: Infectivity of rtK32R variant on HepaRG cells. HepaRG cells were infected with wild-type HBV or rtK32R variant at an MOI of 100 vp/cell. Ten days after infection, cccDNA, intracellular rcDNA and extracellular rcDNA were measured from cell lysates or supernatants.

3.3.2 Novel rtM204 mutations with reduced susceptibility to adefovir and tenofovir

The signature mutations in YMDD motif, rtM204I and rtM204V, are well-documented resistance determinants against lamivudine and entecavir, but not against adefovir and tenofovir. Meanwhile, further rtM204 mutations were reported in patients under adefovir or tenofovir treatment, but their clinical significance in resistance development remains unclear.

Literature mining and sequence databank screening were first performed and seven rtM204 variants were identified in patients with treatment failure or suboptimal response to adefovir or tenofovir (**Table 14**). In order to address the hypothesis that YMDD mutations also mediate resistance against antivirals other than lamivudine we therefore for the first time systematically analyzed the *in vitro* resistance pattern of YMDD mutations and the corresponding viral fitness.

Table 14: rtM204 mutations identified in literature mining

RT Mutation	Treatment	Patient status	Corresponding HBsAg mutation	Associated RT mutation	Reference
rtM204V	LAM | ADV TDF	Resistance Partial response	sI195M	rtL180M	[131, 132] [133]
rtM204I	LAM | ADV TDF	Resistance Partial response	sW196L	rtL180M/-	[132, 134] [133]
rtM204A	ADV	Resistance	sI195M+sW196R	-	[135]
rtM204K	LAM | ADV	No response	sW196R	-	[136]
rtM204L	TDF	Partial response	-	-	[137]
rtM204S	LAM	Resistance	sW196V	rtL180M	[138]
rtM204Q	ADV | TDF	Partial response	sW196R	rtA181T/-	[139]
rtM204T	ADV	Resistance	sW196R	rtI169T	[140]

-, none; |, treatment switch.

For an extensive evaluation, a series of rtM204 mutations (rtM204A/I/K/L/Q/S/T/V) were introduced into pCH-9/3091 by site-directed mutagenesis and phenotypic analysis were performed to characterize the resistance profiles of different rtM204 variants (**Table 15**). As expected, rtM204V/I displayed a 45-fold to more than 1000-fold resistance against lamivudine, consistent with previously published results. Interestingly, rtM204Q and rtM204K had an 80-fold and 500-fold higher resistances compared to wild type, respectively, while variants rtM204A/L/T and rtM204S showed a 10- to 20-fold resistance against this drug. Resistance against entecavir was moderate for M204I (30-fold) and relatively low for all other variants ranging from sensitivity to a 3-fold resistance compared to wild type. Similar results were obtained for phenotypic resistance against adefovir and tenofovir which were low to moderate for all tested variants (RF 1.3-10) but which was strikingly high for rtM204K with resistance factor above the measurable non-toxic dosage range of both drugs.

Table 15: *In vitro* drug susceptibilities of tested rtM204 variant.

Recombinant	LAM		ETV		ADV		TDF	
	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF	IC_{50}	RF
WT	0.1	1.0	0.003	1.0	1.3	1.0	0.6	1.0
rtM204I	>100	>1000	0.1	30	2.5	1.9	1.1	1.8
rtM204V	4.5	45	0.005	1.6	6.5	5.0	6.0	10
rtM204S	1.0	10	0.003	1.0	2.6	2.0	2.4	4.0
rtM204A	1.0	10	0.003	1.0	1.6	1.2	0.8	1.3
rtM204T	2.0	20	0.003	1.0	2.0	1.5	1.8	3.0
rtM204Q	8.0	80	0.003	1.0	2.5	1.8	2.5	4.0
rtM204L	1.0	10	0.002	0.5	5.2	4.0	4.5	7.5
rtM204K	50	500	0.005	1.8	>128	>100	>64	>100

RF, resistance factor; IC_{50} in µM.

This latter effect of multi-resistance conferred by the single point mutation rtM204K was shown more in detail in **Figure 26**. Dose-inhibition curves showing the viral replication in relation to increasing concentrations of antiviral drugs were presented for LAM, ETV, ADV and TDF, respectively; even with high doses of ADV or TDF, no significant inhibition of viral replication could be achieved.

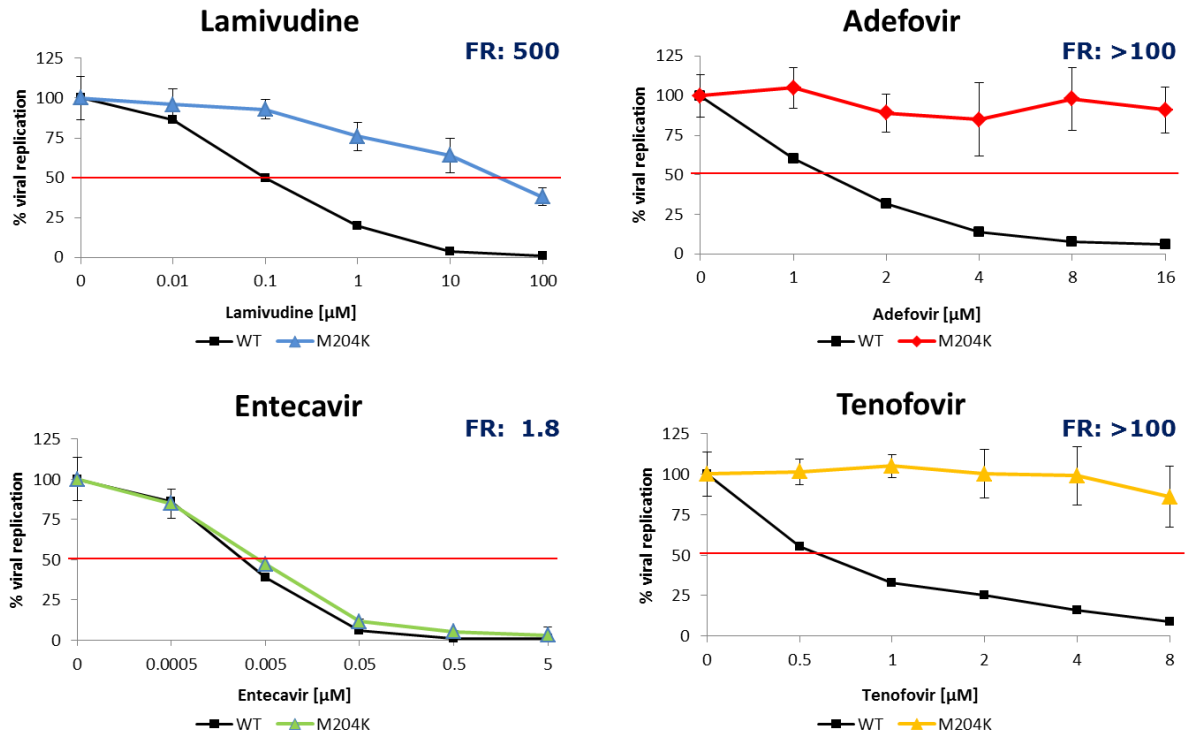


Figure 26: ***In vitro*** **susceptibility of rtM204K variant to four drugs.** Huh-7 cells were transfected with wild-type (WT) or rtM204K HBV plasmid and treated with lamivudine, adefovir, entecavir or tenofovir in different concentrations as indicated. The percentage of HBV DNA production is based on the comparison with the level of HBV DNA in the absence of drug. Dose–inhibition curves for the two HBV strains were used to estimate the IC_{50} values for each drug.

