# CHARACTERIZATION OF HEPATITIS B VIRAL DRUG RESISTANCE IN INDIAN SUBCONTINENT PATIENTS WITH CHRONIC LIVER DISEASE

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*by*

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# CERTIFICATE

This is to certify that the thesis entitled **"Characterization of hepatitis B viral drug resistance in Indian subcontinent patients with chronic liver disease"** is based on the results of the work carried out by Mr. A. Mohamed Ismail for the Doctor of Philosophy degree under our supervision and guidance.

The candidate has independently reviewed the literature, standardized the methodology and carried out the techniques towards the completion of the thesis work.

This thesis has not been submitted for the award of any degree or diploma of any other university.

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# 1. INTRODUCTION

## 1. INTRODUCTION

Globally, an estimated two billion people are infected with hepatitis B virus (HBV) and around 350 million live with chronic infection [1]. Approximately 75% of these patients reside in the Asia-Pacific region, India harbouring the second largest pool of about 50 million chronic HBV carriers [2, 3]. About 15% to 40% of HBV infected subjects develop complications leading to cirrhosis, decompensated cirrhosis and hepatocellular carcinoma (HCC) contributing to over 1 million deaths per year [4, 5]. Thus HBV associated liver diseases is considered to be of public health importance, emphasizing the need for the prevention and control of disease progression.

Hepatitis B virus is a circular, partially double-stranded DNA virus of the family *Hepadnaviridae*. The virus is classified into 8 major genotypes and several subgenotypes with an intergenotypic diversity of 8% and intra-genotypic diversity of 4% respectively [6]. These genotypes have known to show a geographical pattern in their distribution and have been used to trace the migration of populations from geographically distant regions [7, 8]. The HBV strains are also distinguished into nine major subtypes based on their antigenic determinants in the major hydrophilic region [9]. Hepatitis B virus genotypes and subtypes have been reported to influence disease progression and treatment response [10-13]. Therefore, determination of HBV genotypes and subtypes is important for disease monitoring and clinical outcome.

Currently there are 7 approved therapies for HBV including 2 formulations of interferon, i.e., standard interferon alfa-2b (IFN- α-2b) and pegylated interferon alfa-2a (pegIFN-α-2a) and 5 nucleos(t)ide analogues, i.e., lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir. Interferons are immunomodulatory drugs that are administered for a finite period of treatment. Due to their adverse side-effects, subcutaneous injection and cost, nucleos(t)ide

analogues remains the drug of choice and are widely used in this country. The nucleos(t)ide analogues lack the 3′-hydroxyl group and the incorporation of these analogues prevent the formation of phosphodiester linkage that is essential for DNA elongation. It inhibits the enzymatic action of HBV reverse transcriptase (rt) and thus acts as a chain terminator of DNA synthesis [32].

The goal of antiviral treatment is to reduce the progression of disease. In patients diagnosed with chronic HBV infection, the aim is to prevent cirrhosis or progression of cirrhosis to decompensated liver disease and HCC. The progression of liver disease is prevented or delayed by the suppression of viral DNA. This is very well evidenced by the Risk Evaluation of Viral Load Elevation and Associated Liver Disease (REVEAL)-HBV study, which showed HBV DNA levels to be a strong predictor for the risk of disease progression [14]. Therefore, therapeutic monitoring of HBV DNA levels is critical in the management of HBV. In addition to HBV DNA, serum ALT levels are also useful in categorizing biochemical breakthrough and response [15-17].

Though there are several options for the treatment of chronic hepatitis B infection, management of HBV still remains a major challenge. Unlike IFN, nucleos(t)ide analogues require long term and continuous treatment. Over time, the virus evolves strategies to counteract the selection pressure and thereby escapes the antiviral action. Therefore, antiviral resistance is a clinically relevant issue in the therapeutic monitoring of patients with chronic hepatitis B.

Antiviral resistance is primarily mediated by mutations in the antiviral target sites thereby altering the drug interactory mechanism. Identification and characterization of these resistant mutations is important for appropriate tailoring of therapy and the design of newer drugs to challenge the resistant strains.

The typical mutations associated with lamivudine resistance are rtL80I, rtI169T, rtV173L, rtL180M, rtA181T/S, rtM204V/I and rtQ215S [18, 19]. The incidence of lamivudine resistance is reported to be 10-32% at 1 year and increases up to 69-80% after 5 years of therapy [20-24].

The primary adefovir-resistant mutations are rtN236T and rtA181T/V [25, 26]. The incidence of adefovir resistance is reported to be 3% at 24 months and increases up to 29% after 5 years of therapy [27]. Although adefovir is less effective than lamivudine, the rate of resistance is much lower and thus remains the suitable drug of choice for the long term treatment.

Entecavir resistance mutations require combinations of substitutions at positions rtI169T, rtL180M, rtT184G, rtS202I, rtM204V or rtM250V [28, 29]. The incidence of entecavir resistance is comparatively lower and studies have documented a resistance rate of 1.2% at 3 years of therapy [30]. Telbivudine resistance mutations are similar to those associated with lamivudine resistance rtM204I, rtL80I/V and rtL180M mutations [31]. The incidence of telbivudine resistance at the end of 2 years is up to 25% [32]. There are no conclusive reports of tenofovir resistance in HBV [33-36]. Since these are newly approved drugs the antiviral efficacy and resistance patterns are still evolving.

Computational methods like molecular modeling and docking studies have helped researchers to understand the structural features of protein, drug-protein interaction and the effect of resistance mutations and drug interaction [37-39]. Knowledge of HBV reverse transcriptase (HBVrt) structure would thus be valuable for understanding the molecular basis of drug resistance. Since there is a good sequence homology between the catalytic sites (A-G) of HBV and human immunodeficiency virus-1 (HIV-1) rt, the homology modeling of HBVrt is built using HIV-1rt template [40]. Molecular modeling has shown that rtM204V/I mutations conferred resistance to lamivudine due to steric hinderance that altered the binding

efficiency of lamivudine to the viral polymerase [18, 40]. Likewise, rtN236T adefovir resistance mutation is due to the disruption of hydrogen bonds between rtN236 and rtS85, rtN236 and adefovir diphosphate respectively [41]. Mutations at positions rtT184 and rtS202 were predicted to reduce the entecavir triphosphate binding pocket [42]. Together the *in silico* analyses of HBV polymerase model have brought out valuable information that might help in the development of newer antiviral strategies.

The frequency of rtM204I/V mutations in lamivudine-experienced Indian patients was found to be 6% at month 12 and 29% at 18 months [43]. Data on additional patterns of resistance mutations associated with lamivudine therapy is not available. So far the antiviral efficacy of adefovir and entecavir therapy and the resistance mutations associated with treatment failure have not been addressed in this population. The evolutionary rate of HBV is high and there are several reports of prevailing HBV genotypes and subtypes. Therefore, determination of HBV genotypes and subtypes might reveal newer circulating strains from geographically distant regions. Moreover, knowledge of HBV genotypes and subtypes and studying their association with treatment response to the available drugs will help in the successful management of HBV.

In this study, it was attempted to do an extensive sequence analysis covering the entire reverse transcriptase domain to identify all the possible mutations that might affect the antiviral efficacy of three major drugs i.e., lamivudine, adefovir and entecavir. Since these drugs have been available over a period of 6-13 years, the transmission of antiviral resistant mutants from the treatment failure patients is possible. Therefore the presence of antiviral resistant mutations in treatment-naive subjects was also studied to identify if baseline monitoring of HBVrt sequence is a requisite before initiation of therapy. The three-dimensional (3D) polymerase model of HBV can assist in understanding the interactions

between HBV polymerase and the antiviral agent. The structure-based approach was also explored to predict the effect of antiviral resistance mutations against the currently used anti-HBV drugs.

**Hypothesis**

This thesis embodies the testing of the following hypothesis:

1. Lamivudine resistant hepatitis B virus infection is associated with mutations in the reverse transcriptase (rt) region of the viral polymerase gene in Indian subcontinent patients

2. Mutations occurring in the B and D domain of the HBV polymerase gene is associated with resistance to adefovir dipivoxil in Indian subcontinent patients

3. Certain mutational patterns in the rt region of the polymerase gene confers resistance to entecavir in HBV infected Indian subcontinent patients

4. Molecular modeling and characterization of hepatitis B virus polymerase protein can reveal the mechanism of viral resistance to lamivudine, adefovir and entecavir

5. Indian subcontinent patients with chronic hepatitis B infection show a genotype-dependent response to antiviral therapy

6. Certain HBV subtypes in Indian subcontinent patients can influence the response to antiviral treatment

# 2. AIMS & OBJECTIVES

## 2. AIMS AND OBJECTIVES

### 2.1  Overall aim:

To detect the predominant mutations that confers drug resistance to HBV in Indian subcontinent patients

### 2.2 Specific Objectives:

1. To analyse the predominant mutations in the reverse transcriptase (rt) region of hepatitis B virus (HBV) polymerase gene associated with resistance to lamivudine therapy

2. To describe the predominant mutations occurring  in the B and D domain of the  HBV polymerase gene associated with resistance to adefovir dipivoxil

3. To determine the occurrence of variants in the rt region of HBV polymerase gene associated with resistance to entecavir therapy

4. To investigate the interactory mechanisms between HBV drug resistant mutations and antiviral agents such as lamivudine, adefovir and entecavir using molecular modeling

5. To investigate the role of HBV genotypes in  response to antiviral therapy

6. To study the influence of HBV subtypes on response to antiviral treatment among Indian subcontinent patients

# 3. REVIEW OF LITERATURE

## 3. REVIEW OF LITERATURE

### 3.1 Hepatitis B virus Discovery

Historically, two types of hepatitis transmission were classified: type A, transmitted by the faecal-oral route and type B, transmitted parenterally [44]. In 1937, Findlay and Maccallum documented cases of hepatitis in British soldiers injected with yellow fever vaccine containing human serum [45, 46]. The parenteral transmission of hepatitis was thus evidenced even before the identification of virus.

Decades later, on the search for identifying novel polymorphism in blood proteins, a mysterious protein from an Australian aborigine then named as Australia (Au) antigen was identified by Baruch Blumberg in 1967 [47]. Intriguingly, this Au antigen was more prevalent in patients with multiple transfusions than in healthy blood donors. This suggested that the antigen might be an infectious agent. Later in 1971, Dane *et al.* [48] using electron microscopy showed the presence of viral particles in serum and liver of hepatitis patient which provided the causal relation of the Au antigen and hepatitis. The accidental discovery of Au antigen thus significantly contributed in reducing the rate of post-transfusion hepatitis by the introduction of blood donors screening for the antigen.

Two years later, Blumberg and Irving Millman postulated that the non-infectious Au antigen particles from the plasma of hepatitis B virus (HBV) infected individuals can prevent the disease transmission [49, 50]. Thus the first cancer vaccine was developed which has prevented millions of HBV infection and its related liver cancer. Employing this strategy, current HBV vaccines are developed by genetic engineering.

The discovery of hepatitis B has helped in identification of other hepatitis viruses A, C, D and E and is the most important findings for hepatology research today [51].

## 3.2 Genomic organization of hepatitis B virus

HBV is a circular, partially double-stranded DNA virus of the family *Hepadnaviridae*. The genome size is between 3182 and 3221 nucleotides long and contains four open reading frames (ORF) [44, 52]. The genomic organization of HBV is shown **(Figure 1)** [53]. HBV genes overlap giving a compact genome structure. ORF P encodes the viral DNA polymerase, reverse transcriptase (rt) and other accessory proteins. The HBV reverse transcriptase (HBVrt) gene contains several regions that are homologous to other RNA-dependent polymerases and are designated as domains A to G [54, 55]. ORF S encodes for pre-S1, pre-S2 and hepatitis B surface antigen (HBsAg) and is completely located within the ORF P. ORF C codes for hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg). ORF X codes for the regulatory X protein. ORF C and ORF X overlap partially with ORF P [44]. The genome contains two directly repeated sequences, DR1 and DR2 at the viral (+) and (-) strands. The integration of HBV DNA occurs via these specific viral DNA sequences [56].

## 3.3 Clinical significance of HBV encoded proteins

Viral proteins of clinical importance include HBsAg, HBcAg and HBeAg. The first serological marker for HBV infection is HBsAg. It can be detected in 1-10 weeks after infection with HBV and 2-8 weeks before the onset of infection [57]. Persistence of serum HBsAg for more than 6 months is characterized as chronic infection [16].

Antibody to HBsAg (anti-HBs) is a protective antibody and appears in individuals who have resolved HBV infection. In addition, anti-HBs antibody is also produced in response to hepatitis B vaccination. The minimum anti-HBs titre of 10 mIU/mL is said to be protective against HBV infection [58].

**Figure 1. Genomic Organization of hepatitis B virus**
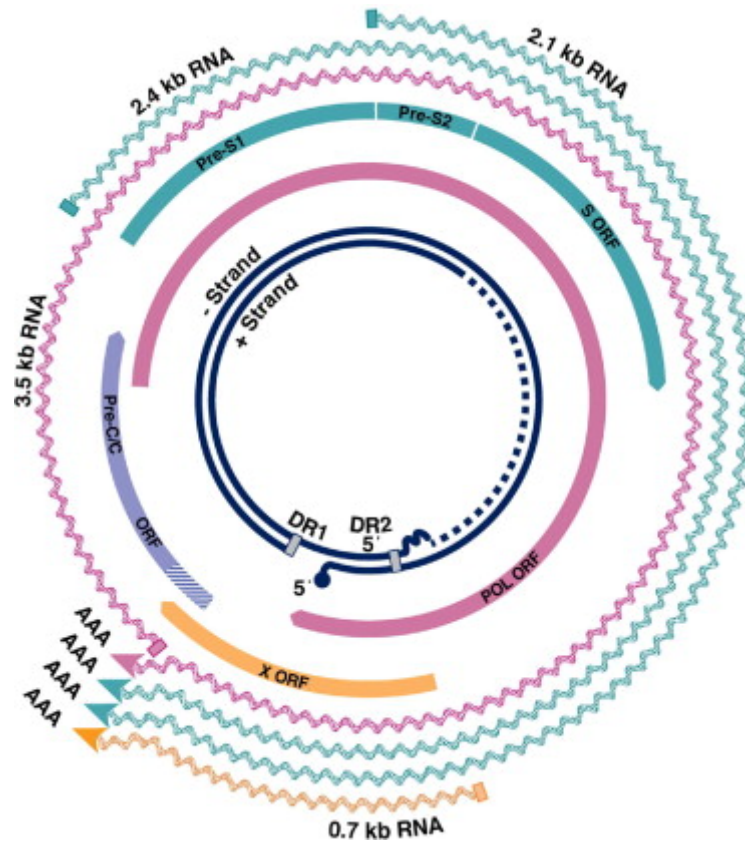


The inner cycle depicts the partially double-stranded DNA genome. Two directly repeated sequences (DR1 and DR2) are important for strand-specific synthesis. The four open reading frames (ORF) consist of the pre-core/core genes (ORF C), the polymerase gene (POL ORF), the pre-S1, pre-S2 and S genes (S ORF), and the X gene (X ORF). The outer circle shows the 4 major viral mRNAs, the 3.5-kilobase (kb) core or pre-genomic RNA, the 2.4-kb pre-S1 mRNA, 2.1-kb pre-S2/S mRNA and the 0.7-kb X mRNA. Figure adapted from Ghany and Liang [53].

HBcAg is associated with the intact virion and is not detectable in the serum. During acute HBV infection, the IgM antibody to HBcAg (IgM anti-HBc) appears 1-2 weeks after HBsAg and persists for 6 to 12 months and is the most sensitive antibody marker of acute HBV infection. The IgG anti-HBc gradually replaces IgM anti-HBc and persists life-long in individuals exposed to HBV infection [57].

HBeAg is a serological marker of active viral replication and is accompanied by high levels of HBV DNA. HBeAg persistence for more than 12 weeks indicates chronicity and early seroconversion with loss of HBeAg and development of antibodies to HBeAg (anti-HBe) indicates recovery [59].

### 3.4 Natural course of chronic hepatitis B infection

The natural course of chronic HBV infection is classified into four disease phases based upon ALT levels, HBeAg status, HBV DNA levels and host immune response **(Figure 2)** [60-62].

### 3.4.1 Immune tolerant phase

The immune tolerant phase also known as "HBeAg-positive chronic hepatitis" is characterized by the presence of HBeAg, high serum HBV DNA levels and normal levels of aminotransferases with mild or no liver injury.

### 3.4.2 Immune clearance phase

The immune clearance phase is characterized by the presence of HBeAg, decrease in serum HBV DNA levels, persistent or intermittently increased ALT levels and active inflammation of the liver. The ALT levels can flare to over five fold the upper limit of normal (ULN). This higher ALT levels is associated with heightened immune response and hepatocyte damage [63]. The immune clearance phase is also characterized by anti-HBe seroconversion which normally occurs at a rate of 2-20% per year [64-68].

**Figure 2. Natural course of chronic hepatitis B infection**
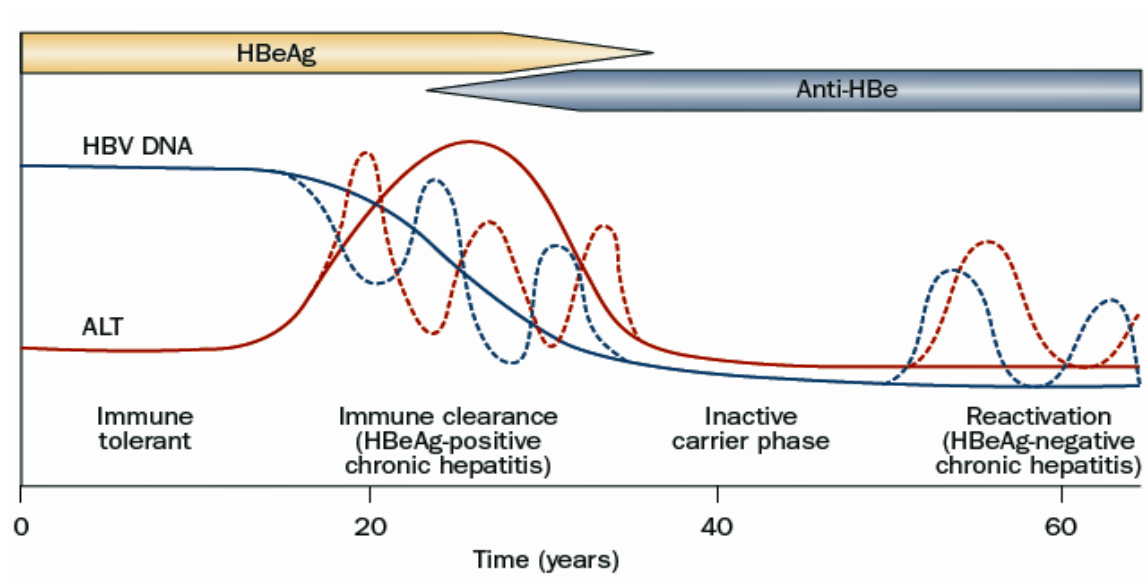


ALT - alanine aminotransferases, HBeAg - Hepatitis B e antigen, Anti-HBe - Hepatitis B e antibody. Figure adapted from Kwon and Lok [62].

### 3.4.3 Inactive HBV carrier phase

The inactive carrier phase is characterized by anti-HBe seroconversion, normal ALT levels and low or undetectable serum HBV DNA levels.

### 3.4.4 Reactivation phase

The reactivation phase also known as "HBeAg-negative chronic hepatitis" is characterized by anti-HBe seroconversion; periodic reactivation with fluctuating HBV DNA and ALT levels; and active inflammation in the liver. Most of the subjects in this phase of infection harbour HBV variants with pre-core or basal core promoter mutations that decreases the level of HBeAg synthesis [69].

### 3.5 HBV Replication

Hepatitis B virus replicates and infects hepatocytes, the major cells of the liver. The early events in viral replication involve attachment to the speculated heparin sulphate proteoglycans receptor [70, 71]. The pre-S1 segment of the HBV envelope protein was identified to be the cell surface receptor binding site [72, 73]. After entry, the virus uncoats and the relaxed circular DNA is transported to the nucleus. In the nucleus, host and viral polymerases repair the partial circular genome to double stranded covalently closed circular DNA (cccDNA). This cccDNA remains the stable form of viral DNA and act as a template for the transcription of messenger RNA's and pre-genomic RNA (pgRNA). All viral RNA's are transported to the cytoplasm. The messenger RNA translates the viral envelope proteins and the X protein. The pgRNA serves both as the template for reverse transcription of genomic DNA and for the translation of core and polymerase proteins [74].

Reverse transcription begins upon binding of viral reverse transcriptase (rt) enzyme to the encapsidation signal called epsilon or stem loop structure on the pre-genomic RNA [75]. This viral rt-epsilon complex serves as the primer and template for synthesis of DNA [76]. Viral

replication occurs after the binding of core proteins to form viral nucleocapsids. Reverse transcription of pgRNA results in negative-strand DNA synthesis and the RNA is degraded by the RNase H activity of viral polymerase, followed by positive-strand DNA synthesis. Once replication is completed, the nucleocapsid assembles with envelope proteins (HBsAg) in the endoplasmic reticulum to form mature virions and is secreted from the cell. Some viral nucleocapsids are transported to the nucleus to form additional cccDNA molecules [77]. The stages of replication is shown in **Figure 3** [78].

## 3.6 Long-term sequelae of chronic hepatitis B

The progression of liver disease is largely influenced by the age of HBV acquisition. A child who acquires the infection within one year of age has 90% risk to become a chronic carrier and the risk reduces to 30% for children 1 to 5 years of age. Subsequently, the risk of chronicity decreases to <5% for children above 5 years and adults [79].

Cirrhosis and hepatocellular carcinoma (HCC) are two major long-term sequelae of chronic hepatitis B. The cumulative probability of subjects diagnosed with chronic hepatitis B developing cirrhosis is 15-20% at the end of 5 years [80, 81]. The cumulative incidence of HCC in subjects with chronic hepatitis B without cirrhosis is 1-3% and in subjects with compensated cirrhosis is 10-17% at the end of 5 years [82]. This illustrates the importance to consider antiviral treatment for the prevention and control of disease progression in subjects diagnosed with chronic hepatitis B.

## 3.7 Antiviral drugs and mechanisms of action

Currently there are 7 approved therapies for HBV including 2 formulations of interferon, i.e., standard interferon alfa-2b (IFN-α-2b) and pegylated interferon alfa-2a (pegIFN-α-2a) and 5 nucleos(t)ide analogues, i.e., lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir disoproxil. Interferons are immunomodulatory drugs that are administered for a

**Figure 3. Stages of HBV replication and molecular targets of nucleos(t)ide analogues**
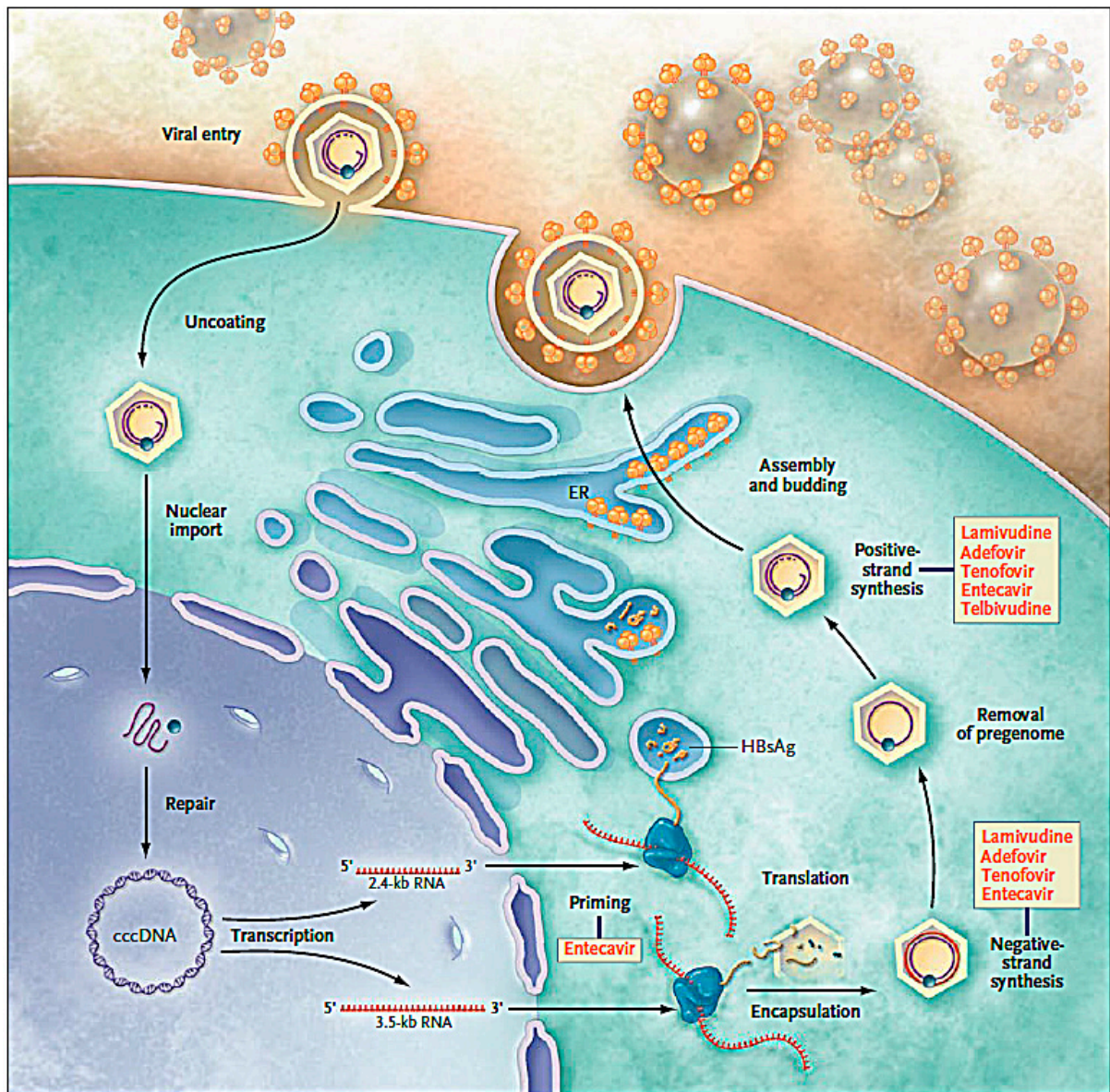


cccDNA – covalently closed circular DNA, ER - endoplasmic reticulum and HBsAg - hepatitis B surface antigen. Figure adapted from Dienstag [78].

finite period of treatment. The nucleos(t)ide analogues lack the 3′-hydroxyl group and the incorporation of these analogues prevents the formation of phosphodiester linkage that is essential for DNA elongation. It inhibits the enzymatic action of HBV reverse transcriptase and thus acts as a chain terminator of DNA synthesis [83]. The nucleos(t)ide analogues and their targets of HBV replication cycle is shown in **Figure 3**.

Interferon- α was the first therapeutic agent used for the treatment of HBV infection. It is known to function by antiviral and Immunomodulatory activity. The antiviral activity is mainly mediated by protein kinase R activation that blocks the synthesis of viral proteins; production of 2′-5′ oligoadenylate that degrades mRNA by ribonuclease-L activation and MxA protein guanosine triphosphate hydrolases (GTPases) that affects the activity of viral polymerases [84-87]. The immunomodulatory effect of IFN is mediated by MHC class I upregulation and viral antigen presentation to CD8+ cytotoxic T cells that results in the destruction of infected cells; by activating natural killer cells and enhances humoral activity by increasing B cell proliferation [88-90]. IFN-α-2b or standard IFN is administered daily or three times a week. Over time, pegylated IFN-α-2a replaced standard IFN, which was shown to have a long-lasting effect and administered once a week. It was also shown to be more effective than standard IFN [91].

Lamivudine, an analogue of cytidine was the first oral drug approved by Food and Drug Administration (FDA) for the treatment of chronic hepatitis B. It still remains the first line therapy due to administration efficacy, easy intake, clinical safety and cost.

Adefovir, a nucleotide analogue of adenosine is another orally administered drug which is reported to be effective against both wild type and lamivudine resistant strains of HBV [92]. Both lamivudine and adefovir have shown to inhibit the positive and negative strand synthesis of HBV DNA **(Figure 3)**.

In March 2005, Entecavir was approved by the FDA for the treatment of chronic HBV. It is a guanosine analogue that competes with the natural substrate deoxyguanosine triphosphate and inhibits the reverse transcriptase activity. Entecavir is structurally distinct from the other oral drugs and allows incorporation of additional nucleotides before chain termination. Entecavir displays activity against the priming function and was shown to affect both the positive and negative strand DNA synthesis (**Figure 3**) [93]. This pseudo or non-obligate chain terminator thus differs from the action of obligate terminators such as lamivudine and adefovir.

Telbivudine and tenofovir are the recently approved drugs for the treatment of chronic hepatitis B. Telbivudine is a thymidine nucleoside analogue and is shown to have a more potent activity on HBV DNA suppression and higher response rate than lamivudine [32, 94]. It directly targets the synthesis of positive strand DNA and thereby hinders the replication cycle of HBV [78].

Tenofovir a nucleotide analogue, is shown to be very effective and is an alternate drug of choice in subjects who failed to show response for lamivudine, adefovir and entecavir treatment [95]. Tenofovir inhibits the replication cycle of HBV by targeting both the positive and negative strand DNA synthesis which is related to the antiviral action of lamivudine and adefovir (**Figure 3**) [78].

### 3.7.1 Goals of antiviral therapy

The complete eradication of HBV is difficult due to the persistence of cccDNA in the nucleus of infected hepatocytes. However, antiviral drugs help to reduce the progression of liver disease. In subjects diagnosed with chronic HBV infection, the aim is to prevent cirrhosis or progression of cirrhosis to decompensated liver disease and hepatocellular carcinoma (HCC). The progression of liver disease is prevented or delayed by the suppression of viral DNA.

This is very well evidenced by the Risk Evaluation of Viral Load Elevation and Associated Liver Disease (REVEAL)-HBV study, which showed HBV DNA levels to be a strong predictor for the risk of disease progression [14, 96]. The odds ratio of developing cirrhosis for HBV DNA levels <300 to $10^4$ copies/mL, $10^4$ to $10^5$ copies/mL, $10^5$ to $10^6$ copies/mL and >$10^6$ copies/mL were 1.4 (95% confidence interval [CI], 0.9–2.2), 2.5 (95% CI, 1.6–3.8), 5.9 (95% CI, 3.9–9.0) and 9.8 (95% CI, 6.7-14.4) respectively. Similarly, the odds ratio of developing hepatocellular carcinoma (HCC) for these viral loads at were 1.1 (95% CI, 0.5–2.3), 2.3 (95% CI, 1.1–4.9), 6.6 (95% CI, 3.3–13.1), and 6.1 (95% CI, 2.9–12.7) respectively [14]. This shows that the relative risk of cirrhosis and HCC increased with increasing serum HBV DNA levels. Suppression of HBV DNA is therefore the major goal in HBV management.

### 3.7.2 End-points of therapy

The end-points for the therapeutic management of HBV can be categorized as biochemical, histological and virological responses [61].

### 3.7.2.1 Biochemical response

Biochemical responses are usually measured by the serum alanine aminotransferases (ALT) and aspartate aminotransferases (AST) levels. A biochemical response is defined as the normalization of serum aminotransferases. The normal range for ALT and AST is 5-35 U/L and 8-40 U/L respectively [97]. However, there is no widely accepted criterion for the normal range of serum enzyme levels. It has been proposed that upper limit of ALT for men and women to be 30 U/L and 19 U/L respectively [98]. In another report from our center, the upper limit of normal ALT in blood donors was determined to be 64 U/L [99]. The ALT levels is usually monitored at 3 to 6 months interval over the course of therapy and in

subjects with elevated ALT levels (1-2 ULN), the frequency of monitoring is 1 to 3 months [100].

### 3.7.2.2 Histological Response

Histological responses are measured by the scoring systems for the inflammation grade and fibrosis stage of chronic hepatitis [101-103]. Histological improvement is defined as a two point decrease in the histological activity index (HAI) between the baseline and end-of-treatment liver biopsies. However, liver biopsy is an invasive and painful procedure and is limitedly used in clinical practice [61].

### 3.7.2.3 Virological response

Virological response is critically monitored with HBV DNA levels. There are several time points at which response is measured for nucleos(t)ide analogues over the course of therapy.

**Early virological response**

Early virological response (EVR) is measured after 3-6 months of therapy and are categorized as

- **Complete virological response** defined as undetectable HBV DNA [104]
- **Partial virological response** with a reduction in HBV DNA $\geq 1$ $\log_{10}$ IU/mL from baseline [15, 104].
- **Primary treatment failure or non-response** to HBV is the lack of reduction of HBV DNA $\geq 1$ $\log_{10}$ IU/mL [15, 17].

**End-of-treatment response**

End-of-treatment response (ETR) is measured after 12 months of therapy and are categorized as

- **Virological response** defined as undetectable HBV DNA [15, 105]

- **Secondary treatment failure or virological breakthrough** with $\geq 1$ $\log_{10}$ IU/mL increase in HBV DNA compared with the lowest value during antiviral treatment (nadir), in two consecutive time-points of therapy [15, 17, 106].

**Maintained response**

Maintained response is an on-treatment measurement of HBV DNA to monitor the virological response (undetectable HBV DNA) in long-term therapy [61].

**Sustained virological response**

Sustained virological response is the off-treatment measurement of HBV DNA levels and is indicated by the persistent loss of HBV DNA after 6 or 12 months of treatment discontinuation (SVR-6 or SVR-12) [16].

**3.7.3 HBV DNA quantification assays and therapeutic monitoring**

HBV DNA kinetics have helped clinical researchers in framing guidelines and definitions for the assessment of treatment responses to HBV drugs. Virological monitoring of HBV DNA is therefore the best predictor in the management of hepatitis B [61, 104, 107]. **Figure 4** shows the dynamic ranges of some of the widely used HBV DNA quantification assays [61, 108]. Hybridization assays, initially used for HBV DNA quantification were sensitive for viral load above $10^4$ IU/mL. With the high sensitivity and broad dynamic range, real-time PCR has gradually replaced other signal amplification and target amplification technologies for HBV DNA detection [109]. As more HBV DNA quantitative assays become available, it is important to use an accurate HBV virological tool for monitoring HBV DNA levels. A standardized approach for use of HBV DNA assays in clinical practice has been recommended for efficient management of HBV. In order to ensure comparability between the assays, HBV DNA levels should be universally reported in IU/mL after calibration with the World Health Organization (WHO) International Standard for HBV DNA [17]. One

**Figure 4. Dynamic ranges of widely used HBV DNA quantification assays**
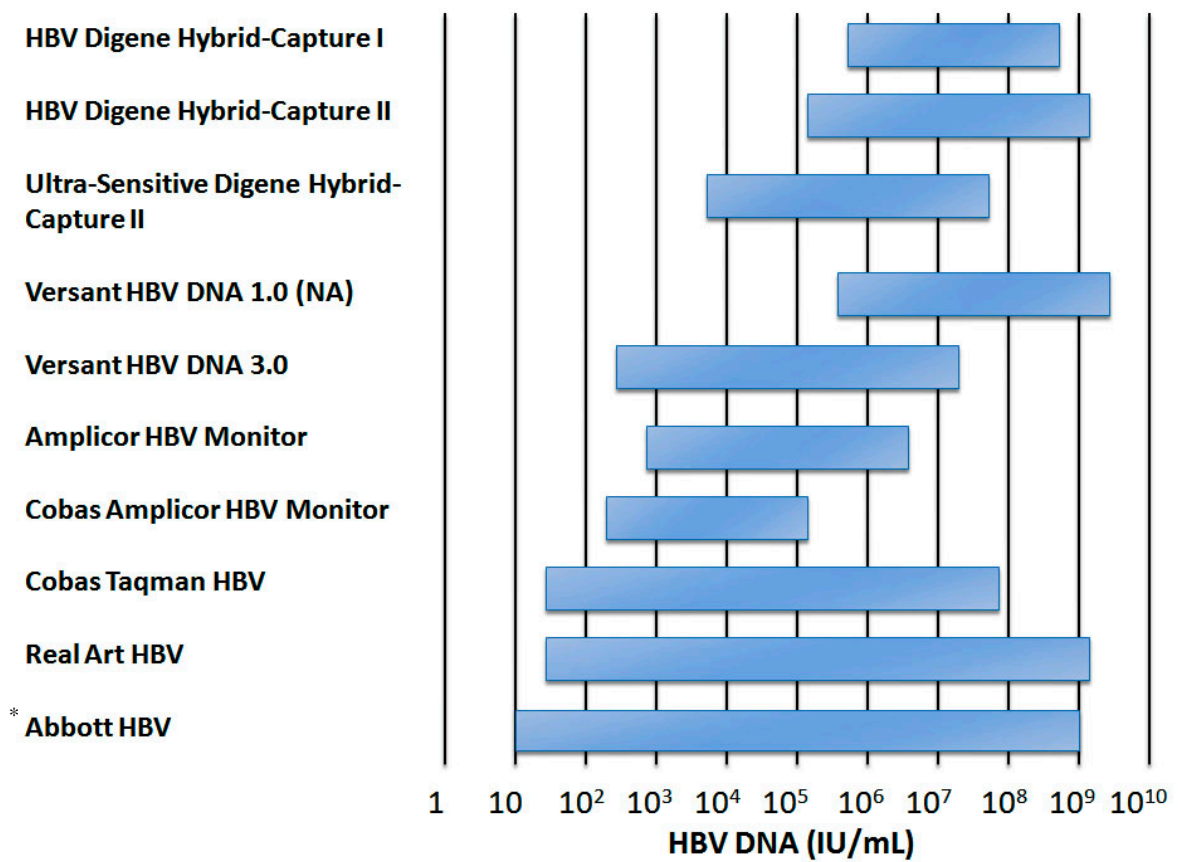
*Manufacturers claim of dynamic range [61]

Figure adapted and modified from Hoofnagle *et al.* (2007) [108].

IU/mL of HBV DNA is approximately equivalent to five genome equivalents/mL and therefore a multiplication factor of five is applied to convert IU/mL into copies/mL [110, 111]. In therapeutic monitoring of HBV, a more sensitive assay with a lower limit of detection (LLD) of 10 IU/mL is recommended for early detection of viral rebound [15, 107]. As there are assay to assay variations in quantification of HBV DNA, the use of same assay for a given subject is important in clinical practice to precisely monitor the antiviral efficacy of any given drug [15, 107].

### 3.7.4 HBV Antiviral resistance

Though there are several options for the treatment of chronic hepatitis B infection, management of HBV still remains a major challenge. Unlike IFN, nucleos(t)ide analogues require long term and continuous treatment. Overtime, the virus evolves strategies to counteract the selection pressure and thereby escapes the antiviral action. Therefore, antiviral resistance is a clinically relevant issue in the therapeutic monitoring of patients with chronic hepatitis B.

In order to have a consensus in stating antiviral resistant HBV mutations in the polymerase region, the nomenclature proposed by Stuyver *et al.* (2001) [112] is followed. Mutations are annotated by the gene region i.e., rt, followed by the wild-type amino acid symbol, its position and the mutant amino acid symbol [17].

Signature mutations that are associated with antiviral resistance to the currently used nucleos(t)ide analogues are shown in **Figure 5** [106].

### 3.7.4.1 Lamivudine resistance mutations

The typical mutations considered as primary mutations conferring lamivudine resistance involves amino acid substitution from methionine to valine or isoleucine at codon 204 in the

**Figure 5. Hepatitis B virus polymerase domain showing primary antiviral resistance mutations**



| Catalytic domain | I (G) | II (F) | A | B | C | D | E |
|---|---|---|---|---|---|---|---|
| aa position | aa 24-36 | aa 37-47 | aa 75-90 | aa 159-182 | aa 200-210 | aa 230-241 | aa 247-257 |
| Lamivudine resistance | | | rtL80V/I | rtV173L rtL180M rtA181T | rtM204V/I/S | | |
| Adefovir resistance | | | | rtA181T/V | | rtN236T | |
| Entecavir resistance | | | rtI169T | rtL180M rtT184* rtM204V | rtS202$ | rtM250I/V | |
| Telbivudine resistance | | | | | rtM204I | | |
| Tenofovir resistance | | | | rtA194T # | | | |
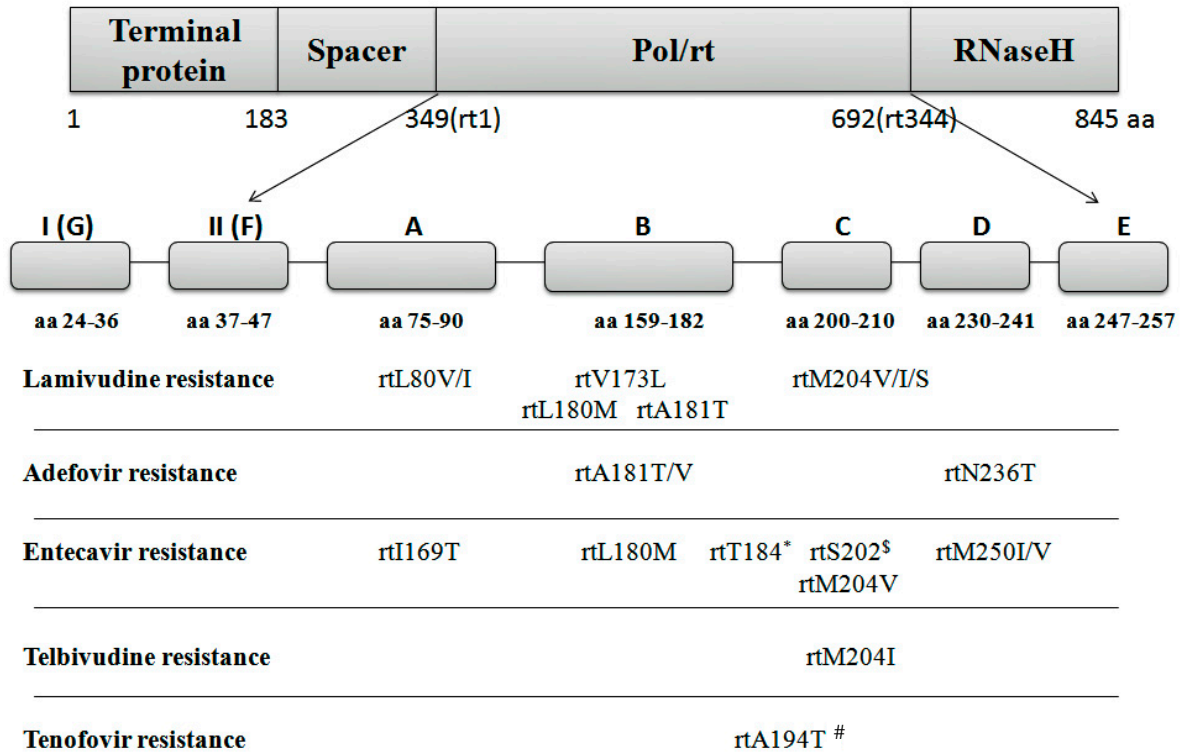
The polymerase domain has four functional domains (terminal protein, spacer, pol/rt and RNaseH) and seven catalytic domains A-G. The primary antiviral drug resistance mutations to each drug are shown. Figure adapted and modified from Zoulim and Locarnini [106].

aa- amino acid positions

rt- reverse transcriptase region (aa numbering according to the nomenclature of Stuyver *et al.* (2001) [112].

*rtT184S/A/I/L/G/C/M

$rtS202C/G/I

#rtA194T- the role of this mutation to confer tenofovir resistance is contradictory

highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the C domain (rtM204V or rtM204I) [18]. Other aminoacid substitutions at sites rtL80I, rtI169T, rtV173L, rtL180M, rtA181T/S and rtQ215S occur during lamivudine therapy in order to restore the replication capability  and they are called secondary or compensatory mutations [19].

The incidence of lamivudine resistance is 10-32% at 1 year, 37-48% at 2 years, 52-60% at 3 years, 60-67% at 4 years and 69-80% at 5 years respectively [20-24].

### 3.7.4.2 Adefovir resistance mutations

The primary adefovir-resistant mutations are rtN236T and rtA181T/V [25]. A pooled analysis from four major adefovir dipivoxil clinical trials confirmed these mutations to be the only HBV polymerase mutations significantly associated with treatment failure [26]. Although adefovir is less effective than lamivudine, the rate of resistance is much lower and thus remains the suitable drug of choice for the long term treatment.

The cumulative probability of mutations associated with resistance to adefovir is 0%, 3%, 11%, 18%,and 29% after 1, 2, 3, 4, and 5 years respectively [27].

### 3.7.4.3 Entecavir resistance mutations

Entecavir related mutations require combinations of substitutions at positions rtI169T, rtL180M, rtT184G/S/A/I/L/C/M, rtS202C/G/I, rtM204V/I and rtM250V [28, 29, 113]. The incidence of entecavir resistance at 1, 2, 3,4 and 5 years was found to be 0.2%, 0.5%, 1.2%, 1.2% and 1.2%  respectively [30, 114].

### 3.7.4.4 Telbivudine resistance mutations

Telbivudine resistance mutations are similar to those associated with lamivudine resistance rtM204I, rtL80I/V and rtL180M mutations [31]. The incidence of telbivudine resistance at

the end of 24 months in HBeAg-positive and-negative individuals was found to be 25.1% and 10.8% respectively [32].

### 3.7.4.5 Tenofovir resistance mutations

HBV rtA194T is the only mutation shown to be associated with tenofovir resistance [35]. However, there are no conclusive reports of tenofovir resistance in HBV [33-36]. Since tenofvir is a newly approved drug the antiviral efficacy and resistance patterns are still evolving.

### 3.7.5 Genetic barrier to resistance

In drugs with low antiviral pressure or in absence of antiviral pressure, the viral replication is active and there is a sustained prevalence of wild type population. As antiviral activity increases, the viral replication is lowered. However, the incomplete drug pressure leads to the selection of mutants and results in resistance development. In drugs with high selection pressure, there is a complete suppression or very low levels of viral replication that limits the chance of mutant selection and resistance development **(Figure 6)** [115, 116]. Therefore, drugs with low or incomplete antiviral pressure have a higher chance for resistance development and are termed as having low genetic barrier to resistance and vice versa for drugs with high genetic barrier to resistance.

The genetic barrier to resistance can also be defined by the number of primary mutations required for the development of antiviral resistance [116-118]. Accordingly, lamivudine, adefovir and telbivudine that confer antiviral resistance by single nucleotide mutations are classified as low-genetic barrier drugs. In contrast, entecavir that requires a combination of at least three rt mutations (L180M, M204V/I and T184G or S202I or M250V) is classified as drug with high genetic barrier to resistance [116, 119].

**Figure 6. Antiviral selection pressure and drug resistance**
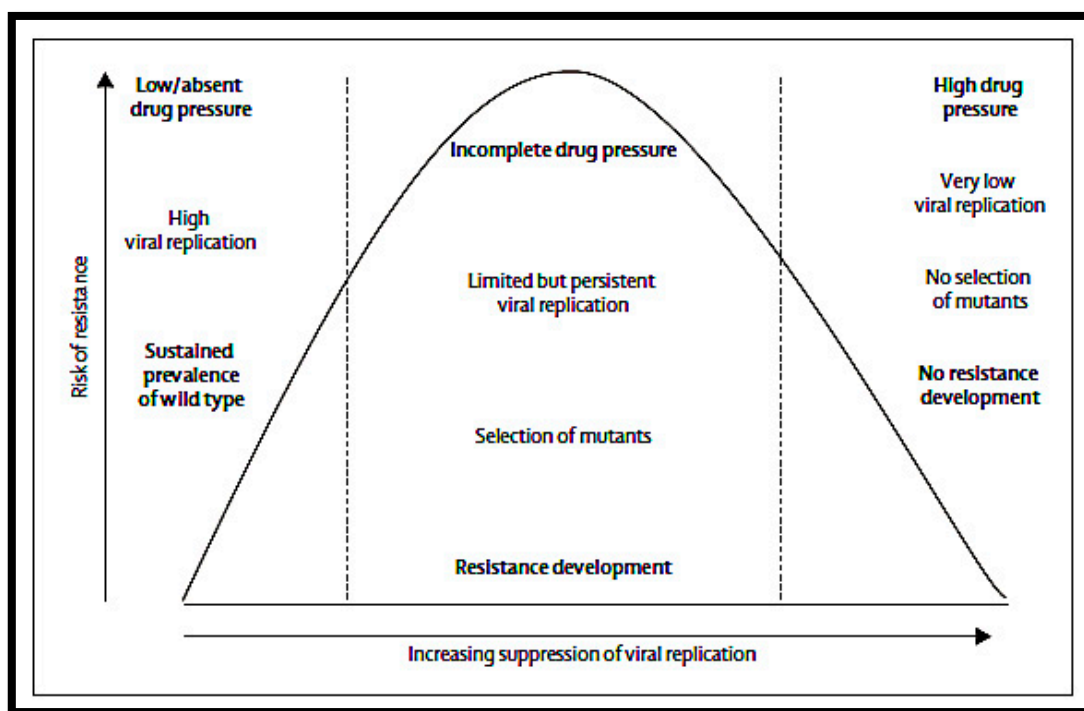


This figure adapted from Gish *et al.* (2012) [116] shows the relation between antiviral drug activity and the development of drug resistance.

**3.7.6 Pre-existing antiviral resistance mutations in treatment-naive subjects**

In a study to determine the pre-existing antiviral resistance related mutations, adefovir related rtN236T (1.1%) and tenofovir related rtA194T (2.3%) amino acid substitutions were identified in Turkish patients [120]. A case report also showed the pre-existence of tenofovir related rtA194T substitution [121]. Another study documented the presence of rtM204V/I, rtL180M, rtA181T/V amino acid substitutions in a total of 3.9% treatment-naive subjects [122]. In a report from China, none of the subjects had primary resistance amino acid substitutions. However additional compensatory substitutions were shown in 31% of the subjects [123]. In all these studies, the impact of these pre-existing antiviral related amino acid substitutions on treatment follow-up were not shown.

**3.7.7 Clinical outcome and antiviral resistance: Experience of lamivudine monotherapy**

A prospective, randomized, double-blind, placebo-controlled study showed anti-HBe seroconversion and HBeAg loss in 17% and 32% of lamivudine-experienced group versus 6% and 11% in the placebo group at the end of 12 months [21]. Serum ALT levels were normalized in 41% of lamivudine group as compared to 7% in the placebo group. Serum HBV DNA was undetectable by hybridization assay in 98% of the lamivudine group and 33% in the placebo group. Loss of HBsAg occurred in only 2% of the lamivudine-experienced subjects. Histological improvement was seen in 52% and 23% of the lamivudine group and controls respectively. Together this data showed the efficacy of lamivudine as the first line of treatment for chronic hepatitis B. However, the study also reported higher proportion (32%) of mutations in the YMDD motif of HBV polymerase region in American population as compared to 14% in Asians at the end of 12 months treatment [124]. These two studies helped to consider lamivudine as first line drugs in the treatment of chronic hepatitis B.

Since then there are a number of studies which have shown the efficacy of lamivudine for the treatment of chronic hepatitis B. It was considered to be clinically safe and effective. All studies showed fairly similar rates of response. In HBeAg positive subjects, HBeAg seroconversion was documented in about 16-21% subjects, undetectable serum HBV DNA (<300 to 400 copies/mL) were shown in 36-44%, ALT normalization in 41-75%, HBsAg loss ≤1% and histological improvement in 49-62% at the end of 48 to 52 weeks of lamivudine treatment [21, 94, 124-126]. In HBeAg negative subjects, undetectable serum HBV DNA (<300 to 400 copies/mL) in 60-73%, ALT normalization in 62-79%, HBsAg loss ≤1% and histological improvement was shown in 60-66% of subjects at the end of 48 to 52 weeks treatment [127-129]. Altogether, there existed a difference in response rates based on HBeAg status with HBeAg negative subjects showing better therapeutic outcome to lamivudine. However, some studies have showed similar response rates in HBeAg-positive and HBeAg-negative subjects [128].

### 3.7.7.1 Long-term outcome

Extended lamivudine treatment have revealed continued viral suppression, increased HBeAg seroconversion rates, sustained ALT normalization and histological improvement [130-133]. In HBeAg positive subjects, HBeAg seroconversion was increased upto 50% at 5 years of lamivudine treatment [24]. Loss of HBsAg was seen in 3.5% subjects after 3 years of treatment [134]. There is insufficient data on maintained suppression of HBV DNA in HBeAg positive subjects with long term lamivudine treatment. In HBeAg negative subjects, continued suppression of HBV DNA was seen in 6 to 39% and HBsAg loss <1% at the end of 4 years treatment [62, 78, 135, 136].

However, the major concern in the long-term lamivudine treatment is the development of antiviral resistance [24].The incidence of lamivudine resistance increases with the treatment

duration of 10-32% at 1 year, 37-48% at 2 years, 52-60% at 3 years, 60-67% at 4 years and 69-80% at 5 years respectively [20-24]. Differences in lamivudine resistance rates exist between HBeAg-positive and HBeAg-negative subjects with the rate of 39.5% and 25.9% respectively at 24 months therapy [32].

**3.7.8 Clinical outcome and antiviral resistance: Experience of adefovir monotherapy**

A randomized, double blind, placebo-controlled study evaluated the effects of 10 mg and 30 mg doses of adefovir in HBeAg-positive subjects. Adefovir at a dosage of 10 mg was very well tolerated and shown to be effective. Therefore this dosage was recommended and currently used for treatment of chronic hepatitis B. At week 48, 21% of the subjects in the 10 mg group had undetectable serum HBV DNA (<400 copies/mL) as compared with 0% in the placebo group. Loss of HBeAg occurred in 24% in the adefovir 10 mg group as compared to 11% in the controls. HBeAg seroconversion occurred in 12% as compared to 6% in placebo. In the adefovir 10 mg experienced group 48% had normal ALT levels and 16% in placebo group at week 48. Histological improvement was seen in 53% in adefovir 10 mg group and 25% in the placebo group. None of these subjects were detected with antiviral resistance mutations [137].

A randomized, double blind, placebo-controlled study in HBeAg-negative chronic hepatitis B subjects showed significant virological, biochemical and histological improvement at 48 weeks of adefovir treatment. Fifty one percent of the subjects in the adefovir group had undetectable HBV DNA (<400 copies/mL) as compared with 0% in the placebo group. Normalized ALT levels were seen in 72% vs. 29% in the adefovir and placebo groups respectively. More subjects in the adefovir group showed histological improvement as compared to placebo (64%vs 33%). None of the subjects were detected with adefovir

resistance mutations [138]. Similar to lamivudine, HBeAg-negative subjects showed higher rate of response than HBeAg-positive subjects.

As reviewed by Dienstag *et al.* [78], HBeAg seroconversion was documented in about 12% subjects, undetectable serum HBV DNA (<300 to 400 copies/mL) were shown in 13-21%, ALT normalization in 48-61%, HBsAg loss 0% and histological improvement in 53-68% at the end of 48 to 52 weeks of adefovir treatment in HBeAg positive subjects. In HBeAg-negative subjects, undetectable serum HBV DNA (<300 to 400 copies/mL) were shown in 51-64% subjects, ALT normalized in 48-77% subjects, loss of HBsAg not seen and histological improvement in 61-66% subjects at the end of 48 to 52 weeks adefovir treatment.

### 3.7.8.1 Long term outcome

Long-term treatment of chronic hepatitis B with adefovir has showed increasing rates of HBeAg seroconversion of 48%, undetectable serum HBV DNA (<1000 copies/mL) in 39%, HBsAg loss in 2% and histological improvement of necroinflammation and fibrosis were seen in 67% and 60% of subjects at the end of 5 years in HBeAg-positive subjects [139]. The typical adefovir related rtA181V and rtN236T mutation was detected in 20% at the end of 5 years.

In HBeAg negative subjects, undetectable serum HBV DNA (<1000 copies/mL) was documented in 67% of the subjects, ALT normalized in 69%, histological improvement of necroinflammation and fibrosis were seen in 83% and 73% respectively at the end of 5 years treatment. Adefovir related rtA181V and rtN236T mutation was detected in 29% of subjects at the end of 5 years treatment [27].

Overall, the incidence of adefovir resistance is reported to be 3% at 24 months and increases upto 29% after 5 years of therapy [27]. Among the HBV drugs, adefovir is shown to be a less

efficacious drug; lacks early virological response, delays the suppression of HBV DNA and have low anti-HBe seroconversion rates [78, 140]. However, addition of adefovir (add-on therapy) is shown to be effective in subjects with lamivudine-resistant chronic hepatitis B [141, 142]. Therefore, the use of adefovir monotherapy in the management of chronic hepatitis B is very limited.

**3.7.9 Clinical outcome and antiviral resistance: Experience of entecavir monotherapy**

In a phase III, double-blind, randomized trial, the efficacy of entecavir over lamivudine was compared in HBeAg positive subjects [143]. This "Benefits of Entecavir for Hepatitis B liver Disease" (BEHoLD) study showed entecavir to have higher response rates over lamivudine and demonstrated its primary benefit in the management of HBeAg positive subjects. The results of entecavir treated subjects showed HBeAg seroconversion in 21% of the subjects, undetectable HBV DNA (<300 copies/mL) in 67%, normalization of ALT levels in 68% and histological improvement in 72% of the subjects at the end of 48 weeks. Moreover, none of the HBVrt substitutions analysed showed resistance to entecavir.

The BEHoLD group also showed the efficacy of entecavir in HBeAg-negative subjects [129]. At week 48 of entecavir therapy, undetectable serum HBV DNA (<300 copies/mL) was seen in 90%, normalization of ALT levels in 78% and histological improvement in 70% of the subjects. None of the subjects in the study presented with entecavir resistance mutations. Subsequent studies on entecavir also showed similar results [30, 144].

A recent meta-analysis comparing the efficacy of entecavir over adefovir at 48 week therapy also showed increased response rates of entecavir [145]. In all these studies, entecavir was found to be more potent in suppression of viral DNA and normalization of ALT levels. However, entecavir induced HBeAg seroconversion rates were similar with that of lamivudine and adefovir seroconversion rates [143, 145].

**3.7.9.1 Long-term outcome**

The results of long-term treatment of entecavir up to 5 years in HBeAg-positive subjects showed undetectable HBV DNA (<57 IU/mL) and normal ALT levels in 94% and 80% respectively. In addition to 31% and 5% who showed HBeAg seroconversion and HBsAg loss respectively at year 2, 23% achieved HBeAg seroconversion and 1.4% lost HBsAg subsequently at the end of 5 years. Entecavir resistance mutations were detected in 1 of 146 subjects (0.68%) who were initially treated with 0.5mg entecavir for 1 year and followed with 1.0 mg entecavir subsequently [146].

There were no specific data on long-term effects of entecavir in HBeAg-negative subjects However, Yuen *et al.* [30] showed three years data on entecavir therapy in both HBeAg-positive and HBeAg-negative (59.5%) subjects. In HBeAg positive subjects, undetectable serum HBV DNA (<12 IU/mL), HBeAg seroconversion and ALT normalization was observed in 82.9%, 43.9% and 97.1% at the end of 3 years respectively. In HBeAg-negative subjects, undetectable HBV DNA (<12 IU/mL) and ALT normalization were documented in 98.3% and 85.7% respectively. Entecavir-resistant mutation was detected in 1.2% (HBeAg positive) subjects at 3 years. Overall, the incidence of entecavir resistance was 0.2%, 0.5%, 1.2%, 1.2% and 1.2% for 1, 2, 3,4 and 5 years respectively [30, 114].

Recently Manns *et al.* [147] showed the long-term safety and tolerability of entecavir in chronic hepatitis B subjects with or without previous experience to other nucleos(t)ide analogues. The adverse effects to entecavir over median treatment duration of 184 weeks (3.54 years) were ≤10%. Together, with the low adverse effects and high barrier to resistance and long term efficacy, entecavir was shown to be the suitable drug of choice in management of chronic hepatitis B.

### 3.7.10 Experience with other HBV drugs

IFN has showed higher sustained response rates, improved serological responses such as HBeAg seroconversion and loss of HBsAg as compared with oral drugs. It was not shown to cause viral resistance [127, 148-150]. However, significant side effects and cost-constraints have reduced its use in HBV management, especially in resource poor settings.

In the 2-year GLOBE trial (multi-centric International phase 3 trial) on comparison of telbivudine and lamivudine, telbivudine showed to have a more potent activity on HBV DNA suppression and higher response rate at 12 and 24 months respectively. The incidence of telbivudine resistance at the end of 24 months in HBeAg-positive and HBeAg-negative individuals was found to be 25.1% and 10.8% respectively [32]. Tenofovir a nucleotide analogue closely related to adefovir is shown to be very effective and alternate drug of choice in the treatment failure subjects [95].

In 2010, a meta-analysis was performed that evaluated the efficacy of five nucleos(t)ide analogues for treatment of chronic hepatitis B [151]. This meta-analysis showed that in HBeAg-positive subjects, 94% of tenofovir treated subjects had undetectable HBV DNA (<300 copies/mL) in comparison to 38% for lamivudine, 49% for adefovir, 63% for telbivudine and 73% for entecavir after 1 year of treatment. The HBeAg seroconversion rates were between 22% to 27% and there was no significant difference between the nucleos(t)ide analogues. This analysis had insufficient evidence for the comparison of nucleos(t)ide analogues efficacy in HBeAg-negative subjects and was not demonstrated.

### 3.7.11 Predictors of antiviral response to HBV

In the investigations for the successful management of HBV certain baseline and on-treatment predictors of subsequent response to nucleos(t)ide analogues have been identified. Low serum HBV DNA levels of <7 $\log_{10}$ IU/mL, high serum ALT levels (3 to 5 times the

ULN) and high activity scores on liver biopsy was shown to be the baseline predictors of response [15, 152, 153]. Complete loss of HBV DNA at 6 and 12 months of therapy and anti-HBe seroconversion are shown to be associated with maintained virological response over the course of therapy and serve as an on-treatment predictors of response [15, 27, 31].

**3.7.12 Predictors of antiviral resistance to HBV**

Significant predictors of antiviral resistance included male gender, older age, higher baseline ALT levels, HBeAg-positivity, higher baseline HBV DNA and high histological scores [23, 154-159]. Persistence of HBV DNA during antiviral therapy, an episode of virological breakthrough and longer treatment duration was shown as on-treatment factors associated with development of antiviral resistance [24, 154, 158, 160, 161].

**3.7.13 Characterization of hepatitis B virus drug resistance: Molecular modeling approach**

Computational methods like molecular modeling and docking studies have helped scientists to understand the structural features of protein, drug-protein interaction and the effect of resistance mutations and ligand binding [37-39, 162]. Knowledge of HBV reverse transcriptase (HBVrt) structure would thus be valuable for understanding the molecular basis of drug resistance. The high resolution structure of HBV polymerase protein is not yet available. Since there is a good sequence homology between the catalytic sites (A-G) of HBVrt and human immunodeficiency virus 1 (HIV-1) rt, drug resistance in HBVrt is studied using the HIV-1 rt template by homology modeling [40].

The p66 polymerase domain of HIV-1 rt is known to have a right-handed structure with finger, palm and thumb subdomains (**Figure 7**) [163]. The three aspartate amino acids that form the catalytic sites in HIV-1 rt is well conserved in HBVrt at positions 85, 203 and 204. Likewise, most of the amino acids interacting with the template primer and the incoming

**Figure 7. The p66 polymerase domain model of HIV-1 reverse transcriptase**
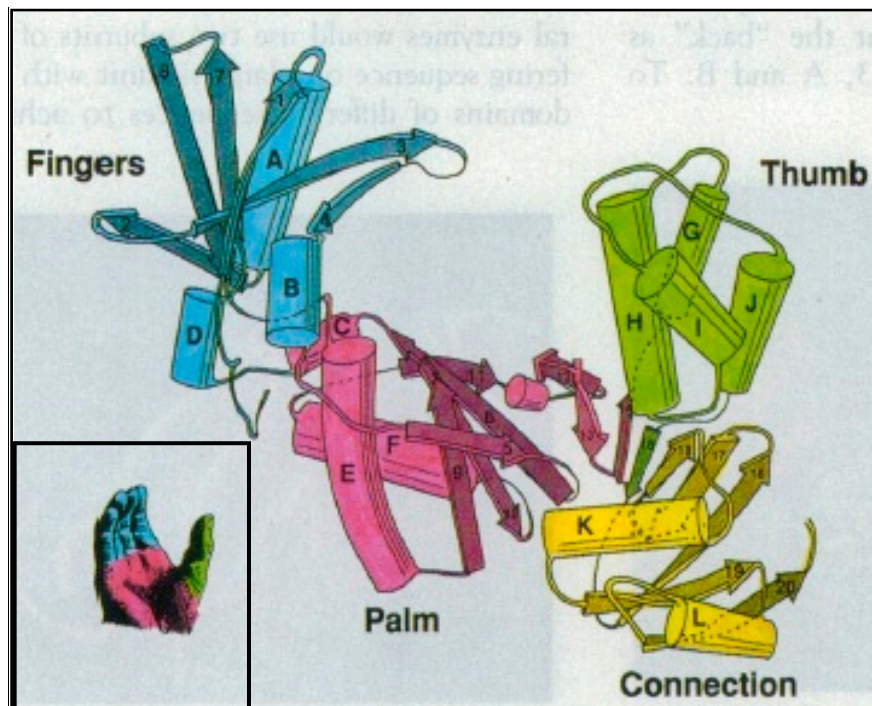


The figure shows a right hand structure with finger, palm and thumb subdomains. The α-helix is shown by alphabets and the β-sheets are numbered. Figure adapted from Kohlstaedt *et al*. (1992) [163].

dNTP substrates are conserved both in HIV and HBVrt [40]. Moreover, the nucleos(t)ide analogues lamivudine, adefovir and tenofovir used for chronic HBV treatment were initially developed for HIV infection and their drug interactory mechanisms are very well documented [164, 165]. Therefore, molecular modeling and docking studies of HBV using HIV-1rt template would be a suitable model for the prediction of drug resistance.

### 3.7.13.1 Prediction of lamivudine resistance

The primary mutation (M204V/I) associated with lamivudine resistance is located adjacent to the two aspartate residues in the YMDD motif. Modeling has shown that substitution of valine or isoleucine for methionine results in steric hindrance between the sulphur atom in lamivudine and the substituted amino acid side chains. This hindrance for lamivudine binding is therefore predicted as the cause for lamivudine resistance [18, 40]. This mechanism of lamivudine resistance is also postulated for HIV [165]. It was also proposed that side chains of the substituted amino acids partially fills the hydrophobic pocket and excludes lamivudine binding **(Figure 8).** This change creates a small hole in the HBVrt and also affects the replication of the virus [166].

In vitro studies have shown that HBV bearing the primary resistant mutation cannot replicate as efficiently to wild type virus [40, 42, 167, 168]**.** In order to restore the replication capacity, the virus evolves additional substitutions called compensatory mutations. The mechanisms of three such lamivudine resistance compensatory mutations are well studied by HBV modeling.

### 3.7.13.1.1 Effects of rtL180M compensatory mutation

The most common rtL180M compensatory mutation reorients the surrounding rtM204V/I and rtF88 amino acid side chains [40]. The rtF88 residue interacts with the incoming dNTPs and undergoes conformational changes during polymerization reaction. The conformational changes to rtF88 might happen in a way that enhances the replication fitness of the virus

**Figure 8. Homology model of hepatitis B virus reverse transcriptase for prediction of lamivudine resistance**
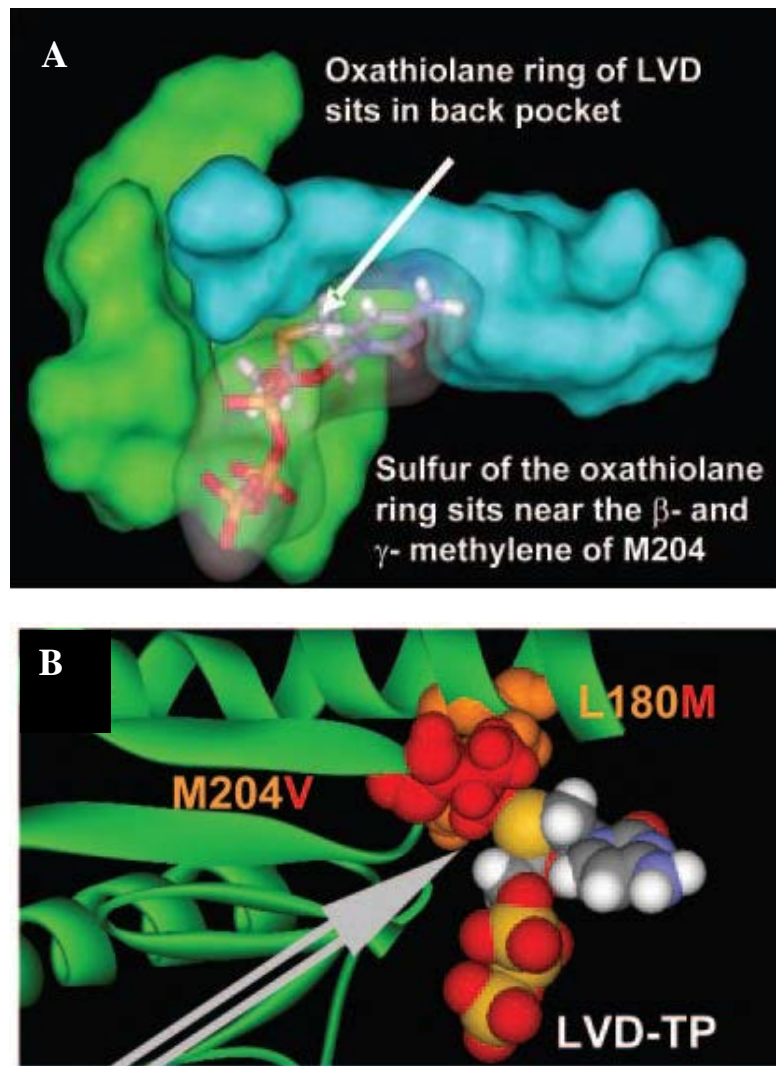


Homology model of HBVrt shows (A) a hydrophobic pocket at the rear of the dNTP binding site (protein, green and DNA, cyan). The oxathiolane ring of lamivudine fits within the pocket. (B).The rtM204V mutation reduces the pocket size (indicated by arrow) and prevents lamivudine triphosphate (LVD-TP) binding. Figure adapted from Langley *et al.*(2007) [166].

[168]. Also, rtL180M mutation eliminates the hole created by primary rtM204V/I mutations and restores some efficiency to the lamivudine resistant HBVrt [166].

**3.7.13.1.2 Effects of rtL80I/V compensatory mutation**

The rtL80I/V mutation affects the relative positioning of the aspartate amino acid at the catalytic site rtD83. It also alters the rtT240 positioning that in turn affects the conformation of dNTP binding site and results in the decreased space for lamivudine binding [167].

**3.7.13.1.3 Effects of rtV173L compensatory mutation**

It was proposed that rtV173 together with the adjacent glycine residues (rtG172 and rtG174) is crucial for the positioning of template strand. The rtV173L mutation affects the template strand positioning and also alters the rtF88 residue enhancing the polymerization efficiency [168].

**3.7.13.2 Prediction of adefovir resistance**

Molecular modeling has shown that rtN236 is crucial for stabilizing the γ-phosphate of adefovir. In the rtN236T mutation, the loss of two hydrogen bonds between rtN236 and γ-phosphate of adefovir and between rtN236 and rtS85 results in decreased binding affinity to adefovir [41].

**3.7.13.3 Prediction of entecavir resistance**

Mutations at positions rtT184 and rtS202 were predicted to reduce the entecavir triphosphate binding pocket. In addition to rtL180M and rtM204V mutations, rtS202G mutation further restricts the binding sites for entecavir. Likewise, rtT184 substitution caused similar changes for entecavir binding.

In addition to the rtM204V and rtL180M mutations, rtM250V/L mutation altered the dNTP binding site as well as the primer positioning [42]. Thus several mutations together cause resistance to entecavir. This study also highlighted the importance of including magnesium ($Mg^{2+}$) in the model. Modeling dNTP's or inhibitors in the presence of $Mg2^+$ ions produced conformations that were in close agreement to HIV rt structure. However, in absence of $Mg^{2+}$ ions, the conformations varied.

## 3.8 Genetic variability of HBV

### 3.8.1 HBV genotypes and subgenotypes

HBV is classified into genotypes and subgenotypes with an intergenotypic diversity of 8% and intra-genotypic diversity of 4% respectively. Accordingly, eight major HBV genotypes (A-H), two novel genotypes (I and J) and several subgenotypes within the genotypes are identified [6, 169, 170]. So far, HBV subgenotypes A1-A4, B1-B8, C1-C7, D1-D7 and F1-F4 are well described. Further extensive investigations may lead to the recognition of yet unidentified HBV variants as suggested by earlier reports [6, 7, 171-183].

### 3.8.1.1 Geographical distribution of HBV genotypes

The HBV genotypes have known to show a geographical pattern in their distribution. Genotype A is distributed in Europe, India, Africa and North America; genotypes B and C are common Asia; genotype D is widely spread worldwide and predominantly found in the Mediterranean area and eastern regions; genotype E is more prevalent in Western sub-Saharan areas; genotype F is common in Central and South America and Polynesia; genotype G is found in the USA and Europe and genotype H is prevalent in Central America [6]. The new genotype 'I' is reported in three south East Asian countries Vietnam, Laos and India [170, 184, 185]. The more recently introduced genotype J was reported from Japan [169].

Certain genotypes are also associated with high-risk groups like intravenous drug users and homosexual men [8, 186]. Therefore, HBV genotypes have been used to trace the migration of populations from geographically distant regions and also to identify the route of transmission.

### 3.8.1.2 Standardised criteria for genotyping and subgenotyping

Recently, certain criteria were proposed for defining new genotypes and subgenotypes [7, 180]:

- New genotypes or subgenotypes should be identified by complete genome sequence analysis

- Intra-genotypic variability of less than 4% with distinct phylogenetic clustering should be defined as clades within subgenotypes

- Evidence of recombination should be considered for identification of new subgenotype or clade of genotype.

- The determination of new genotype should be supported by its epidemiological, virological or clinical characteristics.

### 3.8.1.3 HBV genotypes and disease progression

It is been evident that HBV genotypes are associated with progression of disease. An African case-control study showed that individuals with genotype A had 4.5 fold higher risk for hepatocellular carcinoma (HCC) than those infected with genotype D and E [187].In contradiction, a longitudinal study showed that genotype A was significantly associated with sustained biochemical remission and clearance of HBV DNA and HBsAg than those with genotype D in chronic HBV infection [188]. HBV genotype B was suggested to be an important etiological factor for non-cirrhotic hepatoma in chronic HBV carriers [189]. HBV genotype C was shown to be associated with higher risk of liver inflammation, liver fibrosis,

cirrhosis and HCC compared to genotype B [190-193]. A study from the Indian subcontinent showed the association of genotype D with severe liver disease and HCC as compared to genotype A in young adults [10]. The influence of other genotypes in progression of disease is not well characterized.

### 3.8.1.4 HBV genotypes and antiviral response

HBV genotypes have shown to influence the therapeutic outcome and this is very well evidenced for interferon treatment. In a study measuring the sustained response rate to standard IFN, HBV genotype A was significantly associated with higher sustained response rate than HBV genotype D (49% vs 26%) [194]. HBV genotype A is also showed to have higher rate of HBeAg seroconversion for IFN treatment in comparison to genotype D (37% vs.6%) [195]. Another study showed the loss of HBeAg on interferon treatment to be significantly higher in genotype B compared with genotype C (41% vs 15%) [196]. The association of genotype B for better response to IFN therapy was also shown in a retrospective analysis, where 39% showed antiviral response as compared to 17% of genotype C [197]. In a study that investigated the loss of HBsAg with Peg-IFN treatment α-2b, there existed difference in HBsAg loss between genotypes; 14% for genotype A, 9% for genotype B, 3% for genotype C, and 2% for genotype D respectively [198]. Overall, genotype A and B is associated with better virological response to interferon treatment than genotype C and D. This was also evidenced in a recent meta-analysis [199].

Unlike IFN, there is insufficient data for genotype associated response to nucleos(t)ide analogues. Chien *et al.* [200] reported that genotype B subjects have a higher sustained response to lamivudine treatment than those infected with genotype C (61% vs 20%). This study also showed genotype to independently determine sustained HBeAg response [odds ratio (OR) of 5.922 (CI 1.61-21.77; p=0.07)]. HBV genotype A was shown to be significantly

associated with loss of HBsAg at 3 years of telbivudine therapy [201]. A meta-analysis on HBV genotype and antiviral response showed lack of association for genotypes A to D and nucleos(t)ide analogues response in both HBeAg-positive and HBeAg-negative subjects. However, this analysis did not account for different therapeutic endpoints and therefore requires careful interpretation [199].

**3.8.1.5 HBV genotypes, subgenotypes and antiviral resistance**

A study from Taiwan reported no significant difference between genotype B and genotype C for the development of lamivudine resistance [202]. Likewise, Akuta *et al.* [203] in a study to identify the influence of HBV genotypes on lamivudine resistance, showed lack of association for genotypes A, B and C. However, lamivudine resistance was significantly higher for subgenotype Ba and Bj (now recognised as B2 and B1 respectively [7]). In a prospective study, genotype C was shown to be associated with lower frequency of lamivudine resistance in univariate analysis. When entered into a multivariate model, genotypes did not show any association with lamivudine resistance [159].

**3.8.2 HBV subtypes**

Based on the antigenic determinants of HBsAg, HBV strains are divided into nine subtypes; *ayw1*, *ayw2*, *ayw3*, *ayw4*, *adw2*, *adw3*, *adw4*, *ayr* and *adr* respectively [9]. There exist significant association between HBV genotypes and subtypes. HBV subtype *adw2* is mainly identified in genotypes A, B and G and less commonly in genotypes C and D. Subtype *ayw1* is determined in genotype A and B. Subtypes *adr* and *ayr* is restricted to genotype C. Subtype *adw4* was shown to occur only in genotypes F and H. Subtypes *ayw2* and *ayw3* is always associated with genotype D. Subtype *ayw4* is specific to genotype E [6]. However, HBV genotypes and subtypes are shown to co-exist in other combinations [9, 204, 205].

### 3.8.2.1 HBV subtypes: Antiviral response and resistance

There are few reports that showed the association of antiviral response and resistance development with certain HBV subtypes. The study by Zollner *et al.* [12] showed subtype *adw* to have a 20-fold increased risk of lamivudine resistance compared to subtype *ayw*. Subsequently it was shown subtype *adw* to have a better response for lamivudine than subtype *ayw* [206]. Later, Buti *et al.* [11] showed that the subtype-dependent risk of lamivudine resistance is not associated with prolonged treatment for 2-3 years and pointed out that lamivudine resistance takes longer time to emerge in subtype *ayw*.

### 3.9 Costs of HBV therapy

A one year course of peg-IFN is estimated to cost approximately $18,000. Among the oral drugs, lamivudine is estimated to be the cheaper drug available for HBV treatment. It costs approximately $2,500 for one year of treatment. The cost for other HBV antivirals adefovir, tenofovir and telbivudine approximately ranges between $6000 and $6500 per year. Entecavir is the expensive oral drug (approximately $8,700) and it adds up to further costs for more than one-year course of therapy [61].

### 3.10 HBV treatment: Indian Scenario
### 3.10.1 Experience with Interferon treatment

A randomized-control trial showed loss of HBV DNA (<1 pg HBV DNA by dot-blot hybridization assay) and HBeAg in 50% of subjects treated with IFN-α-2b for 4 months as compared to spontaneous clearance of 4.8% in the placebo. The response was accompanied by decrease in ALT levels. Additionally, anti-HBe seroconversion was shown in 35% of the IFN-group as compared to 4.8% in the placebo group. Sustained response increased to 65% of the subjects after 12 months-off therapy. Anti-HBe seroconversion and HBsAg loss was shown in 50% and 15% respectively [207]. Another study in HBeAg-negative subjects

treated with IFN-α-2b treated for 4 months showed loss of HBV DNA (<1 pg HBV DNA by dot-blot hybridization assay) in 72% subjects. However, the relapse rate was high after stopping therapy [208].

### 3.10.2 Experience with oral drugs

In a randomized study comparing sequential therapy with IFN+lamivudine combination to lamivudine monotherapy in HBeAg positive subjects, sequential therapy was found to be more efficacious [209]. After one year of treatment, HBeAg loss with or without anti-HBe seroconversion and undetectable HBV DNA ($<1.4 \times 10^5$ copies/mL) were shown in 26% and 14% respectively. ALT levels normalized in 48% and 41% respectively. Most of the subjects who had sequential therapy also showed higher maintained response rate at the end of 72 weeks. Lamivudine resistant rtM204V/I mutations were shown in 16% and 8% respectively and there was no significant difference between the treatment groups. Moreover, the relapse rate was significantly higher in subjects with lamivudine monotherapy. In another randomized control study, the effect of initial lamivudine therapy for 4 weeks followed by peg-IFN for 24 weeks in HBeAg positive subjects was studied. In comparison to peg-IFN alone treated subjects, initial lamivudine therapy followed by peg-IFN treated subjects showed better sustained virological response [210].

In a cross-sectional analysis of 17 subjects on lamivudine therapy, one subject with 12 months of lamivudine and five subjects with 18 months of lamivudine were detected to carry rtM204V/I mutation. This is the first and only Indian report that documented a prevalence of 6% and 29% of lamivudine resistance mutations at 12 and 18 months of treatment respectively [43]. In 32 HBeAg subjects, end-of treatment response (ETR) and sustained virological response (SVR-6) was documented in 25% and 22% lamivudine-experienced subjects. The ETR and SVR-6 in 22 HBeAg-negative subjects was found to be 48% and 40%

respectively. In this study, they have also shown 16 types of lamivudine-induced surface gene mutations [211]. In other 17 subjects with chronic hepatitis B, both core promoter and YMDD motif mutations was shown to be associated with virological breakthrough (HBV DNA $>10^6$ copies/ml) for long-term lamivudine treatment of ≥12 months. In an open labelled trial, lamivudine-induced HBeAg seroconversion rates were documented to be 29%, 37% and 40% at years 1-3 respectively [212].

In a pilot study comparing lamivudine and adefovir for the treatment of chronic hepatitis B, there was no sufficient evidence to show the therapeutic advantage of one drug over another [213]. There are very few reports showing the experience of oral antiviral drugs for HBV management in the Indian subcontinent. The major limitation of all these studies is the small sample size.

### 3.11 Distribution of HBV genotypes and subgenotypes in India

HBV genotypes A, C and D are the major genotypes identified in India. Genotypes A and D are documented to be the prevailing genotypes in mainland India [10, 214-216]. The evidence of genotype C in eastern India was first reported by our laboratory and now is known to be the common genotype circulating in this region [178, 216-218]. Among the genotypes, subgenotype A1; subgenotype C1, subgenotype D1, D2, D3 and D5 are the currently reported subgenotypes in India [178, 218-221]. A community study reported a unique subgenotype D5 in primitive tribals in Eastern India. On complete genome analysis, 27 amino acid residues specific to subgenotype D5 were identified and are considered to be the signature substitutions that will enable subgenotype D5 classification. Based on the estimated divergence time, this study also showed subgenotype D5 to be the most ancient subgenotype compared to subgenotypes D1 to D4 [222]. A study from Haryana state of North India showed the existence of genotype E (5%) by surface gene sequences [223].