In order to evaluate the effect of the rtM204 mutations on viral fitness, normalized transient transfection in HuH-7 cells was performed to determine the replication capacity. To avoid the influence of corresponding mutation on HBsAg/virion secretion and detection, all rtM204 strains were further modified by introducing a premature stop codon into each L/M/S ORF (LMS-), and additionally cotransfected with a wild-type HBsAg construct for complementation. By using HBsAg quantification for normalization, relative replication of each variant was determined as percentage ratio against the wild-type strain which was set to 100% (**Figure 27**). Remarkably, the mutation rtM204K strongly impaired the viral replication capacity, while rtM204Q and rtM204L may slightly increase it. The remaining variants also displayed a significant reduction in their replication capacity although the effect was less pronounced than in case of the rtM204K.

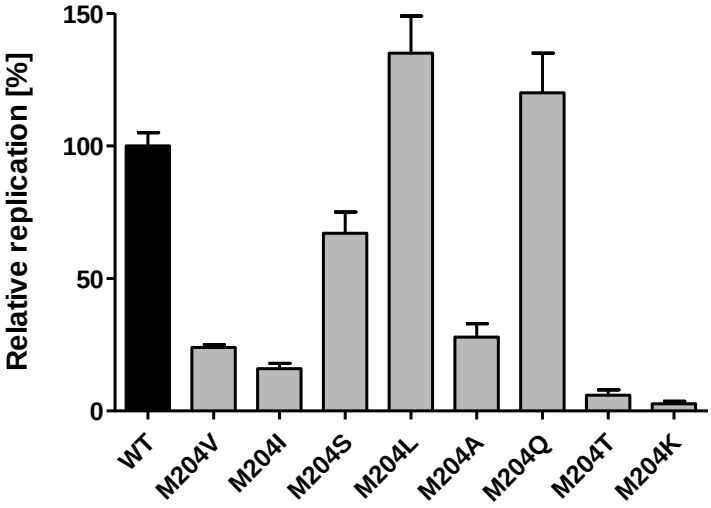


Figure 27: Replication capacity of rtM204 variants. Each rtM204 variant or wild-type HBV construct, all HBsAg knockout (LMS-), was co-transfected with a wild-type HBsAg expressing construct into HuH-7 cells. HBV DNA in supernatants were measured by qPCR and normalized by HBsAg quantification. Variant replication capacity was reported as the percentage ratio in comparison to wild-type replication (set to 100%).

Having observed the impaired replication capacity of the rtM204K variant the question was addressed to what extent known compensatory mutations, such as rtL180M or rtL80I, can restore the replication fitness of the rtM204K variant. Interestingly, our results indicate that neither the mutation rtL80I nor the mutation rtA181T were able to increase the viral replication capacity. Moreover, the mutation rtL180M even led to an almost complete deficit of the viral replication.

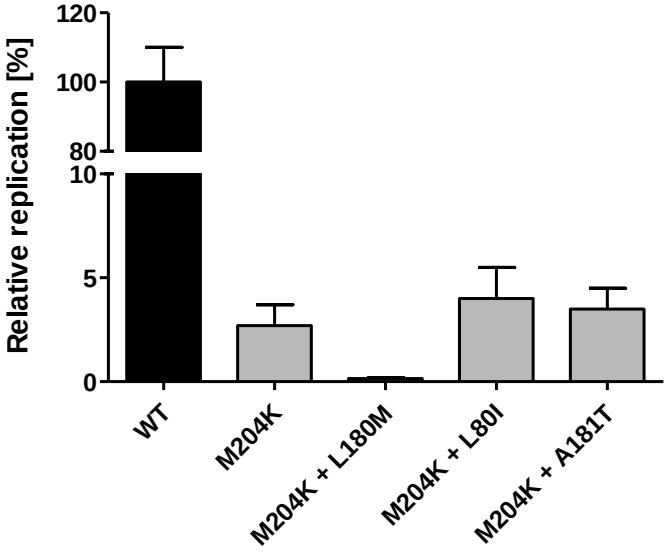


Figure 28: Impact of additional mutations on replication capacity of rtM204K. HBV variants containing rtM204K and its combinations with additional mutations (rtL80I, rtL180M or rtA181T), respectively, were tested for replication capacity and compared to wild-type HBV**.**

The rtM204 mutation may have a negative effect on the integrity of HBsAg structure which is encoded by an overlapping reading frame and is responsible for proper secretion of itself and viral particles. Therefore we analyzed whether the rtM204 mutations can affect the secretion due to the simultaneous change in the HBsAg.

As shown in **Figure 29**, no significant effect on the HBsAg secretion was observed for the variants rtM204V, rtM204I, rtM204S and rtM204L, while the secretion of HBsAg was severely blocked for the variants rtM204A, rtM204K, rtM204Q and rtM204T. All these 4 secretion-impaired rtM204 variants (A/K/Q/T) resulted in a sW196R substitution in the HBsAg. The substitution leads to an integration of a hydrophilic and positive-charged residue into a hydrophobic and nonpolar cluster. On the contrary, the unaffected rtM204 variants shared tolerable substitution of amino acids with similar properties.

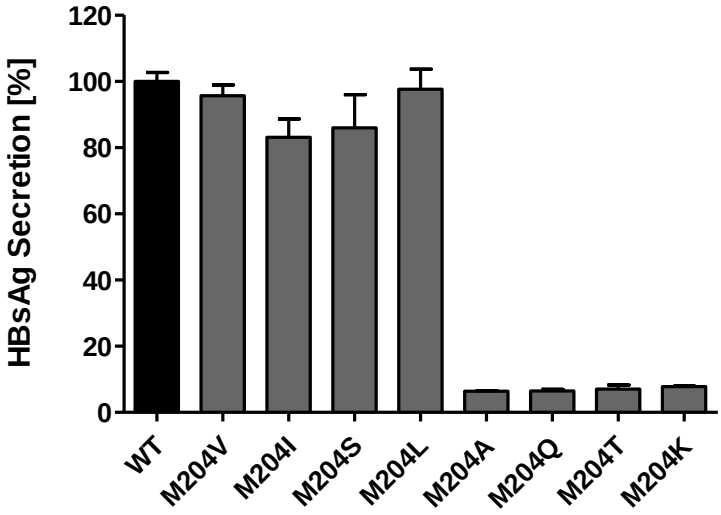


Figure 29: HBsAg secretion of different rtM204 variants. Each rtM204 variant plasmid was transfected into Huh-7 cells and the HBsAg in the supernatants were quantified using AxSYM HBsAg assay and compared to that of wild type.

HBV virion shares the similar secretory pathway as for HBsAg. The effect of sW196R on virion secretion was further examined by comparison of HBV particle production in the supernatant using wild-type, sW196R/rtM204Q and HBsAg knockout (LMS-)/rtM204Q constructs, respectively, for transfection followed by density gradient centrifugation to separate different HBV particles in different fractions. The real-time PCR quantification showed (**Figure 30**) that the wild type construct produced a large proportion of enveloped virion in lower density fractions (7-8) and a minor amount of nonenveloped DNA-containing capsid in higher density fractions (3-5). However, rtM204Q/sW196R only produced

nonenveloped DNA-containing capsids exactly to the same manner as rtM204Q (LMS-) did in the absence of HBsAg by nature (**Figure 30**). Therefore, sW196R variants induced by rtM204 mutation A/K/Q/T conferred a strong secretion defect for HBV virion as well as for HBsAg.

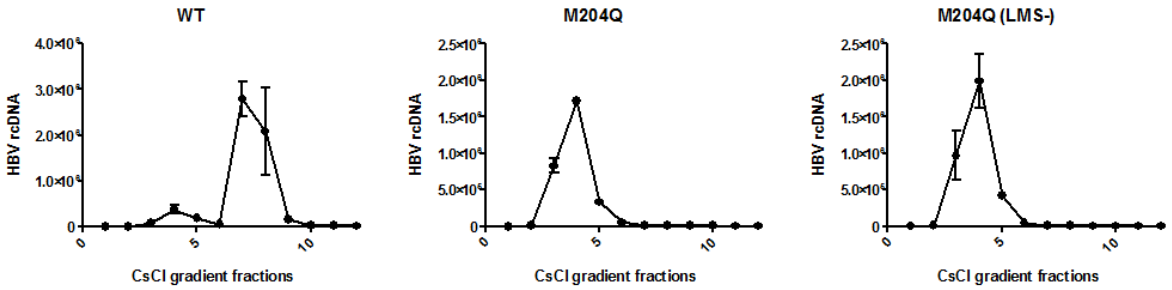


Figure 30: HBV particle production by wild-type and rtM204Q variants. Supernatants were collected from Huh-7 cells transfected with wild-type, rtM204Q or rtM204Q (LMS-) (HBsAg knockout), and submitted to CsCl density gradient centrifugation. HBV DNA from each fraction was measured by qPCR after purification.

In order to mimic the *in vivo* situation where wild-type and mutated virus commonly coexist in one cell, co-transfection of constructs encoding wild-type and rtM204K/sW196R with different knockout was performed to evaluate the trans-complementation effect for virion secretion. The results showed that the sW196R mutation conferred no effect on secretion of coexpressed wild-type HBsAg, but a dominant negative effect on HBV virion secretion when coexpressed with the wild-type HBsAg (**Figure 31**).

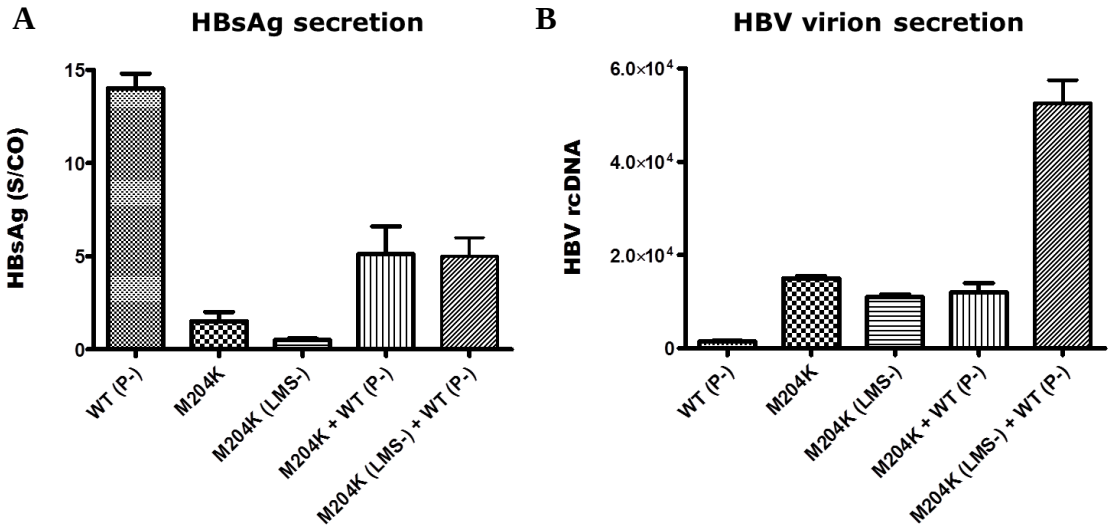


Figure 31: HBsAg and virion secretion in the setting of cotransfection. HuH-7 cells were transfected with WT (P-), rtM204K, rtM204K (LMS-), respectively, or co-transfected with WT (P-) + rtM204K or WT (P-) + rtM204K (LMS-) in a 1:1 ratio. HBsAg and HBV DNA in the supernatants were measured as described above.

In an *in vitro* infectivity assay based on infection of HepaRG cells the real viral fitness in a real infection status was analyzed. Most of rtM204 variants had a reduced or even eliminated infectivity with respect to the HBV markers, including cccDNA, HBeAg, intra- and extracellular rcDNA measured 10 days post inoculation (**Figure 32**). The only observed exception was rtM204L, which contained no corresponding HBsAg mutation and conferred comparable replication capacity to the wild type strain. On the contrary, despite the comparable replication capacity, rtM204Q representing rtM204A/K/T showed no infectivity at all probably due to the inability of producing mature virion with proper envelope.

In the infectivity assay, the cccDNA quantification correlated well with the HBeAg level, and the intra- and extracellular rcDNA levels also correlated closely with each other. In general, the cccDNA level represented the efficiency of viral entry which was largely affected by HBsAg mutations, while intra- and extracellular rcDNA levels represented the replication-coupled infectivity which was a combined effect determined by both HBsAg and polymerase substitutions.