Another study from North India, reported a case of occult hepatitis B virus with genotype G infection but this was only shown by partial surface gene sequences [224]. Recently, a novel recombinant genotype was identified in a primitive tribe from Arunachal Pradesh state in Eastern India. This genotype clustered with Vietnam and Laos genotype and was confirmed to be genotype I by complete genome sequencing and recombination analysis [185]. Thus the eastern part of India is shown to be of greater epidemiological importance for HBV because of the routes of transmission (higher intravenous drug use) and the presence of established genotype with poorer prognosis (genotype C) and novel genotype.

**3.12 Distribution of HBV subtypes in India**

HBV subtype *adw2* of genotype A; subtype *ayw3* and *ayw2* of genotype D; subtype *adr* of genotype C [178, 225] were documented in India. The presence of genotype C was cited in Indian reports starting from year 2006. Subtype *adr* which is always known to be associated with genotype C was earlier reported in 1991 from high risk groups and blood donors in Bombay [226].

**3.13 Costs of HBV therapy: Indian scenario**

One of the practical difficulties in the management of hepatitis B in the Asia-pacific region is the cost of antiviral drugs [227]. Treatment with standard IFN costs approximately INR 140,000 and for peg-IFN therapy costs to approximately INR 660,000 for one year [228]. Using the Markov transitional probability model, IFN therapy was not shown to be a cost-effective drug in developing countries like India [229]. The cost of lamivudine and adefovir ranges from INR 3000 to 7000 per year and seems to be the pragmatic therapy options for HBV treatment [230]. A one year course of entecavir, telbivudine or tenofovir is estimated to be around INR 80,000, 65,000 and 16,000 respectively [228]. Due to cost constraints the use of entecavir and telbivudine may be limitedly used for long-term treatment. Hence, treatment

of HBV largely depends on oral drugs particularly lamivudine despite the limitations and challenges in using this antiviral agent in the Indian subcontinent.

# 4. MATERIALS & METHODS

## 4. MATERIALS AND METHODS

### 4.1 Sample size calculation

The sample size was calculated using the statistical software for epidemiology, Epi Info version 6 (Epi6).

In an earlier study from India, the prevalence of lamivudine resistant mutants (rtM204I/V) at 12 months was 6% [43]. Taking this as the prevalence for the calculation of sample size with the precision of 4% and 95% confidence interval (CI), a sample size of 136 lamivudine treated chronic hepatitis B subjects was required.

An equal number of 136 treatment-naive chronic hepatitis B subjects were required for baseline characteristics.

In India, reports on adefovir and entecavir resistance mutations for HBV are still evolving. These drugs are relatively sparingly used in our centre for the treatment of chronic HBV infection. Therefore, at least 30 subjects in each group, considered as minimal number for statistical analysis was required.

### 4.2 Study design

The study has two parts: a cross-sectional analysis and a prospective analysis. The algorithm of study subjects recruited and analysed is shown in **Figure 9**.

### 4.3 Ethics approval and funding

The study was approved by the Institutional Review Board (EC Min. No. IRB (EC)-10-16-01-2008) and informed written consent was obtained from all the subjects. Subjects also gave consent to use archived samples if available **(Appendix II and III)**.

**Figure 9. Algorithm of subjects recruited and analysed**
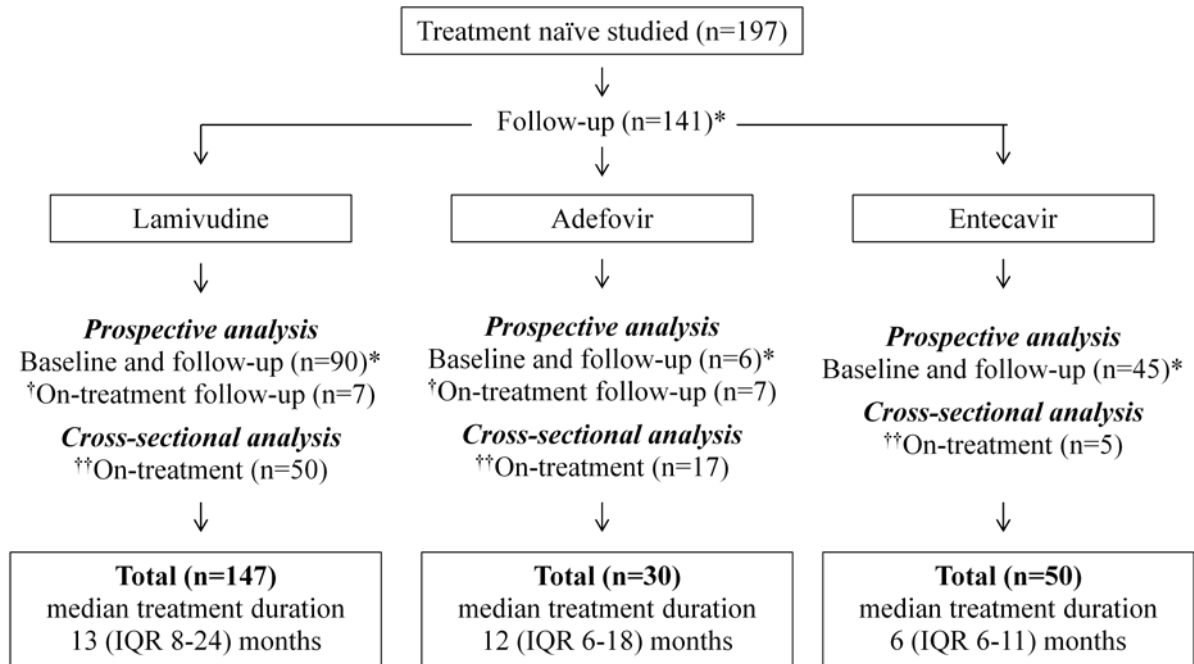


n- indicates number of subjects

*indicates number of subjects recruited with treatment naive samples and followed up with lamivudine or adefovir or entecavir treatment. Remaining subjects did not follow-up or did not meet the inclusion criteria.

On-treatment subjects were of two categories: [†]those who were part of prospective analysis with follow-up samples and [††]those who had only one-time sampling

IQR- Interquartile range

Apart from routine HBV DNA quantification and serology, drug resistance testing was entirely funded by Indian Council of Medical Research (ICMR), India (Ref. No. 5/8/7/7/2008-ECD-1).

## 4.4 Subjects

The study subjects comprised of individuals attending the liver clinic of Christian Medical College, a tertiary care teaching hospital in Vellore, South India. These subjects were referred to the department of Clinical Virology for HBV DNA testing and were recruited between January 2007 and November 2011.

The inclusion criteria were chronic HBV infection with documented evidence of HBsAg positivity for more than 6 months and at least one sample (pre or post therapy) with detectable HBV DNA for sequence analysis. Treatment compliance was checked by verbal questioning and by reviewing the clinical records. Patients who reported to adhere strictly to the treatment schedule without any interruption were only recruited.

The exclusion criteria were history of previous treatment with other HBV antivirals and immunomodulators (switch-off therapy); add-on or combination therapy; Infection with hepatitis C, hepatitis D or human immunodeficiency virus (HIV); use of immunosuppressive drugs and chemotherapy.

## 4.5 Collection and processing of samples

Blood samples (8-10 mL) were collected by venipuncture in vacutainer tubes containing di-potassium ethylene diamine tetra acetate-$K_2$EDTA (Beckton Dickinson, Plymouth, UK). The plasma was separated on the day of collection after centrifugation at 2500 rpm ($1000 \times g$) for 10 mins at room temperature and stored in aliquots at -60°C until testing.

## 4.6 Biochemical tests

Serum alanine transaminase (ALT) and serum aspartate transaminase (AST) levels were obtained from the subject's hospital records. The normal range for ALT and AST levels were 8-40 U/L and 5-35 U/L respectively [97].

## 4.7 Serology markers

Hepatitis B surface antigen (HBsAg) was tested in any one of these assays: AxSYM (Abbott,Weisbaden, Germany), ARCHITECT (Abbott, Weisbaden, Germany) and Monolisa HBsAg ULTRA (Bio-Rad, Marnes-la-coquette, France). HBeAg and anti-HBe testing was performed in an enzyme immunoassay (EIA) (Diasorin S.P.A., Saluggia, Italy). HCV antibody (Ab), HDV Ab and HIV were screened in Ortho HCV 3.0 (Ortho Clinical Diagnostics, Raritan, N.J., USA), IgM anti-HD EIA (Diasorin S.P.A., Saluggia, Italy) and AxSYM or ARCHITECT HIV Ag/Ab combo (Abbott, Weisbaden, Germany) respectively. The manufacturer's instruction was strictly followed for all the procedures.

## 4.8 Molecular methods

All molecular methods were performed in unidirectional workflow taking appropriate precautions as prescribed by Kwok and Higuchi [231] and Ratcliff *et al.* [232].

## 4.8.1 DNA isolation

DNA was extracted from plasma using the QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) as per manufacturer's instructions. Briefly, twenty micro litres (µl) of Qiagen protease, 200 µl of plasma sample and 200 µl of lysis buffer (AL) were added into 1.7mL micro centrifuge tube. The tubes were pulse vortexed for 15 seconds and briefly spun down and incubated at 56°C for 10 minutes in a dry bath (Genei, Bangalore, India). After the incubation, tubes were centrifuged at 8000 rpm ($6000 \times g$) for 1 minute. Two hundred micro litres of absolute alcohol was added and pulse vortexed for 15 seconds and briefly spun

down. The entire content was transferred to the Qiagen spin column (silica-gel membrane) and centrifuged at 8000 rpm (6000 × *g*) for 1 minute. Five hundred micro litres of reconstituted wash buffer (AW1) was added and centrifuged at 14,000 rpm (20,000 × *g*) for 3 minutes. Five hundred micro litres of reconstituted wash buffer (AW2) was added and centrifuged at 14,000 rpm (20,000 × *g*) for 3 minutes. The spin columns were placed into micro centrifuge tubes and 50 µl of elution buffer (AE) was added and incubated at room temperature for 5 minutes. The spin columns with microcentrifuge tubes were centrifuged at 8000 rpm (6000 × *g*) for 1 minute and the eluted DNA was stored in aliquots at -80°C.

### 4.8.2 HBV DNA quantification

**Principle**

The quantification of HBV DNA utilizes TaqMan probe principle which relies on the 5′-3′ nuclease activity of *Taq* polymerase to cleave a dual-labelled probe during hybridization to the complementary target sequence and fluorophore-based detection. In real-time PCR method, the resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the PCR.

The artus® HBV RG PCR assay (Qiagen GmbH, Hilden, Germany) constitutes a ready to use system for the detection of HBV DNA using PCR in Rotor-Gene™ 3000 or 6000 platform (Corbett Research, Mortlake, Vic., Australia). The HBV RG master mix contains reagents and enzymes for the specific amplification of 134 base pair (bp) region of the HBV genome. The amplification of HBV DNA is detected by the emission of fluorescence signal from 6-carboxyfluorescein (FAM). The assay has an additional second heterologous amplification system, an internal control (IC) to identify PCR inhibition by measurement of fluorescence signal from 6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxyfluorescein (JOE). The assay employs the use of five Quantitation Standards (HBV RG QS 1-5) that is calibrated

using the 1<sup>st</sup> WHO International standard for HBV DNA. The system software uses the standards to generate a standard curve for the absolute quantification of viral load.

**Amplification and quantification of HBV DNA**

The cooling block (Corbett Research, Mortlake, Vic., Australia) was pre-cooled at +4°C for 30 minutes and 0.2mL flat capped PCR tubes were placed. The HBV RG master mix and HBV RG IC were equilibrated to room temperature and mixed gently and centrifuged briefly. Thirty micro litres of HBV RG master mix was mixed with 2 µl of IC per reaction in a microcentrifuge tube. Thirty micro litres of PCR mix was dispensed into each PCR tube and 20 µl of extracted sample DNA and standards were added into appropriate tubes. The PCR tubes were loaded into the 36 well rotors in a Rotor-Gene PCR platform and the amplification was performed with the following cycling conditions: pre denaturation at 95°C for 10 minutes and 45 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 15 seconds.

The artus HBV RG PCR assay is European Conformity (CE)-marked and *in vitro* diagnostics (IVD)-licensed. The manufacturer's claimed lower limit of detection (LLD) is 20 IU/mL. The LLD in consideration of DNA purification with Qiamp DNA Blood Mini kit according to our determination was 82 IU/mL (95% detection limit) [233].

**Quality controls**

Two samples with known HBV DNA levels (low and high positive controls) and multiple sterile milliQ water (negative template controls) spaced for every three samples were included in all assays. The assay was considered valid, only if the positive controls showed $\leq$ $\pm 0.5$ $\log_{10}$ IU/ml difference from previously determined 20 data points (acceptable range = median HBV DNA of 20 data points $\leq$ $\pm 0.5$ $\log_{10}$ IU/ml) and when the negative controls

were negative. In addition, assays were considered valid if coefficient of variation (CV) for the five standards was less than 10%.

Samples that showed FAM and JOE signals were reported positive and samples that showed only JOE signal were reported negative. PCR inhibition was indicated by absence of both FAM and JOE signals. The samples showing internal control inhibition were repeated in a subsequent assay.

**4.8.3 HBV polymerase/reverse transcriptase gene PCR**

HBV polymerase gene covering the entire reverse transcriptase (rt) region was amplified (1323 bp) using Platinum® *Taq* DNA polymerase high fidelity (Invitrogen, Carlsbad, Calif., USA) as described previously [28]. The PCR was performed for all samples that were positive in HBV quantification PCR. The primer sequences were custom synthesized at Sigma-Aldrich (Bangalore, India) and are shown in **Table 1**.

The PCR reaction mix contained 5-10 µl (upto 200 ng) of DNA template, 1X high fidelity PCR buffer, 0.2mM dNTPs, 2mM $MgSO_4$, 0.2µM of each primers and 1 unit of Platinum® *Taq* high fidelity. The total volume of the mix was made up to 50 µl with sterile milliQ water. The amplification reactions were carried out on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, Calif., USA) or MyCycler™ (BioRad, Hercules, Calif., USA) with the following cycling conditions: Initial denaturation 94°C for 2 minutes, followed by 40 cycles of 94°C for 15 seconds, 55°C for 15 seconds and 68°C for 3 minutes.

The amplicons were mixed in a 1:6 concentration with loading dye containing bromophenol and sucrose. The amplified products were then run on a 1.5% agarose gel (Seakem® LE agarose, Lonza, Rockland, ME, USA) containing ethidium bromide (0.5µg/mL) and visualized by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel

**Table 1. HBV polymerase/reverse transcriptase gene amplification and sequencing primers**

| Primer | Primer Sequence (5′-3′) | [*]Nucleotide positions |
|---|---|---|
| **Amplification Primers** [28] | | |
| Forward | CCT CAG GCC ATG CAG TGG AA | 3196-3215 |
| Reverse | CCT GCT GCG CGC AAA ACA AGC GGC TAG GAG TTC CGC AGT ATG GA | 1308-1265 |
| **Sequencing primers** [28, 234] | | |
| SP1 | CTC CAG TTC AGG AAC AGT AAA CCC | 67-90 |
| ISP2 | CGA ACC ACT GAA CAA ATG GC | 704-685 |
| HBVFS4 | TGT ATT CCC ATC CCA TC | 599-615 |
| HBV4 | GCT AGG AGT TCC GCA GTA TGG A | 1286-1265 |

[*]Nucleotide positions according to the ECORI site between the pre-S1 and pre-S2 region of HBV genome (Gen Bank accession No. X04615)

documentation system (BioRad, Hercules, Calif., USA). Sterile milliQ water was used as negative control in each run.

### 4.8.4 DNA purification and sequencing

### 4.8.4.1 Pre-cycle sequencing clean-up

The amplified PCR products were purified by Multiscreen HTS PCR plate (Millipore, Billerica, Mass., USA). The purification before setting up the sequencing reaction helps to remove dNTPs and primers. Briefly, the amplified product was made up to 100 µl using nuclease-free water (Ambion, Austin, TX, USA). The diluted product was then transferred to the Millipore pre-sequencing PCR plate and placed on the Millipore vacuum manifold. Vacuum pressure was applied until the well is completely dried. The step was again repeated by adding 100 µl of nuclease-free water. Upon complete drying of the wells, 20µl of nuclease-free water was added and mixed in titer plate shaker (Barnstead International, Dubuque, IA, USA) for 5 minutes. Finally, the contents were transferred to PCR tubes.

### 4.8.4.2 Automated nucleotide sequencing by capillary electrophoresis

**Principle**

Automated cycle sequencing procedure using dye terminator chemistry incorporates dideoxynucleotide tri-phosphates (ddNTPs), each tagged with different fluorescent dye. Each dye emits a unique wavelength when excited by light. Thus the fluorescent dye on the extension product identifies the 3′ terminal dideoxynucleotide as adenine (A), cytosine (C), guanine (G) or thymine (T).

During the cycle sequencing, the AmpliTaq DNA polymerase FS (Applied Biosystems, Foster City, Calif., USA) extends the primer, incorporating dideoxynucleotide tri-phosphates (ddNTPs) that stop the extension reactions. This process generates fragments randomly that differ in length by one base.

In the ABI PRISM 310 Genetic Analyzer, the autosampler brings each sample successively into contact with the cathode electrode and one end of a glass capillary filled with performance optimized polymer-6 (POP-6). An anode electrode at the other end of the capillary is immersed in buffer.

As current flows from the cathode to the anode, a portion of the sample enters the capillary by electrokinetic injection. The sample forms a tight band during this injection and the end of the capillary near the cathode is then placed in buffer. When electrophoresis happens the negatively charged DNA molecules move through the polymer in an electric field and the DNA fragments are separated by size.

When the nucleotides reach a detector window in the capillary, a laser excites the fluorescent dye labels and the emitted fluorescence is recorded by a charge-coupled device (CCD) camera. The data collection software collects the raw data and the sequencing analysis software converts the data to a colour coded electropherogram in which blue represents C, green represents A, black represents G and red represents T.

**DNA cycle sequencing**

The sequencing reaction was carried out using the ABI Prism BigDye® terminator v3.1 cycle sequencing ready reaction reagents (Applied Biosystems, Foster City, Calif., USA). The primer sequences are showed in **Table 1** [28, 234]. The primers were custom synthesized and HPLC purified at Sigma-Aldrich (Bangalore, India). Briefly, 1 µl of the purified PCR product is mixed with 1.6 pmol of the primer, and 1 µl of the ready reaction mix with 2 µl of the sequencing buffer, making the volume to 10 µl with nuclease-free water. The cycling conditions consisted of 25 cycles of 96°C for 15 seconds, 50°C for 20 seconds and 60°C for 4 minutes.

### 4.8.4.3 Post-cycle sequencing clean-up

Excess salts and dye terminators were removed from the sequencing mixture using Montage SEQ 96 filtration (Millipore Billerica, Mass., USA). Briefly, the sequence reaction samples were made up to 40 µl using injection solution. The diluted reactions were then transferred to the $SEQ_{96}$ plate wells and placed on the Millipore vacuum manifold. Vacuum was applied until the wells are completely dried. The step was repeated by adding 40 µl of injection solution. Upon complete drying of the wells, 30 µl of injection solution was added and the DNA was completely resuspended by shaking for 5 minutes on a titer plate shaker (Barnstead International, Dubuque, IA, USA). Finally the contents were transferred to genetic analyzer sample tubes and sealed with septa (Applied Biosystems, Foster City, Calif., USA).

### 4.8.4.4 DNA sequencing and sequence analysis

The sequencing reactions were run on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster City, Calif., USA). The nucleotide sequences generated with a good read length of at least 550-600 bps were taken for analysis. Sequences with low signal and poor read lengths were repeated appropriately. Obtained bidirectional sequences were analyzed using BioEdit v7.0.9 and the consensus sequence was generated.

### 4.8.4.4.1 HBVrt sequence database

The generated sequences were submitted to the HBVSeq program for HBV drug resistance in Stanford database (http://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html) [235]. The database assigns a genotype to each sequence and compares the amino acid sequence to the corresponding consensus reference genotype amino acid sequence. The results are then displayed showing the difference between the submitted sequence and the database consensus sequences which are interpreted as mutations.

The output of the submitted sequence shows the following **(Figure 10)**

a) HBV genotype and list of well described drug resistance mutations.

b) Sequence quality assessment (QA) indicating the positions that contains stop codons, frame shifts, insertions, deletions, highly ambiguous nucleotides and mutations at highly conserved regions.

Sequences with any of the QA problems are shown as short red lines, polymorphic mutations as short blue lines and drug resistance mutations as tall blue lines at corresponding amino acid positions respectively.

c) A tabular display of mutations is shown according to genotype and treatment

Three-hundred and seventy two sequences generated from this study have been deposited in GenBank database under accession numbers GU798963 to GU799059 and JQ514280 to JQ514554.

**4.8.5 HBV genotypes, subgenotypes and subtype analysis**

The distribution pattern of HBV genotypes, subgenotypes and subtypes were determined for the total 296 chronic hepatitis B subjects (147 in lamivudine group, 30 in adefovir group, 50 in entecavir group and 69 treatment-naive subjects with no follow-up).

**4.8.5.1 Determination of HBV genotypes**

HBV genotypes were determined by HBVrt sequence analysis in the Standford database as described in section 4.8.4.4.1

**4.8.5.2 Genetic diversity of HBV genotypes**

An analysis of number of base substitutions per site between sequences [genetic distance ($d$)] was conducted using the Maximum Composite Likelihood method in MEGA4. The number of synonymous substitutions per synonymous site ($d$S) and the number of non-synonymous

# Figure 10. Schematic representation of hepatitis B virus reverse transcriptase sequence analysis in HBVseq Standford database

**SeqID: Rx Experienced follow-up aligned.fas_21 07H 157   Date: 12 March 2012**

## Summary Data

Sequence includes RT: codons: 1 - 344

*Genotype and % similarity to closest reference isolate: A (98.7%)*

*Mutations at established drug resistance positions: 180M, 204V*

## Sequence Quality Assessment

| Gene | QA Problem | Codons |
|------|------------|--------|
| RT | Stop Codons, Frame Shifts: | None |
| RT | B,D,H,V,N: | None |
| RT | Unusual Residues: | None |

RT

25  50  75  100 125 150 175 200 225 250 275 300 325

*Red lines indicate QA problems; Blue lines indicate differences from consensus genotype A sequence; Tall blue lines indicate sites associated with drug resistance.*

## Mutation Prevalence According to Genotype and Treatment

(a) Column headers contain the number of persons with isolates according to genotype and drug class exposure. (b) Table entries list the amino acid differences from genotype consensus and their frequency (%). (c) Detailed information on isolates containing a specific mutation can be obtained by clicking the mutation. (d) The table containing the rates of mutation at positions 1-344 in RT can be obtained by clicking here.

| Pos | NA | AA | NRTI Naive Persons | | | | | | | | | | NRTI Treated Persons | | |
|-----|----|----|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | A 439 | B 535 | C 707 | D 758 | E 227 | F 82 | G 23 | H 23 | I 32 | pooled 2806 | L-Nucl. 465 | Acyclic PO4 93 | L-Nucl. + Acyclic PO4 191 |
| 128 | AMC | NT | T $N^{2.5}$ $A^{0.9}$ $I^{0.5}$ $P^{0.5}$ $H^{0.2}$ $S^{0.2}$ | T $N^{1.9}$ $I^{1.9}$ $A^{1.7}$ $S^{0.4}$ $P^{0.4}$ | T $A^{7.4}$ $N^{0.4}$ $P^{0.3}$ $I^{0.1}$ | T $N^{2.0}$ $I^{1.2}$ $P^{0.4}$ $S^{0.3}$ $A^{0.1}$ | T $P^{0.4}$ | T $S^{2.4}$ | T | T $S^{4.3}$ | T | T $A^{2.4}$ $N^{1.4}$ $I^{0.8}$ $P^{0.4}$ $S^{0.3}$ $H^{0.0}$ | T $N^{2.5}$ $I^{2.2}$ $A^{1.2}$ | T $I^{7.0}$ $N^{5.8}$ $A^{1.2}$ | T $N^{3.7}$ $A^{1.6}$ $I^{1.1}$ $S^{0.5}$ $P^{0.5}$ |
| 129 | CTG | L | M $L^{44.1}$ $V^{1.4}$ $P^{0.5}$ $K^{0.2}$ | M $L^{1.1}$ $V^{0.4}$ | M $L^{8.4}$ | M $L^{10.0}$ $V^{0.3}$ $I^{0.1}$ | L $M^{0.9}$ $H^{0.4}$ | M $L^{22.0}$ | L $M^{4.3}$ | L | M $L^{25.8}$ | M $L$ $V^{0.4}$ $P^{0.1}$ $H^{0.0}$ $I^{0.0}$ $K^{0.0}$ | M $L$ $V^{0.5}$ | M $L$ | M $L$ |
| 180 | ATG | M | L $W^{0.2}$ | L $W^{0.2}$ | L $M^{0.3}$ | L $M^{0.1}$ $R^{0.1}$ | L | L | L $M^{4.3}$ | L | L | L $M^{0.1}$ $W^{0.1}$ $R^{0.0}$ | L $M^{34.4}$ | L $M^{2.2}$ | L $M^{33.5}$ |
| 204 | GTG | V | M $I^{0.9}$ | M $I^{0.2}$ | M $I^{0.4}$ $V^{0.3}$ $L^{0.1}$ | M $V^{0.1}$ | M | M | M $V^{4.3}$ | M | M | M $I^{0.3}$ $V^{0.1}$ $L^{0.0}$ | M $V^{24.0}$ $I^{20.6}$ $S^{0.2}$ | M $I^{1.1}$ $V^{1.1}$ | M $V^{25.7}$ $I^{16.0}$ |

L-Nucl. Lamivudine, entecavir and/or telbivudine; Acyclic PO4- adefovir or tenofovir

substitutions per non-synonymous site ($d$N) were calculated using the Nei-Gojobori model with Jukes-Cantor correction in MEGA4.

### 4.8.5.3 Determination of HBV subgenotypes

The study sequences were aligned with published sequences representing all known HBV subgenotypes [6, 180, 222, 236, 237]. Multiple sequence alignment was performed using the built-in CLUSTALW integrated in MEGA4 [238]. HBV subgenotypes were determined by phylogenetic analysis in MEGA4 using the neighbour joining method with a bootstrap test of 1,000 replicates and maximum composite likelihood algorithm.

### 4.8.5.4 Determination of HBV subtypes

A new programme for HBV subtype determination was developed in Microsoft Visual Basic (VB6). The overlapping surface gene sequence of HBVrt (155 to 835 nucleotides) was translated to the corresponding surface gene amino acids using BioEdit tool. The subtypes were then determined by the subtype programme that examines every combination of amino acids at position 122,160,127,159 and 140 (in this order) as deduced by Purdy *et al.* [9]. The algorithm used to determine the HBV subtypes is shown in **Figure 11**.

### 4.8.6 Clonal analysis

Clonal analysis was performed by TOPO TA cloning (Invitrogen, Carlsbad, Calif., USA) for one sample suspected with mixed HBV genotype infection.

### 4.8.6.1 Addition of 3′A-overhangs post-amplification

The PCR amplified HBVrt region was purified by Multiscreen HTS PCR plate (Millipore, Billerica, Mass., USA). This step was performed to ensure the removal of high fidelity *Taq* used for HBVrt amplification, as the presence of *Taq* polymerase with proof reading activity will remove the 3′A-overhangs required for TOPO TA cloning. After the purification, poly-A

**Figure 11. Algorithm used to determine hepatitis B virus subtypes**
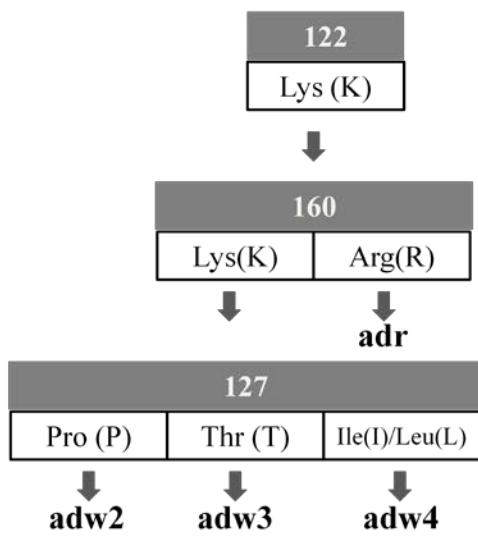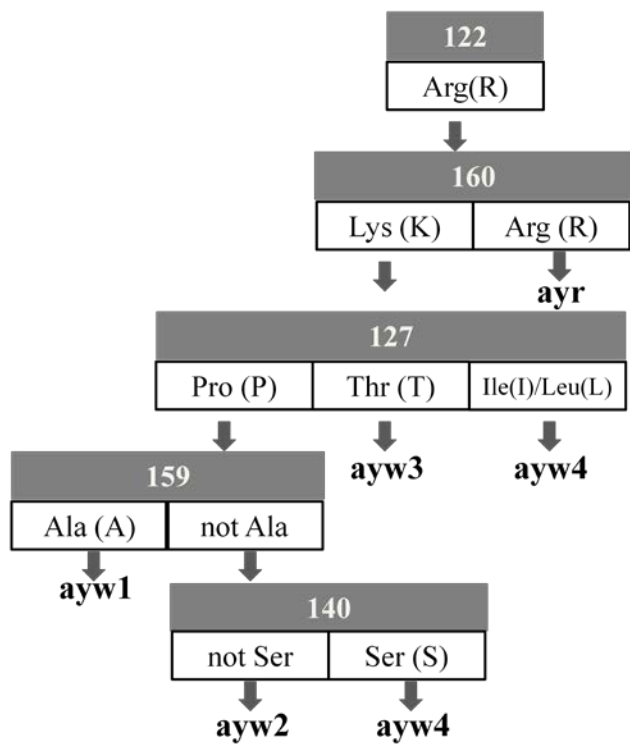


The HBV subtypes were determined using the surface gene codons as described by Purdy *et al.*(2007) [9]

tailing step was performed by adding 1 unit of SupraTherm™ *Taq* polymerase (GeneCraft, Munster, Germany) and 0.2 mM dATP (New England BioLabs, Ipswich, Mass., USA). The poly A-mix was then incubated at 72°C for 20 minutes**.**

### 4.8.6.2 TOPO cloning reaction

To 2 µl of the poly-A tailed product, 1 µl of the salt solution (1.2 M NaCl and 0.06 M MgCl$_2$) and 1 µl of the TOPO Vector (Invitrogen, Carlsbad, Calif., USA) were added. The total volume of the mix was made up to 6 µl with the supplied PCR grade water. The tubes were mixed by swirling and incubated at room temperature for 30 minutes.

### 4.8.6.3 Transformation

The cloned product is transformed into TOP10 *Escherichia coli* competent cells (Invitrogen, Carlsbad, Calif., USA) by chemical transformation according to the manufacturer's protocol. Briefly, two micro litres of the cloned product was added to the thawed competent cells and incubated for 15 minutes in ice. Heat-shock treatment was given to the cells for 40 seconds at 42°C in an equilibrated water bath (Thermo Fischer Scientific, Newington, USA). The tubes were immediately transferred to ice. The cells were supplemented with 250 µl of room temperature SOC medium and incubated at 37°C for 90 minutes in a shaking platform at 200 rpm (Tarsons, India). The transformed cells were then spread in prewarmed LB plates containing 25 µg/mL of ampicillin overlaid with 40 µl of X-gal (Invitrogen, Carlsbad, Calif., USA). The plates were then incubated at 37°C overnight.

### 4.8.6.4 Analyzing transformants

The recombinant clones were selected by blue-white screening using X-gal. All recombinants were white due to the insertional inactivation of lacZ gene which in wild type colonies produces beta-galctosidase that reacts with X-gal and produces blue colour. The X-gal screening thus helps to distinguish self ligated vectors and vectors with desired fragment of

interest. At least twenty colonies were individually selected and cultured overnight in LB medium containing 25 µg/mL of ampicillin.

### 4.8.6.4.1 Plasmid isolation

After overnight incubation, plasmid isolation was performed using PureLink™ Quick Plasmid kit (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol. Briefly, 5 mL of overnight culture was spun down at 4000 rpm (3000 × $g$) at 4°C for 10 minutes. The pellet was completely resuspended in 250 µl of resuspension buffer (R3) with RNase A. After addition of 250 µl lysis buffer (L7), the tubes were mixed gently by inverting and incubated for 5 minutes at room temperature. Three fifty micro litres of precipitation buffer (N4) was added to the lysate and mixed by inverting to make the solution homogenous. The tubes were then centrifuged at 11,000 rpm (12,900 × $g$) for 10 minutes at room temperature. The supernatant was transferred to the spin column and centrifuged at 11,000 rpm (12,900 × $g$) for 1 minute at room temperature. Seven hundred micro litres of wash buffer (W9) with ethanol was added and centrifuged at 11,000 rpm (12,900 × $g$) for 1 minute at room temperature. The centrifugation step was repeated with a fresh wash tube to remove residual wash buffer, if any. The spin columns were placed into microcentrifuge tubes and 50 µl of preheated elution buffer (TE, 65°C) was added and incubated at room temperature for 2 minutes. The spin columns with microcentrifuge tubes were centrifuged at 11,000 rpm (12,900 × $g$) for 2 minutes. The eluted DNA was used to analyze the transformants by HBVrt PCR amplification and sequencing (as shown in section 4.8.3 and 4.8.4).

### 4.8.6.5 Phylogenetic analysis

Hepatitis B virus reverse transcriptase sequences of respective clones were aligned with reference HBV genotype sequences. Phylogenetic analysis was performed in MEGA4 using

the neighbour joining method with a bootstrap test of 1,000 replicates and maximum composite likelihood algorithm to identify mixed HBV genotype infection.

## 4.9 Molecular modeling and docking studies

Schematic diagram of homology modeling and docking analysis used to predict HBV antiviral drug resistance mutations is shown in **Figure 12**.

### 4.9.1 Homology model of hepatitis B virus polymerase/reverse transcriptase

A homology model of HBVrt was built in MODELLER 9v8 using the crystal structure of HIV-1rt template (Protein Data Bank, PDB code: 1RTD chain A). HBVrt nucleotide sequence was translated into the amino acid sequences using BioEdit. The translated target sequence was aligned with the HIV-1rt template using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The target-template alignment was used to build the three-dimensional model of target protein. At least five models were generated for each target and the model with lowest Discrete Optimization Protein Energy (DOPE) is selected.

### 4.9.2 Structure Validation

The structure validation was performed in PROCHECK using the Structure Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/) to check the stereochemical quality of the protein structure.

### 4.9.3 Docking studies

To the modelled protein, the two magnesium ($Mg^{2+}$) ions and the template primer DNA duplex [d(GCXCCGGCGCTC)-d(GAGCGCCGG)] were located based on the co-ordinates of PDB: 1RTD chain A of HIV-1rt. The 'X' in the DNA duplex was substituted to the complementary base of the incoming nucleotide or nucleoside reverse transcriptase inhibitors

**Figure 12. Schematic diagram showing the process of homology modeling and docking analysis for the prediction of hepatitis B virus drug resistance mutations**
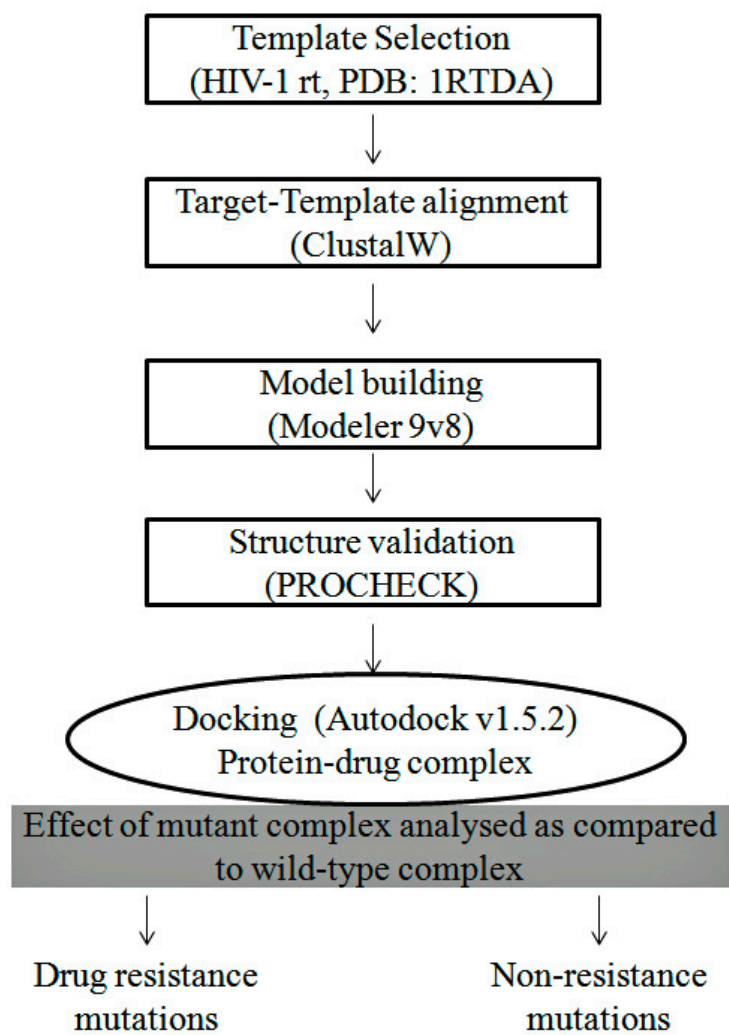


PDB- Protein Data Bank Code

ClustalW- Multiple-sequence alignment tool

PROCHECK- checks the stereochemical quality of the protein structure

(NRTI): lamivudine (cytidine analogue) X=G, adefovir (adenosine analogue) X=T and Entecavir (guanosine analogue) X=C respectively. The generated model and the respective NRTIs for which the effect should be studied were then docked using Autodock tools (v1.5.2). The amino acids interaction in the modelled protein and the drug binding efficiency was visualized using PYMOL (DeLano Scientific LLC, San Carlos, Calif., USA).

## 4.10 Statistical analysis

Continuous variables were summarized using means and standard deviations, if normally distributed. Medians with interquartile range (IQR) were used to describe variables with skewed distribution.

Correlation between HBV DNA and ALT levels, HBVrt amino acid substitutions and age were analyzed using Spearman's correlation coefficient.

Comparison of study variables (ALT, AST, HBV DNA, HBeAg, anti-HBe, treatment duration, HBVrt amino acid substitutions, $d$, $dS$ and $dN$) was done using non-parametric tests; Kruskal-Wallis test, Chi-square test, Wilcoxon rank-sum test or Wilcoxon signed-rank test as appropriate. A p-value of <0.05 was considered statistically significant.

All variables in the univariate analysis significantly associated were entered into the multivariate analysis. Multivariable logistic regression analysis was performed to assess the predictive factors of treatment response and antiviral resistance. The cumulative proportion of resistance mutations was showed by Kaplan-Meier analysis.

All analysis was done using STATA 11 (StataCorp, College Station, Tex., USA).

**4.11 Definitions**

**Normalization of ALT and AST levels** is defined as the decrease in serum ALT and AST levels to normal range of 5-35 U/L and 8-40 U/L respectively

**Early virological response**

Early virological response (EVR) is measured at the median treatment duration of 6 months and are categorized as

- **Complete virological response** defined as undetectable HBV DNA (<82 IU/mL)

- **Partial virological response** defined as reduction in HBV DNA levels $\geq 1$ $\log_{10}$ IU/mL from baseline

- **Primary treatment failure or non-response** defined as the lack of reduction of HBV DNA to $\geq 1$ $\log_{10}$ IU/mL from baseline

The lower limit of detection (LLD) for artus HBV RG real-time PCR according to our determination was 82 IU/mL (95% detection limit) [233].

**End-of-treatment response**

End-of-treatment response (ETR) is measured at the median treatment duration of 12 months and are categorized as

- **Virological response** defined as undetectable HBV DNA (<82 IU/mL)

- **Secondary treatment failure or virological breakthrough** defined as $\geq 1$ $\log_{10}$ IU/mL increase in HBV DNA levels compared with the lowest HBV DNA levels during therapy (nadir value)

**Maintained response**

Maintained response is measured at the median treatment duration of 24 and 41 months and subjects who continued to show virological response (undetectable HBV DNA, <82 IU/mL) were classified as responders

**Sustained virological response**

Sustained virological response is defined as undetectable HBV DNA (<82 IU/mL) measured at the median duration of 6 (SVR-6), 12 (SVR-12) or 18 (SVR-18) months after cessation of therapy

**HBeAg seroconversion** is defined as loss of HBeAg and detection of anti-HBe in subjects who were previously HBeAg positive

# 5. RESULTS & ANALYSIS

## 5. RESULTS AND ANALYSIS

### 5.1 Treatment-naive group

A total of 198 HBV DNA positive subjects who have never been exposed to any HBV antivirals were studied. Among these subjects, 166 (84%) were male and 32 (16%) were female; their median age was 36 (IQR 27-47) years.

### 5.1.1 Biochemical parameters

Among the 198 treatment-naive subjects, normal serum ALT levels (5-35 U/L) were seen in 77 (39%) subjects, 69 (35%) had ALT levels of 1-2 ULN (35-70 U/L) and 52 (26%) had >2 ULN (>70 U/L). Likewise, normal serum AST levels (8-40 U/L) were seen in 93 (47%) subjects, 63 (32%) had AST levels of 1-2 ULN and 42 (21%) had >2 ULN (>80 U/L). The median levels for ALT and AST were 42 (IQR 27-73) U/L and 42 (IQR 28-74) U/L respectively; and showed good correlation (Spearman's rho, r=0.77, p<0.0001).