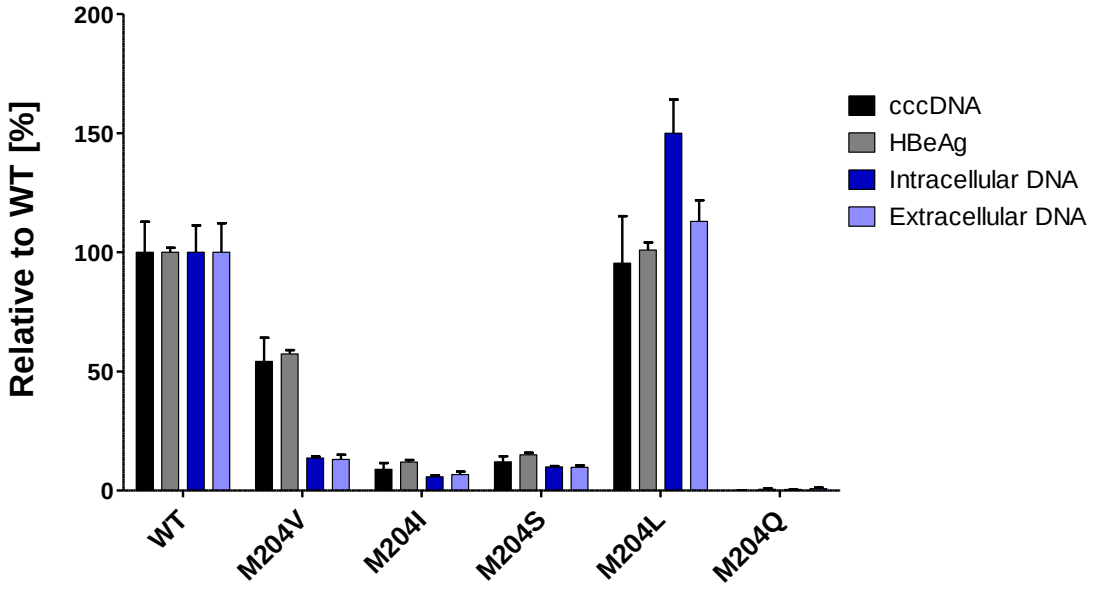


Figure 32: Infectivity test of rtM204 variant on HepaRG cells. HuH-7 cells were transfected with different mutation constructs, and the secreted virions were purified by heparin affinity column chromatography and concentrated by ultrafiltration. 10 days after infection of HepaRG cells, expression of different HBV markers were determined for each variant and compared to the wild-type virus.

4. Discussion

Antiviral drug resistance, the major hurdle to the success of chronic hepatitis B therapy with nucleos(t)ide analogues, is conferred by the selection of polymerase gene variants with reduced susceptibility to the applied drugs. Fundamental issues regarding antiviral resistance include the criteria for defining drug-resistant mutations and the method for diagnosing drug resistance. *In vitro* phenotypic assay remains the "gold standard" to confirm genotypic antiviral resistance [27]. Compared to the usage in the HIV field, phenotypic testing for HBV is still at its early stage and lacks a standardized platform so far. In particular, the conventional methodology is usually time consuming and labor intensive due to the missing of a convenient cell culture system, a specific and sensitive quantification method and the need to use specific HBV replication competent clones. In addition, multiple substitutions or sequences elsewhere in the HBV genome may influence the result. The lack of an efficient HBV phenotyping assay for analyzing virus clinical isolates had been a hindrance for drug resistance surveillance in nucleos(t)ide anti-HBV inhibitor clinical studies and for monitoring patients undergoing antiviral therapy. In this study, substantial progress in favor of high throughput analysis was made towards the clinical implementation.

4.1 Advantages and possible improvements of the new phenotypic assay

As a start point, the mutant genomes can be generated either by site-directed mutagenesis or by cloning of the naturally occurring variants. Also, depending on the methodology used in this study, the *in vitro* phenotype of either a single viral species or a mixture of species representing the natural quasispecies observed in patients can be determined.

For the cloning procedure, a combination strategy taking advantage of two previously described methods [98, 141] together with a self-developed one was established. Depending on the viral load in patient sample and the genetic information that needs to be covered, cloning of the full length HBV genome, the nearly whole polymerase gene (aa 8-811out of aa 1-832), or the entire HBV RT region (aa 1–344) , respectively, can be adapted. This flexibility allows the use in large-scale clinical trials. Given that amplifying and cloning of the entire HBV genome can be technically challenging, and the RT region alone lacks the potentially important information within the other polymerase domains, our self-developed method, whole polymerase cloning, represents a decent compromise/balance between technical feasibility and information capacity. It has a high PCR sensitivity similar to that of the RT cloning method, and in addition provides >97% the sequence content of the full polymerase

equivalent to >76% of the complete genome. Although all known resistance mutations reside within the RT region, evidences do exist indicating possible roles of mutations outside the RT in resistance development [142, 143]. Besides, this method could also be used for evaluation of new HBV drug candidates against polymerase such as RNaseH inhibitors [144] and the related possible resistance mutations. However, the BspEI restriction site used for cloning may occur more than once inside the polymerase gene specifically of genotype C, resulting in an improper HBV clone with a truncated sequence after digestion and insertion. Therefore, quality controls for the correct polymerase size were included to ensure a replication competent expression vector.

For mutation detection and capture, a two-step sequencing approach is applied as a standard procedure. In the first step, direct population sequencing will suffice for simple mutation patterns, but does not adequately describe linkage between different substitutions. Variant frequency in mixed populations is not proportionally represented by changes in drug susceptibility, possibly due to differing replication capacities [72, 133, 145]. Clonal analysis as the second step should be performed if resistance patterns are complex, multiple resistance pathways exist or population sequencing does not explain viral breakthrough. Firm conclusions regarding linkage of mutations must be based on multiclonal analysis and verified by sequencing of products/clones generated from multiple independent polymerase chain reactions. A minimum of 20 clones should be sequenced to collect and evaluate resistance mutations identified with population sequencing, resulting in a detection frequency of ~10-15% with a 90% CI. Sequencing of more clones might be necessary to characterize low-frequency variants, but meanwhile, could be time, labor, and cost intensive. Using the bioinformatics program QSVanalyser [146], the relative proportions of different variants present in the quasispecies can be inferred directly from the sequencing electropherograms, thereby significantly facilitating the estimation for multiclonal analysis. However, the early detection of resistance would require more sensitive and quantitative methods such as ultra-deep sequencing [70, 73, 147].

In the absence of a robust infectable cell culture system, replication and drug susceptibility assays are based on the transfection of hepatoma cell lines (e.g. HepG2 and HuH-7) that are able to replicate and secrete both enveloped and unenveloped nucleocapsids. A close comparison had been made, demonstrating the superiority of expression vector containing 1.1× fold overlength HBV genome driven by a strong mammalian promoter over the two other previously reported methods [96, 99]. This vector design ensures a constantly high level of pgRNA synthesis, the prerequisite for a higher level of viral DNA synthesis, to

facilitate the determination of drug susceptibility by standard procedures. Therefore, transfection of HuH-7 cells with a standard amount of pCH-HBV1.1 is a reproducible and efficient method to analyze the phenotypic status of HBV viral populations. Furthermore, using pCH-9/3091 with genotype D laboratory reference strain as a uniform backbone facilitates the downstream phenotypic analysis of cloned POL/RT from low replicating genotypes.

Another important issue with respect to the efficacy and sensitivity of phenotypic assays lies in the method used for detection and measurement of HBV DNA synthesis in transfected cells. Until recently, the method used to this end was Southern blot analysis [92, 99, 145]. This method is relatively insensitive, time consuming (3-4 weeks), fastidious to perform and requires large amounts of cell cultures. In addition, the use of radioactive material requires protective equipment and potentially dangerous handling procedures. As a result, it cannot be implemented into a clinical service activity such as large- scale phenotyping compatible with a therapeutic decision.

Real-time PCR is a superior alternative to measure the amount of intra- or extracellular HBV DNA. One of the big advantages of real-time PCR compared to Southern blot is the higher sensitivity resulting in a scale-down of the cell culture to 96-well plate format. This format is the requirement for a possible automatization of the method, which is more compatible with high throughput studies that include numerous samples. However, unambiguous quantification of newly synthesized HBV rcDNA in the presence of excess HBV plasmid DNA is not technically trivial, especially by normal real-time PCR approaches. Our highly selective qPCR strategy provides a much more convenient and efficient solution to overcome this major drawback than previously described methods. In contrast, other options including frequent medium changes [148], intensive DNase treatments [149], and specific capsid immunocapture [49] require much more extra handling and may still often be insufficient especially in case of dealing with low replicating genotypes (genotype A/G) or clones. On average, only 40%-60% of the isolated clones from patients were found to be competent for viral DNA synthesis in transfected cells [150], even less for certain genotypes with weak replication *in vitro*. Due to the high background coming from the residual input plasmid, those real-time PCR quantification are not able to accurately measure the drug susceptibility of clones that replicated at low levels (generally <10% of wild type), necessitating a prior selection for competent clones. Our selective qPCR gives a significantly higher sensitivity and is able to test the clones that replicated even <1% of wild type, which makes the analysis of complex mutation combinations or rare mutations feasible.

Furthermore, it can be used on either cell lysates or supernatants. It also removes the effects of overlapping HBsAg envelope changes, as HBV DNA from secreted enveloped and unenveloped nucleocapids are generally extracted and measured alike.

Unlike the normal HBV real-time PCR methods, the selective qPCR can also distinguish HBV rcDNA from single (minus) stranded DNA replication intermediates. Considering that the synthesis of single-stranded intermediates are affected and targeted differently from the synthesis of second (plus) strand and the consequent forming of rcDNA, cautions must be taken when evaluating the phenotypes simply by quantifying the HBV total DNA. For example, telbivudine and clevudine preferentially inhibit HBV second strand (DNA-dependent) compared to first strand (RNA-dependent) DNA synthesis, which is preferentially inhibited by lamivudine [92, 151, 152]. Meanwhile, HBV mutations may have different impacts on DNA synthesis of different strands. More importantly, several resistance mutations such as rtM250V/L decrease drug susceptibility in a template-dependent manner [153]. Reduced susceptibility conferred by those mutations was found to be manifested primarily during RNA-directed minus strand DNA synthesis, as the IC_{50} for the first (minus) strand was significantly increased relative to that for the second (plus) strand. In each case, the preferential inhibition of the second-strand DNA synthesis results in an overproportion of the single-stranded intermediates finally leading to an overestimation of the resistance and replication phenotypes if the normal real-time PCR for total HBV DNA is applied. Altogether, the selective qPCR, specifically amplifying HBV rcDNA, enables a more convenient and highly accurate measurement for phenotypic testing.

HBV DNA extraction from cultured cells and/or supernatants for real-time PCR analysis using commercially available kits is technically robust and easy to establish. In this study, an automated DNA extraction optimized on a Tecan robotic platform significantly increases the throughput with a consistent and reliable performance. However, the relatively high costs of kits with consumables for numerous samples and especially the need of expensive robotic system largely limit the use for a routine test in normal laboratories or in a resource-limited region. A rapid and simple method for extracting HBV DNA in a more cost-efficient way is under development and would further extend the practicality of our new phenotypic assay.

4.2 HBx-deficient vector for potential escape variants

During phenotypic and viral fitness analysis, a considerable amount of recombinant virus is produced and handled normally in a biosafety level (BSL) 2 environment. However, some special variants, especially vaccine-escape strains, require a higher biosafety level due to the

reduced efficacy of protective antibody. Indeed, vaccine escape mutations such as sP120T and sG145R in combination with resistance mutations are frequently selected in HBV monoinfected patients following antiviral treatment [154]. Moreover, some of these resistance mutations have also been shown to induce reduced antigenicity of the overlapping HBsAg. It was shown that common lamivudine resistant substitutions (rtM204V/sI195M, rtM204I/sW196S/L and rtV173L/sE164D + rtL180M + rtM204V/sI195M) resulted in reductions in the reactivity of the altered HBsAg with vaccine-induced antibody against HBsAg. In particular, the triple mutational pattern (rtV173L+ rtL180M+rtM204V) causes two amino acid changes in the overlapping surface gene (sE164D+sI195M) which reduce anti-HBs binding to levels seen only with the vaccine escape mutant sG145R [155]. These results have been independently confirmed using different *in vitro* models [156], and further extended to *in vivo* analyses. In a chimpanzee model, the triple mutation variant, rtV173L+rtL180M+rtM204V, which displays sE164D+sI195M in HBsAg, successfully infected hepatitis B-immunized chimpanzees that carried high titers of circulating anti-HBs pre-challenge [157]. All these results certainly raise an inconvenient biosafety issue regarding the phenotypic testing of those escape-competent variants. To avoid the need for BSL3 facilities, which are less frequent than BSL2 laboratories and cause higher running costs, without reducing the safety we used an attenuated, HBx-deficient HBV vector for phenotypic and infectivity assay.

Many studies have indicated the importance of HBx in the HBV life cycle using different models. In the context of human HBV infection it was demonstrated very recently using human hepatocyte chimeric mice [158] and relevant cellular models of HBV infection [127]. Indeed, HBx deficient HBV developed measurable viremia only in HBx-expressing livers. In addition, it was shown that HBx-deficient HBV genomes are strongly attenuated for HBV replication using HBV transgenic mice [159], hydrodynamically-injected mice [160], and/or cell culture models [160, 161]. Interestingly, HBx-deficiency had little effect on HBV replication in HBV 1.1×unit plasmid-transfected human hepatoma HuH-7 cells (**Figure 19**). Taken together, HBx is essential to initiate and constantly required to maintain productive HBV infection. HBx-deficient viral vector cannot initiate and maintain viral gene expression and DNA replication after infection, therefore is strongly attenuated *in vitro* and *in vivo*, but can still be used in transfection-based phenotypic and fitness testing, which can be performed in a routine BSL2 laboratory.

Given that HBx-deficient virus is still capable of forming cccDNA, the reservoir for persistent infection which may allow genotypic reversion to wild type as observed in the

woodchuck model [162], two individual premature stop codons were introduced into the HBx ORF to minimize the possibility. On the other hand, the attenuated virus should be capable of inducing protective immunity after accidental infection and quickly eliminated from infection by existing or induced immune responses.

A notable alternative strategy for a better biosafety would be using a Pre-S1 knockout vector, which produces no infectious virions due to the lack of L protein but nonenveloped DNA-containing capsids by transfection. This is a totally safe system even for BSL1 laboratory, phenotypic and replication capacity tests can still be performed by quantifying intracellular DNA. However, no newly produced virus in cell supernatant or infectivity can be determined, whereas, for HBx-deficient vector, a complete phenotypic characterization of the variant by using an HBx-expressing HepaRG cell line for trans-complementation is possible [127]. Therefore, only the HBx-deficient vector was used in this study.