### 5.1.2 Virological parameters

### 5.1.2.1 Serology

All subjects were positive for HBsAg. Hepatitis B e-antigen was positive in 120 (61%) subjects and 78 (39%) were HBeAg-negative. Four (3%) HBeAg positive subjects in the anti-HBe seroconversion phase and 71 (91%) HBeAg-negative subjects were positive for anti-HBe antibody.

### 5.1.2.2 Molecular testing

### 5.1.2.2.1 HBV DNA quantification

A representative amplification plot of real-time PCR and standard curve generated for HBV DNA quantification are shown in **Figure 13a and Figure 13b**. The HBeAg-positive subjects had median HBV DNA levels of 6.7 (IQR 5-7.48) $\log_{10}$ IU/mL and was significantly higher

**Figure 13a. A representative amplification plot of HBV DNA quantification in plasma**
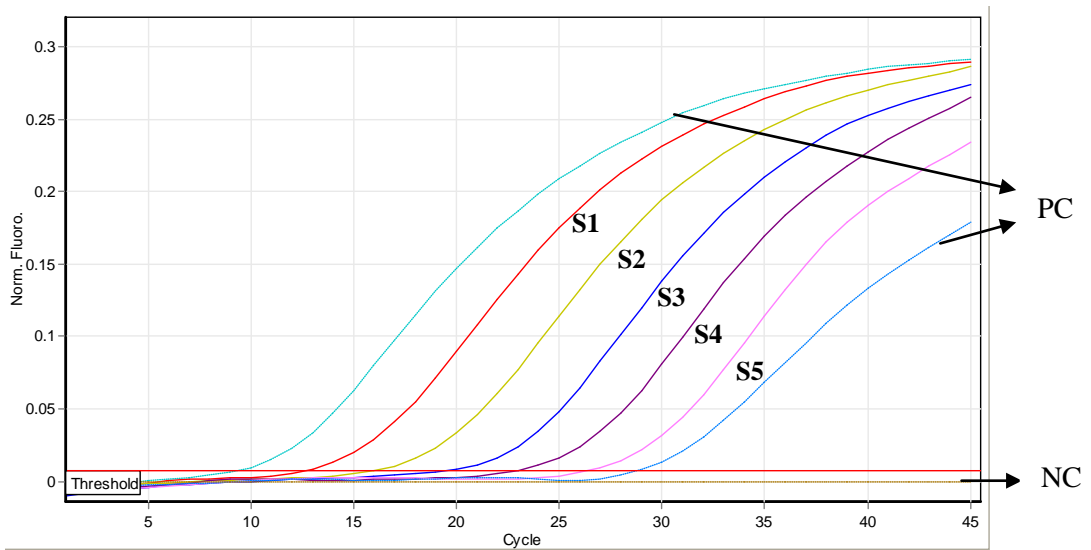


The X axis shows the number of cycles performed and Y axis shows the fluorescence intensity. The horizontal line across the graph is the threshold. The sigmoid curve S1 to S5 indicates HBV DNA standards used to generate the standard graph. PCs and NC above and below the threshold indicates the positive and negative controls used in the run

**Figure 13b. A representative standard curve generated by logistic regression analysis**
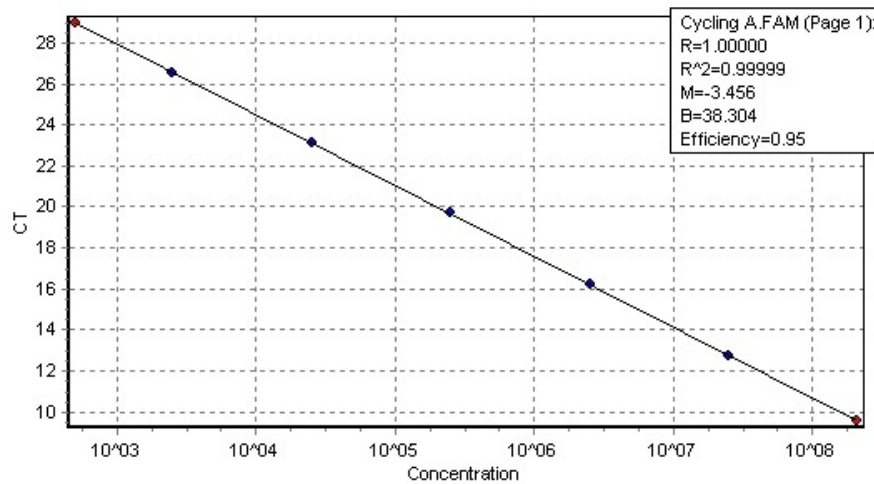


The standard curve is generated using the HBV DNA standards (ranging from $2.5 \times 10^3$ to $2.6 \times 10^7$ IU/mL). The concentration (IU/mL) is shown on the X axis and $C_t$ (threshold cycle) in the Y axis. The standards are shown as blue dots and samples are shown as red dots.

than HBeAg-negative subjects with the median HBV DNA levels of 4.39 (IQR 3.48-5.6) $\log_{10}$ IU/mL (Wilcoxon rank-sum, p<0.0001).

**5.1.2.2.2 HBV polymerase/rt gene amplification and sequence analysis**

A representative agarose gel (1.5%) electrophoresis of PCR amplified HBVrt product is shown in **Figure 14**. Among the 198 samples analyzed, no known "hot-spot" HBVrt mutations L80I/V, I169T, V173L, L180M, A181T/V, T184S, A194T, S202G, M204I/V, N236T or M250I/L/V that can independently affect the antiviral susceptibility to any of the drugs were seen. However, other amino acid substitutions were identified and are classified as:

- **Putative amino acid substitutions** that are possibly related to antiviral resistance
- **Compensatory amino acid substitutions** widely seen in treatment-experienced subjects that restore the replication fitness of the virus
- **Atypical amino acid substitutions** with unusual amino acid residues in HBVrt positions that are crucial for antiviral action
- **Naturally occurring polymorphisms** that show poor response to antiviral drugs and
- **Novel amino acid substitutions** that are different from the Stanford database consensus sequence

Additionally there were other amino acid substitutions not related to any of the above categories.

**5.1.2.2.2.1 Putative, compensatory, atypical amino acid substitutions and naturally occurring polymorphisms related to antiviral resistance**

Adefovir related rtI233V putative amino acid substitution was seen in 5 (2.5%) subjects. An antiviral resistant compensatory amino acid substitution (rtS213T) that restores the replication fitness during lamivudine and entecavir treatment was seen in 6 (3%) subjects.

**Figure 14. A representative agarose gel electrophoresis of PCR amplified HBVrt product**
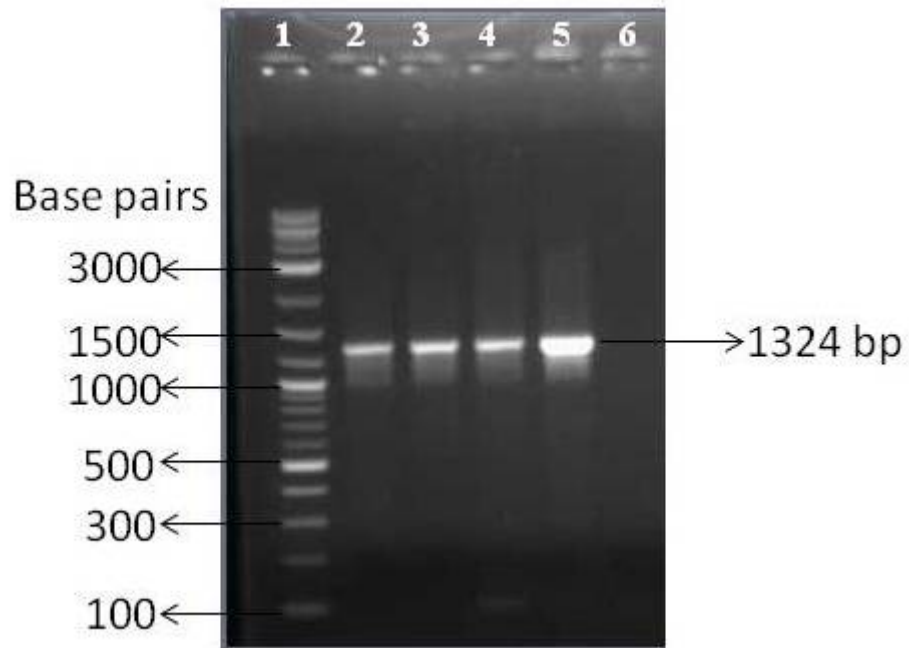


Lane 1:  Molecular ladder (2-log DNA ladder, 0.1-10 Kb)

Lane 2-5: HBVrt positive samples

Lane 6:  Negative control ($H_2O$)

All samples tested positive in HBV real-time PCR was used and therefore, a separate positive control was not included

Other putative and compensatory substitutions rtT128N, rtV214A, rtQ215S and rtN238T/S/D were identified in 1 (0.5%), 4 (2%), 5 (2.5%) and 7 (3.5%) subjects respectively.

Atypical amino acid substitution at positions that are crucial for antiviral action i.e., rtV84L, rtL180S and rtL180W+rtA181G substitutions were detected exclusively in 3 (1.5%) subjects. Naturally occurring polymorphisms (rtI91L and rtL217R) that respond poorly to lamivudine and adefovir were seen in 16 (8%) and 4 (2%) subjects respectively.

On follow-up analysis of 32 subjects with these pre-existing amino acid substitutions, 25 (78%) responded to lamivudine or entecavir treatment with $\geq 1$ $\log_{10}$ reduction in HBV DNA levels with a median treatment duration of 6 months (EVR, Early virological response) or loss of HBV DNA with a median treatment duration of 12 months (ETR, End-of-Treatment Response). The effect of rtI233V substitution could not be followed-up with adefovir therapy. However, two of the subjects with pre-existing rtI233V substitution showed EVR or ETR to lamivudine and entecavir subsequently. None of the pre-existing antiviral resistance related amino acid substitutions observed in treatment-naive subjects showed specific association with subsequent non-response.

**5.1.2.2.2.2 Novel HBVrt amino acid substitutions**

On comparison with the Stanford database consensus sequence, 22 unusual amino acid residues were identified in 20 subjects and were considered as novel HBVrt amino acid substitutions. The list of novel amino acid substitutions are shown in **Table 2**. All these subjects with novel HBVrt substitutions at baseline had follow-up samples. Among these 20 subjects, 14 (70%) showed EVR or ETR to lamivudine and entecavir subsequently. The remaining 6 (30%) subjects with rtW79C+rtF183L+rtL209M, rtR167G, rtK168R, rtQ182H, rtI266R amino acid substitutions and rtK333 stop codon mutation showed non-response to subsequent lamivudine or entecavir treatment.

**Table 2**. **Novel HBVrt amino acid substitutions in treatment-naive subjects and subsequent virological response**

| $HBVrt amino acid substitutions | Treatment | †Subsequent virological response |
|---|---|---|
| G4E | Entecavir | Response |
| F28L | Lamivudine | Response |
| W79C, F183L, L209M | Entecavir | Non-response |
| K154N | Entecavir | Response |
| H156Q | Lamivudine | Response |
| Y158D | Entecavir | Response |
| R167G | Lamivudine | Non-response |
| K168R | Lamivudine | Non-response |
| P170L | Lamivudine | Response |
| L175R/V/G | Entecavir | Response |
| Q182H | Lamivudine | Non-response |
| H216Q | Entecavir | Response |
| H264Q, L308F | Entecavir | Response |
| I265V | Entecavir | Response |
| I266R | Lamivudine | Non-response |
| K333N | Entecavir | Response |
| K333* | Entecavir | Non-response |

This table shows the novel hepatitis B virus reverse transcriptase (HBVrt) amino acid substitutions identified in treatment-naive subjects who subsequently showed response or non-response to lamivudine and entecavir treatment.

†Virological response is classified based on subsequent reduction in HBV DNA levels to $\geq 1$ $\log_{10}$ IU/mL within a median treatment duration of 6 months or undetectable HBV DNA (<82 IU/mL) at the median treatment duration of 12 months. Non-response is subjects who did not meet these criteria

*indicates stop codon mutation

$All substitutions were identified in single subjects except L175R/V/G (n=4)

On statistical analysis there was a significant positive correlation between age of subjects and number of HBVrt amino acid substitutions (r=0.31, p<0.0001). The median number of HBVrt amino acid substitutions were significantly higher in HBeAg-negative subjects [7 (IQR 5-10)] than in HBeAg-positive subjects [5 (IQR 4-7)]; p<0.001.

Nucleotide sequences generated from this analysis have been deposited in GenBank database under accession numbers GU798963 to GU799059 and JQ514280 to JQ514379.

**5.2 Lamivudine-experienced group**

A total of 147 lamivudine-experienced subjects were studied. Among these subjects, 119 (81%) were male and 28 (19%) were female; their median age was 39 (IQR 24-50) years. All adults received lamivudine at a dosage of 100 mg/day and children <10 years received 3mg/kg/day up to 100 mg/day.

**Categorization of study subjects**

An overview of subjects in cross-sectional and prospective analysis is outlined in **Figure 9** (Materials and Methods)

Among the 147 lamivudine-experienced subjects, 90 (61%) had their treatment-naive (baseline) and subsequent follow-up samples for testing, 9 (6%) had consecutive on-treatment samples and 48 (32%) had only one on-treatment sample available for testing. Overall 297 pre-and-post-therapy samples of these 147 subjects were tested. The number of subjects studied at varying treatment duration is shown in **Figure 15**.

In addition to baseline characteristics, on-treatment samples available for testing were grouped into any one or more of the following categories

- **Measurement of early virological response (EVR)** at the median treatment duration of 6 months (n=77)

- **Measurement of end-of-treatment response (ETR)** at the median treatment duration of 12 months (n=71)

- **Measurement of maintained response** at the median treatment duration of 24 (n=36) and 41 months (n=19)

- **Measurement of sustained virological response (SVR)** at the median duration of 18 months after cessation of therapy (n=4)

**Figure 15. Flow chart of lamivudine experienced subjects studied**
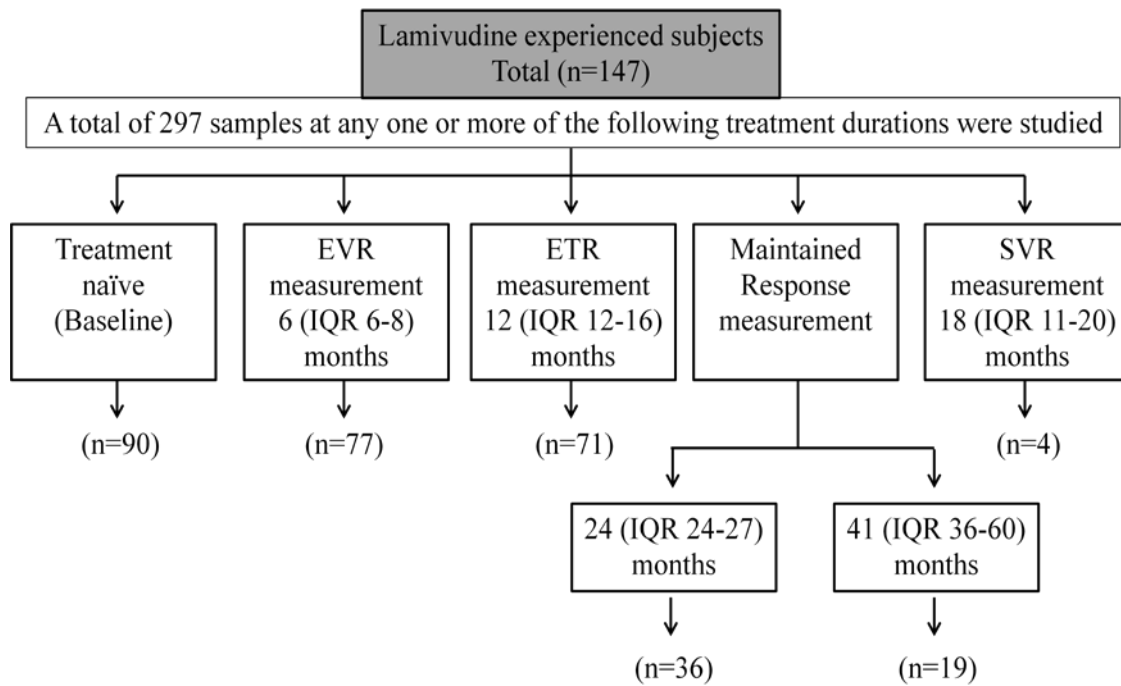


EVR – Early virological response

ETR – End-of-treatment response

SVR – Sustained virological response

**Ascertainment of clinical outcomes**

Virological response and antiviral resistance development were the two major outcomes determined in this study. In the samples analyzed, baseline variables were used to identify the predictive factors of response and on-treatment variables were used to identify factors associated with response.

**5.2.1 Baseline characteristics**

**5.2.1.1 Biochemical parameters**

Among the 90 treatment-naive samples followed-up with lamivudine treatment, 30 (33%) subjects had normal ALT levels, 28 (31%) had 1-2 ULN and 32 (36%) had >2 ULN. Likewise, 27 (30%) subjects had normal AST levels, 34 (38%) had 1-2 ULN and 29 (32%) subjects had >2 ULN. The median ALT and AST levels were 48 (IQR 30-97) U/L and 62 (35-95) U/L respectively.

**5.2.1.2 Virological parameters**

Among the 90 subjects, 55 (61%) were HBeAg-positive and 35 (39%) were HBeAg-negative. Two (4%) HBeAg-positive subjects in the anti-HBe seroconversion phase and 28 (80%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA levels in HBeAg-positive and HBeAg-negative subjects were 6.78 (IQR 5-7.6) and 4.3 (IQR 3.48-5.6) $\log_{10}$ IU/mL respectively (p<0.0001).

**5.2.1.3 Baseline factors for prediction of lamivudine response**

Among the 90 baseline samples analyzed, 48 (53%) responded to subsequent lamivudine treatment and 42 (47%) were non responders. To identify baseline factors that would predict the virological response to lamivudine, Kruskal-Wallis test for age, gender, ALT, AST, HBV DNA, HBeAg and anti-HBe was performed **(Table 3)**. In the factors analysed by univariate analysis, AST is the only factor that showed significant association for the prediction of

**Table 3. Univariate analysis of baseline factors for prediction of lamivudine response**

|  | Responders (n=48) | Non-responders (n=42) | p value |
|---|---|---|---|
| Age, years[†] | 44 (29-52) | 38 (24-49) | 0.308 |
| Gender, male[*] | 35 (73) | 37 (88) | 0.073 |
| ALT (U/L)[†] | 54 (32-151) | 46 (28-68) | 0.092 |
| AST (U/L)[†] | 77 (35-150) | 51 (35-74) | 0.037 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 5.3 (3.8-6.8) | 5.7 (4.3-7.6) | 0.168 |
| HBeAg Pos[*] | 25 (52) | 30 (71) | 0.060 |
| HBeAg Neg[*] | 23 (48) | 12 (29) | |
| Anti-HBe Pos[*] | 19 (40) | 11 (26) | 0.179 |
| Anti-HBe Neg[*] | 29 (60) | 31 (74) | |

Values are [†]median (Interquartile range, IQR) or [*]number (%)

virological response (p=0.037). The median AST level in the responders was 77 (IQR 35-150) U/L and in the non-responders was 51 (IQR 35-74) U/L **(Figure 16)**.

**5.2.1.4 Baseline factors for prediction of lamivudine resistance**

To predict antiviral resistance development, univariate analysis of baseline factors including age, gender, ALT, AST, HBV DNA, HBeAg and anti-HBe was performed. There was no significant difference between the subjects who developed antiviral resistance (n=16) and who have not developed resistance (n=74) as showed in **Table 4**.

**5.2.2 Measurement of early virological response (EVR)**

Seventy-seven lamivudine-experienced subjects with the median treatment duration of 6 (IQR 6-8) months were analysed.

**5.2.2.1 Biochemical parameters**

Among the 77 subjects, 39 (50.6%) had normal ALT levels, 29 (37.7%) had 1-2 ULN and 9 (11.7%) had >2 ULN. Likewise, 37 (48.1%) had normal AST levels, 35 (45.5%) had 1-2 ULN and 5 (6.5%) had >2 ULN. The median ALT and AST levels were 41 (IQR 32-61) U/L and 35 (IQR 25-51) U/L respectively.

**5.2.2.2 Virological parameters**

All 77 subjects continued to be positive for HBsAg. Hepatitis B e-antigen was positive in 47 (61%) subjects and 30 (39%) were HBeAg negative. Four (9%) HBeAg-positive subjects in the anti-HBe seroconversion phase and 28 (93%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 3 (IQR 0-4) $\log_{10}$ IU/mL.

Early virological response (EVR) measured at the median treatment duration of 6 (IQR 6-8) months were categorized as

- **Complete virological response** defined as undetectable HBV DNA (<82 IU/mL)

**Figure 16. Box and Whisker plot showing the baseline serum aspartate transaminase (AST) levels in lamivudine responders (n=48) and non-responders (n=42).**
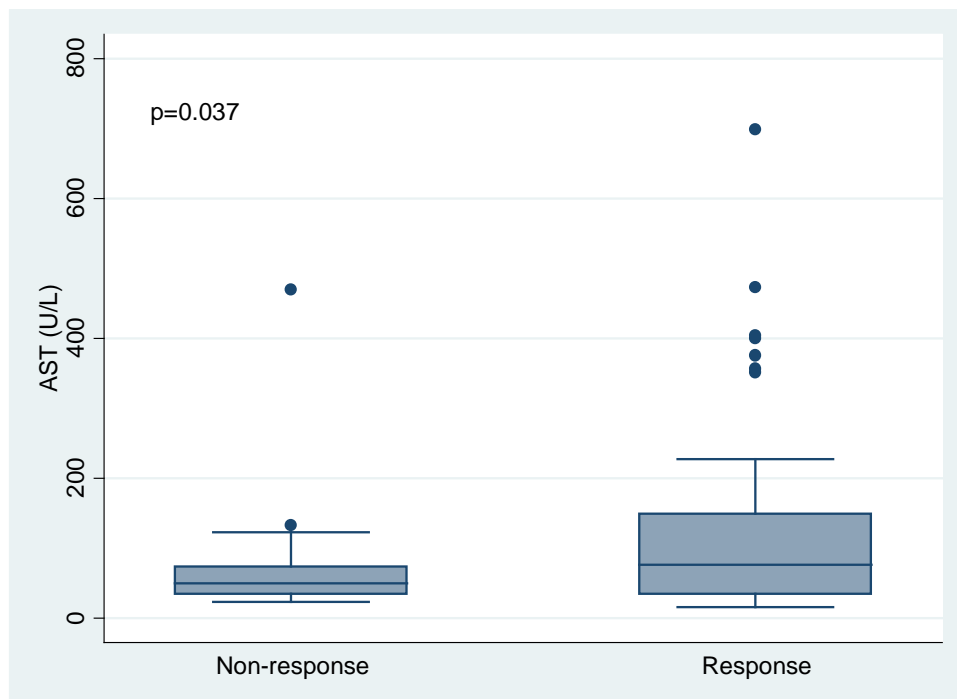


The box represents the median and inter-quartile range. The areas covered by the whiskers indicate 1-99 percentile and the dots are outliers. The level of significance p=0.037

**Table 4. Univariate analysis of baseline factors for prediction of lamivudine resistance**

| | No antiviral resistance (n=74) | Antiviral resistance (n=16) | p value |
|---|---|---|---|
| Age, years[†] | 40 (29-49) | 43 (15-55) | 0.903 |
| Gender, male[*] | 57 (77) | 15 (94) | 0.129 |
| ALT (U/L)[†] | 47 (30-102) | 50 (33-74) | 0.788 |
| AST (U/L)[†] | 66 (35-102) | 52 (38-73) | 0.339 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 5.39 (4.3-6.95) | 6.09 (4.35-7.6) | 0.243 |
| HBeAg Pos[*] | 44 (59) | 11 (69) | 0.489 |
| HBeAg Neg[*] | 30 (41) | 5 (31) | |
| Anti-HBe Pos[*] | 25 (34) | 5 (31) | 0.845 |
| Anti-HBe Neg[*] | 49 (66) | 11 (69) | |

Values are [†]median (IQR) or [*]number (%)

- **Partial virological response** defined as reduction in HBV DNA levels to $\geq 1$ $\log_{10}$ IU/mL from baseline.

- **Primary treatment failure or non-response** defined as the lack of reduction in HBV DNA levels to $\geq 1$ $\log_{10}$ IU/mL.

Among the 77 subjects, 24 (31%) showed complete virological response and 26 (34%) showed partial virological response. Ten (13%) subjects were classified as non-responders. The virological response for the remaining 17 (22%) subjects could not be categorised as all these subjects continued to be positive for HBV DNA and their baseline HBV DNA was not available to measure the significant reduction in viral load **(Figure 17)**.

### 5.2.2.2.1 Antiviral resistance mutations

Among the 53 samples positive for HBV DNA in real-time PCR (partial response, n=26; non-response, n=10 and uncategorised 17), 40 samples amplified in HBVrt PCR and 13 samples with low HBV DNA levels (0.6-2.59 $\log_{10}$ IU/mL) failed to amplify using primers specific for the rt region. On sequence analysis of 40 samples, 6 (15%) were identified with rtL80I+rtM204I, rtL180M+rtM204I, rtL180I+rtA181C, rtL80V+rtL180M+rtM204V, rtA181T and atypical rtA181G lamivudine resistance mutations (Figure 17). Interestingly, two of the subjects who presented with rtA181T and rtL80V+rtL180M+rtM204V resistance mutations showed partial virological response with a reduction of 1.4 and 3.6 HBV DNA $\log_{10}$ IU/mL from baseline levels. In the additional HBVrt amino acid substitutions identified, there was no specific pattern of mutations related to non-response.

In summation, among the 77 samples analysed in the EVR measurement, 13 samples failed to amplify in HBVrt PCR. Excluding these samples, the cumulative proportion of lamivudine-experienced subjects who developed resistance at the median treatment duration of 6 months was 9% (6/64).

**Figure 17. Early virological response and antiviral resistance mutations in lamivudine experienced subjects**
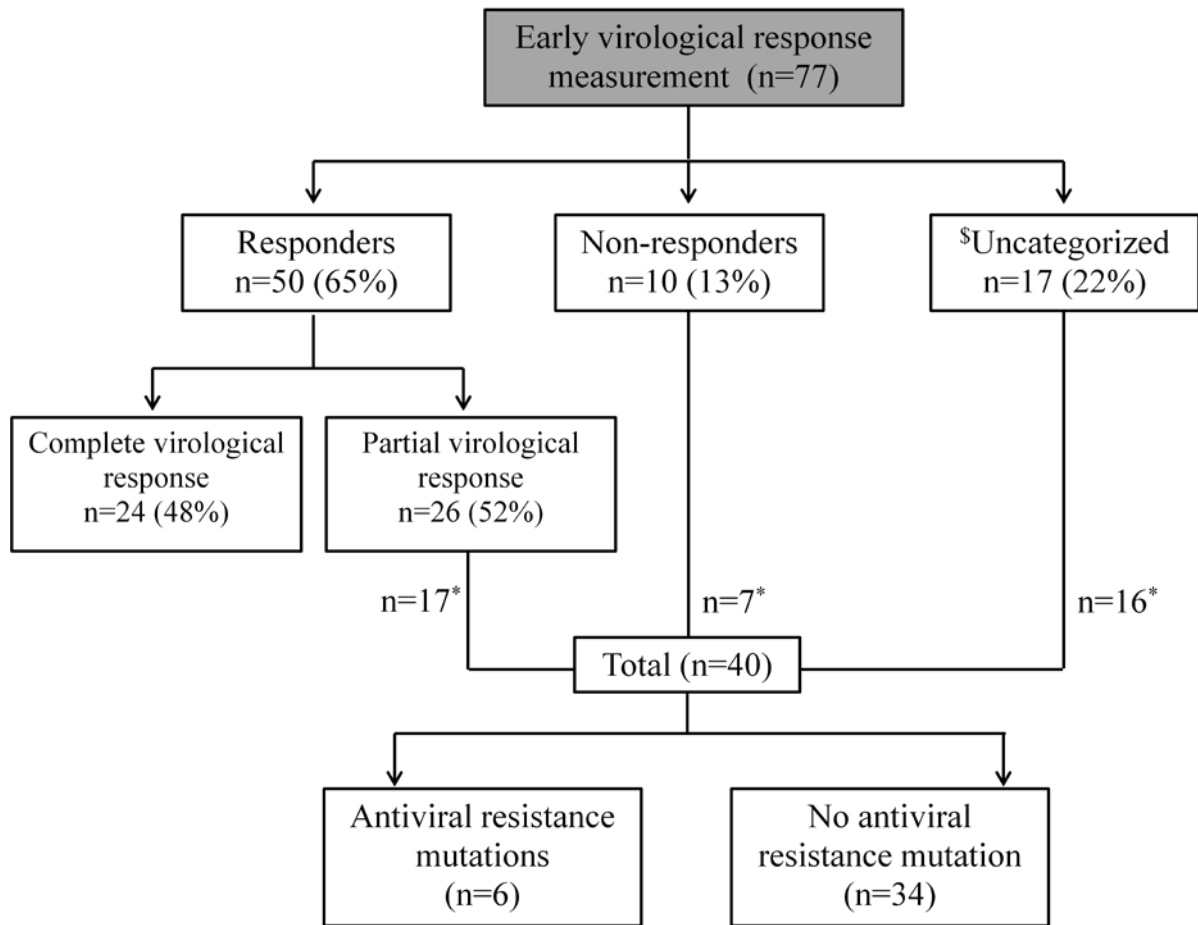


Early virological response measured at the median treatment duration of 6 (IQR 6-8) months

Complete virological response - undetectable HBV DNA (<82 IU/mL)

Partial virological response - reduction in HBV DNA to $\geq 1$ $\log_{10}$ IU/mL from baseline

Non-responders are subjects who failed to show reduction in HBV DNA to $\geq 1$ $\log_{10}$ IU/mL

[$] In the total 77 subjects with EVR measurement, 22 did not have baseline HBV DNA. Five of these subjects showed complete virological response and were classified as responders. The remaining 17 subjects continued to be positive for HBV DNA and the significant reduction in viral load could not be measured. Therefore the virological response was uncategorised

[*] Number of samples amplified in HBVrt PCR

### 5.2.2.3 EVR measurement and baseline characteristics

Among the 77 subjects with EVR measurement, 55 (71%) had baseline characteristics and the differences between the observations are shown in **Table 5**. A Wilcoxon sign-rank test was performed to compare the pre and post-treatment variables and there existed a significant difference between the variables analyzed (p=0.0006 to <0.0001).

### 5.2.2.4 EVR measurement for prediction of subsequent virological response

To assess whether EVR can predict subsequent virological response, subjects who had EVR measurements with follow-up samples were analyzed. Among the 31 subjects with follow-up, 11 subjects had showed partial virological response, 16 subjects had showed complete virological response and 4 subjects had showed non-response. Subsequently, 2 (18%) partial virological response subjects and 8 (50%) complete virological subjects maintained response with undetectable HBV DNA (<82 IU/mL) at the median treatment duration of 17 (IQR 12-22) months. All four non-responders in the EVR measurement failed to show lamivudine response subsequently **(Figure 18)**. However, there was no significant association between EVR and subsequent response (p=0.074).

### 5.2.3 Measurement of end-of-treatment response (ETR)

Seventy-one lamivudine-experienced subjects with the median treatment duration of 12 (IQR 12-16) months were analysed.

### 5.2.3.1 Biochemical parameters

Among the 71 subjects, 41 (58%) had normal ALT levels, 19 (27%) had 1-2 ULN and 11 (15%) had >2 ULN. Likewise, 41 (58%) had normal AST levels, 23 (32%) had 1-2 ULN and 7 (10%) had >2 ULN. The median ALT and AST levels were 37 (IQR 26-46) U/L and 32 (IQR 24-45) U/L respectively.

**Table 5. Differences between baseline and EVR measurements for lamivudine therapy**

| Variables studied (n=55) | Baseline measurement | EVR measurement[$] | p value |
|---|---|---|---|
| ALT (U/L)[†] | 47 (28-90) | 33 (24-49) | 0.0006 |
| AST (U/L)[†] | 63 (32-102) | 40 (31-54) | <0.0001 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 5.3 (4.3-6.84) | 1.77 (0-3.47) | <0.0001 |
| HBeAg Pos[*] | 34 (62) | 32 (58) | 0.0001 |
| HBeAg Neg[*] | 21 (38) | 23 (42) | |
| Anti-HBe Pos[*] | 16 (29) | 24 (44) | 0.0001 |
| Anti-HBe Neg[*] | 39 (71) | 31 (56) | |

[$]Measurement of early virological response (EVR) at the median treatment duration of 6 (IQR 6-8) months

Values are [†]median (IQR) or [*]number (%)

**Figure 18. Early virological response measurement and subsequent response rates to lamivudine treatment**
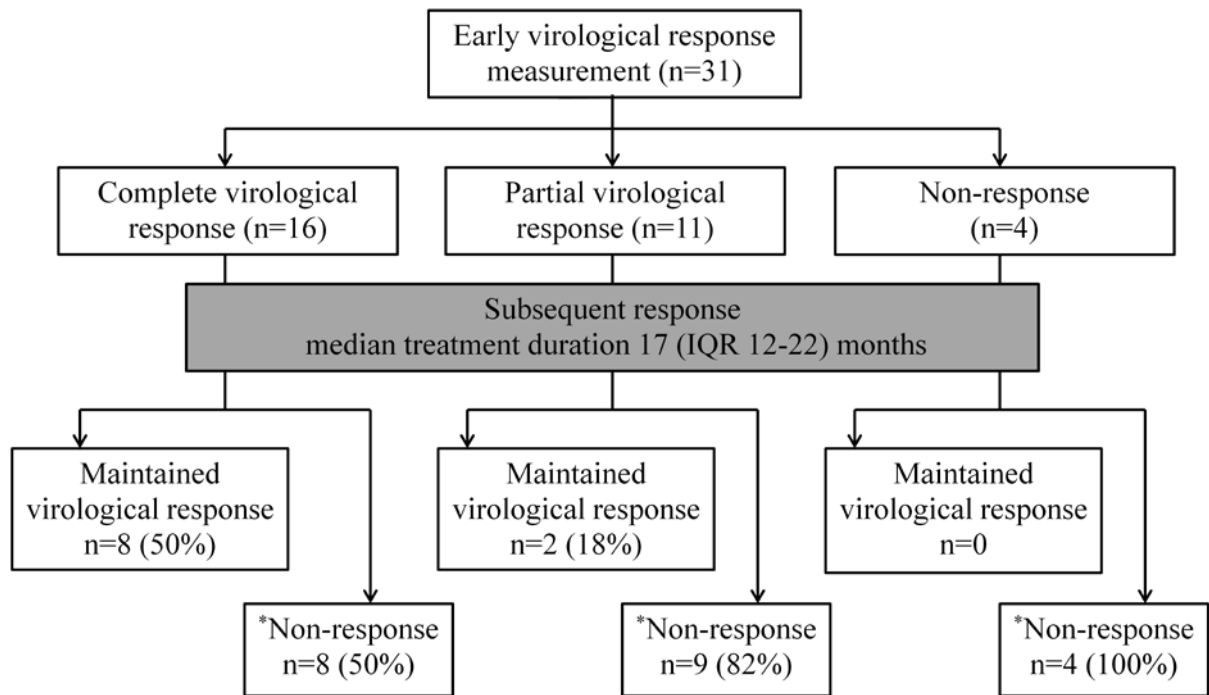


Early virological response measured at the median treatment duration of 6 (IQR 6-8) months

Complete virological response - undetectable HBV DNA (<82 IU/mL)

Partial virological response - reduction in HBV DNA to $\geq 1$ $\log_{10}$ IU/mL from baseline

Maintained virological response - subjects who showed undetectable HBV DNA (<82 IU/mL) after median treatment duration of 17 (IQR 12-22) months

[*]Non-response are subjects who failed to show undetectable HBV DNA (<82 IU/mL) after median treatment duration of 17 (IQR 12-22) months

### 5.2.3.2 Virological parameters

All 71 subjects continued to be positive for HBsAg. Hepatitis B e-antigen was positive for 39 (55%) subjects and 32 (45%) were HBeAg-negative. Three (8%) HBeAg-positive subjects in the anti-HBe seroconversion phase and 26 (81%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 2.5 (IQR 0-5) $\log_{10}$ IU/mL. Among these subjects, 27 (38%) with undetectable HBV DNA (<82 IU/mL) were classified as experiencing ETR and 44 (62%) with persistence of HBV DNA were classified as non-responders. There existed a significant difference in age, gender, ALT, AST, HBeAg and anti-HBe status between the responders and non-responders **(Table 6)**. Multivariate analysis could not be performed as the numbers were limited in the responders group.

### 5.2.3.2.1 Antiviral resistance mutations

Among the 44 non-responders, 36 samples amplified in HBVrt PCR and 8 samples with low viral load (1-2.09 $\log_{10}$ IU/mL of HBV DNA) failed to amplify. On sequence analysis of 36 samples, 14 (39%) were identified with typical lamivudine resistance rtL80I/V, rtI169L, rtL180M, rtA181V and rtM204V/I or rtM250L mutations. None of the additional HBVrt mutations showed specific association with non-response.

In summation, among the 71 samples analysed in the ETR measurement, 8 samples failed to amplify in HBVrt PCR. Excluding these samples, the cumulative proportion of lamivudine-experienced subjects who developed resistance at the median treatment duration of 12 months was 22% (14/63).

### 5.2.4 Measurement of maintained response (median treatment duration 24 months)

Maintained response measurement with the median treatment duration of 24 (IQR 24-27) months was performed for 36 subjects.

**Table 6. End-of-Treatment response and non-response to lamivudine**

| Variables studied | Responses (n=27) | Non-responses (n=44) | p value |
|---|---|---|---|
| Age, years[†] | 46 (28-52) | 33 (22-48) | 0.042 |
| Gender, male[*] | 16 (60) | 40 (91) | 0.002 |
| ALT (U/L)[†] | 28 (18-34) | 36 (28-65) | 0.002 |
| AST (U/L)[†] | 29 (22-42) | 40 (30-53) | 0.006 |
| HBeAg Pos[*] | 5 (19) | 34 (77) | <0.0001 |
| HBeAg Neg[*] | 22 (81) | 10 (23) | |
| Anti-HBe Pos[*] | 19 (70) | 10 (23) | <0.0001 |
| Anti-HBe Neg[*] | 8 (30) | 34 (77) | |

End-of-treatment response is defined as undetectable HBV DNA (<82 IU/mL) at the median treatment duration of 12 (IQR 12-16) months. Non-response is subjects who did not meet these criteria

Data are [†]median (IQR) or [*]n (%)

### 5.2.4.1 Biochemical parameters

Among the 36 subjects, 24 (67%) had normal ALT levels, 8 (22%) had 1-2 ULN and 4 (11%) had >2 ULN. Likewise, 22 (61%) had normal AST levels, 8 (22%) had 1-2 ULN and 6 (17%) had >2 ULN. The median ALT and AST levels were 36 (IQR 29-58) U/L and 30 (IQR 22-42) U/L respectively.

### 5.2.4.2 Virological parameters

All 36 subjects continued to be positive for HBsAg. Hepatitis B e-antigen was positive in 22 (61%) subjects and 14 (39%) were HBeAg-negative. Three (14%) HBeAg-positive subjects in the anti-HBe seroconversion phase and 13 (93%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 4.2 (IQR 1.3-6.4) $\log_{10}$ IU/mL. Among these subjects 8 (22%) maintained virological response (undetectable HBV DNA; <82 IU/mL) and 28 (78%) with detectable HBV DNA were classified as non responders.

### 5.2.4.2.1 Antiviral resistance mutations

Among the 28 non-responders, 26 samples amplified in HBVrt PCR and were carried for sequencing. In the samples analyzed, lamivudine resistance rtL80I/V, rtL180M/H or rtM204V/I mutations were identified in 15 (58%) subjects. Additional HBVrt mutations identified did not show any association with virological non-response.

In summation, among the 36 samples analysed in the maintained response measurement, 2 samples failed to amplify in HBVrt PCR. Excluding these samples, the cumulative proportion of lamivudine-experienced subjects who developed resistance at the median treatment duration of 24 months was 44% (15/34).

### 5.2.5 Measurement of maintained response (median treatment duration 41 months)

Additionally, maintained response measurement for 19 subjects with the median treatment duration of 41 (IQR 36-60) months was performed.

### 5.2.5.1 Biochemical parameters

Among the 19 subjects, 9 (47.4%) had normal ALT levels, 5 (26.3%) had 1-2 ULN and 5 (26.3%) had >2 ULN. Likewise, 9 (47%) had normal AST levels, 7 (37%) had 1-2 ULN and 3 (16%) had >2 ULN respectively. The median ALT and AST levels were 45 (IQR 34-58) U/L and 42 (IQR 23-78) U/L respectively.

### 5.2.5.2 Virological parameters

All 19 subjects continued to be positive for HBsAg. Hepatitis B e-antigen was positive in 16 (84%) subjects and 3 (16%) were HBeAg-negative. Two (13%) HBeAg positive subjects in the anti-HBe seroconversion phase and all 3 (100%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 5.8 (IQR 3.9-7.1) $\log_{10}$ IU/mL Among the 19 subjects, 1 (5%) subject who showed non-response at ETR measurement with 2.09 $\log_{10}$ IU/mL of HBV DNA showed undetectable HBV DNA (<82 IU/mL) after 42 months of treatment (delayed response). The remaining 18 (95%) subjects were non-responders.

### 5.2.5.2.1 Antiviral resistance mutations

Among the 18 non-responders, 16 samples amplified in HBVrt PCR were carried for sequencing. Lamivudine resistance rtL80I/V, rtV173L, rtL180M, rtA181V, rtM204V/I or rtM250L mutations were identified in 12 (75%) subjects.

In summation, among the 19 samples analysed in the maintained response measurement, 2 samples failed to amplify in HBVrt PCR. Excluding these samples, the cumulative proportion

of lamivudine-experienced subjects who developed resistance at the median treatment duration of 41 months was 71% (12/17).

**5.2.6 Last follow-up analysis**

Altogether, last follow-up on-treatment samples of the total 147 lamivudine-experienced subjects were separately analysed. The median treatment duration was 13 (IQR 8-24) months.

**5.2.6.1 Biochemical parameters**

Among the 147 subjects, 78 (53%) had normal ALT levels, 47 (32%) had 1-2 ULN and 22 (15%) had >2 ULN. Likewise, 79 (54%) had normal AST levels, 49 (33%) had 1-2 ULN and 19 (13%) had >2 ULN. The median ALT and AST levels were 33 (IQR 24-49) U/L and 40 (30-54) U/L respectively.

**5.2.6.2 Virological parameters**

All 147 subjects continued to be positive for HBsAg till the last follow-up analysis. Hepatitis B e-antigen was positive in 93 (63%) subjects and 54 (37%) were HBeAg-negative. Seven (7.5%) HBeAg-positive subjects in the anti-HBe seroconversion phase and 47 (87%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 3.6 (IQR 0.5.84) $\log_{10}$ IU/mL. Among the 147 subjects, 50 (34%) were classified as responders who showed $\geq 1$ $\log_{10}$ IU/ml of HBV DNA reduction within 6 months of therapy and/or undetectable HBV DNA (<82 IU/mL) at the end of 12 months therapy. Eighty-four (57%) were non responders showing <1 $\log_{10}$ IU/mL reduction of HBV DNA in 6 months therapy or continued to be positive for HBV DNA with a median treatment duration of 12 months. The remaining 13 (9%) had only one-time point of sampling and HBV DNA continued to be positive with $\leq$ 9 months of lamivudine. The virological response for these 13 (9%) subjects was not categorized as their baseline HBV DNA is not available.

**5.2.6.2.1 Antiviral resistance mutations**

Among the 97 subjects (non-responders, n=84 and uncategorized, n=13) analysed, 40 presented with typical lamivudine resistance mutations. The primary rtM204V/I mutation was exclusively detected in 9 (22.5%) subjects. The rtL180M and rtM204V combination was the predominantly identified mutation, n=12 (30%) followed by L80I and rtM204I combination, n=9 (22.5%). The rtM204V/I mutation was also detected with rtL80V, rtL180M, rtA181V, rtV173L or rtM250L compensatory mutations. Especially, the rtV173L mutation was identified in 2 subjects with longer treatment duration of 72 months. Additionally, rtI169L, rtA181V antiviral resistant mutations and rtA181G atypical mutations were detected exclusively in one subject each.

In summation, among the total 147 lamivudine-experienced subjects analysed, 40 (27%) were identified with resistance mutations at the last follow-up with the median treatment duration of 13 (IQR 8-24) months.

The distribution pattern of lamivudine resistance HBVrt mutations with varying treatment duration is detailed in **Table 7**. Kaplan-Meier analysis was performed to show the cumulative rates of lamivudine resistance over the course of therapy (range 0-84 months; **Figure 19**). When a subject had more than one post-therapy sequence, the mutation identified was counted for each time-point of therapy. Cumulatively, 6/64 (9%), 14/63 (22%), 15/34 (44%) and 12/17 (71%) subjects were detected with resistance mutations in the median treatment duration of 6 (IQR 6-8), 12 (IQR 12-16), 24 (IQR 24-27) and 41 (IQR 36-60) months respectively (**Figure 20**).

**Table 7. Profile of lamivudine resistance HBVrt mutations with varying treatment duration**

| HBVrt mutations | Treatment duration in months median (IQR) | | | |
|---|---|---|---|---|
| | 6 (6-8) | 12 (12-16) | 24 (24-27) | 41 (36-60) |
| **No. of samples tested** | **64** | **63** | **34** | **17** |
| L80I+M204I | 1 | 3 | 5 | 1 |
| L180M+M204I | 1 | - | - | 1 |
| L80V+L180M+M204V | 1 | - | - | - |
| *L180I+A181C | 1 | - | - | - |
| A181T | 1 | - | - | - |
| *A181G | 1 | - | - | - |
| M204I | - | 1 | 3 | 3 |
| L80V+M204I | - | 1 | - | 1 |
| L80V+M204V | - | 1 | - | - |
| I169L | - | 1 | - | - |
| L180M+A181V+M204V | - | 1 | - | - |
| M204I+M250L | - | 1 | - | - |
| L180M+M204V | - | 5 | 5 | 1 |
| M204V | - | - | 1 | 1 |
| L80V+L180H+M204V | - | - | 1 | - |
| L80I | - | - | - | 1 |
| A181V | - | - | - | 1 |
| V173L+L180M+M204V | - | - | - | 1 |
| V173L+L180M+M204I+M250L | - | - | - | 1 |
| L80I+L180M+M204V | - | - | - | - |
| **Frequency** | **6 (9)** | **14 (22)** | **15 (44)** | **12 (71)** |

*Atypical mutations with unusual amino acid substitutions in HBV reverse transcriptase (HBVrt) positions that are crucial for antiviral action

IQR- Interquartile range

Values in frequency parenthesis represent percentages

**Figure 19. Kaplan-Meier analysis showing the cumulative rates of lamivudine resistance over the course of treatment**
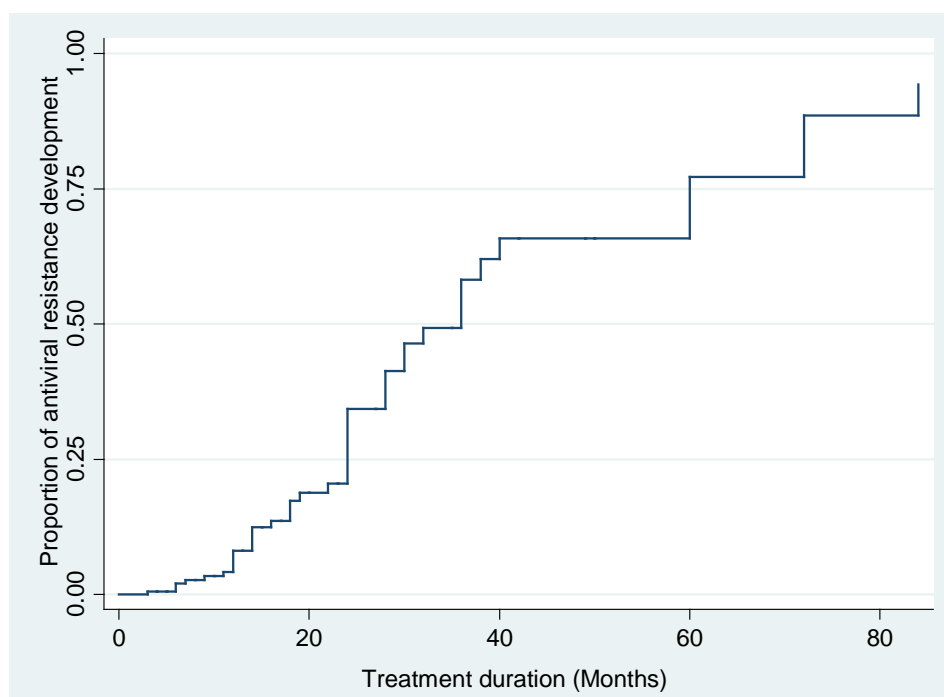
**Figure 20. Percentage frequency of lamivudine resistance mutations and end-points of treatment**
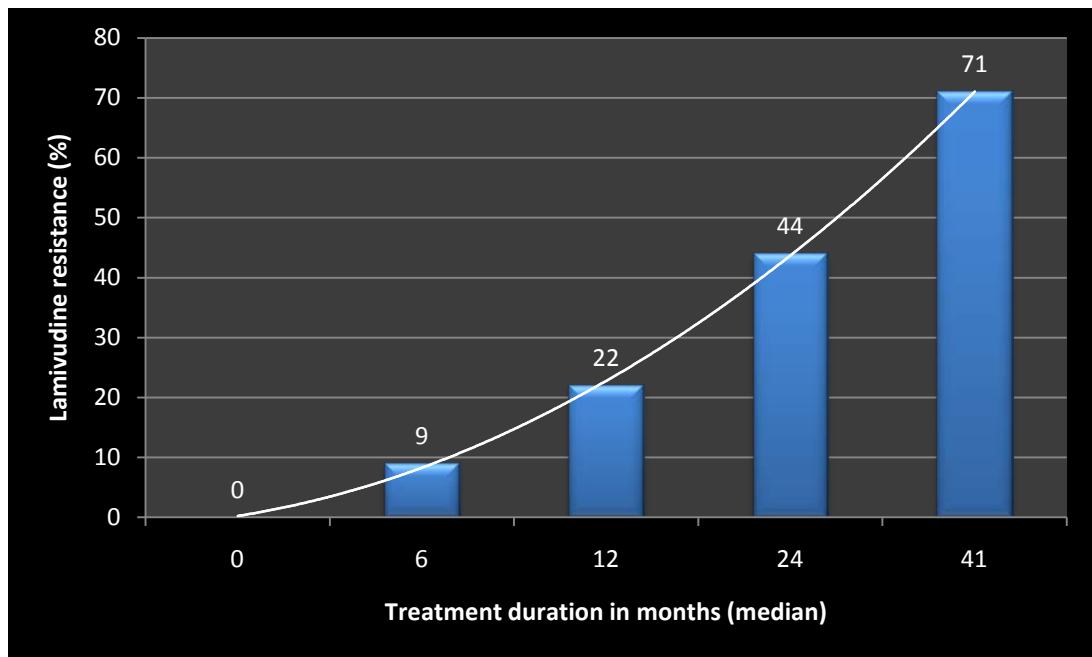


0- Treatment naive (baseline)

6- Early virological response measured at median treatment duration of 6 months

12- End-of-treatment response measured at median treatment duration of 12 months

24- Maintained virological response measured at median treatment duration of 24 months

41- Maintained virological response measured at median treatment duration of 41 months

**5.2.6.3 Factors associated with clinical outcome at last follow-up examination**

**5.2.6.3.1 Virological response**

Virological response and non-response is categorised based on HBV DNA status and treatment duration as described earlier. To identify other factors associated with virological response, univariate analysis for age, gender, ALT, AST, HBeAg and anti-HBe was performed. On analysis, male gender, higher ALT and AST levels, HBeAg-positive and anti-HBe-negative status was significantly associated with non-response **(Table 8)**.

All variables in univariate analysis significantly associated (p<0.05) with virological response were entered into the multivariate model. Gender (male versus female), ALT levels (≤2 ULN versus >2 ULN; ≤70 and >70 U/L), AST levels (≤2 ULN versus >2 ULN; ≤80 and >80 U/L) and anti-HBe status (positive versus negative) did not differ significantly with virological response but HBeAg status (positive versus negative) showed significant association with virological response. Compared with HBeAg negative subjects, HBeAg positive subjects had reduced rate of lamivudine response [OR 0.2, 95% CI (0.06-0.68); p=0.01; **Table 9**].

**5.2.6.3.2 Antiviral resistance**

Among the 147 lamivudine-experienced individuals, 40 (27%) subjects were presented with resistance mutations. The frequency of antiviral resistance mutations did not differ significantly with age, gender, ALT and AST levels, but was significantly lower in subjects with low HBV DNA levels [2.84 (IQR 0-5) $\log_{10}$ IU/mL] than those with higher virus loads [5.95 (IQR 4.75-7.15) $\log_{10}$ IU/mL]; those with shorter treatment duration [12 (IQR 6-18) months] when compared to longer treatment duration [24 (IQR 15-34) months] and in HBeAg-negative and anti-HBe-positive subjects **(Table 10)**.

**Table 8. Univariate analysis of factors associated with lamivudine response and non-response at last follow-up[#]**

| | Response (n=50)[$] | Non-response (n=84)[$] | p value |
|---|---|---|---|
| Age, years[†] | 44 (28-52) | 39 (26-49) | 0.269 |
| Gender, male[*] | 35 (70) | 69 (86) | 0.024 |
| ALT (U/L)[†] | 28 (20-44) | 36 (27-59) | 0.006 |
| AST (U/L)[†] | 34 (22-44) | 40 (32-57) | 0.008 |
| HBeAg Pos[*] | 17 (34) | 67 (80) | <0.0001 |
| HBeAg Neg[*] | 33 (66) | 17 (20) | |
| Anti-HBe Pos[*] | 31 (62) | 19 (23) | <0.0001 |
| Anti-HBe Neg[*] | 19 (38) | 65 (77) | |

[$]Among 147 lamivudine-experienced subjects, the virological response for 13 subjects could not be categorized and were not included for analysis

[#]Last follow-up is measured at the median treatment duration of 13 (IQR 8-24) months

Values are [†]median (IQR) or [*]number (%)

**Table 9. Multivariate analysis of lamivudine response at last follow-up[#]**

| Lamivudine response | Multivariate | | |
|---|---|---|---|
| | OR | 95% CI | p value |
| Gender | 1.94 | 0.71-5.3 | 0.194 |
| ALT (U/L) | 1.58 | 0.36-6.89 | 0.539 |
| AST (U/L) | 0.21 | 0.04-1.2 | 0.080 |
| HBeAg | 0.20 | 0.06-0.68 | 0.010 |
| Anti-HBe | 1.89 | 0.56-6.36 | 0.306 |

Multivariate analysis stratified by Gender (male versus female), ALT ($\leq$70 and >70 U/L), AST ($\leq$80 and >80 U/L), HBeAg and anti-HBe status

[#]Last follow-up is measured at the median treatment duration of 13 (IQR 8-24) months

OR- odds ratio

CI- confidence interval

**Table 10. Univariate analysis of factors associated with lamivudine resistance at last follow-up**

| | No antiviral resistance (n=107) | Antiviral resistance (n=40) | p value |
|---|---|---|---|
| Age, years[†] | 37 (26-49) | 46 (26-54) | 0.123 |
| Gender, male[*] | 85 (80) | 31 (84) | 0.630 |
| Treatment duration, months[†] | 12 (6-18) | 24 (15-34) | <0.0001 |
| ALT (U/L)[†] | 33 (24-49) | 36 (23-65) | 0.472 |
| AST (U/L)[†] | 39 (30-54) | 41 (31-64) | 0.395 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 2.84 (0-5) | 5.95 (4.75-7.15) | <0.0001 |
| HBeAg Pos[*] | 59 (55) | 34 (85) | 0.001 |
| HBeAg Neg[*] | 48 (45) | 6 (15) | |
| Anti-HBe Pos[*] | 47 (44) | 7 (18) | 0.003 |
| Anti-HBe Neg[*] | 60 (56) | 33 (82) | |

Values are [†]median (IQR) or [*]number (%)

Last follow-up is measured at the median treatment duration of 13 (IQR 8-24) months

Multivariate analysis stratified by HBV DNA levels ($\leq 4$ $\log_{10}$ IU/mL and $>4$ $\log_{10}$ IU/mL); treatment duration (median treatment duration of 6, 13, 24 and 41 months); HBeAg status (positive versus negative) and anti-HBe status (positive versus negative) was performed. The risk of antiviral resistance increased with HBV DNA levels [OR 5.9, 95% CI (1.94-17.7); p=0.002] and treatment duration [OR 2.8, 95% CI (1.71-4.57); p<0.001; **Table 11**].

### 5.2.7 Measurement of sustained virological response (SVR)

Sustained virological response measurement was available for four subjects. All four subjects initially showed virological response and were continuing treatment for up to a median duration of 25 (IQR 21-29) months. None of these subjects showed sustained virological response after stopping treatment at a median duration of 18 (IQR 11-20) months. HBeAg seroconversion and normal serum aminotransferase levels were seen in one and two subjects respectively.

### 5.2.7.1 Antiviral resistance mutation

Among the four subjects with SVR measurement, one subject with HBV DNA level of 2.76 $\log_{10}$ IU/mL failed to amplify in HBVrt PCR. In the remaining 3 samples analysed, 1 (33%) was identified with L80I+L180M+M204V lamivudine resistance mutation.

Nucleotide sequences generated from this analysis have been deposited in GenBank database under accession numbers JQ514380 to JQ514499.

**Table 11. Multivariate analysis of factors associated with lamivudine resistance at last follow-up[#]**

| Lamivudine resistance | Multivariate | | |
|---|---|---|---|
| | OR | 95% CI | p value |
| Treatment duration | 2.8 | 1.71-4.57 | <0.001 |
| HBV DNA ($\log_{10}$ IU/mL) | 5.9 | 1.94-17.7 | 0.002 |
| HBeAg | 1.2 | 0.22-7.14 | 0.794 |
| Anti-HBe | 0.64 | 0.13-3.29 | 0.595 |

Multivariate analysis stratified by treatment duration [median 6 (IQR 6-8), 12 (IQR 12-16), 24 (IQR 24-27) and 41 (IQR 36-60) months]; HBV DNA ($\leq$4 and >4 log IU/mL); HBeAg and anti-HBe status

[#]Last follow-up is measured at the median treatment duration of 13 (IQR 8-24) months

OR- odds ratio

CI- confidence interval

**5.3 Adefovir-experienced subjects**

A total of 30 adefovir-experienced subjects were studied. Among these subjects, 28 (93%) were male and 2 (7%) were female; their median age was 42 (IQR 30-44). All received adefovir at a standard dosage of 10mg/day.

**Categorization of study subjects**

An overview of subjects in cross-sectional and prospective analysis is outlined in **Figure 9** (Materials and Methods)

Among the 30 subjects, 6 had baseline and subsequent follow-up samples for testing, 7 had consecutive on-treatment samples and had only one on-treatment sample available for testing. Overall, 49 samples of these 30 subjects with varying treatment durations were tested. The number of subjects studied at varying treatment duration is shown in **Figure 21.**

In addition to baseline characteristics, on-treatment samples available for testing were grouped into any one or more of the following categories

- **Measurement of early virological response (EVR)** at the median treatment duration of 5 months (n=18)

- **Measurement of end-of-treatment response (ETR)** at the median treatment duration of 12 months (n=16)

- **Measurement of maintained response** at the median treatment duration of 24 months (n=8)

- **Measurement of sustained virological response (SVR)** at the median duration of 6 months after cessation of therapy (n=1)

**Figure 21. Flow chart of adefovir experienced subjects studied**
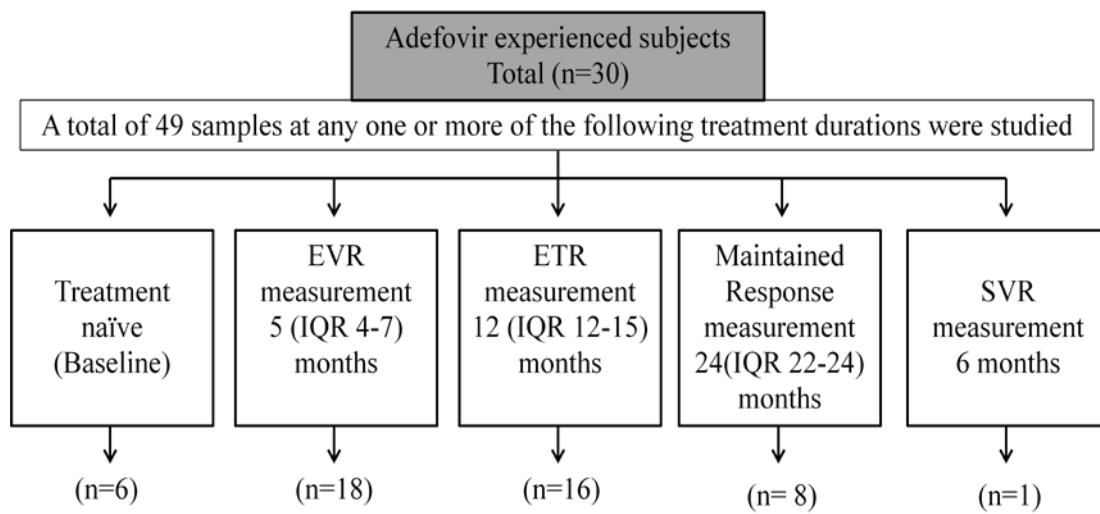


EVR – Early virological response

ETR – End-of-treatment response

SVR – Sustained virological response

### 5.3.1 Baseline characteristics

### 5.3.1.1 Biochemical parameters

In the 6 treatment-naive samples followed-up with adefovir treatment, 3 (50%) subjects had normal ALT levels, 1 (17%) had 1-2 ULN (39 U/L) and 2 (33%) had >2 ULN. Likewise, 3 (50%) subjects had normal AST and 3 (50%) subjects had 1-2 ULN AST levels. The median ALT and AST levels were 34 (IQR 20-65) U/L and 34 (IQR 28-42) U/L respectively.

### 5.3.1.2 Virological parameters

Among the 6 subjects, 2 (33%) were HBeAg-positive and 4 (67%) were HBeAg-negative. All 4 HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 3 (IQR 2.14-3) $\log_{10}$ IU/mL.

### 5.3.2 Measurement of early virological response (EVR)

Eighteen adefovir-experienced subjects with the median treatment duration of 5 (IQR 4-7) months were analysed.

### 5.3.2.1 Biochemical parameters

Among the 18 subjects, 5 (28%) subjects had normal ALT levels, 9 (50%) had 1-2 ULN (39 U/L) and 4 (22%) had >2 ULN. Likewise, 6 (33%) subjects had normal AST levels, 10 (56%) had 1-2 ULN (39 U/L) and 2 (11%) had >2 ULN AST levels. The median ALT and AST levels were 47 (IQR 34-68) U/L and 42 (IQR 33-70) U/L respectively.

### 5.3.2.2 Virological parameters

All samples were positive for HBsAg. Hepatitis B e-antigen was positive in 11 (61%) subjects and 7 (39%) were HBeAg-negative. One (9%) HBeAg positive subjects in the anti-HBe seroconversion phase and 6 (86%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 3.82 (IQR 2.84-4.73) $\log_{10}$ IU/mL.

Early virological response (EVR) measured at the median treatment duration of 5 (IQR 4-7) months of therapy and are categorized as

- **Complete virological response** defined as undetectable HBV DNA (<82 IU/mL)

- **Partial virological response** defined as reduction in HBV DNA levels to $\geq 1$ $\log_{10}$ IU/mL from baseline.

- **Primary treatment failure or non-response** defined as the lack of reduction in HBV DNA levels to $\geq 1$ $\log_{10}$ IU/mL.

Among the 18 subjects, 3 (17%) showed complete virological response and 1 (6%) subject was classified as non-responder. None of the subjects showed virological breakthrough with $\geq 1$ $\log_{10}$ IU/mL increase from nadir (lowest HBV DNA levels during therapy). The virological response for the remaining 14 (77%) subjects could not be categorised as the baseline HBV DNA was not available.

### 5.3.2.2.1 Antiviral resistance mutations

Among the 15 samples positive for HBV DNA in real-time PCR (non-response, n=1 and uncategorised, n=14), 14 samples amplified in HBVrt PCR. On sequence analysis of these 14 samples, 1 (7%) was identified with rtI169L antiviral resistance mutation. In the additional HBVrt mutations identified, there were no specific patterns of mutations related to non-response.

In summation, among the 18 samples analysed in the EVR measurement, 1 sample failed to amplify in HBVrt PCR. Excluding this sample, the cumulative proportion of adefovir-experienced subjects who developed resistance at the median treatment duration of 5 months was 6% (1/17).

### 5.3.3 Measurement of end-of-treatment response (ETR)

Sixteen adefovir-experienced subjects with the median treatment duration of 12 (IQR 12-15) months were analysed.

### 5.3.3.1 Biochemical parameters

The median ALT and AST levels were 37 (IQR 27-46) U/L and 32 (IQR 29-36) U/L respectively. Among the 16 subjects, 7 (44%) subjects had normal ALT levels, 8 (50%) had 1-2 ULN (39 U/L) and 1 (6%) had >2 ULN (74 U/L). Likewise, 13 (81%) subjects had normal AST levels, 3 (19%) had 1-2 ULN AST levels.

### 5.3.3.2 Virological parameters

All samples were positive for HBsAg. Hepatitis B e-antigen was positive in 9 (56%) subjects and 7 (34%) were HBeAg-negative. One (11%) HBeAg-positive subject in the anti-HBe seroconversion phase and 6 (86%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 4.24 (IQR 2.84-6.2) $\log_{10}$ IU/mL. Among these subjects, 2 (12.5%) with undetectable HBV DNA (<82 IU/mL) were classified as ETR and 14 (87.5%) with persistence of HBV DNA were classified as non-responders.

### 5.3.3.2.1 Antiviral resistance mutations

Among the 14 non-responders, 13 samples amplified in HBVrt PCR. On sequence analysis of 13 samples, 1 (8%) was identified with typical adefovir resistance rtA181V mutation. None of the additional HBVrt mutations showed specific association with non-response.

In summation, among the 16 samples analysed in the ETR measurement, 1 sample failed to amplify in HBVrt PCR. Excluding this sample, the cumulative proportion of adefovir-experienced subjects who developed resistance at the median treatment duration of 12 months was 7% (1/15).

### 5.3.4 Measurement of maintained response

Maintained response measurement with the median treatment duration of 24 (IQR 22-24) months were available for 8 subjects.

### 5.3.4.1 Biochemical parameters

The median ALT and AST levels were 48 (IQR 30-70) U/L and 40 (IQR 22-42) U/L respectively. Among the 8 subjects, 4 (50%), 2 (25%) and 2 (50%) had normal, 1-2 ULN and >2 ULN ALT and AST levels respectively.

### 5.3.4.2 Virological parameters

All samples were positive for HBsAg. Hepatitis B e-antigen was positive in 4 (50%) subjects and 4 (50%) were HBeAg-negative. All 4 (100%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA levels were 4.8 (IQR 2.15-5.55) $\log_{10}$ IU/mL. Among these 8 subjects, one subject who had showed ETR subsequently maintained response (undetectable HBV DNA; <82 IU/mL) and one subject with HBV DNA level of 4.48 $\log_{10}$ IU/mL at 12 months of adefovir showed delayed virological response after 24 months of adefovir. The remaining 6 subjects continued to be positive for HBV DNA and were non-responders. One of this subjects who showed complete virological response at 7 months of adefovir showed virological breakthrough with increase in HBV DNA levels of 6.78 $\log_{10}$ IU/mL after 24 months of adefovir therapy.

### 5.3.4.2.1 Antiviral resistance mutations

On sequence analysis of the 6 non-responders, none of the subjects were detected with adefovir resistance mutations including the subject who had virological breakthrough.

### 5.3.5 Last follow-up analysis

Altogether, last follow-up on-treatment samples of the total 30 adefovir-experienced subjects were separately analysed. The median treatment duration was 12 (IQR 6-18) months.

### 5.3.5.1 Biochemical parameters

Among the 30 subjects, 12 (40%) subjects had normal ALT levels, 12 (40%) had 1-2 ULN and 6 (20%) had >2 ULN (74 U/L). Likewise, 15 (50%) subjects had normal AST levels, 9 (30%) had 1-2 ULN and 6 (20%) subjects had >2 ULN. The median ALT and AST levels were 29 (IQR 43-65) U/L and 29 (IQR 42-62) U/L respectively.

### 5.3.5.2 Virological parameters

All samples were positive for HBsAg till the last follow-up analysis. Hepatitis B e-antigen continued to be positive in 15 (50%) subjects and 15 (50%) were HBeAg-negative. Four (27%) of the HBeAg-positive subjects in the anti-HBe seroconversion phase and 6 (40%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA levels were 3 (IQR 4.5-5.6) $\log_{10}$ IU/mL.

Among the 30 subjects, 2 (7%) showed virological response till the last follow-up analysis and 19 (63%) were non-responders. The remaining 9 (30%) had only one-time point of sampling and HBV DNA continued to be positive with $\leq$ 9 months of adefovir. The virological response for these 9 (30%) subjects was not categorized as their baseline HBV DNA is not available.

### 5.3.5.2.1 Antiviral resistance mutations

Among the 28 samples (non-responders, n=19 and uncategorized, n=9) analysed, 25 samples amplified in HBVrt PCR and were carried for sequence analysis. In the samples analysed, 2 subjects were identified with rtI169L and the typical adefovir resistance rtA181V mutation.

One subject with rtN238K amino acid substitution at baseline was subsequently detected with this mutation after 10 months of therapy. Likewise, atypical rtV214E mutation was identified in 2 subjects at 4 and 30 months of treatment. No additional patterns of amino acid substitutions associated with adefovir non-response could be identified.

Cumulatively, 1/17 (6%) and 1/15 (7%) subjects were detected with resistance mutations in the median treatment duration of 5 (IQR 4-7) and 12 (IQR 12-15) months respectively.

**5.3.6 Measurement of sustained virological response (SVR)**

One virological response (ETR) subject showed SVR after stopping therapy for 6 months (Figure 21). However, this subject was subsequently detected with 0.7 and 2.1 $\log_{10}$ IU/mL of HBV DNA at SVR measurements of 12 and 18 months respectively. The anti-HBe antibody continued to be positive in this subject from baseline to the last follow-up analysis at 30 months.

Nucleotide sequences generated from this analysis have been deposited in GenBank database under accession numbers JQ514500 to JQ514530.

**5.4 Entecavir-experienced subjects**

A total of 50 entecavir-experienced subjects were studied. Among these subjects, 46 (92%) were male and 4 (8%) were female; their median age was 34 (IQR 25-50) years. All subjects received entecavir at a standard dosage of 0.5mg/day.

**Categorization of study subjects**

An overview of subjects in cross-sectional and prospective analysis is outlined in **Figure 9** (Materials and Methods).

Among the 50 subjects, 45 had their baseline and consecutive on-treatment samples for testing and 5 subjects had only one on-treatment sample available for testing. Overall 114 pre-and-post-therapy samples of these 50 subjects were analysed. The number of subjects studied at varying treatment durations are shown in **Figure 22**.

In addition to baseline characteristics, on-treatment samples available for testing were grouped into any one or more of the following categories

- **Measurement of early virological response (EVR)** at the median treatment duration of 6 months (n=47)

- **Measurement of end-of-treatment response (ETR)** at the median treatment duration of 12 months (n=15)

- **Measurement of maintained response** at the median treatment duration of 24 months (n=3)

- **Measurement of sustained virological response (SVR)** at the median duration of 6 or 12 months after cessation of therapy (n=4)

**Figure 22. Flow chart of entecavir experienced subjects studied**
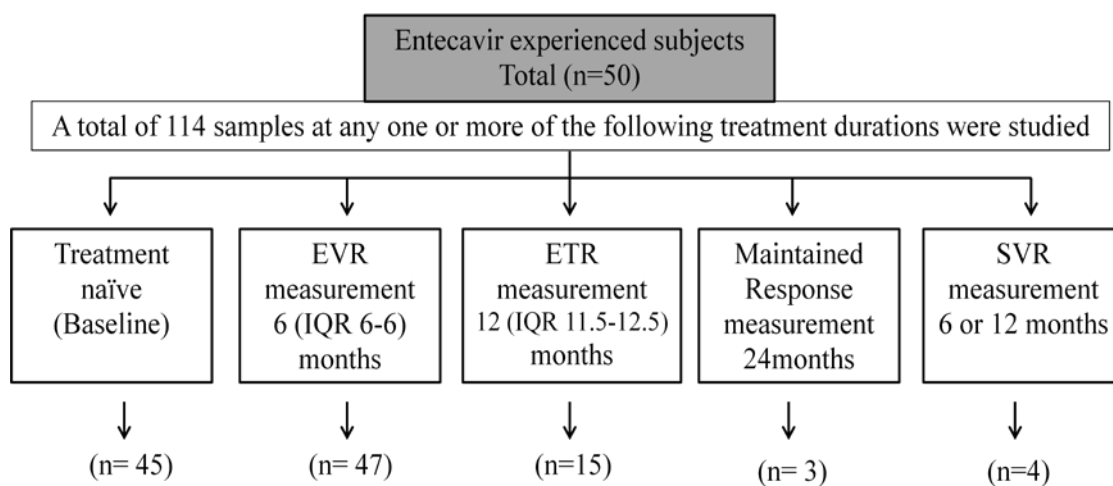


EVR – Early virological response

ETR – End-of-treatment response

SVR – Sustained virological response

**Ascertainment of clinical outcomes**

Virological response and antiviral resistance development were the two major outcomes determined in this study. In the samples analyzed, baseline variables were used to identify the predictive factors of response and on-treatment variables were used to identify factors associated with response.

## 5.4.1 Baseline characteristics

Forty-five subjects had their baseline sample and were prospectively analysed over the course of entecavir therapy.

### 5.4.1.1 Biochemical parameters

Among the 45 treatment-naive subjects followed-up with entecavir treatment, 16 (35.5%) had normal ALT levels, other 16 (35.5%) had 1-2 ULN and 13 (29%) had >2ULN. Likewise, 24 (53.3%) had normal AST levels, 11 (24.4%) had 1-2 ULN 10 (22.2%) had >2ULN AST levels respectively. The median ALT and AST levels were 47 (IQR 30-81) U/L and 37 (IQR 29-64) U/L respectively.

### 5.4.1.2 Virological parameters

Among the 45 subjects, 25 (56%) were HBeAg-positive and 20 (44%) were HBeAg-negative. One (4%) HBeAg-positive subjects in the anti-HBe seroconversion phase and 19 (95%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA levels in HBeAg-positive and HBeAg-negative subjects were 7.3 (IQR 6.48-7.48) and 4.5 (IQR 3.74-5.74) $\log_{10}$ IU/mL respectively (p=0.0001).

### 5.4.1.3 Baseline factors for prediction of virological response

Among the 45 subjects who had baseline measurement, 38 (84%) responded (EVR or ETR) to entecavir subsequently and 7 (16%) were non-responders. To identify baseline factors that

would predict the virological response to entecavir, univariate analysis for age, gender, ALT, AST, HBV DNA, HBeAg and anti-HBe was performed **(Table 12)**. In the factors analysed, HBeAg and anti-HBe antibody showed significant association for the prediction of virological response (p=0.01). All 7 non-responders were HBeAg-positive and anti-HBe negative. Twenty HBeAg-negative subjects with anti-HBe response at baseline responded subsequently.

### 5.4.2 Measurement of early virological response

Forty-seven entecavir-experienced subjects with the median treatment duration of 6 (IQR 6-6) months were analysed.

### 5.4.2.1 Biochemical parameters

Among the 47 subjects, 29 (62%) had normal ALT levels, 16 (34%) had 1-2 ULN and 2 (4%) had >2 ULN. Likewise, 32 (68%) had normal AST levels, 13 (28%) had 1-2 ULN and 2 (4%) had >2 ULN. The median ALT and AST levels were 29 (IQR 43-65) U/L and 29 (IQR 42-62) U/L respectively.

### 5.4.2.2 Virological parameters

All subjects were positive for HBsAg. Hepatitis B e-antigen was positive in 25 (53%) subjects and 22 (47%) were HBeAg-negative. Two (8%) HBeAg positive subjects in the anti-HBe seroconversion phase and all 22 (100%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 1.79 (IQR 0-3.15) $\log_{10}$ IU/mL.

Early virological response (EVR) measured at the median treatment duration of 6 (IQR 6-6) months of therapy and are categorized as:

- **Complete virological response** defined as undetectable HBV DNA (<82 IU/mL)

**Table 12. Univariate analysis of baseline factors for prediction of entecavir response**

|  | Responders (n=38) | Non-responders (n=7) | p value |
|---|---|---|---|
| Age, years[†] | 38 (25-50) | 25 (19-41) | 0.240 |
| Gender, male[*] | 35 (92) | 7 (100) | 0.442 |
| ALT (U/L)[†] | 38 (27-85) | 54 (47-63) | 0.316 |
| AST (U/L)[†] | 38 (33-44) | 37 (28-67) | 0.661 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 5.74 (3.95-7.3) | 7.3 (3.95-7.3) | 0.266 |
| HBeAg Pos[*] | 18 (47) | 7 (100) | 0.01 |
| HBeAg Neg[*] | 20 (53) | 0 | 0.01 |
| Anti-HBe Pos[*] | 20 (53) | 0 | 0.01 |
| Anti-HBe Neg[*] | 18 (47) | 7 (100) | 0.01 |

Values are [†]median (IQR) or [*]number (%)

- **Partial virological response** defined as reduction in HBV DNA to $\geq 1$ $\log_{10}$ IU/mL from baseline.

- **Primary treatment failure or non-response** defined as the lack of reduction of HBV DNA to $\geq 1$ $\log_{10}$ IU/mL.

Among the 47 subjects, 19 (40%) showed complete virological response and 23 (49%) showed partial virological response. The virological response for the remaining 5 (11%) subjects could not be categorised as the baseline HBV DNA was not available.

### 5.4.2.2.1 Antiviral resistance mutations

Among the 28 samples positive for HBV DNA in real-time PCR (partial virological response, n=23 and uncategorised, n=5), 18 samples amplified in HBVrt PCR. On sequence analysis of 18 samples, 1 was exclusively identified with rtV173L mutation. This subject showed partial virological response with reduction in HBV DNA level of 1.8 $\log_{10}$ IU/mL at 7 months therapy. In the additional HBVrt mutations identified, there were no specific patterns of mutations related to non-response.

### 5.4.2.3 EVR measurement and baseline characteristics

Among the 47 subjects with EVR measurement, 42 had their baseline characteristics and the differences between the observations are shown in **Table 13**. A Wilcoxon sign-rank test was performed to compare the pre and post-treatment variables and there existed a significant difference between all the variables analysed (p=0.0005 to <0.0001).

### 5.4.2.4 EVR measurement for prediction of subsequent virological response

To assess whether EVR can predict subsequent virological response, subjects who had EVR measurements with follow-up samples were analyzed. Among the 10 subjects with follow-up, 5 subjects had showed partial virological response and 5 subjects had showed complete

**Table13. Differences between baseline and EVR measurements for entecavir therapy**

| Variables studied (n=42) | Baseline measurement | EVR measurement[$] | p value |
|:---:|:---:|:---:|:---:|
| ALT (U/L)[†] | 49.5 (32-85) | 29 (22-43) | 0.0005 |
| AST (U/L)[†] | 43.5 (29-67) | 33 (23-42) | 0.0002 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 6.58 (4.6-7.3) | 1.41 (0-2.82) | <0.0001 |
| HBeAg Pos[*] | 23 (55) | 22 (52) | <0.0001 |
| HBeAg Neg[*] | 19 (45) | 20 (48) | |
| Anti-HBe Pos[*] | 19 (45) | 22 (52) | <0.0001 |
| Anti-HBe Neg[*] | 23 (55) | 20 (48) | |

[$]Measurement of Early Virological Response (EVR) at the median treatment duration of 6 (IQR 6-8) months

Values are median (IQR) or n (%)

virological response. Subsequently, 1 (20%) subject who showed partial virological response and 4 (80%) subjects who showed complete virological response maintained response with undetectable HBV DNA (<82 IU/mL) at the median treatment duration of 13 (IQR 12-24) months. However, there was no significant association between EVR and subsequent response (p=0.058).

### 5.4.3 Measurement of end-of-treatment response (ETR)

Fifteen subjects with the median treatment duration of 12 (IQR 11.5-12.5) months were analysed.

### 5.4.3.1 Biochemical parameters

Among the 15 subjects, 7 (47%) had normal ALT levels, 5 (33%) had 1-2 ULN and 3 (20%) had ≥2 ULN respectively. Likewise, 11 (73%) had normal AST levels, 3 (20%) had 1-2 ULN and 1 (7%) had AST levels of ≥2 ULN (82 U/L). The median ALT and AST levels were 29 (25-43) U/L and 36 (21-40) U/L respectively.

### 5.4.3.2 Virological parameters

All subjects were HBsAg positive. Hepatitis B e-antigen was positive in 7 (47%) subjects and 8 (53%) were HBeAg-negative. All HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 0 (IQR 0-2.55) $\log_{10}$ IU/mL. Among these subjects, 10 (67%) showed end-of-treatment response and the remaining 5 (33%) were non-responders.

### 5.4.3.2.1 Antiviral resistance mutations

On sequence analysis of 5 non-responders, none of the subjects were identified with typical entecavir resistance mutations. None of the additional HBVrt mutations identified showed specific association with non-response.

### 5.4.4 Measurement of maintained Response

Maintained response measurement with the treatment duration of 24 months was available for 3 subjects and all were non-responders. Two subjects were anti-HBe-negative and had normal ALT and AST levels. One subject was anti-HBe-positive and had raised ALT and AST levels of 175 and 135 U/L. The HBV DNA levels in these individuals were 1.23, 1.64 and 2.16 $\log_{10}$ IU/mL. Antiviral resistance testing could not be performed in these samples due to low viral load.

### 5.4.5 Last follow-up analysis

Altogether, last follow-up on-treatment samples of the total 50 entecavir-experienced subjects were separately analysed. The median treatment duration was 6 (IQR 6-11) months.

### 5.4.5.1 Biochemical parameters

Among the 50 subjects, 33 (66%) had normal ALT levels, 13 (26%) had 1-2 ULN and 4 (8%) had >2 ULN. Likewise, 33 (66%) had normal AST levels, 14 (28%) had 1-2 ULN and 3 (6%) had >2 ULN. The median ALT and AST levels were 28 (IQR 22-41) U/L and 31 (23-44) U/L respectively.

### 5.4.5.2 Virological parameters

All subjects were HBsAg positive. Hepatitis B e-antigen was positive in 27 (54%) subjects and 23 (46%) were HBeAg-negative. Two (7%) HBeAg subjects in the anti-HBe seroconversion phase and all 23 (100%) HBeAg-negative subjects were positive for anti-HBe antibody. The median HBV DNA level was 1.75 (IQR 0-2.98) $\log_{10}$ IU/mL. Among the 50 subject, 38 (76%) were classified as responders who showed $\geq 1$ $\log_{10}$ IU/ml of HBV DNA reduction within median treatment duration of 6 months (partial virological response) or

undetectable HBV DNA (<82 IU/mL) at the median treatment duration of 12 months (complete virological response). Seven (14%) were non responders showing <1 $\log_{10}$ IU/mL reduction of HBV DNA in median treatment duration of 6 months or continued to be positive for HBV DNA after median treatment duration of 12 months. The remaining 5 (10%) had only one-time point of sampling and HBV DNA continued to be positive between 3 to 7 months of therapy. The virological response for these 5 subjects was not categorized as their baseline HBV DNA is not available.