4.3 Complex resistance mutations and potential interactions

Potent antiviral with a high resistance barrier, such as entecavir or tenofovir, as a first-line therapy has revolutionized the clinical management of chronic hepatitis B. However, long term treatment, suboptimal dosing or preexisting mutations, can result in the selection of HBV variants with several complex mutation patterns [139, 163, 164]. A more complex scenario may emerge during unsuccessful sequential or combination therapies and substantially confers a multi-drug resistant phenotype [35, 165-168]. Extensive analysis of these complex resistance mutations is of high importance to design and develop efficient rescue or combination therapies in the clinical context of insufficient entecavir or tenofovir response. Several individual studies have been conducted with focus on the major clinical isolates but are largely limited by small sample sizes. These results cannot be directly compared and seamlessly integrated for further analysis due to the non-standardized assays performed in different studies. A more comprehensive evaluation of entecavir resistance mutations was recently performed using a uniform platform for all possible substitutions at position rtT184, rtS202 or rtM250, respectively, in the rtL180M+rtM204V lamivudine resistance HBV backbone [49]. However, only single substitutions at these three positions were evaluated for their susceptibility to entecavir, without further assessment on additional combinations and cross-resistance profiles.

In our study, the effects of single mutations and their combinations were carefully assessed in proper genetic context so that potential interactions with co-existing mutations are taken into account. In total, eight codons in HBV polymerase are thus associated with primary

drug resistance to nucleos(t)ide analogues: rtI169, rtL180, rtA181, rtT184, rtS202, rtM204, rtN236, rtM250. We could confirm that resistance to lamivudine can arise from a single substitution at position rtM204 in the YMDD motif, the active site loop of HBV polymerase. A secondary change at position rtL180 further increases the lamivudine resistance as well as the replication capacity of the primary variant. The significant fitness gain by combination can well explain why rtM204V mutation is always accompanied by rtL180M [94, 169]. This lamivudine resistant backbone confers only low-level reduced susceptibility to entecavir, consistent with the clinical observations that entecavir is still active against lamivudine resistant HBV but with a reduced efficacy [170]. Within this signature mutation backbone, additional substitutions at specific positions (rtI169T, rtV173L, rtT184G, rtS202I/G, rtM250V), which have been regarded as entecavir resistance associated mutations as summarized from previously published data [26], display different replication and resistance phenotypes. These results can be explained at least partially by molecular modeling of HBV RT [153, 171], which predicts a decreased recognition and binding of entecavir- triphosphate as well as the natural dGTP substrate by these resistant HBV polymerase mutants, albeit to different extents. Notably, inconsistent with the current hypothesis that rtI169T mainly confers an ancillary or adaptive change analogous to substitution rtV173L for compensation, our results can demonstrate its dual effects of significantly increasing both resistance and replication phenotypes on the lamivudine resistance backbone. This entecavir resistance determining effect of rtI169T can be further enhanced when additionally combined with rtS202I/G but not with rtT184G or rtM250V, implying a position specific interaction between residues affecting the active binding pocket. Along the line, interaction between rtM250V and rtT184G or rtS202I/G resulting in a strong negative effect suggests an incompatibility of these mutations within the limited resistant pocket scaffold. These results indicate that the strength of resistance is not necessarily based on the number of resistance mutations combined in one genome, but moreover, is the result of a complex interaction between the occurring mutations and needs to be evaluated to allow valuable predictions. Besides, our new collection and further understanding of potential mutation interactions provide an optimized framework for refining existing HBV RT models towards a predictable three-dimensional illustration.

Genotypic resistance mutations to tenofovir remain to be identified. Adefovir resistance mutations rtA181T/V and rtN236T confer low-level reduced susceptibility to tenofovir. The combinations rtA181T/V+rtN236T have been associated with insufficient response to tenofovir treatment [46, 48]. In this study, several combinations have been identified with even higher resistance levels to adefovir/tenofovir such as rtL180M+rtA181V+rtN236T,

rtI169T+rtM204V+rtN236T, and rtK32R+rtN236T. These mutation combinations also confer interesting cross-resistance profiles to lamivudine, indicating the possibility of being selected during long-term combination therapy despite the relatively high genetic barrier. Tenofovir plus entecavir should be considered in this case for an efficient viral inhibition. On the other hand, entecavir resistance mutations commonly confer low-level reduced susceptibility to tenofovir while the subsequent introduction of additional rtA181T/V or rtN236T does not further increase the resistance to tenofovir significantly but may reduce its resistance to entecavir. This result suggests that using tenofovir as rescue therapy in entecavir resistant patients is probably sufficient to suppress the mutated HBV and to prevent further selection of multi-drug resistant variants.

4.4 Impact of newly identified potential resistance mutations

The selection and emergence of drug resistance mutations during long-term therapy with nucleos(t)ide analogues was believed almost inevitable because of the high adaptability and the quasispecies nature of HBV, based on the experience gained from HIV. Surprisingly, long-term follow-up studies of HBV resistance with 2 new drugs, entecavir and tenofovir, showed incredibly low cumulative resistance rates in treatment-naive patients (**Table 4**). Although several entecavir resistance-associated mutations have been identified, the resistance mutations to tenofovir remain controversial. It has been reported that the rtA194T mutation is associated with TDF resistance in two HBV-HIV coinfected patients [172], which was supported by *in vitro* drug susceptibility analysis [173]. However, several subsequent reports failed to confirm this finding [174, 175], as did ours. Indeed, no resistance to tenofovir was detected following up to 5 years of treatment in a recent trial indicating a high genetic barrier for tenofovir resistance [36]. To date, no selection of HBV polymerase gene mutations associated with breakthrough of HBV replication during long-term tenofovir treatment has been reported in any large-scale trials. On the other hand, due to high structure similarity, tenofovir shares a similar resistance profile with adefovir *in vitro* and *in vivo*. The adefovir resistance mutations rtA181T/V and rtN236T also confer low level reduced susceptibility to tenofovir. However, clinical data suggested that tenofovir is still active against those variants, probably due to a much higher therapeutic dose of 300 mg per day as compared to a dose of 10 mg per day for adefovir [48, 139], which may cause toxicity at substantially higher doses (data on file, Gilead Sciences).

The sequence, structure and functional similarity between HBV RT and HIV RT provides a new spotlight on tenofovir resistance, since tenofovir was initially developed and approved

for HIV as a first-line antiretroviral drug with more resistance information available [176]. Several comparative approaches have been applies to identify potential tenofovir resistance mutations for HBV based on the known crystal structure of HIV RT complexed with DNA template and tenofovir in the active site [177, 178]. Notably, during HIV therapy, tenofovir can select the K65R resistance mutation in HIV RT conferring 2-5 fold reduced susceptibility resulting in treatment failure [179]. Using our new phenotypic assay, we have successfully shown that the HBV mutation rtK32R homologous/analogous to the K65R in HIV RT confer >10 fold reduced susceptibility to tenofovir, a considerable discrimination of inhibitor from natural substrate.