**5.4.5.2.1 Antiviral resistance mutations**

Among the 23 HBV DNA positive samples in real-time PCR, 13 samples amplified in HBVrt PCR. On sequence analysis of 13 samples, none of the subjects were identified with signature entecavir resistance mutations. However, one subject was identified with rtV173L mutation and its impact on subsequent response could not be studied as the subject was lost to follow-up. None of the additional HBVrt mutations identified showed specific association with entecavir non-response.

**5.4.5.2.2 Factors associated with clinical outcome at last follow-up examination**

To identify factors associated with virological response, univariate analysis for age, gender, ALT, AST, HBeAg and anti-HBe was performed **(Table 14)**. On analysis, anti-HBe is the only factor associated with response (p=0.034).

**5.4.6 Sustained virological response measurement**

Four subjects who showed initial EVR had SVR measurement. All four subjects showed HBV DNA reactivation after 6 months or 12 months cessation of therapy. The median increase in HBV DNA level was 3.13 (IQR 1.57-6.3) $\log_{10}$ IU/mL.

**Table 14. Univariate analysis of factors associated with entecavir response at last follow-up[#]**

| | Responses (n=38) | Non-responses (n=7) | p value |
|---|---|---|---|
| Age, years[†] | 38 (25-50) | 25 (18-43) | 0.240 |
| Gender, male[*] | 35 (92) | 7 (100) | 0.442 |
| ALT (U/L)[†] | 26 (22-36) | 29 (19-91) | 0.406 |
| AST (U/L)[†] | 31 (23-43) | 30 (25-54) | 0.802 |
| HBeAg Pos[*] | 18 (47) | 6 (86) | 0.062 |
| HBeAg Neg[*] | 20 (53) | 1 (14) | |
| Anti-HBe Pos[*] | 22 (58) | 1 (14) | 0.034 |
| Anti-HBe Neg[*] | 16 (42) | 6 (86) | |

[#]Last follow-up is measured at the median treatment duration of 13 (IQR 8-24) months

Among 50 entecavir-experienced subjects, the virological response for 5 subjects could not be categorized and were not included for analysis

Values are [†]median (IQR) or [*]number (%)

Nucleotide sequences generated from this analysis have been deposited in GenBank database under accession numbers JQ514531 to JQ514554.

## 5.5 Molecular Modeling

The three dimensional (3D) model of HBV polymerase for the wild type study sample (GenBank accession: GU798963) was built by homology modeling using the HIV-1 rt as the template.

### 5.5.1 Structure validation

The model was evaluated by PROCHECK and the stereochemical quality of the structure was good with the overall G factor of -0.24. The Ramachandran plot **(Figure 23)** shows the phi ($\varphi$)-psi ($\psi$) torsion angles for all residues except glycine and proline in the structure. The distribution of $\varphi$, $\psi$ angles showed 82.3% residues in the most favourable core region (shown in red), 15.9% of residues in allowed region (yellow) and 0.6% residues in the generous region (third level) . Overall 98.8% of the residues were within the allowed region.

### 5.5.2 Overview of the model

The constructed HBVrt model is shown in **Figure 24**. As described for HIVrt the modelled HBV polymerase has fingers, palm and thumb subdomains. According to the nomenclature of Stuyver *et al.* [112] the fingers subdomain covers the HBVrt codons 1 to 55 and 121 to 171, palm region extends between 56 to 92 and 172 to 265 and thumb subdomain occupies position 266 to 344. The two magnesium ($Mg^{2+}$) ions, thymidine triphoshate and the DNA template were located using the co-ordinates of Protein Data Bank code (PDB): 1RTD chain A of HIV-1rt.

**Figure 23. Ramachandran plot of HBV polymerse/reverse transcriptase model**



Plot statistics

| | | |
|---|---|---|
| Residues in most favoured regions  [A,B,L] | 269 | 82.3% |
| Residues in additional allowed regions  [a,b,l,p] | 52 | 15.9% |
| Residues in generously allowed regions  [~a,~b,~l,~p] | 2 | 0.6% |
| Residues in disallowed regions | 4 | 1.2% |
| | ---- | ------- |
| Number of non-glycine and non-proline residues | 327 | 100.0% |
| Number of end-residues (excl. Gly and Pro) | 2 | |
| Number of glycine residues (shown as triangles) | 22 | |
| Number of proline residues | 24 | |
| | ---- | |
| Total number of residues | 375 | |

Ramachandran plot showing the phi (φ)-psi (ψ) torsion angles for all the HBVrt aminoacid residues in the structure. Glycine and proline residues are shown as triangles (▲) and are not restricted to the regions of plots. The distribution of φ, ψ angles showed 82.3% residues in the most favourable core region (shown in red), 15.9% of residues in allowed region (yellow) and 0.6% residues in the generous region (third level). The plot was generated in PROCHECK using the Structure Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/)

**Figure 24. Homology model of hepatitis B virus reverse transcriptase (HBVrt)**
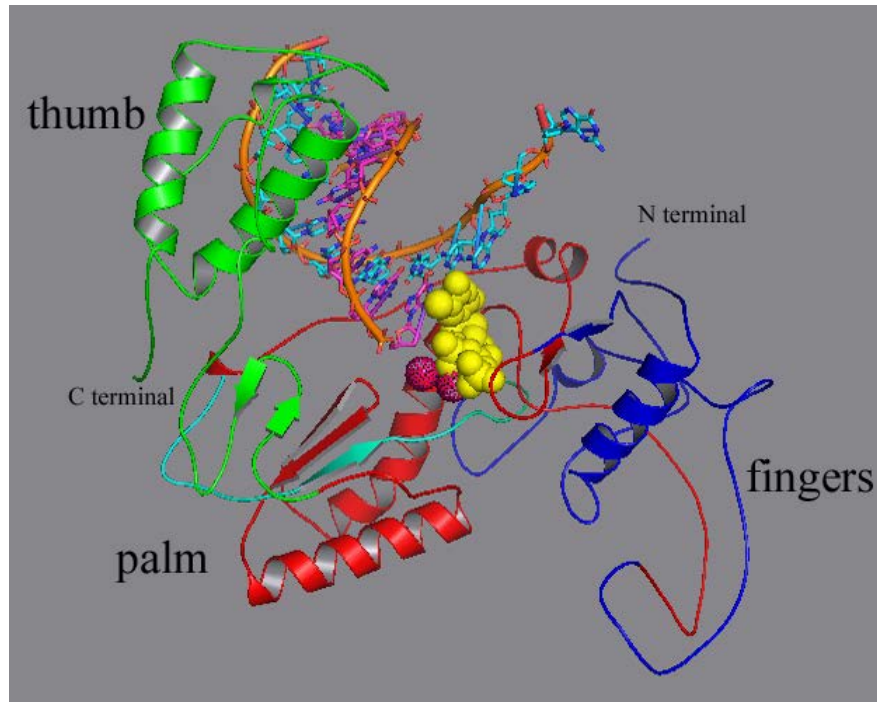


The model was constructed for the wild type study HBVrt sequence (GenBank accession: GU798963) using HIVrt template. The fingers, palm and thumb subdomains are showed in blue, red and green respectively. The DNA template is presented in the sticks mode and the dNTP (thymidine triphosphate) binding site is shown as yellow spheres. The two $Mg^{2+}$ ions required for polymerase activity is shown as dotted spheres in pink.

### 5.5.3 Effect of rtM204V mutation and lamivudine action

To validate and study the effect of wild type and mutant HBV polymerase, the well described lamivudine associated rtM204V primary resistance mutation was modelled using one of the representative study sequence and docking analysis performed. The rtM204V mutation showed decreased space for lamivudine binding, when compared to the wild type model (**Figure 25**). Therefore, the spatial constraint for lamivudine resistance is further evidenced in this study.

### 5.5.4 Effect of rtI233V mutation and adefovir action

The role of rtI233V mutation and adefovir response remains contradictory. This mutation pre-existed in four of our treatment-naive subjects and it was attempted to investigate its impact by molecular modeling. The modelled structure showed the amino acid position rt233 to be located away from the drug interactory site. The substitution of isoleucine to valine did not show to affect the catalytic sites of aspartate residues at HBVrt positions 83, 205 and 206.

It is been proposed that residues 235 to 240 form a bent structure and stabilizes the binding of incoming dNTPs. The wild type isoleucine (rtI233) is just located three amino acids away from the crucial adefovir resistance amino acid position asparagines (rtN236), which in-part forms the bent structure. It was attempted to study whether rtI233V substitution would alter the relative positions of neighbouring residues and alter the conformation. In wild type model the relative distance of the bent structure formed by the HBVrt amino acids L235, N236, P237, N238, K239 and T240 is 7.8 angstrom (Å). Substitution of valine reduced its relative distance to 7.7 Å. The overall conformation of the bent structure is maintained and the 0.1 Å difference in relative distance may not impose a spatial constraint to dNTP binding (**Figure 26**). Therefore, the rtI233V substitution in the reverse transcriptase domain may not affect the antiviral action of adefovir.

**Figure 25. Effect of rtM204V mutation and lamivudine binding**
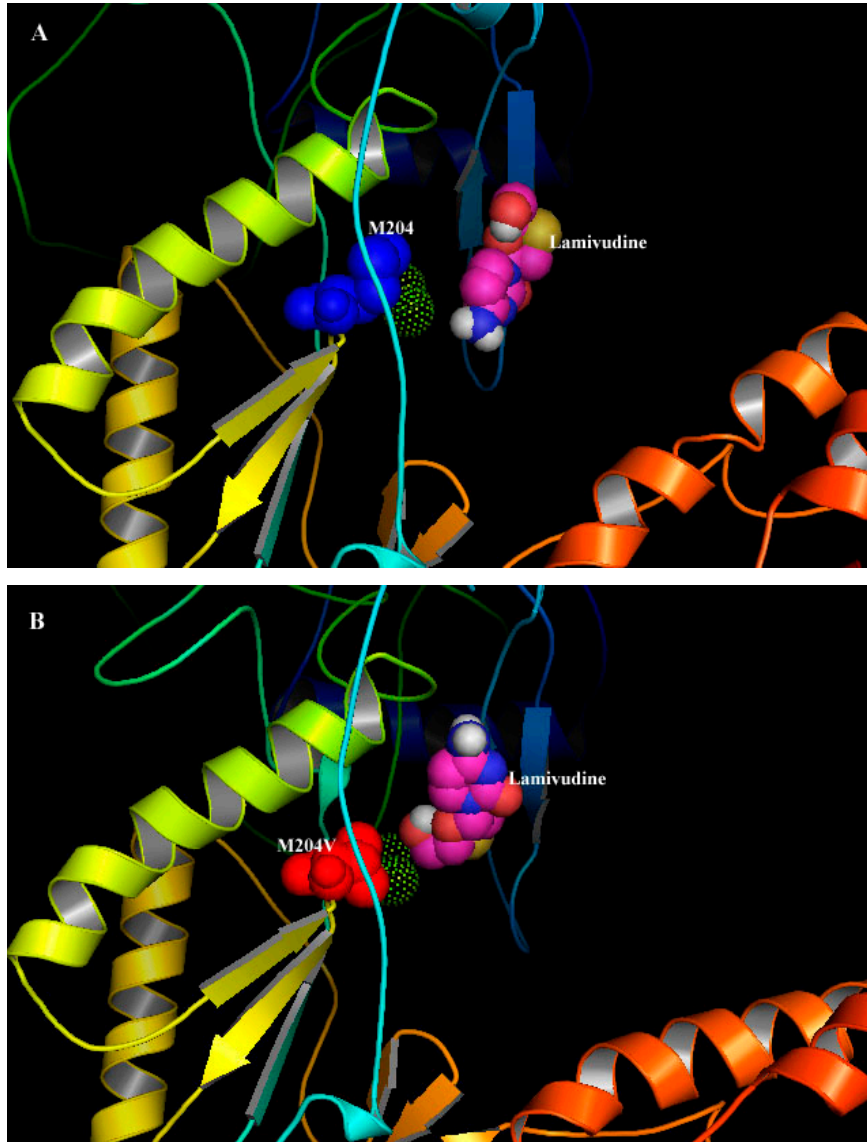


Homology model of HBV polymerase/rt was constructed for a representative study sequence to study the effect of rtM204 wild type and rtM204V mutation in lamivudine binding. In comparison to wild type rtM204 (A), the positional effect of rtM204V mutant (B) induced a spatial constraint for lamivudine binding. The $Mg^{2+}$ ions are shown as dotted spheres.

**Figure 26. Effect of rtI233V mutation and adefovir binding**
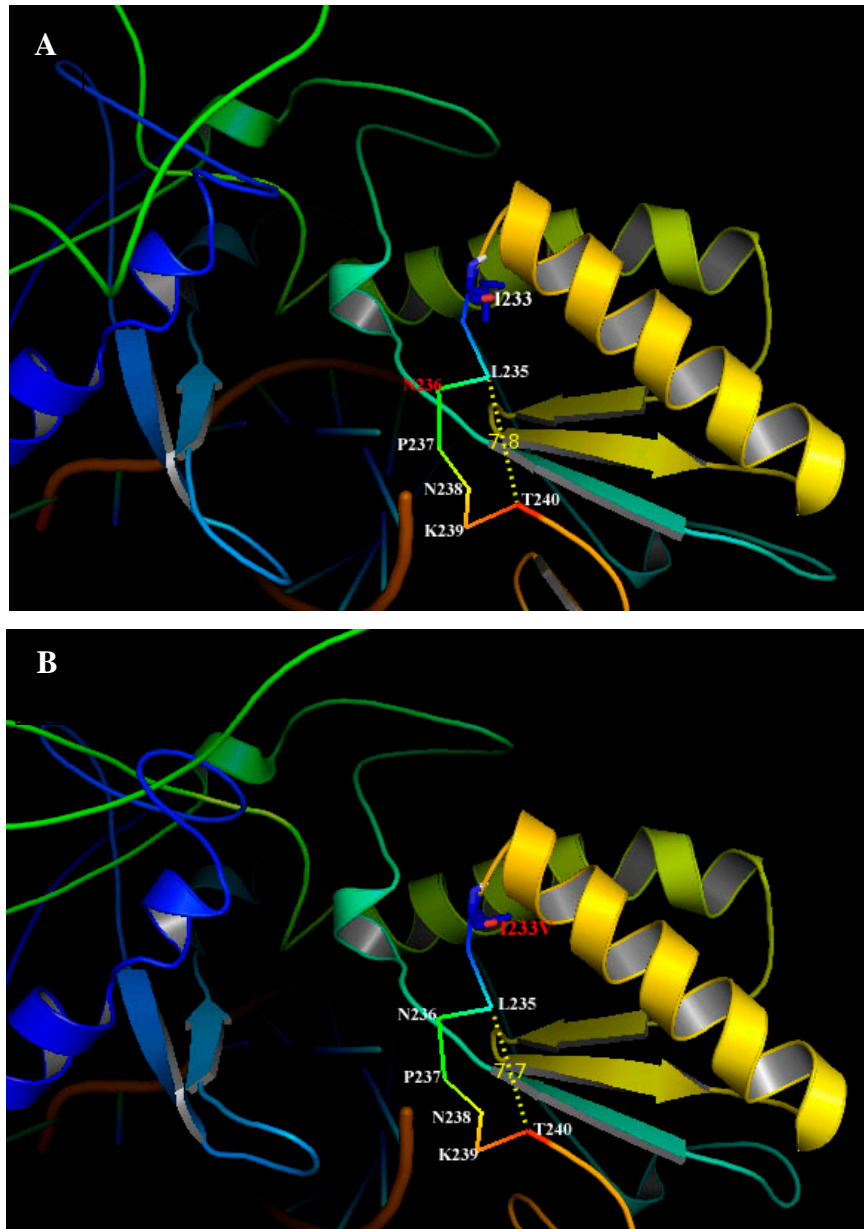


Homology model of HBV polymerase/rt wild type rtI233 (A) was compared with the rtI233V (B) mutation. The relative distance between the residues 235 to 240 crucial for dNTP binding that form the bent structure is shown as yellow dots. Substitution of valine for isoleucine (rtI233V) reduced its relative distance by a difference of only 0.1 Angstrom.

**5.5.5 Effect of rtV173L mutation and Entecavir action**

Entecavir resistance is shown to occur by the combination of three or more amino acid substitutions in the HBVrt region. One of the entecavir-experienced study subjects exclusively presented with rtV173L mutation at the end of 7 months treatment. However, this subject showed partial EVR with the reduction in HBV DNA levels of 1.8 $\log_{10}$ IU/mL from baseline. To understand the effect of rtV173L mutation and entecavir efficacy, HBVrt model of the study sequence (GenBank accession: JQ514535) was constructed and docked with entecavir. Modeling showed the residue rt173 to be located below the DNA template binding region. The rtV173L mutation did not show to alter the entecavir binding or the relative position of F88 that interacts with dNTP substrate **(Figure 27)**. In addition to the clinical evidence, modelling revealed rtV173L mutation cannot independently confer resistance to entecavir or alter the relative amino acid residues.

**5.6 HBV genotypes and subgenotypes**

Overall 296 subjects (147 in lamivudine group, 30 in adefovir group, 50 in entecavir group and 69 treatment-naive subjects with no follow-up) with chronic hepatitis B were enrolled in this study. These study subjects were from different parts of Indian subcontinent and categorised into southern, western, eastern and north-eastern population. The subjects from Tamil Nadu, Kerala, Karnataka and Andhra Pradesh region and one subject from the Maldives island in the south-west of India represented the south-Indian subcontinent population (n=92). Two subjects were from the western Indian states of Maharashtra and Rajasthan. The subjects of east India (n=115) consisted of West Bengal, Bihar, Jharkhand and Orissa states. The subjects from the states of Assam, Manipur, Meghalaya, Tripura, Arunachal Pradesh and the adjacent countries of Bangladesh and Bhutan represented the north-eastern (n=87) region.

**Figure 27. Effect of rt173L mutation and entecavir binding**
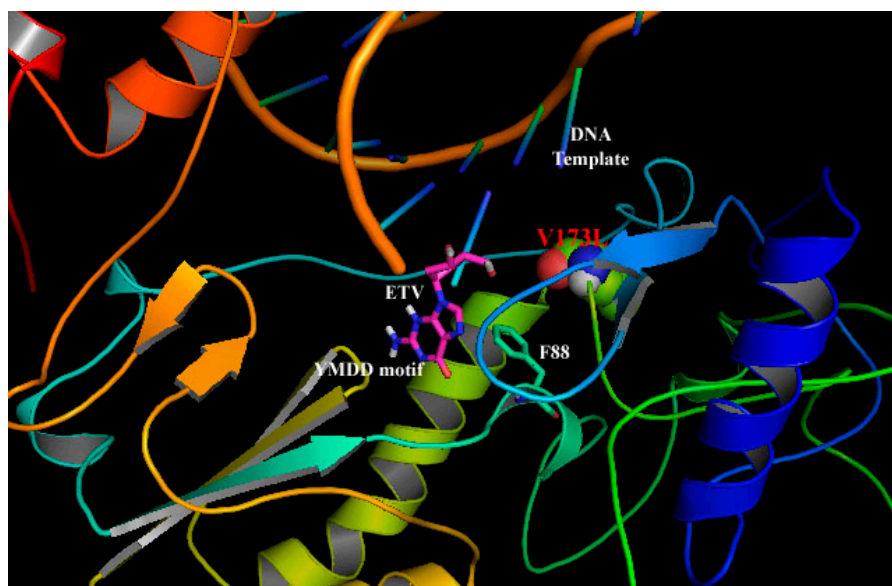


Homology model of HBV polymerase/rt of a study sequence identified with rtV173L mutation was constructed (GenBank accession: JQ514535). The location of rtV173L is shown. Also shown are the YMDD motif and entecavir (ETV) binding sites. The rt173 just lies below the DNA template region and the rtV173L mutation did not appear to alter entecavir binding or rtF88 positioning that interacts with the dNTP substrate.

Three major HBV genotypes A, C, D and a recently identified genotype I were distributed in this population. HBV genotype D was found to be the predominantly circulating genotype followed by genotype C and A in 175 (59.1%), 67 (22.6%) and 53 (17.9%) subjects respectively. Additionally, 1 (0.3%) subject was identified to be infected with genotype I.

### 5.6.1 Region-wise distribution of HBV genotypes

There existed a distinct pattern of HBV genotype circulation between the three main regions of study population.

Among the 92 subjects from southern India, HBV genotype D was the predominant genotype, circulating in 82 (89.1%) subjects. Genotypes A and C were the less-prevalent genotypes identified in 6 (6.5%) and 4 (4.4%) subjects respectively.

Among the 115 subjects from eastern India, HBV genotype D was identified in 58 (50.4%) subjects. The other common genotypes were genotype A and genotype C identified in 37 (32.2%) and 20 (17.4%) respectively.

Among the 87 subjects from north-eastern region, genotype C was identified in 43 (49.4%) subjects, genotype D in 33 (37.9%) subjects and genotype A in 10 (11.5%) subjects respectively. Additionally, this region had 1 (1.2%) subject with genotype I.

HBV genotype D was identified in 2 subjects from western India.

### 5.6.1.1 Analysis of newly identified HBV genotype I

On phylogenetic analysis with genotypes A to G as reference sequences, the new genotype initially clustered with genotype G in a distinct branch **(Figure 28)**. The Genafor/Arevir-geno2pheno prediction tool (Genafor, Bonn, Germany; http://hbv.bioinf.mpi-inf.mpg.de/index.php) also determined the sequence as genotype G **(Figure 29)**. As mixed

**Figure 28. Phylogenetic determination of HBV genotypes with A-G reference sequences**
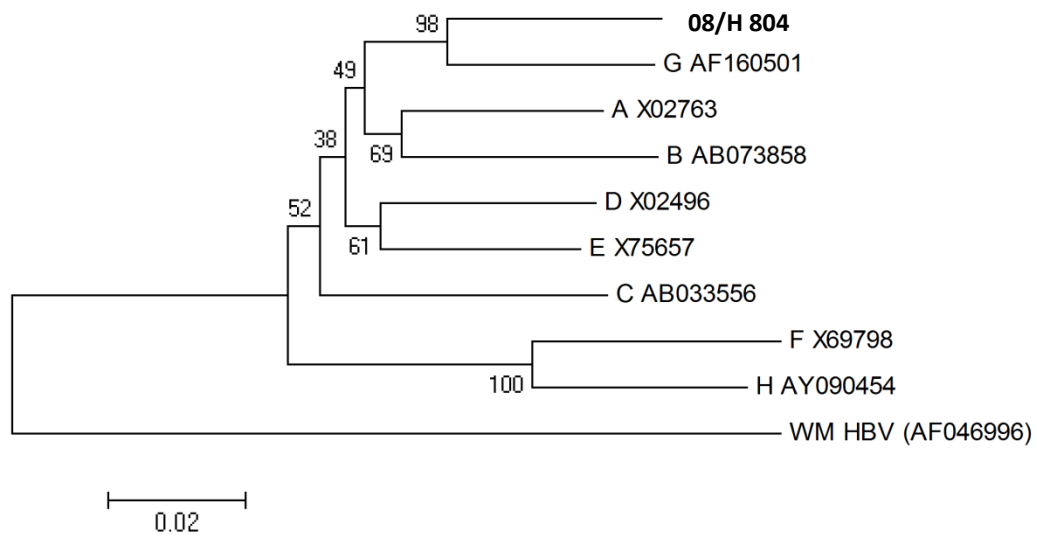


Phylogenetic analysis of HBV reverse transcriptase nucleotide sequences (1085 positions) was conducted in MEGA4 using the neighbour joining and maximum composite likelihood model. GenBank reference sequences are shown by HBV genotype and accession number. The study sequence is designated by study number (08/H 804). The number on the branches are bootstrap values (1000 replicates; values less than 50% are not shown). Woolly monkey HBV was used as an out-group.

**Figure 29. Genafor/Arevir-geno2pheno tool for HBV genotype determination**

Arevir

## HBV Drug Resistance Test

| I. General Information | |
|---|---|
| **Patient:** | |
| Birth date: | Viral load: |
| Sample received: | Sample collected: |
| Sample ID:<br>**08/H** | Current therapy: |
| Sample type: | Physician: |
| Reported by: | |

| II. Results |
|---|
| Genotype:<br>**G** |
| Substitutions *pol* frame:<br>**53I, 54H, 91L, 118N, 153Q** |
| Substitutions *surface protein* frame:<br>**45S, 46T, 49L, 51Q, 63T, 68I, 122R, 145R, 168A, 194V, 200F** |
| Escape mutations *surface protein* frame:<br>**145R** |

| III. Interpretation | | |
|---|---|---|
| Drugs | Scored mutations | Resistance analysis |
| Lamivudine, Zeffix® | none | susceptible |
| Adefovir, Hepsera® | none | susceptible |
| Entecavir, Baraclude® | none | susceptible |
| Tenofovir DF | none | susceptible |
| Telbivudine, Tyzeka®, Sebivo® | none | susceptible |

Screenshot of Genafor/Arevir-geno2pheno tool used for hepatitis B virus (HBV) genotype determination of study sequence (08/H 804). As indicated the sequence was initially predicted as HBV genotype G (last accessed on Dec 2nd 2011).

HBV genotype infection has been commonly reported with genotype G, clonal analysis was also performed for the sequence. All clones clustered as a separate branch with genotype G and no co-infecting genotypes were identified (**Figure 30**).

A recent study from eastern India reported novel recombinants between HBV genotype A, G and C with sequence identity to Vietnam and Laos strains which have been designated as genotype I. Our study sequence was then re-analysed including the newly identified recombinant HBV genotype I. Phylogenetic analysis showed this sequence to cluster with genotype I of Vietnam/Laos and the recently reported eastern Indian strain (**Figure 31**). This was further confirmed using the HBVSeq program in Stanford database. The overlapping surface gene analysis showed this sample to carry the classical HBV vaccine escape mutant sG145R.

### 5.6.2 Subgenotype analysis

The HBV genotypes were further categorised into respective subgenotypes by aligning the study sequences with the reference sequences of all known subgenotypes. To determine the subgenotypes for genotype A, a phylogenetic tree was constructed using 30 HBV reference sequences from countries of different origin representing the subgenotyes A1 to A3. There were a total of 665 nucleotide positions of HBV surface gene in the final dataset. All the HBV 53 genotype A sequences in this study were identified as subgenotype A1 (**Figure 32**). Likewise, genotype C sequences were aligned with 39 reference sequences of subtypes C1 to C7 with countries of different origin. There were a total of 644 nucleotide positions of HBV surface gene in the final dataset. Among the 67 genotype C sequences, 58 (86.6%) clustered to C1 and 6 (8.9%) clustered to C2 subgenotyes respectively (**Figure 33a**). The subgenotyes could not be determined in 3 (4.5%) subjects. The phylogenetic tree of three unassigned samples with bootstrap values is separately shown in **Figure 33b.** One sequence (07/H 935)

**Figure 30. Phylogenetic analysis of HBV clones for identification of mixed HBV genotype infection**
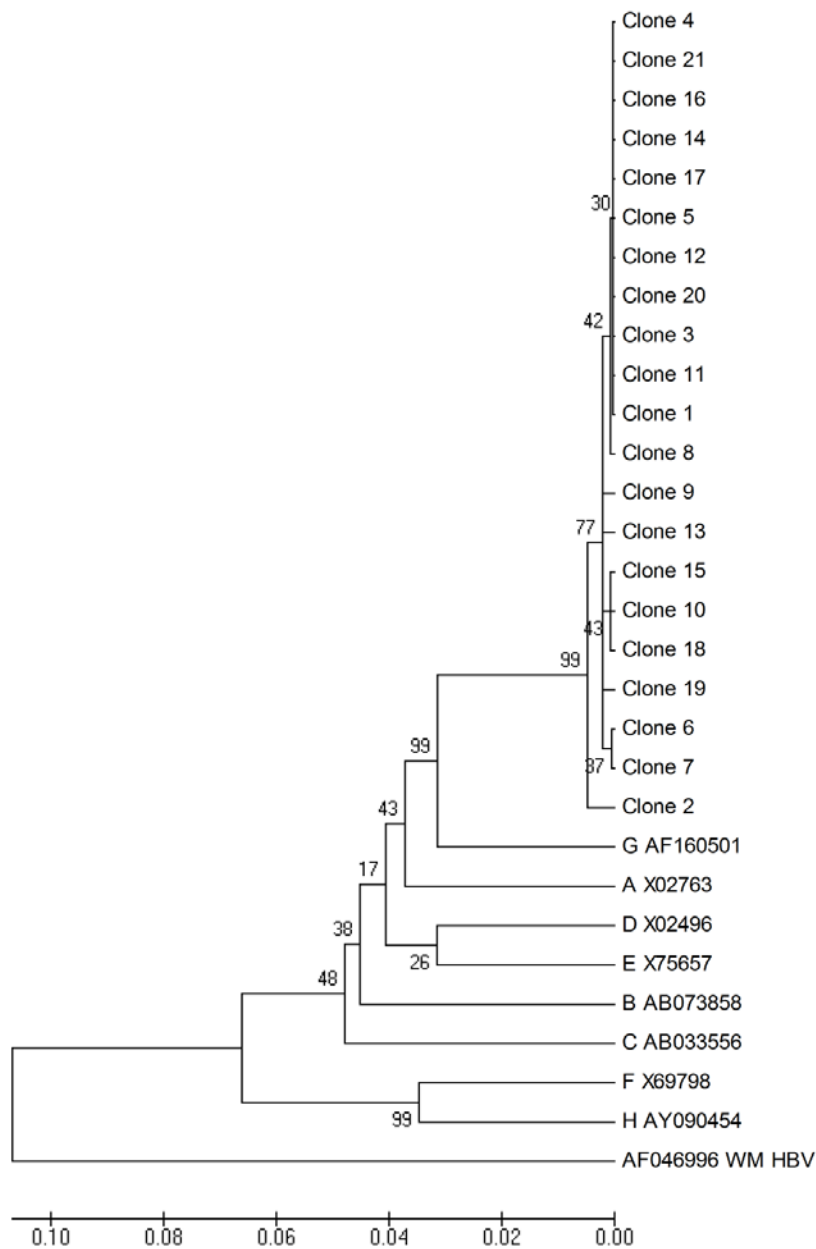


Phylogenetic analysis of HBV reverse transcriptase nucleotide sequences (1027 positions) was conducted in MEGA4 using the neighbour joining method with a bootstrap test of 1000 replicates and maximum composite likelihood model. GenBank reference sequences are shown by HBV genotype and accession number. A total of 21 clones for sample (08/H 804) was analysed and are indicated as clone 1 to 21. The numbers on the branches are bootstrap values. Woolly monkey HBV was used as an out-group.

**Figure 31. Phylogenetic determination of initially classified HBV genotype G sequence (08/H 804) as newly identified HBV genotype I**
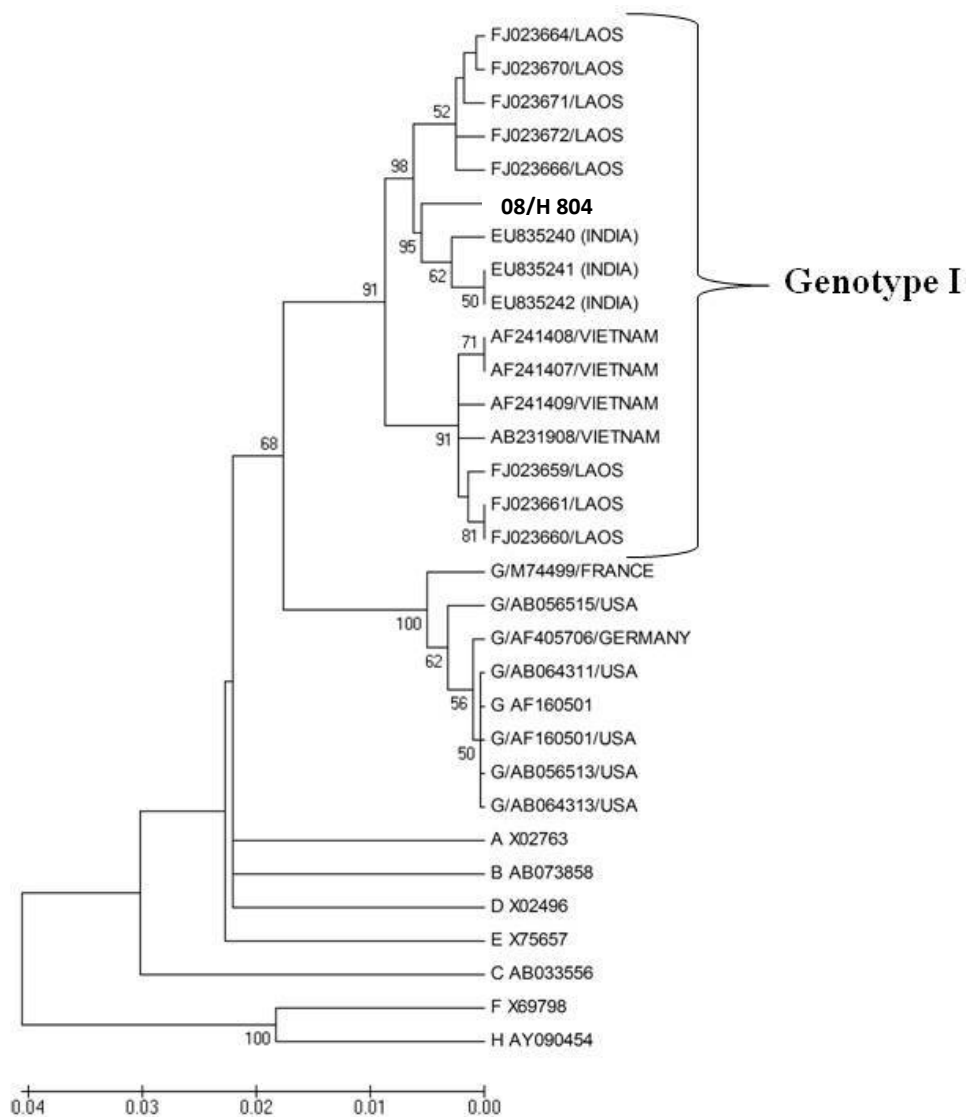


Phylogenetic analysis of HBV reverse transcriptase nucleotide sequences (702 positions) was conducted in MEGA4 using the neighbour joining method and maximum composite likelihood model. GenBank reference sequences are shown by HBV genotype, accession number and country of origin. The study sequence is designated by study number (08/H 804). The numbers on the branches are bootstrap values (1000 replicates; values less than 50% are not shown). Woolly monkey HBV was used as an out-group.

**Figure 32. Phylogenetic determination of HBV subgenotypes of genotype A**



Phylogenetic analysis of HBV surface gene nucleotide sequences (665 positions) was conducted in MEGA4 using the neighbour joining method with a bootstrap test of 1000 replicates and maximum composite likelihood model. GenBank reference sequences are shown by HBV subgenotype, accession number and country of origin. Study sequences are designated by study number. Woolly monkey HBV was used as an out-group.

**Figure 33a. Phylogenetic determination of HBV subgenotypes of genotype C**



Phylogenetic analysis of HBV surface gene sequences (644 positions) was conducted in MEGA4 using the neighbour joining method with a bootstrap test of 1000 replicates and maximum composite likelihood model. GenBank reference sequences are shown by HBV subgenotype, accession number and country of origin. Three samples that did not cluster with any of the known subgenotypes are shown as shaded circles. Study sequences are designated by study number. Woolly monkey HBV (WM HBV) was used as an out-group.

**Figure 33b. Phylogenetic analysis of uncategorised genotype C sequences**



Phylogenetic analysis of three genotype C samples (shaded circles) where the subgenotype could not be assessed at the surface gene level is separately shown. The tree was constructed in MEGA4 using the neighbour joining method and maximum composite likelihood model. The numbers on the branches are bootstrap values (1000 replicates; values less than 50% are not shown). One sample with a bootstrap support of 82% requires further analysis to group with C7 subgenotype. There were a total of 660 HBV surface gene nucleotides compared in the final dataset. GenBank reference sequences are shown by subgenotype, accession number and country of origin. Woolly monkey HBV (WM HBV) was used as an out-group.

clustered with C7 subgenotype with a bootstrap support of 82% and other two samples (07/H 552 and 10/H 1843) formed a new clade in genotype C. These three sequences require further analysis.

To determine the subgenotypes for 175 genotype D sequences, 26 GenBank D1 to D8 subgenotype sequences of different origin were aligned with the study sequences and phylogenetic analysis was performed. The study sequences clustered into subgenotypes D1, D2, D3 and D5 in 20 (11.4%), 112 (64%), 15 (8.6%) and 27 (15.4%) sequences respectively **(Figure 34a and 34b)**. The subgenotype could not be determined in 1 (0.6%) genotype D sample (09/H 230). The sequence clustered with D4 subgenotype with a bootstrap support of 66% and requires further analysis **(Figure 34c)**.

**5.6.2.1 Distribution of hepatitis B virus subgenotypes**

The frequency and distribution of HBV subgenotypes are shown in **Table 15**.

To some extent, the subgenotypes C and D differed in their regional distribution. Among six subgenotypes C2 identified in the study, 5 (83.3%) were found to be circulating in the Arunachal Pradesh state of north-eastern region. This region is bordered by China in the north where subgenotype C2 is more prevalent. Subgenotypes D1 and D3 were identified in 65% and 86.6% of subjects from Eastern India respectively. HBV subgenotype D2 was found to be predominantly circulating in the South Indian region (68.7%). HBV subgenotype D5 was predominantly identified in eastern India (51.9%) and north-eastern subcontinent (40.7%).