So far rtK32R has not been reported in patients treated with adefovir or tenofovir. One of the possible reasons could be that the position rtK32 is far from the hotspots of known resistance mutations between rt160-rt250 of HBV RT. Therefore, it might be largely overlooked in routine practice or even completely omitted in diagnosis using the only commercially available sequence-based HBV genotypic resistance test, which just covers rt99-rt280 (Trugene HBV Genotyping Kit, Siemens Medical Solutions Diagnostics). On the other hand, the intrinsic characters of rtK32R mutation being largely attenuated in replication and infection may also limit its selection *in vivo*.

The novel rtM204 mutations identified in this study present another surprising aspect concerning potential tenofovir resistance. The classical substitutions in the YMDD motif at position rtM204 have seldom been associated with resistance to adefovir or tenofovir and typically seen as fully sensitive to these two drugs. However, extensive analysis of rtM204 variants revealed a complication of resistant profiles. Among them, rtM204K confers the highest resistance level to adefovir/tenofovir but severely impaired replication capacity, while rtM204L/Q confers low resistance level with highly favored replication capacity. Notably, patients with rtM204L/Q showed a persistent detectable viremia during long-term tenofovir treatment similar to those tenofovir treated patients with adefovir resistance mutations rtA181T/V and/or rtN236T, suggesting a partial/delayed response to tenofovir, whereas no virological breakthrough was observed [46, 48, 137, 139]. These clinical observations indicated that HBV variants with reduced susceptibility to tenofovir may further become selected during tenofovir treatment, retain the persistent viral replication at low level without rebound/breakthrough due to the incomplete resistance or impaired replication capacity. Despite this, the clinical significance shouldn’t be overlooked due to the potential selection of additional resistance mutations or compensatory mutations, which may restore replication

fitness. Therefore, a combination therapy consisting of tenofovir and a nucleoside analogue (lamivudine, entecavir or emtricitabine) might be the rationale for treating those patients.

4.5 Importance of viral fitness in resistance development

The mechanism of emergence of specific mutant strains in the viral quasispecies during treatment is crucial to the understanding of resistance. Generally, if a particular viral strain can emerge in the quasispecies within a particular environment, it is most likely because its fitness has become superior to other strains. The rapidity of selection of drug-resistant variants depends on their replication capacity and fitness, their level of resistance, and free liver space available for infection by these variants [180]. This might partially explain the differences in the rate of resistance for the different drugs that are clinically available.

In a simple way, viral fitness can be defined as the sum of parameters that quantify the adaptation of a viral strain to a given environment. The virological parameters of HBV fitness are the capacity of a strain to synthesize its genome, to produce infectious particles and to (re)infect cells (enter and deliver the genome to nucleus) [57]. Cell culture systems or animal models capable of full replication and propagation of the virus are necessary to properly measure the fitness of a viral strain. However, such models do not exist for HBV. In this study as well as most of the others, the 'viral fitness' was narrowly seen as the replication capacity of a given strain, as compared with a reference or wild-type viral strain, and was measured within the phenotypic assay (or independently) based on transfection of hepatoma cells. Besides, the infectivity could be determined by infection of HepaRG cells for selected variants individually. The results might be markedly influenced by system dependent factors such as cell lines used and time frames applied [181], and therefore might not fully reflect the real status *in vivo.* For example, rtA181T and rtV173L+rtL180M+rtM204V showed significantly higher replication capacity in comparison to wild type *in vitro*, inconsistent with the *in vivo* findings that resistant strains did not replicate as well as their wild-type counterparts in the liver. But still, the relative high replication fitness of these two variants is crucial to their prevalence in patients even without antiviral selection pressure, indicating a strong clinical relevance. On the other side, the variant rtL180M+rtS202I+rtM204V with severely impaired replication capacity is rarely observed in entecavir resistant patients while rtL180M+rtS202G+rtM204V with similar resistance factor but better replication fitness can be commonly detected during virological breakthrough [50]. This replication fitness dependence of resistance selection may also explain the absence of rtK32R+rtN236T or rtM204K in common diagnosis.

Fitness selection can be more complex and profound *in vivo*. An interesting example is rtM204L, which has a high level replication capacity as well as an unaffected infectivity, similar to wild-type strain *in vitro*. Although rtM204L confers reduced susceptibility to lamivudine, adefovir and tenofovir without fitness cost *in vitro*, its low prevalence and frequency in patients indicate an 'invisible hand' limiting the turnover. A recent study by immune epitope analysis *in vivo* showed that rtM204L enhances the HLA binding affinity and induces significantly stronger interferon-γ producing CTL response while rtM204V/I have the ability to escape from host immune surveillance by decreasing their immunogenicity [182]. Despite the need for further investigations, this provides a sound example of virus-host interaction as well as its potential impact on viral fitness in resistance development.

4.6 Clinical significance of resistance mutations associated with secretion defect

During antiviral therapy, it has been observed that not only polymerase gene mutations but also S gene mutations are selected [53, 54, 183], mainly due to the overlap of the two ORFs. Some of these S gene mutations lead to major alterations of the antigenicity of HBsAg as discussed previously, while others may even severely affect the integrity and/or intrinsic properties of HBsAg. For example, rtS87T, rtA181T and rtM204I lead to sC69*, sW172* and sW196* (dependent on codon usage) nonsense mutations in the S gene, which generate C-terminal truncated forms of HBsAg. Among them, rtA181T/sW172* has been relatively well characterized, which is of particular importance because it confers cross-resistant to most antivirals at different levels. *In vitro* experiments indicated that the rtA181T/sW172* variant confers a dominant negative effect on the secretion of HBsAg and viral particles [55], consistent with the clinical observation in a hepatoma patient infected with rtA181T/sW172* variant showing retention of HBsAg in the hepatocytes [184]. A potentially hazardous effect of this variant is that intracellular retention of HBsAg could generate significant endoplasmic reticulum (ER) stress. This leads to continuous liver cell damage and regeneration and could on the long run favor HCC [185]. Moreover, the intracellularly retained L and M proteins due to C-terminal truncation have been implicated in the progression to HCC as they possess transactivational activity [186]. A molecular mechanism for the transcriptional transactivation function has been described, and is dependent on the cytoplasmic localization of the pre-S2 with an increased oncogenic potential [187].

We have shown that antiviral treatment using lamivudine, adefovir or tenofovir may result in the emergence of HBV variants encoding rtM204/sW196R, typically as a mixed population with wild-type virus. The relevance of the rtM204/sW196R mutations in the

clinical situation may be more than reduced sensitivity to antivirals. These variants did not secrete HBsAg due to a tryptophan-to-arginine substitution at codon 196 of the small S protein, resulting in a positively charged, hydrophilic amino acid (R) inside the C-terminal hydrophobic domain [188]. Interestingly, these variants containing the same sW196R mutation also have a defect in secretion of viral particle with intracellular retention of surface proteins, and exert a dominant negative effect on wild-type virion secretion, similar to what has been previously observed on rtA181T/sW172*. Likely, due to this secretory defect, selection of those mutations may result in a progressive and slow increase of viral load, hindering their detection especially at early time point [189]. This non-classical viral rebound could also mask the diagnosis of drug resistance if only viral load is used as the sole criterion for diagnosing viral breakthrough. This hypothesis is consistent with the clinical observations that most rtM204/sW196R variants are detected mainly at low frequency with small proportion in viral population.