**Figure 34a. Phylogenetic determination of HBV subgenotypes of genotype D**



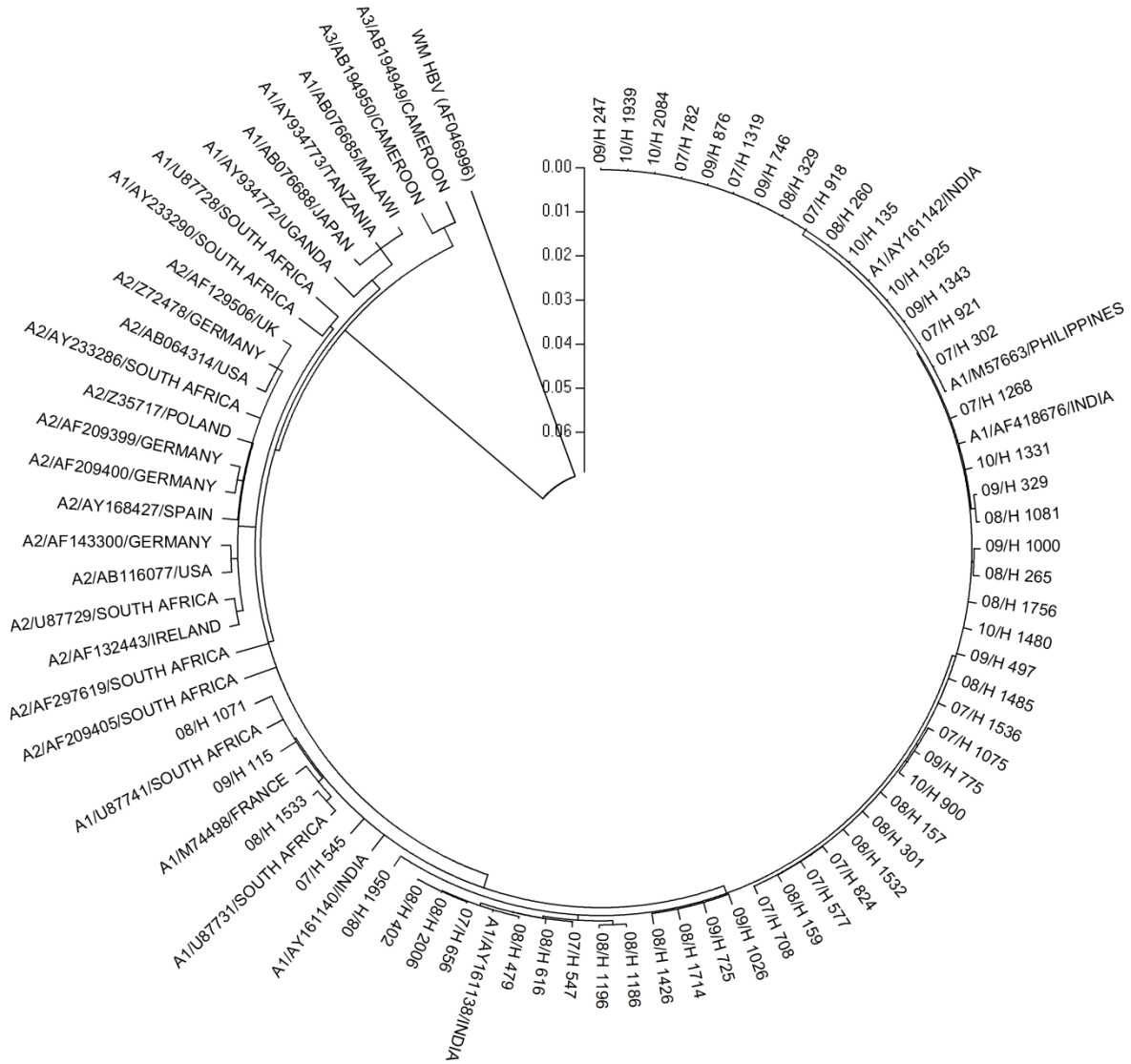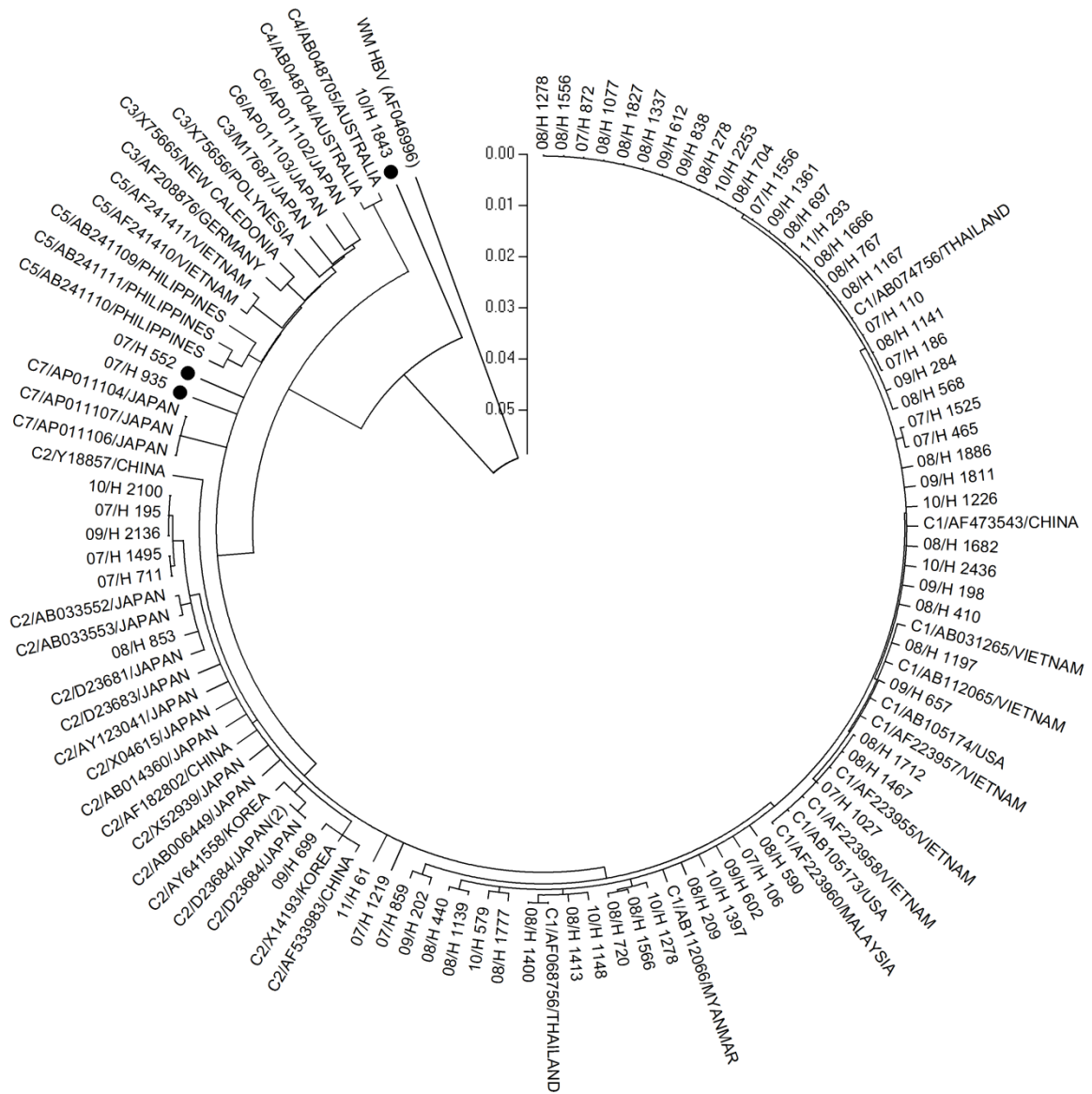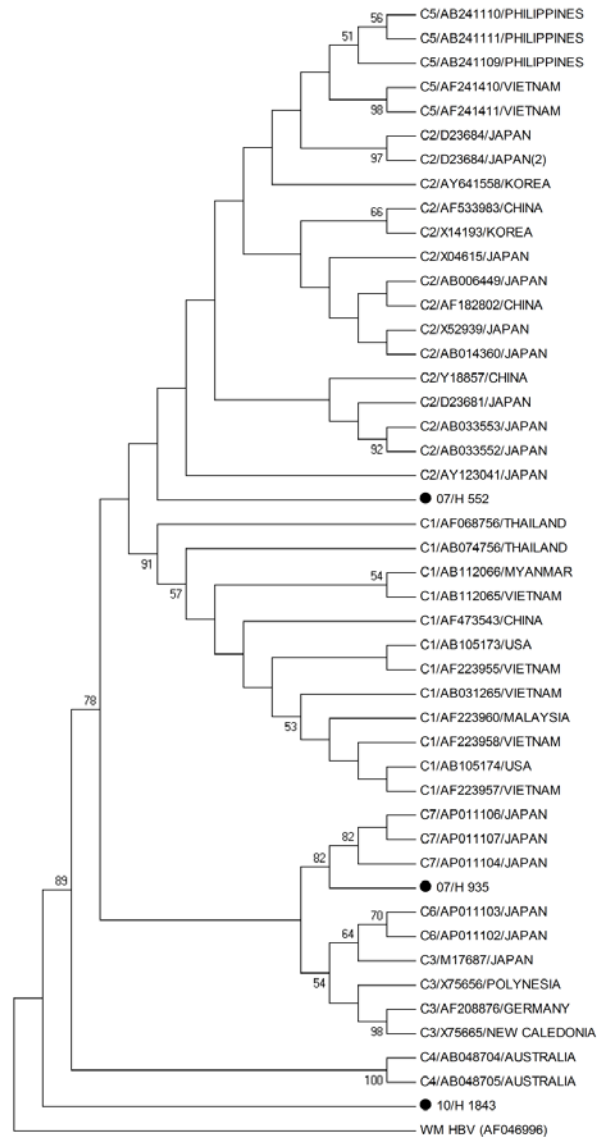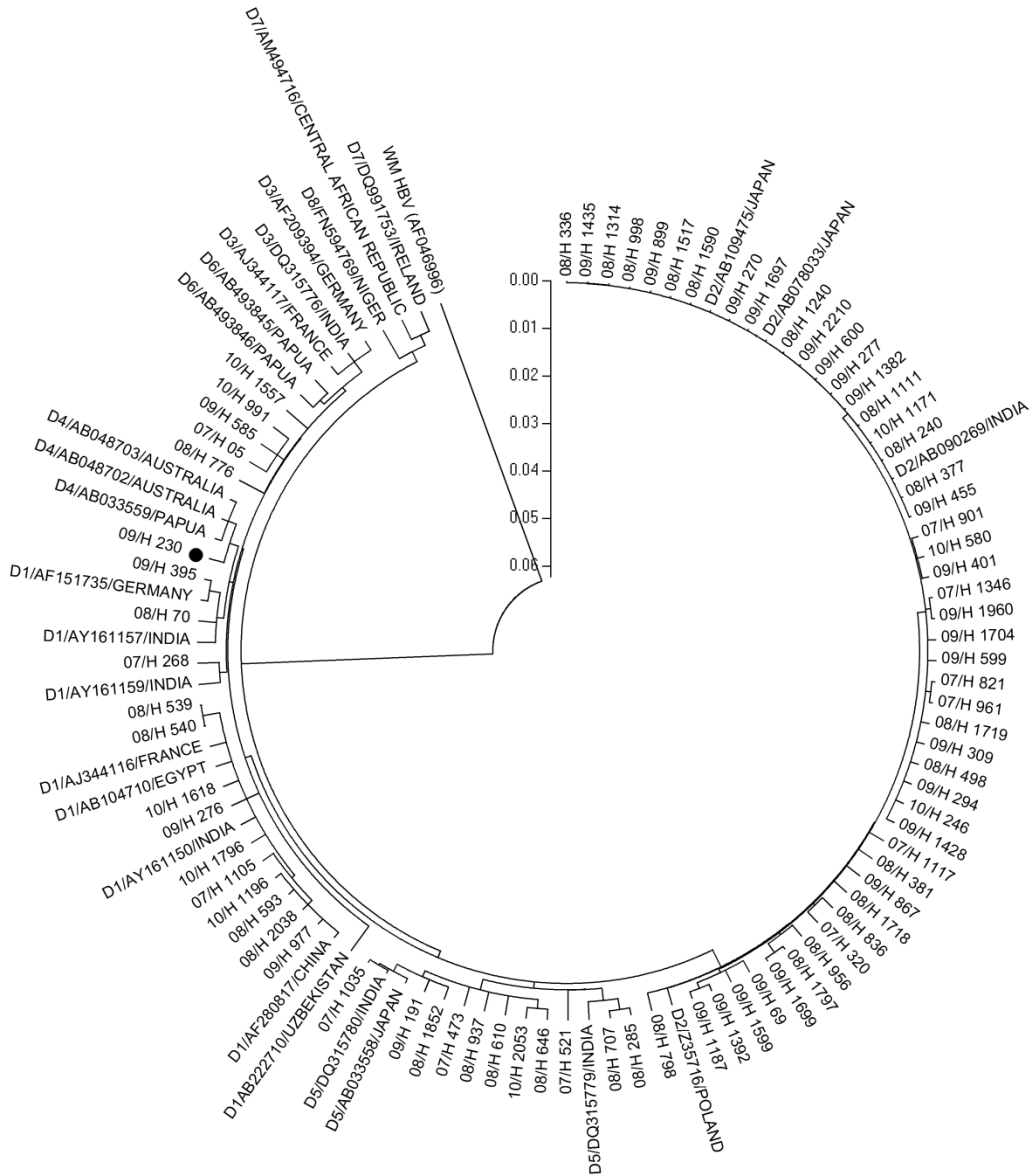Phylogenetic analysis of HBV surface gene nucleotide sequences (655 positions) was conducted in MEGA4 using the neighbour joining method with a bootstrap test of 1000 replicates and maximum composite likelihood model. GenBank reference sequences are shown by HBV subgenotype, accession number and country of origin. Study sequences are designated by study number. One sample that clustered with D4 is shown in shaded circle and requires further analysis. Woolly monkey HBV (WM HBV) was used as an out-group.

**Figure 34b. Phylogenetic determination of HBV subgenotypes of genotype D**
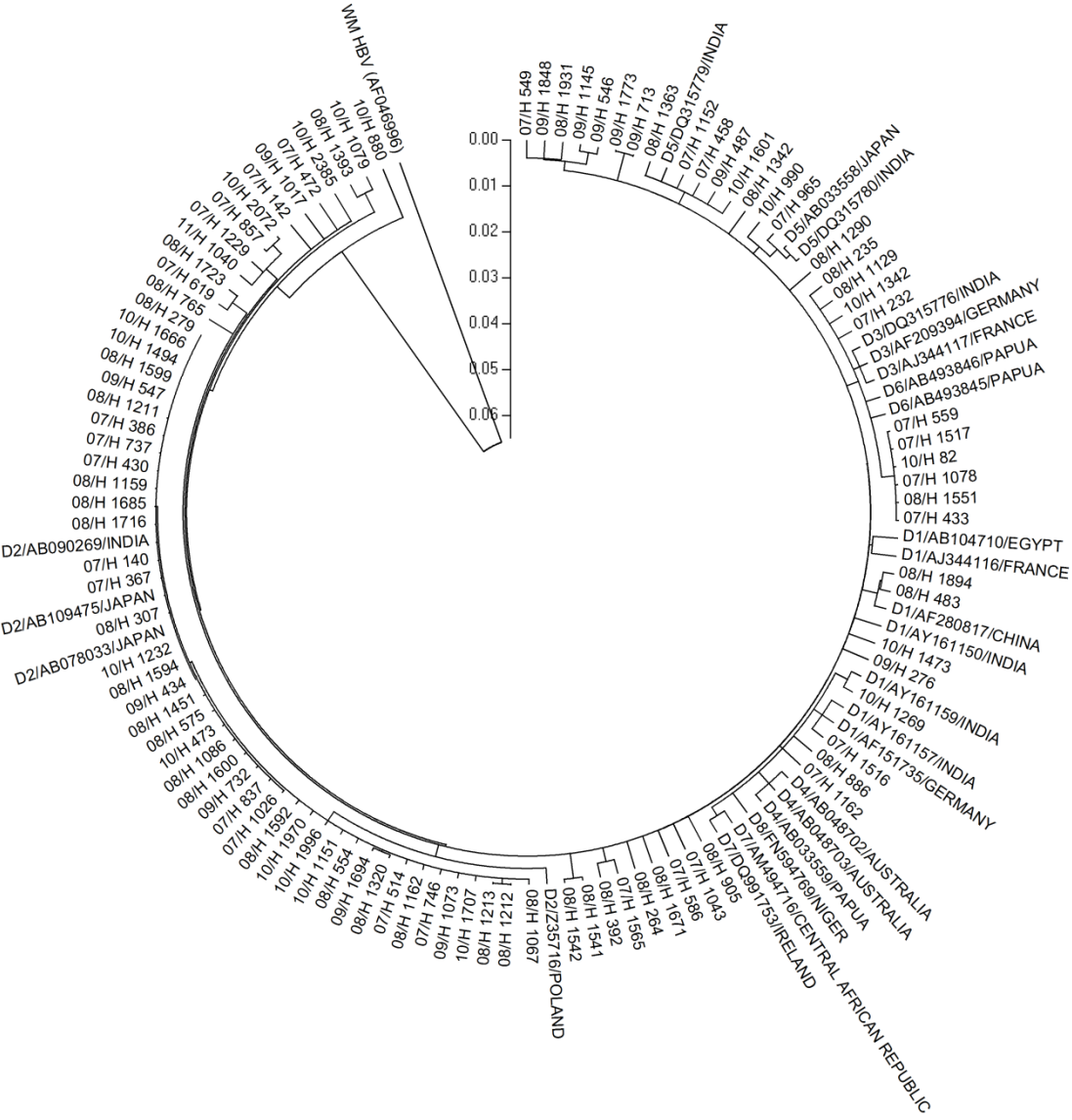


Phylogenetic analysis of HBV surface gene nucleotide sequences (642 positions) was conducted in MEGA4 using the neighbour joining method with a bootstrap test of 1000 replicates and maximum composite likelihood model. GenBank reference sequences are shown by HBV subgenotype, accession number and country of origin. Study sequences are designated by study number. Woolly monkey HBV (WM HBV) was used as an out-group

**Figure 34c. Phylogenetic analysis of uncategorised HBV genotype D sequences**
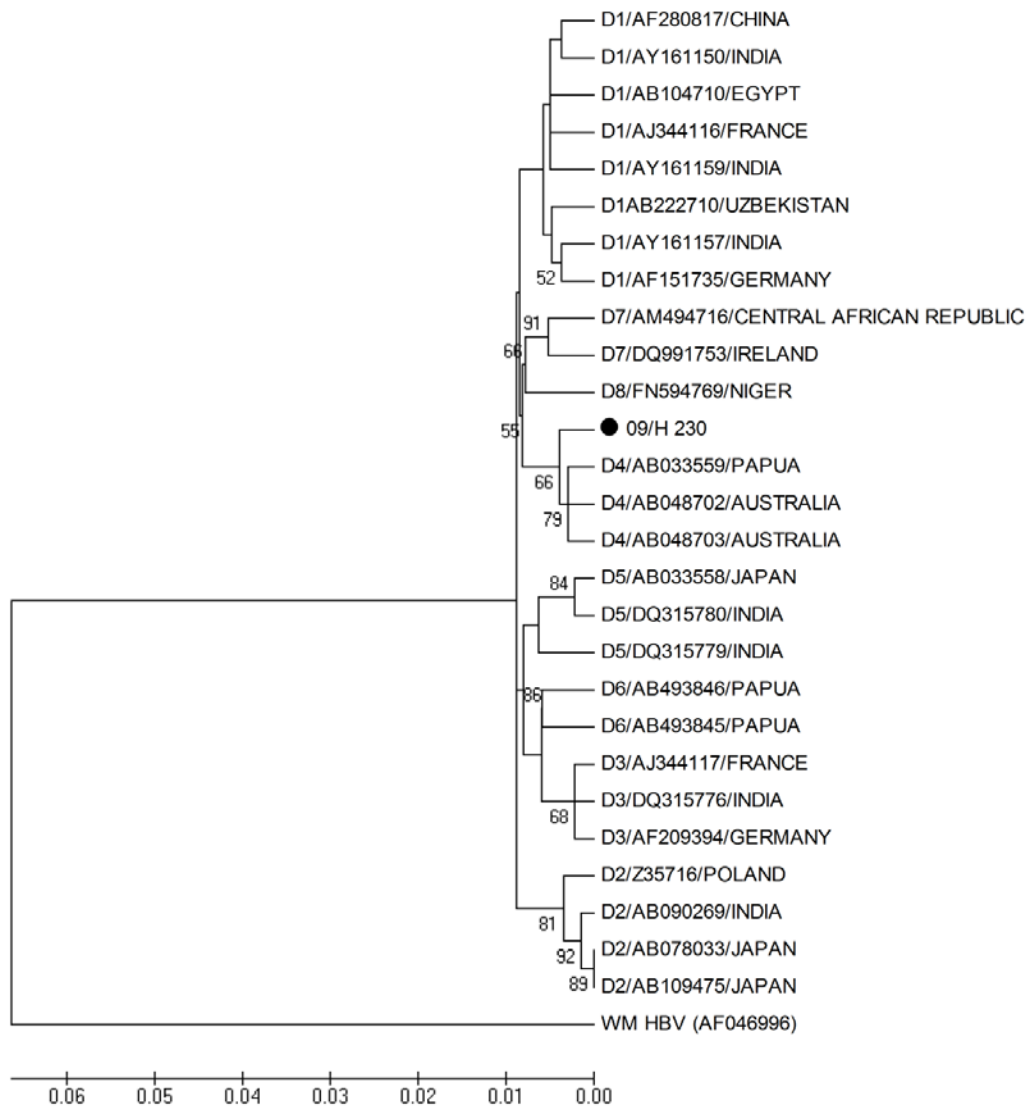


Phylogenetic analysis of genotype D sample (shaded circle) where the subgenotype could not be assessed at the surface gene level is separately shown. The tree was constructed in MEGA4 using the neighbour joining method with a bootstrap test of 1000 replicates and maximum composite likelihood model. The numbers on the branches are bootstrap values (1000 replicates; values less than 50% are not shown). The sample clustered with D4 subgenotype with a bootstrap support of 66% and requires further analysis. There were a total of 681 HBV surface gene nucleotides in the final dataset. GenBank reference sequences are shown by subgenotype, accession number and country of origin. Woolly monkey HBV (WM HBV) was used as an out-group.

**Table 15. Region-Wise distribution pattern of HBV subgenotypes**

| HBV Subgenotypes | Total | Region | | | |
|---|---|---|---|---|---|
| | | Southern (n=92) | Western (n=2) | Eastern (n=115) | North-eastern (n=87) |
| A1 | 53 | 6 (11.3) | 0 | 37 (69.8) | 10 (18.9) |
| C1 | 58 | 4 (6.9) | 0 | 18 (31) | 36 (62.1) |
| C2 | 6 | 0 | 0 | 1 (16.7) | 5 (83.3) |
| *C (unassigned) | 3 | 0 | 0 | 1 (33.3) | 2 (66.7) |
| D1 | 20 | 2 (10) | 1 (5) | 13 (65) | 4 (20) |
| D2 | 112 | 77 (68.7) | 1 (0.9) | 18 (16.1) | 16 (14.3) |
| D3 | 15 | 1 (6.7) | 0 | 13 (86.6) | 1 (6.7) |
| D5 | 27 | 2 (7.4) | 0 | 14 (51.9) | 11 (40.7) |
| *D (unassigned) | 1 | 0 | 0 | 0 | 1 (100) |
| I | 1 | 0 | 0 | 0 | 1 (100) |

*Three HBV genotype C sequences and one genotype D sequence could not be assigned to any known subgenotypes using the surface gene sequences

Values in parentheses represent percentages

### 5.6.3 HBV genotypes and subgenotypes in treatment-naive subjects

Among the 197 treatment-naive subjects studied, HBV genotypes A, C, D and I were identified in 32 (16.2%), 40 (20.2%), 125 (63.1%) and 1 (0.5%) subjects respectively. Due to its lower frequency, HBV genotype I was not included for statistical analysis.

### 5.6.3.1 Baseline characteristics according to HBV genotypes

Baseline HBV DNA, HBeAg and anti-HBe status did not differ significantly between HBV genotypes but ALT, AST levels and number of HBVrt amino acid substitutions were significantly different **(Table 16)**. Serum ALT and AST levels were significantly lower in genotype D subjects as compared to genotypes A and C respectively (p=0.001 and 0.002). Additionally, genotype D showed higher number of HBVrt amino acid substitutions than HBV genotypes A and C respectively (p=0.0001).

### 5.6.3.2 Baseline characteristics according to HBV subgenotypes

Among 40 HBV genotype C in treatment-naive subjects, 39 (97.5%) presented with subgenotype C1 and only 1 (2.5%) was identified with subgenotype C2.

Hepatitis B virus subgenotypes D1, D2, D3 and D5 were seen in 19 (15.2%), 79 (63.2%), 10 (8%) and 16 (12.8%) subjects respectively. Subgenotype for 1 (0.8%) genotype D subject could not be assigned. There was a significant difference between the number of HBVrt amino acid substitutions and the subgenotypes tested. Hepatitis B virus subgenotype D5 showed higher number of amino acid substitution [median 13 (IQR 10-13.5)] as compared to other subgenotypes (p=0.0001) **(Table 17)**.

**Table 16. Baseline characteristics according to HBV genotypes**

| | Genotype A (n=32) | Genotype C (n=40) | Genotype D (n=125) | p value |
|---|---|---|---|---|
| Age, years[†] | 37 (31-48) | 33 (23-41) | 38 (27-49) | 0.152 |
| Gender, male[*] | 31 (19) | 35 (21) | 99 (60) | 0.041 |
| ALT (U/L)[†] | 43.5 (30-131) | 56.5 (40-94) | 37.5 (25-63) | 0.001 |
| AST (U/L)[†] | 55 (31-118) | 59 (35-98) | 37 (27-65) | 0.002 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 5.58 (4.2-7.2) | 5.15 (4.3-6.5) | 5.7 (4-7.3) | 0.412 |
| HBeAg positive[*] | 22 (69) | 24 (60) | 73 (58) | 0.564 |
| HBeAg negative[*] | 10 (31) | 16 (40) | 52 (42) | |
| Anti-HBe positive[*] | 10 (31) | 14 (35) | 51 (41) | 0.553 |
| Anti-HBe negative[*] | 22 (69) | 26 (65) | 74 (59) | |
| HBVrt amino acid substitutions[†] | 4.5 (3-7) | 5 (4-6) | 7 (5-9) | 0.0001 |

Data are [†]median (IQR) or [*]number (%)

**Table 17. Baseline characteristics according to HBV subgenotypes of genotype D**

|  | D1 (n=19) | D2 (n=79) | D3 (n=10) | D5 (n=16) | p value |
|---|---|---|---|---|---|
| Age, years[†] | 31 (23-50) | 39 (26-51) | 34 (33-40) | 42 (25-49) | 0.811 |
| Gender, male[*] | 17 (89) | 59 (75) | 9 (90) | 13 (81) | 0.403 |
| ALT (U/L)[†] | 38 (26-99) | 36 (23-60) | 32 (24-37) | 45 (37-108) | 0.237 |
| AST (U/L)[†] | 41 (29-72) | 34 (26-63) | 33 (25-63) | 43 (36-80) | 0.267 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 4.85 (3.95-7.0) | 6 (4.3-7.48) | 5.19 (3.3-6.85) | 5.85 (4.74-7.39) | 0.581 |
| HBeAg positive[*] | 10 (52) | 47 (59) | 7 (70) | 8 (50) | 0.727 |
| HBeAg negative[*] | 9 (47) | 32 (41) | 3 (30) | 8 (50) | |
| Anti-HBe positive[*] | 8 (42) | 34 (43) | 3 (30) | 6 (37) | 0.867 |
| Anti-HBe negative[*] | 11 (58) | 45 (57) | 7 (70) | 10 (63) | |
| HBVrt amino acid substitutions[†] | 4 (3-7) | 6 (5-8) | 5.5 (5-7) | 13 (10-13.5) | 0.0001 |

Data are [†]median (IQR) or [*]number (%)

**5.6.4 HBV genotypes, subgenotypes in lamivudine-experienced group**

**5.6.4.1 Clinical characteristics of lamivudine-experienced subjects at last follow-up analysis by HBV genotypes**

In 147 lamivudine-experienced subjects, HBV genotype A, C and D were identified in 29 (19.7%), 33 (22.4%) and 85 (57.8%) subjects respectively. Univariate analysis was performed to identify factors that vary across the genotypes tested over the course of treatment. Among the variables analysed only ALT and AST levels were significantly different between the genotypes (p=0.003 and p=0.008). The genotype D subjects had lower ALT and AST levels in comparison to genotype A and C. However, it should be noted that the ALT and AST levels of genotype D subjects was significantly lower in the total 196 treatment-naive subjects studied (p=0.001 and p=0.002; Table 16). Also, on separate analysis of 90 treatment-naive subjects who were subsequently followed-up with lamivudine treatment, the median ALT levels were lower in genotype D subjects [44 (IQR 27-72) U/L] as compared to genotype A [58 IQR (29-155) U/L] and genotype C [83 (IQR 54-110) U/L] respectively (p=0.054). Likewise, the median AST levels were significantly lower in genotype D subjects [53 (IQR 34-78)] U/L as compared to genotype A [81 (IQR 31-129) U/L] and genotype C [85 (IQR 68-102)] U/L respectively (p=0.022). Therefore, the significant difference observed at the last follow-up analysis illustrates that low ALT and AST levels are maintained over the course of therapy.

No other factors showed significant difference across the genotype tested. The number of male subjects in genotype A group were comparatively higher (96%) and the difference did not affect any subsequent analysis. There was no significant difference in treatment duration between the genotypes and this excludes the chance of bias in the analysis performed. **(Table 18)**

**Table 18. Characteristics of lamivudine-experienced subjects at last follow-up analysis according to HBV genotypes**

| | Genotype A (n=29) | Genotype C (n=33) | Genotype D (n=85) | p value |
|---|---|---|---|---|
| Age[†] | 39 (26-49) | 34 (27-47) | 40 (27-52) | 0.503 |
| Gender: male[*] | 28 (96%) | 27 (82%) | 64 (75%) | 0.042 |
| ALT (U/L)[†] | 36 (29-76) | 41 (32-61) | 30 (21-47) | 0.003[£] |
| AST (U/L)[†] | 40 (31-103) | 45 (37-66) | 36 (26-49) | 0.008[£] |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 4 (2.5-5.3) | 3.5 (1-5.6) | 3.6 (0-5.8) | 0.915 |
| HBeAg positive[*] | 19 (66) | 24 (73) | 50 (59) | 0.358 |
| HBeAg negative[*] | 10 (34) | 9 (27) | 35 (41) | |
| Anti-HBe positive[*] | 8 (28) | 12 (36) | 34 (40) | 0.488 |
| Anti-HBe negative[*] | 21 (72) | 21 (64) | 51 (60) | |
| Treatment duration (months)[†] | 12 (6-24) | 12 (7-24) | 13 (10-24) | 0.806 |

[£]The significant difference in ALT and AST levels were also observed in treatment-naive subjects. Therefore, it should be noted that the level of significance did not vary with the influence of treatment but is maintained over the course of therapy.

Data are [†]median (IQR) or [*]number (%)

### 5.6.4.2 HBV genotypes and lamivudine response

Overall the response rate to genotype A, C and D over a median treatment duration of 12 to 13 months were 35%, 31% and 41% respectively There was no significant difference in subjects who showed response and non-response between the genotypes tested (p=0.633). Similarly, 34%, 21%, and 27% of the respective genotypes developed resistance and there was no significant difference in subjects who developed resistance between the genotypes tested (p=0.503) **(Table 19)**.

### 5.6.4.3 HBV subgenotypes and lamivudine response

When HBV subgenotype D subjects were separately analysed, none of the variables showed significant difference between the subgenotypes D1, D2, D3 and D5. The subgenotype for one genotype D could not be typed and was not included in the analysis **(Table 20)**. To identify whether subgenotypes are associated with virological response, univariate analysis was performed for the virological response categorized subjects (n=78; **Table 21**). The analysis showed no subgenotype-dependent response or non-response among the samples tested (p=0.489). Further, on analysis of subgenotypes and lamivudine resistance, there was no significant difference between the subgenotypes tested (p=0.694).

### 5.6.5 HBV genotypes and subgenotypes in adefovir-experienced group
### 5.6.5.1 Clinical characteristics of adefovir-experienced subjects at last follow-up analysis by HBV genotypes

In 30 adefovir-experienced subjects, HBV genotypes A, C and D were identified in 7 (23.3%), 6 (20%) and 17 (57%) subjects respectively. Univariate analysis showed no significant difference between the genotypes and variables analysed **(Table 22)**.

**Table 19. Association of HBV genotypes to lamivudine response and resistance**

|  | Genotype A (n=29) | Genotype C (n=33) | Genotype D (n=85) | p value |
|---|---|---|---|---|
| Response[*#] | 9 (35) | 9 (31) | 32 (41) | 0.633 |
| Non-response[*#] | 17 (65) | 20 (69) | 47 (59) | |
| Resistance[*] | 10 (34) | 7 (21) | 23 (27) | 0.503 |
| No resistance[*] | 19 (66) | 26 (79) | 62 (73) | |
| Treatment duration (months)[†] | 12 (6-24) | 12 (7-24) | 13 (10-24) | 0.806 |

[#]Virological response and non-response categorised for the total 134 subjects were only analysed (section 5.2.6.2)

Data are [†]median (IQR) or [*]number (%)

**Table 20. Characteristics of lamivudine-experienced subjects at last follow-up analysis according to HBV subgenotypes of genotype D**

|  | D1 (n=7) | D2 (n=55) | D3 (n=7) | D5 (n=15) | p value |
|---|---|---|---|---|---|
| Age,years[†] | 55 (50-58) | 39 (23-51) | 40 (28-46) | 40 (26-50) | 0.102 |
| Gender: male | 6 (75) | 41 (75) | 5 (83) | 12 (80) | 0.945 |
| ALT (U/L)[†] | 30 (23-51) | 28 (20-42) | 45 (32-72) | 32 (24-49) | 0.255 |
| AST (U/L)[†] | 37 (32-51) | 34 (23-49) | 42 (36-71) | 39 (26-44) | 0.507 |
| HBV DNA (log$_{10}$ IU/mL)[†] | 0 (0-4.65) | 3.9 (0-5.8) | 2.7 (0-5) | 5.8 (0.8-7) | 0.233 |
| HBeAg positive[*] | 4 (50) | 30 (55) | 4 (67) | 12 (80) | 0.307 |
| HBeAg negative[*] | 4 (50) | 25 (45) | 2 (33) | 3 (20) | |
| Anti-HBe positive[*] | 3 (37) | 25 (45) | 3 (50) | 3 (20) | 0.330 |
| Anti-HBe negative[*] | 5 (63) | 30 (55) | 3 (50) | 12 (80) | |
| Treatment duration (months)[†] | 11 (6-24) | 12 (6-20) | 12 (6-24) | 12 (6-19) | 0.956 |

Data are [†]median (IQR) or [*]number (%)

**Table 21. Association of HBV subgenotypes of genotype D to lamivudine response and resistance**

|  | D1 (n=7) | D2 (n=55) | D3 (n=7) | D5 (n=15) | p value |
|---|---|---|---|---|---|
| Response[*#] | 4 (57) | 20 (40) | 4 (57) | 4 (29) | 0.489 |
| Non-response[*#] | 3 (43) | 30 (60) | 3 (43) | 10 (71) | |
| Resistance[*] | 3 (43) | 15 (27) | 1 (14) | 4 (27) | 0.694 |
| No resistance[*] | 4 (57) | 40 (73) | 6 (86) | 11 (73) | |
| Treatment duration (months)[†] | 11 (6-24) | 12 (6-20) | 12 (6-24) | 12 (6-19) | 0.956 |

Values are n (%) and median (IQR)

Among the 85 genotype D subjects, the one subgenotype D for one subject could not be assigned with the surface gene sequences and was not included in the analysis.

[#]The virological response for 6 subjects (D2=5 and D5=1) could not be categorised and only 78 subjects were included for analysis.

Data are [†]median (IQR) or [*]number (%)

**Table 22. Characteristics of adefovir-experienced subjects at last follow-up analysis according to HBV genotypes**

| | Genotype A (n=7) | Genotype C (n=6) | Genotype D (n=17) | p value |
|---|---|---|---|---|
| Age[†] | 41 (34-56) | 40 (33-43) | 45 (33-57) | 0.693 |
| Gender: male[*] | 7 (100) | 6 (100) | 15 (88%) | 0.441 |
| ALT (U/L)[†] | 58 (43-78) | 51 (37-74) | 34 (25-49) | 0.103 |
| AST (U/L)[†] | 62 (47-80) | 47 (33-56) | 33 (27-47) | 0.194 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 3 (1.7-5.8) | 4.4 (2.5-7.7) | 4.6 (3.3-5.3) | 0.876 |
| HBeAg positive[*] | 5 (71) | 3 (50) | 11 (65) | 0.715 |
| HBeAg negative[*] | 2 (29) | 3 (50) | 6 (35) | |
| Anti-HBe positive[*] | 3 (43) | 2 (33) | 7 (41) | 0.930 |
| Anti-HBe negative[*] | 4 (57) | 4 (67) | 10 (59) | |
| Treatment duration (months)[†] | 12 (4-18) | 11 (10-18) | 12 (5-18) | 0.870 |

Data are [†]median (IQR) or [*]number (%)

### 5.6.5.2 HBV genotypes and adefovir response

Among the 21 subjects categorised for virological response (section 5.3.5.2), 2 subjects with genotype C and D showed virological response and 19 were non-responders. In the 25 subjects analysed for antiviral resistance mutations (section 5.3.5.2.1), two subjects with genotype C and D presented with rtA181V and rtI169L mutation. Since the numbers were few, statistical analysis for virological response and antiviral resistance for adefovir could not be performed.

### 5.6.5.3 HBV subgenotypes and adefovir response

Among 17 HBV genotype D subjects, subgenotypes D1, D2, D3 and D4 were identified in 2 (11.7), 9 (52.9%), 1 (5.9%) and 4 (23.5%) subjects respectively. The subgenotype for 1 (5.9%) subject could not be assigned. Since the numbers were few, statistical analysis for difference in variables between the subgenotypes was not separately performed.

### 5.6.6 HBV genotypes and entecavir response

### 5.6.6.1 Clinical characteristics of entecavir-experienced subjects at last follow-up analysis by HBV genotypes

In 50 entecavir-experienced subjects, HBV genotypes A, C and D were identified in 9 (18%), 12 (24%) and 29 (58%) subjects respectively. Univariate analysis was performed to identify factors that vary across the genotypes tested over the course of treatment. Genotypes D showed considerably lower ALT and AST levels as compared to genotype A and C but were not statistically significant **(Table 23)**.

### 5.6.6.2 HBV genotypes and entecavir response

The virological response was categorised for 45 subjects (section 5.4.5.2). Overall the response rate to genotype A, C and D were 78%, 78% and 89% respectively. There was no

**Table 23. Characteristics of entecavir-experienced subjects at last follow-up analysis according to HBV genotypes**

| | Genotype A (n=9) | Genotype C (n=12) | Genotype D (n=29) | p value |
|---|---|---|---|---|
| Age[†] | 42 (31-47) | 28 (19-33) | 39 (27-51) | 0.126 |
| Gender: male[*] | 9 (100) | 11 (92) | 26 (90) | 0.606 |
| ALT (U/L)[†] | 33 (23-36) | 40 (23-52) | 23 (22-31) | 0.265 |
| AST (U/L)[†] | 37 (27-53) | 36 (31-43) | 27 (22-43) | 0.142 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 0 (0-2.15) | 2.13 (0-4.27) | 1.82 (0-2.89) | 0.553 |
| HBeAg positive[*] | 6 (67) | 7 (58) | 14 (48) | 0.590 |
| HBeAg negative[*] | 3 (33) | 5 (42) | 15 (52) | |
| Anti-HBe positive[*] | 3 (33) | 5 (42) | 17 (59) | 0.334 |
| Anti-HBe negative[*] | 6 (67) | 7 (58) | 12 (41) | |
| Treatment duration (months)[†] | 9 (6-12) | 7 (6-10) | 6 (6-10) | 0.522 |

Data are [†]median (IQR) or [*]number (%)

significant difference in proportion of subjects who showed response and non-response (p=0.602; **Table 24**). On analysis of HBVrt mutations, only one genotype C subject was detected with the exclusive rtV173L mutation and there were no signature entecavir resistance mutations across the genotypes tested in this study.

### 5.6.6.3 HBV subgenotypes and entecavir response

When HBV subgenotype D subjects were separately analysed, none of the variables showed significant difference between the subgenotypes D1, D2, D3 and D5. The subgenotype for one genotype D subject could not be typed and was not included in the analysis **(Table 25)**. Univariate analysis was performed to identify if there is an association of subgenotypes and virological response. The analysis showed no subgenotype-dependent response or non-response among the subgenotypes tested (p=0.603; **Table 26**).

### 5.6.7 Genetic diversity and treatment response

In order to identify the genetic diversity between the HBV genotypes, mean genetic distance (*d*); the number of synonymous substitutions per synonymous site (*d*S) and the number of non-synonymous substitutions per non-synonymous site (*d*N) were studied in 197 treatment-naive subjects. There existed a significant difference between the 3 major HBV genotypes studied (p<0.0001). The *d*, *d*S and *d*N of genotype D was higher as compared to genotypes C and A respectively **(Table 27)**.

Further, to understand if there is a significant association between the baseline sequence and treatment response, baseline samples of subjects who subsequently showed response (n=79) and non-response (n=44) to the nucleos(t)ide analogues (lamivudine or adefovir or entecavir) were analysed. The *d*, *d*S and *d*N was always higher in responders as compared to non-responders irrespective of the genotypes tested (p=0.014 to p<0.0001; **Table 28**).

**Table 24. Association of HBV genotype and entecavir response**

| | Genotype A (n=9) | Genotype C (n=9) | Genotype D (n=27) | p value |
|---|---|---|---|---|
| Response[*] | 7 (78) | 7 (78) | 24 (89) | 0.602 |
| Non-response[*] | 2 (22) | 2 (22) | 3 (11) | |
| Treatment duration (months)[†] | 9 (6-12) | 7 (6-10) | 6 (6-10) | 0.522 |

Data are [†]median (IQR) or [*]number (%)

**Table 25. Characterization of entecavir-experienced subjects at last follow-up analysis according to HBV subgenotypes of genotype D**

| | D1 (n=6) | D2 (n=16) | D3 (n=4) | D5 (n=3) | p value |
|---|---|---|---|---|---|
| Age, years[†] | 32 (25-50) | 45 (28-55) | 34 (25-34) | 47 (39-54) | 0.325 |
| Gender: male[*] | 6 (75) | 41 (75) | 5 (83) | 12 (80) | 0.945 |
| ALT (U/L)[†] | 34 (30-60) | 22 (20-37) | 25 (18-29) | 26 (22-28) | 0.327 |
| AST (U/L)[†] | 40 (34-47) | 26 (21-43) | 24 (21-28) | 23 (23-57) | 0.399 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 0.95 (0-2.88) | 1.52 (0-2.76) | 1.28 (0-3.28) | 4.85 (1.79-5.3) | 0.358 |
| HBeAg positive[*] | 3 (50) | 7 (44) | 3 (75) | 1 (33) | 0.671 |
| HBeAg negative[*] | 3 (50) | 9 (56) | 1 (25) | 2 (67) | |
| Anti-HBe positive[*] | 3 (50) | 10 (63) | 1 (25) | 3 (100) | 0.234 |
| Anti-HBe negative[*] | 3 (50) | 6 (38) | 3 (75) | 0 | |
| Treatment duration (months)[†] | 6 (6-9) | 6 (6-12) | 8 (6-11) | 6 (3-6) | 0.533 |

Data are [†]median (IQR) or [*]number (%)

**Table 26. Association of HBV subgenotypes of genotype D and entecavir response**

| | D1 (n=6) | D2 (n=16) | D3 (n=4) | D5 (n=3) | p value |
|---|---|---|---|---|---|
| Response[*] | 6 (100) | 13 (87) | 3 (75) | 2 (100) | 0.603 |
| Non-response[*] | 0 | 2 (13) | 1 (25) | 0 | |
| Treatment duration (months)[†] | 6 (6-9) | 6 (6-12) | 8 (6-11) | 6 (3-6) | 0.533 |

Data are [†]median (IQR) or [*]number (%)

**Table 27. Genetic diversity of HBV genotypes in treatment-naive subjects**

| | Genotype A (n=32) | Genotype C (n=40) | Genotype D (n=125) | p value |
|---|---|---|---|---|
| $d$ ($10^{-2}$ substitution/site) | 1.262 (0.019) | 1.640 (0.038) | 2.213 (0.0156) | 0.0001 |
| $d$S ($10^{-2}$ substitution/site) | 2.988 (0.055) | 4.460 (0.119) | 6.049 (0.046) | 0.0001 |
| $d$N ($10^{-2}$ substitution/site) | 0.719 (0.129) | 0.641 (0.016) | 0.814 (0.006) | 0.0001 |

The table shows the mean genetic distance ($d$); the number of synonymous substitutions per synonymous site ($d$S); the number of non-synonymous substitutions per non-synonymous site ($d$N) of HBV genotypes A, C and D in treatment-naive subjects (n=197)

Values are mean (standard error in parenthesis)

**Table 28. Genetic diversity of HBV genotypes in treatment-naive subjects and subsequent response to nucleos(t)ide analogues**

| | Responders | Non-responders | p value |
|---|---|---|---|
| **Genotype A** | **(n=15)** | **(n=8)** | |
| $d$ ($10^{-2}$ substitution/site) | 2.24 (0.223) | 0.943 (0.494) | 0.0001 |
| $d$S ($10^{-2}$ substitution/site) | 5.406 (0.565) | 2.389 (0.174) | 0.0001 |
| $d$N ($10^{-2}$ substitution/site) | 1.277 (0.131) | 0 (0) | 0.0001 |
| **Genotype C** | **(n=14)** | **(n=9)** | |
| $d$ ($10^{-2}$ substitution/site) | 3.273 (0.252) | 1.728 (0.188) | 0.0001 |
| $d$S ($10^{-2}$ substitution/site) | 8.884 (0.632) | 4.453 (0.575) | 0.0001 |
| $d$N ($10^{-2}$ substitution/site) | 1.455 (0.144) | 0.819 (0.086) | 0.014 |
| **Genotype D** | **(n=50)** | **(n=27)** | |
| $d$ ($10^{-2}$ substitution/site) | 2.821 (0.040) | 2.196 (0.073) | 0.0001 |
| $d$S ($10^{-2}$ substitution/site) | 6.718 (0.108) | 5.941 (0.212) | 0.0001 |
| $d$N ($10^{-2}$ substitution/site) | 1.480 (0.225) | 0.978 (0.350) | 0.0001 |

The table shows the mean genetic distance ($d$); the number of synonymous substitutions per synonymous site ($d$S); the number of non-synonymous substitutions per non-synonymous site ($d$N) of baseline HBV genotypes A, C and D in responders and non-responders to nucleot(s)ide analogues

Values are mean (standard error in parenthesis)

nucleos(t)ide analogues- lamivudine, adefovir or entecavir

The difference in genetic diversity was also studied in lamivudine-experienced genotype D subjects who showed partial virological response with $\geq 1$ $\log_{10}$ reduction from baseline (n=8) and non-responders who failed to show such decline (n=6) at the early virological response measurement. On analysis, the *d*, *d*S and *d*N of responders were significantly higher than those of non-responders (p<0.0001; **Table 29**).