Hence, during HBV replication, these secretion defective mutants may induce oncogenic transcriptional transactivation and, meanwhile, cause cell stress and damage by their intracellular retention which can trigger the unfolded protein response via ER stress. These effects in hepatocytes can consequently lead to hepatocellular carcinoma as reported for rtA181T/sW172* [190]. The clinical consequences of infection by these S gene variants demand further clarification and close monitoring is advised for those patients.

4.7 Further development and applications of resistance testing

With the development of new anti-HBV molecules, drug susceptibility assays becomes an important tool for the management of patients infected with resistant HBV isolates and forms a crucial component of resistance surveillance. A convenient phenotypic assay will also be of great value for the screening of novel antiviral compounds against viral strains circulating in the infected population and new drug resistant mutants that may emerge in the future. However, HBV phenotypic testing is still at its early stage compared to the usage in HIV field and most importantly lacks a convenient cell culture system or animal model capable of full replication and propagation of the virus.

A functional HBV receptor (NTCP/SLC10A1) has been recently identified and used for generating susceptible human hepatoma cell lines for HBV infection [7]. This breakthrough in HBV life cycle study together with several related findings has lightened the path towards a better infection model which may greatly facilitate the phenotypic and the fitness testing in a natural infection context. Once established, the infection model can also be applied for

screening anti-HBV molecules targeting not only the viral polymerase but the whole life cycle, analyzing the viral-host interaction for more drug targets, and eventually evaluating the new therapy by treatment monitoring. For resistance testing, this novel approach should be used together with our newly established assay whenever possible to gain a complementary knowledge of the specific variants.

5. Summary

This PhD project strived to establish and then apply a new phenotypic testing which easily connects the genotypic resistance with clinical resistance by identifying which HBV genetic variations cause clinical resistance, confer potential cross-resistance to other therapies and/or affect viral replication.

The thesis consists of three main parts. The first part was devoted to the development and standardization of the *in vitro* assay. A rapid PCR amplification of full length HBV genomes, polymerase or reverse transcriptase sequences, respectively, from patient material and cloning into replication competent HBV constructs for all HBV genotypes were designed and optimized successfully to achieve a high sensitivity. This ensures that, even with very low viral titer in patient serum (10^2 IU/mL), the downstream geno- and phenotyping can still be performed efficiently and reliably. The scalable transfection-based assay was adapted to a standardized high throughput 96-well cell culture format in which treatment with 4 drugs in 5 serial dilutions can be analyzed in triplicate at the same time in a very convenient way. To avoid the conventional Southern blot analysis, which is extremely time and labor intensive, a new quantification method using a sensitive, selective qPCR to determine HBV replication by measuring HBV progeny in the cell culture supernatant was successfully established. The selective primers can distinguish HBV rcDNA from input constructs with a selectivity of more than $1:10^{4.5}$, thus overcome the biggest hurdle for using qPCR for phenotypic testing. Overall, the new phenotypic assay is robust and easy to be embedded into clinical practice.

This new assay was, in the second part of the study and the thesis, validated and applied for testing of an extensive set of 100 HBV variants harboring most of the known resistance mutations individually or in combination, generated by site-directed mutagenesis or cloning from patient serum. The first batch of evaluation showed a good correlation and concordance of resistance factors measured to already published data. The whole set presented for the first time the big picture, how these mutations interact with each other in a complex context and what the substantial impacts are in resistance development, especially for entecavir and tenofovir.

Lastly, a great effort was made to identify and characterize potential novel resistance mutations. The phenotypic resistance to adefovir/tenofovir conferred by rtK32R as well as a series of novel rtM204 mutations, especially rtM204K, was clearly demonstrated but their association with impaired viral fitness and infectivity was also observed, respectively. The deep understanding of the different levels of selection pressure during resistance development

helped to evaluate the clinical significance of these mutations. On the other hand, the recently reported mutations, rtI233V found in adefovir resistant patients and rtA194T in tenofovir resistant patients were negated as genotypic resistance mutations, because of their full susceptibility to either drug they showed in phenotypic testing.

All data obtained have been integrated into a database to generate the geno2pheno HBV webserver for HBV mutation analysis. This will improve and ease the medication of chronic HBV infected patients to achieve fast reduction of HBV titers and to reduce treatment cost and adverse effect avoiding ineffective drugs.

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7. Abbreviations

µ	micro (10^{-6})
A	adenine
aa	amino acid
Ab	antibody
ADV	adefovir
ATP	adenosine 5′-triphosphate
bp	base pare
C	cytosine
CMV	cytomegalovirus
cccDNA	covalently closed circular DNA
ddH_2O	double distilled, deionized and filtered water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
dsDNA	double strand DNA
dNTP	desoxyribonucleosid-5´-Triphosphat
EDTA	ethylene diamine tetraacetat, sodium salt
ER	endoplasmatic reticulum
EtBr	ethidium bromide
EtOH	ethanol
ETV	entecavir
FCS	fetal calve serum
Fig.	figure
FTC	emtricitabine
g	gram
G	guanine
GM	gentamicin
h	hour
HBV	hepatitis B virus
HC	hydrocortisone
INS	insulin
IC50	half maximal inhibitory concentration

kb	kilo base pair
kDa	kilo dalton
l	litre
LAM	lamivudine
LdT	telbivudine
L-Glu	L-glutamine
m	meter, milli (10^{-3}),
M	molar
MESA	MOPS-EDTA-sodium acetate buffer
min	minute
MM	molar mass
MU	million units
Mvk	mevalonate kinase
n	nano (10^{-9})
N	nitrogen
NEAA	non-essential amino acids
OD	optic density
p	piko (10^{-12})
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF membrane	polyvinylidin-difluorid-membrane
P/S	penicillin/streptomycin
rcDNA	relaxed circular DNA: partial double-stranded HBV genome
RML	mouse adapted scrapie strain from the Rocky Mountain Laboratory
RNA	ribonucleic acid
RNase	ribonuclease
RPII	RNA polymerase II
rpm	rounds per minute
RT	reverse transcriptase
RT-PCR	reverse transcription-PCR
SP	sodium pyruvate
SDS	sodium dodecylsulfate

sec	second
Strep	streptomycin
T	thymine
TAE	Tris/ Acetate/ EDTA
Taq	Thermus aquaticus
TE	Tris/ EDTA
TDF	tenofovir disoproxil fumarate
TEMED	N,N,N´,N´-Tetramethylethylendiamine
TFV	tenofovir
Tris	tris(hydroxymethyl)aminomethane
U	unit
UV	ultraviolet
WME	William’s Medium E
% (v/v)	volume per volume
% (w/v)	weight pre volume

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