As the numbers of lamivudine responders and non-responders for genotypes A and C were limited, analysis was not performed for genotypes A and C. Likewise, there were no sufficient numbers to compare HBV genetic diversity and treatment response in adefovir and entecavir-experienced subjects and were not analysed.

**5.7 HBV subtypes**

A new programme for HBV subtype determination was developed using Microsoft Visual Basic (VB6). The schematic representation of the programme is illustrated in **Figure 35**. In the subjects studied, six of the nine HBV subtypes *adr*, *adw2*, *adw3*, *ayw1*, *ayw2* and *ayw3* were found to be circulating in the Indian subcontinent. Subtypes *ayw3*, *adr*, *adw2* and *ayw2* were the most common subtypes identified in 134 (54%), 66 (22%), 52 (18%) and 36 (12%) of the study subjects respectively. The remaining two subtypes *ayw1* and *adw3* were identified only in 2 (0.7%) and 1 (0.3%) subjects respectively. The subtypes could not be determined in 5 treatment-experienced (1.7%) subjects, as they presented with unusual amino acid substitutions at surface gene positions that are crucial for subtype determination. Representative samples of HBV subtypes and the corresponding amino acid positions used for identification are shown in **Figure 36**. Hepatitis B virus *ayw3* subtype-dependant amino acid substitution (sT125M) identified in subgenotype D5 subjects is also shown.

**Table 29. Differences in genetic diversity of HBV genotype D in lamivudine-experienced subjects at EVR measurement**

| Genotype D | Responders (n=8) | Non-responders (n=6) | p value |
|---|---|---|---|
| $d$ ($10^{-2}$ substitution/site) | 2.511 (0.279) | 0.746 (0.768) | 0.0001 |
| $d$S ($10^{-2}$ substitution/site) | 5.932 (0.727) | 1.187 (0.186) | 0.0001 |
| $d$N ($10^{-2}$ substitution/site) | 1.396 (0.143) | 0.58 (0.05) | 0.0001 |

The table shows the mean genetic distance ($d$); the number of synonymous substitutions per synonymous site ($d$S); the number of non-synonymous substitutions per non-synonymous site ($d$N) of HBV genotype D in lamivudine responders and non-responders at the early virological response (EVR) measurement at the median treatment duration 6 (IQR 6-8) months

Values are mean (standard error in parenthesis)

**Figure 35. Schematic representation of HBV subtyping tool**
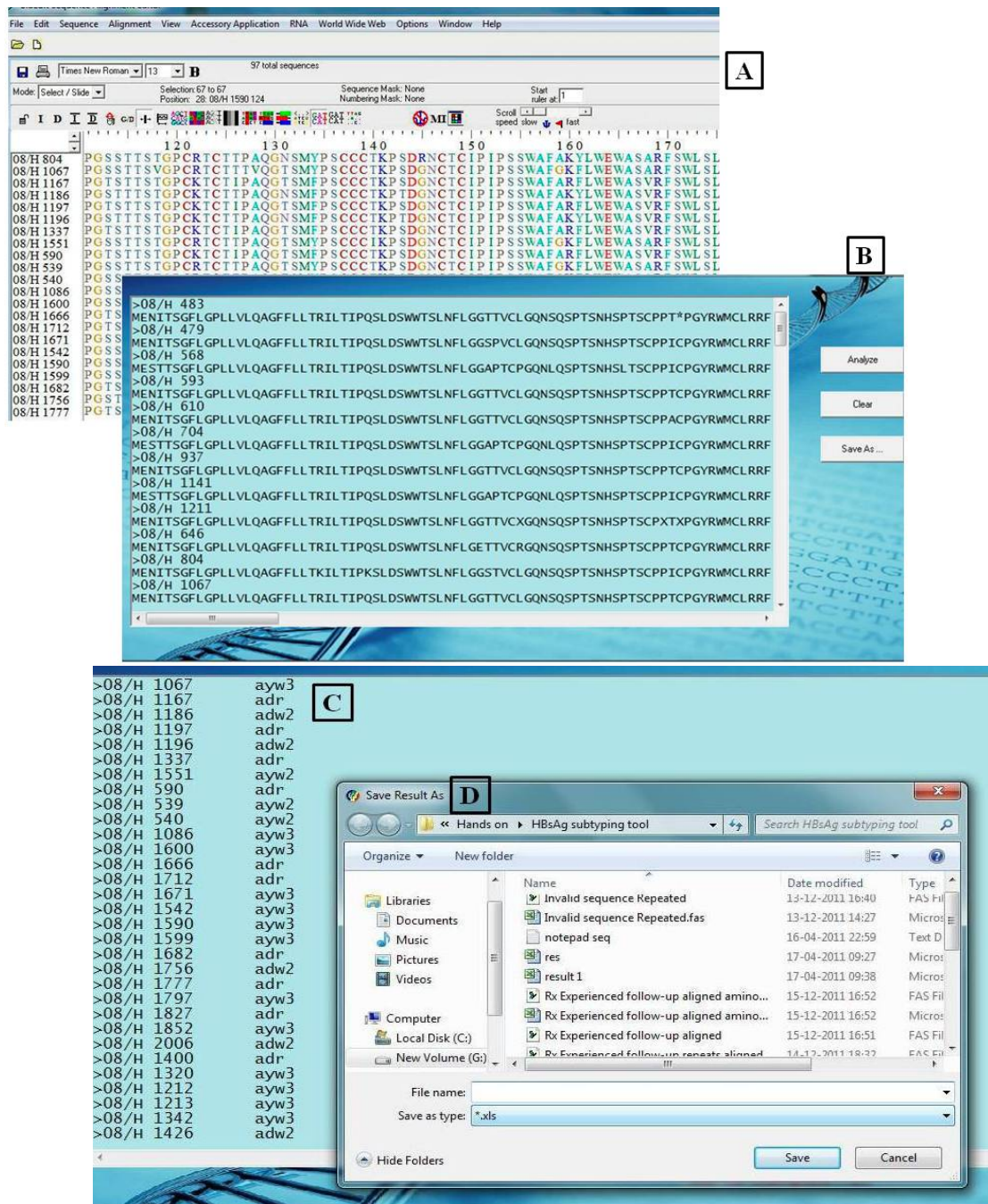


In the sequence alignment editor (A), HBV surface gene sequences (155-835 nucleotides of HBV genome) are translated to the corresponding amino acid sequences. The amino acid codons are then copied and pasted in the HBV subtyping programme (B). This programme examines every combination of amino acids at position 122,160,127,159 and 140 and the resulting HBV subtypes are displayed in the analysis window (C). The generated results are finally added to the excel data sheet (D).

**Figure 36. Representative HBV subtypes identified in this study**



Hepatitis B virus (HBV) surface gene codons showing representative samples of HBV subtype class identified in this study. Amino acid positions in the order 122, 160, 127, 159 and 140 are used for subtype determination. An illustration of ayw3 subtype-dependant amino acid substitution (sT125M) identified in HBV subgenotype D5 subjects is also shown.

### 5.7.1 HBV subtypes and genotype/subgenotype association

There existed a significant association between HBV genotypes and subtypes (P<0.0001). In the analysis performed, HBV subtype *adw2* and *adr* were always found to co-exist with genotype A and genotype C respectively. Likewise, subtype *ayw2* and *ayw3* were always presented in genotype D subjects. Subtype *adw3* was identified in one subject carrying subgenotype D2. Subtype *ayw1* was detected in one subject each with genotype A and newly identified genotype I infection **(Table 30)**.

### 5.7.2 Distribution of HBV genotypes, subgenotypes and subtypes

### 5.7.2.1 HBV genotype A and subtypes

In 53 subjects identified with HBV genotype A, 52 (98.1%) specifically carried subtype *adw2* and the remaining 1 (1.1%) subject presented with unusual *ayw1* subtype which is more common in South African countries. All these subjects were from eastern India (69.8%) and north-eastern region (30.2%).

### 5.7.2.2 HBV genotype C and subtypes

HBV genotype C was always associated with subtype *adr* and was identified predominantly in north-eastern region (63.6%), eastern India (30.3%) and with a low frequency in southern India (6.1%).

### 5.7.2.3 HBV genotype D and subtypes

One subject with *adw3* subtype and D2 subgenotype was from south India. Most of the subtype *ayw2* associated with genotype D were from eastern India (72.2%) with little distribution in north east (16.7%), south (8.3%) and western India (2.8%). The subtype *ayw3* was spread throughout the study population with the distribution of 56.1% in the south, 23.1% in the east, 20.1% in the north east and 0.7% in the central India. Interestingly, all the subjects with subtype *adw3* in the southern region had subgenotype D2.

**Table 30. Frequency and association of HBV subtypes and genotypes/subgenotypes**

| | A1 | C | | D | | | | I |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | C1 | C2 | D1 | D2 | D3 | D5 | |
| *adw2* | 52 (100) | | | | | | | |
| *adw3* | | | | | 1 (100) | | | |
| *adr* | | 58 (87.9)[*] | 6 (9.1)[*] | | | | | |
| *ayw1* | 1 (50) | | | | | | | 1 (50) |
| *ayw2* | | | | 19 (52.8)[#] | 1 (2.8)[#] | 15 (41.6)[#] | | |
| *ayw3* | | | | 1 (0.7) | 106 (79.1) | | 27 (20.2) | |

[*]The subgenotype C could not be assigned for 2 (3%) subjects presented with *adr* subtype

[#]The subgenotype D could not be assigned for 1 (2.8%) subject presented with *ayw2* subtype

HBV subtypes could not be assigned in 5 subjects, 4 (1.35%) in subgenotype D2 and 1 (0.34%) genotype C

The genotype I in one subject was identified to be subtype *ayw1*.

The overall distribution pattern of hepatitis B virus subgenotypes and subtypes identified in this study is depicted in **Figure 37.** In south India, the most predominant HBV subgenotype/subtype was D2/*ayw3* (79%). In eastern India, A1/*adw2* (32%) is the predominant subgenotype/subtype followed by C1/*adr* (16%), D2/*ayw3* (14%), D5/*ayw3* (12%), D3/*ayw2* (11%) and D1/*ayw2* (10%) in almost equal proportions. In north-eastern region, C1/*adr* (41%) is the predominant subtype and with a low frequency of D2/*ayw3* (18%), D5/*ayw3* (13%) and A1/*adw2* (10%). In addition, two subjects from western India had subgenotye/subtype D1/*ayw2* and D2/*ayw3* and one subject from Maldives Island, south-west India was identified with subgenotype/subtype D2/*ayw3*.

### 5.7.3 HBV subtypes in treatment-naive group

In 197 treatment-naive subjects studied, HBV subtypes *adr*, *adw2*, *adw3*, *ayw1*, *ayw2* and *ayw3* were identified in 40 (20.3%), 32 (16.2%), 1 (0.5%), 1 (0.5%), 28 (14.2%) and 95 (48.2%) subjects respectively. Baseline HBV DNA, HBeAg and anti-HBe status did not differ significantly between HBV subtypes but as earlier observed with genotypes, the ALT, AST levels and number of HBVrt amino acid substitution was significantly different among the subtypes tested **(Table 31)**. Serum ALT and AST levels were lower in *ayw2* and *ayw3* subtypes subjects as compared to *adr* and *adw2* subtypes, Subtype *ayw3* had higher number of HBVrt amino acid substitution as compared to other 3 major subtypes.

### 5.7.4 HBV subtypes in lamivudine-experienced group

### 5.7.4.1 Clinical characteristics of lamivudine-experienced subjects at last follow-up analysis according to HBV subtypes

In 147 lamivudine-experienced subjects, HBV subtype *adw2*, *adr*, *ayw2* and *ayw3* were identified in 31 (21.8%), 32 (22.5%), 15 (10.6%) and 64 (45.1%) subjects respectively.

**Figure 37. Region-wise distribution pattern of HBV subgenotypes/subtypes in Indian subcontinent subjects with chronic HBV**
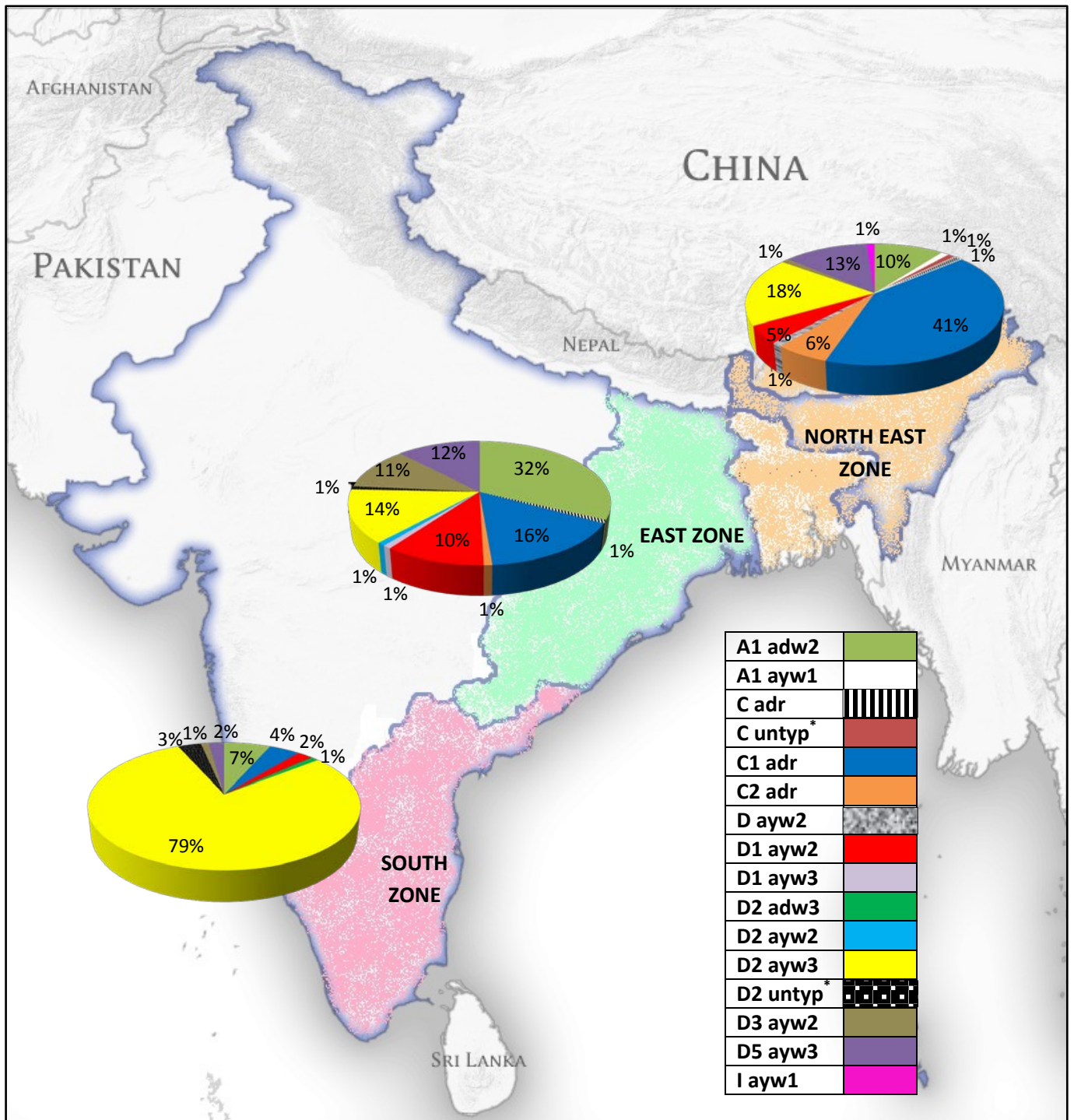


The subgenotype/subtype in two subjects from western India (D1/*ayw2* and D2/*ayw3*) and one subject from Maldives Island, south-west India (D2/*ayw3*) are not shown.

*untyp- untypeable

**Table 31. Baseline characteristics according to HBV subtypes**

| | *adw2* (n=32) | *adr* (n=40) | *ayw2* (n=28) | *ayw3* (n=95) | p value |
|---|---|---|---|---|---|
| ALT (U/L)[†] | 44 (30-131) | 57 (40-94) | 33 (25-49) | 40 (24-67) | 0.014 |
| AST (U/L)[†] | 55 (31-118) | 59 (35-98) | 36 (28-59) | 38 (26-68) | 0.017 |
| HBV DNA (log$_{10}$ IU/mL)[†] | 5.6 (4.24-7.15) | 5.15 (4.3-6.45) | 4.9 (3.6-6.9) | 6.0 (4.39-7.48) | 0.144 |
| HBeAg positive[*] | 22 (69) | 24 (60) | 17 (61) | 56 (58) | 0.655 |
| HBeAg negative[*] | 10 (31) | 16 (40) | 11 (39) | 40 (42) | |
| Anti-HBe positive[*] | 10 (31) | 14 (35) | 11 (39) | 39 (41) | 0.651 |
| Anti-HBe negative[*] | 22 (69) | 26 (65) | 17 (61) | 57 (59) | |
| HBVrt amino acid substitutions[†] | 4.5 (3-7) | 5 (4-6) | 4.5 (3-7) | 7 (5-10) | 0.0001 |

Data are [†]median (IQR) or [*]number (%)

Univariate analysis was performed to identify factors that vary across the subtypes tested over the course of treatment. Among the variables analysed only ALT and AST levels were significantly different between the subtypes (p=0.017 and p=0.01). Among the subtypes analyzed, there existed a decreasing pattern of ALT levels for subtypes *adr*, *adw2*, *ayw2* and *ayw3* with the median ALT levels of 40 (IQR 32-62), 35 (IQR 29-76) 32 (IQR 24-57) and 28 (20-46) U/L respectively (*adr>adw2>ayw2>ayw3*). The median AST levels for the corresponding subtypes were 47 (IQR 36-69), 39 (30-103), 41 (32-52), 34 (23-46) U/L respectively **(Table 32)**. Both the *ayw2* and *ayw3* subtypes showed reduced ALT and AST levels in comparison to other two subtypes. However, it should be noted that the ALT and AST levels of these subjects were significantly lower in the total 196 treatment-naive subjects studied (p=0.014 and p=0.017; shown in Table 31). Also, on separate analysis of 87 of 90 treatment-naive subjects (one *adw3* subtype and three untypeable samples were excluded) who were subsequently followed-up with lamivudine treatment, the ALT and AST levels were lower but not significantly different (p=0.081 and p=0.110) in *ayw2* and *ayw3* subtypes. Therefore, the significant difference observed at the last follow-up analysis illustrates that the lower ALT and AST levels are maintained over the course of therapy.

**5.7.4.2 HBV subtypes and lamivudine response**

Overall the response rate to subtype *adw2*, *adr*, *ayw2* and *ayw3* were 32%, 32%, 53% and 39% respectively. There was no significant difference in proportion of subjects who showed response and non-response to lamivudine (p=0.505). Similarly, 39%, 19%, 27% and 16% of the respective subtypes developed lamivudine resistance and there was no significant difference between the genotypes tested. (p=0.503). There was no significant difference in treatment duration between the subtypes and this excludes the chance of bias in the analysis performed (p=0.869; **Table 33**).

**Table 32. Characteristics of lamivudine-experienced subjects at last follow-up analysis according to HBV subtypes**

| | *adr* (n=32) | *adw2* (n=31) | *ayw2* (n=15) | *ayw3* (n=64) | p value |
|---|---|---|---|---|---|
| Age, years[†] | 34 (27-46) | 39 (25-50) | 46 (39-57) | 39 (24-50) | 0.133 |
| Gender: male[*] | 26 (81%) | 30 (97%) | 11 (73%) | 47 (73%) | 0.053 |
| ALT (U/L)[†] | 40 (32-62) | 35 (29-76) | 32 (24-57) | 28 (20-46) | 0.017 |
| AST (U/L)[†] | 47 (36-69) | 39 (30-103) | 41 (32-52) | 34 (23-46) | 0.010 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 3.48 (0.5-5.72) | 4 (2.52-5.7) | 1.86 (0-4.95) | 4.3 (0-6.15) | 0.317 |
| HBeAg positive[*] | 23 (72) | 20 (65) | 8 (53) | 40 (63) | 0.644 |
| HBeAg negative[*] | 9 (28) | 11 (35) | 7 (47) | 24 (37) | |
| Anti-HBe positive[*] | 12 (37) | 9 (29) | 6 (40) | 24 (38) | 0.837 |
| Anti-HBe negative[*] | 20 (63) | 22 (71) | 9 (60) | 40 (62) | |
| Treatment duration (months)[†] | 14 (6-24) | 12 (7-26) | 24 (10-24) | 13 (9-21) | 0.869 |

HBV subtypes arranged in the decreasing pattern of ALT levels

Data are [†]median (IQR) or [*]number (%)

**Table 33. Association of HBV subtypes to lamivudine response and resistance**

| | *adw2* (n=31) | *adr* (n=32) | *ayw2* (n=15) | *ayw3* (n=64) | p value |
|---|---|---|---|---|---|
| Response[*#] | 9 (32) | 9 (32) | 8 (53) | 23 (39) | 0.505 |
| Non-response[*#] | 19 (68) | 19 (68) | 7 (47) | 36 (61) | |
| Resistance[*] | 12 (39) | 6 (19) | 4 (27) | 16 (25) | 0.333 |
| No resistance[*] | 19 (61) | 26 (81) | 11 (73) | 48 (75) | |
| Treatment duration (months)[†] | 14 (6-24) | 12 (7-26) | 24 (10-24) | 13 (9-21) | 0.869 |

HBV subtype for 4 untypeable subjects and subtype *ayw1* identified in one subject were not included for analysis.

[#]The virological response and non-response categorised for the total 130 subjects were only included

Data are [†]median (IQR) or [*]number (%)

### 5.7.5 HBV subtypes in adefovir-experienced group

### 5.7.5.1 Clinical characteristics of adefovir-experienced subjects at last follow-up analysis by HBV subtypes

In 30 adefovir-experienced subjects, HBV subtype *adw2*, *adr*, *ayw1*, *ayw2* and *ayw3* were identified in 6 (20%), 7 (23.3%), 1 (3.3%), 3 (10%) and 12 (40%) subjects respectively. HBV subtype for 1 (3.3%) subject could not be assigned. Univariate analysis was performed for subtypes *adw2*, *adr*, *ayw2* and *ayw3* to identify factors that vary across the subtypes over the course of adefovir treatment **(Table 34)**. None of the factors showed significant association to specific subtypes analysed. However, there was a decreasing pattern of ALT levels in the order *adr>adw2>ayw2>ayw3* with the median ALT levels of 60 (IQR 37-74), 52 (IQR 43-67) 35 (IQR 28-49) and 32 (22-47) U/L respectively (p=0.212). The median AST levels for the corresponding subtypes were 47 (IQR 36-69), 39 (30-103), 41 (32-52), 34 (23-46) U/L respectively.

### 5.7.5.2 HBV subtypes and adefovir response

Among the 21 subjects categorised for virological response (section 5.3.5.2), 2 subjects with subtypes *adr* and *ayw3* showed response and the remaining 19 were classified as non-responders. In the 25 subjects analysed for antiviral resistance mutations (section 5.3.5.2.1), one subject of subtype *adr* presented with rtA181V mutation and the subtype is not known for one subject who presented with rtI169L mutation. Since the numbers were few, the association of adefovir response and antiviral resistance mutations between the subtypes could not be performed.

### 5.7.6 HBV subtypes in entecavir-experienced subjects

### 5.7.6.1 Clinical characteristics of entecavir-experienced subjects at last follow-up analysis by HBV subtypes

**Table 34. Characteristics of adefovir-experienced subjects at last follow-up analysis according to HBV subtypes**

| | adr (n=7) | adw2 (n=6) | ayw2 (n=3) | ayw3 (n=12) | p value |
|---|---|---|---|---|---|
| Age, years[†] | 41 (33-43) | 47 (34-56) | 45 (18-57) | 51 (30-60) | 0.766 |
| Gender: male[*] | 7 (100) | 6 (100) | 3 (100%) | 10 (83) | 0.412 |
| [*]ALT (U/L)[†] | 60 (37-74) | 52 (43-67) | 35 (28-49) | 32 (22-47) | 0.212 |
| AST (U/L)[†] | 48 (33-61 | 55 (47-64) | 34 (27-39) | 29 (25-72) | 0.245 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 3.95 (2.48-7.77) | 4.39 (1.73-5.77) | 4.78 (0.9-6.77) | 4.53 (3.15-5.39) | 0.999 |
| HBeAg positive[*] | 4 (57) | 4 (67) | 1 (33) | 9 (75) | 0.568 |
| HBeAg negative[*] | 3 (43) | 2 (33) | 2 (67) | 3 (25) | |
| Anti-HBe positive[*] | 2 (29) | 3 (50) | 2 (67) | 4 (33) | 0.627 |
| Anti-HBe negative[*] | 5 (71) | 3 (50) | 1 (33) | 8 (67) | |
| Treatment duration[†] | 10 (4-18) | 10 (4-12) | 16 (11-30) | 16 (5-22) | 0.544 |
| HBVrt amino acid substitutions[†] | 3 (0-3) | 0 (0-2) | 0 (0-1) | 5 (4-6) | 0.001 |

HBV subtypes arranged in the decreasing pattern of ALT levels

Data are [†]median (IQR) or [*]number (%)

In 50 entecavir-experienced subjects, HBV subtype *adw2*, *adr*, *ayw2* and *ayw3* were identified in 12 (24%), 10 (20%), 9 (18%) and 19 (38%) subjects respectively. Univariate analysis was performed to identify factors that vary across the subtypes tested over the course of treatment **(Table 35)**. None of the variables analysed showed significant difference between the subtypes analysed. Among the subtypes there was a decreasing pattern of ALT levels in the order *adr>adw2>ayw2>ayw3* with the median ALT levels of 40 (IQR 23-52), 34 (IQR 23-41) 29 (IQR 21-30) and 23 (22-36) U/L respectively, but was not statistically significant.

### 5.7.6.2 HBV subtypes and entecavir response

Overall the response rate to subtype *adw2*, *adr*, *ayw2* and *ayw3* were 80%, 78%, 89% and 88% respectively. There was no significant difference in proportion of subjects who showed response and non-response. On antiviral resistance analysis, only one subject of subtype *adr* carried rtV173L mutation exclusively. There was no significant difference in treatment duration between the genotypes and this excludes the chance of bias in the analysis performed **(Table 36)**.

### 5.7.7 Genotype, subgenotype and subtype dependant novel HBVrt amino acid substitutions

On analysis of HBVrt sequences, additional amino acid substitutions to the consensus sequence of Stanford database were identified and were considered as novel amino acid substitutions. Among the substitutions identified, some were specifically associated with certain HBV genotypes or subgenotypes and/or subtypes tested. HBVrt amino acid substitutions rtD7A/T, rtY126H, rtM129L, rtV163I, rtR217L, rtI253V and rtN122H were only identified in HBV subgenotype/subtype, A1/*adw2*. Hepatitis B virus C1 subgenotype specifically had rtH9Y, rtH55Q substitutions and C2 subgenotype had a rtL199V

**Table 35. Characteristics of entecavir-experienced subjects at last follow-up analysis according to HBV subtypes**

| | *adr* (n=12) | *adw2* (n=10) | *ayw2* (n=9) | *ayw3* (n=19) | p value |
|---|---|---|---|---|---|
| Age, years[†] | 28 (19-33) | 39 (31-47) | 33 (25-34) | 47 (30-57) | 0.055 |
| Gender: male[*] | 11 (92) | 10 (100) | 9 (100) | 16 (84) | 0.359 |
| ALT (U/L)[†] | 40 (23-52) | 34 (23-41) | 29 (21-30) | 23 (22-36) | 0.212 |
| AST (U/L)[†] | 36 (31-43) | 40 (27-53) | 25 (22-36) | 27 (21-54) | 0.169 |
| HBV DNA ($\log_{10}$ IU/mL)[†] | 2.13 (0-4.27) | 0.82 (0-2.89) | 0 (0.-1.89) | 2.47 (0-3) | 0.373 |
| HBeAg positive[*] | 7 (58) | 7 (70) | 5 (56) | 8 (42) | 0.530 |
| HBeAg negative[*] | 5 (42) | 3 (30) | 4 (44) | 11 (58) | |
| Anti-HBe positive[*] | 5 (42) | 3 (30) | 4 (44) | 13 (68) | 0.202 |
| Anti-HBe negative[*] | 7 (58) | 7 (70) | 5 (56) | 6 (32) | |
| Treatment duration (months)[†] | 7 (6-10) | 8 (6-12) | 6 (6-10) | 6 (6-12) | 0.844 |

HBV subtypes are arranged in the decreasing pattern of ALT levels

Data are [†]median (IQR) or [*]number (%)

**Table 36. Association of HBV subtypes and entecavir response**

| | *adr* (n=9) | *adw2* (n=10) | *ayw2* (n=9) | *ayw3* (n=17) | p value |
|---|---|---|---|---|---|
| Response[*] | 7 (78) | 8 (80) | 8 (89) | 15 (88) | 0.855 |
| Non-response[*] | 2 (22) | 2 (20) | 1 (11) | 2 (12) | |
| Treatment duration (months)[†] | 7 (6-10) | 8 (6-12) | 6 (6-10) | 6 (6-12) | 0.844 |

Data are [†]median (IQR) or [*]number (%)

substitution. In genotype D, rtA/P/S/T54H and rtN123D was commonly identified in all the subgenotypes tested. Additionally, there were certain substitutions that are specific to subgenotypes D2, D3 and D5. The subgenotypes D2 and D5 were found to carry rtA7T substitution. Amino acid substitution rtH248N was mostly detected in subgenotypes D1, D2 and D3. Additionally, rtF122I/L/V was seen in subgenotypes D2, D3 and D5. RtY257W was specifically detected in subgenotype D5. There were also substitutions that are subtype-dependent. RtN/I/S/T53D was specifically detected in *ayw3* subtype of D2 subgenotype. Interestingly, all *ayw3* subtype in subgenotype D2 and the only one subject identified with D1/*ayw3* had rtH/Y126R substitution.  The amino acid substitution rtS135Y was specifically detected in *ayw3* subtype. The frequency of these novel amino acid substitutions is listed in **Table 37**.

There were no other additional genotype or subgenotype or subtype markers identified in the treatment-experienced subjects. None of these genotype specific amino acid substitutions were identified in the unassigned subgenotype or subtypes.

**Table 37. Genotype, subgenotype and subtype specific HBVrt amino acid substitutions**

| HBVrt amino acid substitutions | Frequency in % | HBVrt amino acid substitutions | Frequency in % |
|---|---|---|---|
| **A1/*adw2* (n=31)** | | **D (n=125)** | |
| D7A/T | 48 | 54H | 75 |
| Y126H | 29 | N123D | 14 |
| M129L | 100 | **D2/*ayw3* (n=80)** | |
| V163I | 32 | 53D | 89 |
| R217L | 32 | **D1/D2/*ayw3* (n=81)** | |
| I253V | 29 | H/Y126R | 100 |
| N122H | 32 | **D1/D2/D3 (n=109)** | |
| **C1/*adr* (n=38)** | | H248N | 40 |
| H9Y | 89 | **D2/D5/*ayw3* (n=96)** | |
| H55Q | 13 | A7T | 22 |
| **C2/*adr* (n=2)** | | **D2/D3/D5 (n=107)** | |
| L199V | 100 | F122L/I/V | 16 |
| ***ayw3* (n=108)** | | **D5 (n=16)** | |
| S135Y | 85 | Y257W | 75 |

# 6. DISCUSSION

## 6. DISCUSSION

Antiviral resistance is a major challenge to the treatment currently available for hepatitis B virus (HBV). In this study we have characterized the antiviral efficacy and profile of antiviral resistance mutations for three drugs; lamivudine, adefovir and entecavir which are widely used for the treatment of chronic hepatitis B in the Indian subcontinent.

### 6.1 Antiviral resistance mutations in treatment-naive group

Antiviral drugs for HBV have been available for more than a decade with reports of emerging antiviral resistance [18, 61, 132, 146, 239]. The transmission of antiviral resistant mutants from treatment failure subjects is therefore possible. The presence of pre-existing antiviral resistance mutations may affect subsequent response to treatment. This concept is very well documented in HIV studies [240-242].

Though there are few reports of naturally occurring HBV variants with primary resistance to antiviral drugs, its impact on treatment response is not well documented [121, 122, 243-245]. In order to identify the presence of pre-existing HBV antiviral resistance mutations in our population, we studied a total of 197 treatment-naive subjects who have never been exposed to any HBV antivirals.

In these treatment-naive study subjects, we also analysed the biochemical and virological parameters. As it is widely known, there was a good correlation between serum ALT and AST levels (r=0.77; p<0.0001). Also HBV DNA levels paralleled HBeAg status (p<0.0001). Elevated serum ALT and AST levels (>2ULN) were observed in 26% and 21% of subjects, although most presented with high HBV DNA levels. This suggests that most of the subjects infected with HBV are asymptomatic in spite of having high HBV DNA levels. Therefore, as

suggested by Zoulim *et al.* [159] active screening of HBV is of crucial importance to prevent the complications of HBV related disease.

In our analysis of antiviral resistance mutations, no known "hot-spot" mutations that can independently affect the antiviral susceptibility to any of the stated drugs were seen. However, other additional antiviral resistance related amino acid substitutions were identified and were classified as putative, atypical, compensatory, novel amino acid substitutions and naturally occurring polymorphisms. Their role is still being investigated [243, 246].

Schildgen *et al.* [247] showed adefovir failure in three cases with pre-existing rtI233V mutation and pointed that the naturally occurring HBV variant is primarily resistant to adefovir. Further, they also reported selection of rtI233V mutation in two cases during the course of adefovir therapy and concluded that rtI233V mutation confers resistance to adefovir [248]. On contrary, Curtis *et al*. [249] showed four cases of HBV with pre-existing rtI233V mutation who subsequently responded to adefovir. It has been documented that rtI233V mutation occurs in approximately 2% of treatment-naive chronic hepatitis B virus carriers [250]. Similarly, adefovir related rtI233V putative mutation was identified in 2.5% of treatment-naive subjects in our study. The effect of rtI233V substitution in these subjects could not be followed-up with adefovir therapy. However, two of these study subjects with pre-existing rtI233V substitution showed early virological response (EVR) to entecavir and end-of-treatment response (ETR) to lamivudine subsequently.

Ciancio *et al.* [246] showed that the naturally occurring rtI91L polymorphism respond poorly to lamivudine. In contrast, Yuen *et al.* [251] showed no difference in lamivudine response in subjects presented with rtI91L polymorphism. This polymorphism was identified in 16 (8%) treatment-naive subjects in our study. On follow-up analysis of 4 subjects carrying this polymorphism, 3 failed to respond to lamivudine. Similarly, the rtL217R polymorphism was

speculated to show poor adefovir response [252]. This polymorphism was identified in 4 (2%) treatment-naïve subjects in our study. None of these subjects had subsequent follow-up sample and the impact of rtL217R polymorphism could not be studied.

Among the subjects with pre-existing putative, atypical, compensatory amino acid substitutions or naturally occurring polymorphisms, 78% responded (EVR or ETR) to lamivudine and entecavir subsequently.

In addition, we have identified some novel HBVrt amino acid substitutions in 20 treatment-naive subjects. Among these subjects, 70% responded to lamivudine and entecavir subsequently and the remaining were non-responders. However, the potential role of these novel amino acid substitutions could not be demonstrated as all these novel amino acid substitutions were not frequently distributed among the study subjects. The rtL175R/V/G substitutions were identified in 4 subjects and all four responded (EVR or ETR) to entecavir subsequently **(Table 2)**.

We observed a significant positive relationship between the age of subjects and the number of HBVrt amino acid substitutions (r=0.31, p<0.0001). As proposed by Solomone *et al*. [253] long term carriage of HBV could be the reason for the accumulation of substitutions in older age groups.

We have employed population-based sequencing for detecting drug resistance mutations. A major limitation of this method is its lower sensitivity in the detection of minority variants that are below 20% in the heterogeneous viral population [254]. This might have led to the underestimation of pre-existing amino acid substitutions in our study subjects.

Previously, Solmone *et al*. [253] showed the pre-existence of rtM204I and rtV214A antiviral-resistance related amino acid substitutions in 2 of the 5 treatment-naive cases. Apparently,

these substitutions (minority variants) were detected by ultra deep pyrosequencng and not by direct sequencing and INNO-LiPA assay. Fang *et al*. [255] developed a sensitive real-time allele-specific PCR assay for the detection of minority variants and showed the pre-existence of resistance variants that were not detected by population sequencing. However, both these studies did not show the impact of these pre-existing mutations and subsequent treatment response. Recently, Lee *et al.* [256] identified pre-existence of lamivudine resistance rtL180M and rtM204V/I mutations in four cases using a sensitive dual-priming oligonucleotide primers and all four cases with these pre-existing resistance mutations responded to subsequent lamivudine treatment. The clinical impact of pre-existing minority variants and treatment failure is well described for HIV [241, 257-259]. This warrants the need to extend this approach for HBV studies and understand its clinical significance.

Among 197 treatment-naive subjects studied, the results obtained in 97 subjects have been published [217].

## 6.2 Lamivudine-experienced group

The subjects referred to the Department of Clinical Virology for routine HBV DNA quantification were recruited for this study. Sequential follow-up of subjects who started on antiviral therapy was dependant on their subsequent visit for HBV DNA quantification. With these constraints, we attempted to perform this study assigning the subjects to any one or more of the end-points recommended for therapeutic monitoring.

Virological response and antiviral resistance development were the two major outcomes determined in this study. In the samples analysed, baseline variables were used to identify the predictive factors of response and on-treatment variables were used to identify factors associated with response.

High serum ALT levels and low HBV DNA levels at baseline were previously reported to predict lamivudine response [15, 152, 153]. In our analysis of baseline variables, AST is the only factor that showed significant association for lamivudine response (p=0.037). The baseline AST level was significantly higher in subjects who subsequently responded to lamivudine than in non-responders. In the natural course of HBV infection, the immune clearance phase is characterized by elevated serum aminotransferases. In order to eradicate the virus, the immune system acts on the target hepatocytes and the presence of elevated liver enzymes in serum indicates lysis of infected cells. Therefore, the measure of serum aminotransferases is an indirect measure of hepatocellular damage much of which is mediated by the immune system. The higher baseline AST levels in subjects who subsequently responded to lamivudine indicated a heightened immune response. Therefore, high immune responses together with the antiviral action of lamivudine might have led to the better clinical outcome. This was also postulated in a study with similar findings [260]. Though ALT levels were not significantly different (p=0.092), the baseline ALT levels were higher in responders as compared to non-responders **(Table 3)**.

In our study we did not observe a significant association between baseline HBV DNA levels and subsequent lamivudine response. A previous study by Yuen *et al*. [260] also showed similar observation. Their study suggested that lamivudine response is not solely dependent on the baseline HBV DNA levels as subjects with high baseline HBV DNA levels still showed good response to lamivudine subsequently. This study illustrated that on-treatment reduction of HBV DNA levels is a better predictor of lamivudine response than baseline HBV DNA levels. Another study by Perrillo *et al.* [261] showed less evidence for baseline HBV DNA (p=0.07) as a predictive factor of HBeAg loss for lamivudine treatment when compared to ALT (p<0.001) and HAI score (p<0.001). These studies corroborate the findings of our results.

Earlier studies have reported that male gender, older age, lower baseline ALT levels, HBeAg-positivity and higher baseline HBV DNA to be the predictors of lamivudine resistance [23, 154, 155, 157-159]. In our analysis, none of these baseline parameters showed significant association with lamivudine resistance which might be due to limited numbers (No antiviral resistance, n=74 vs. antiviral resistance, n=16; **Table 4**).

In our study, EVR measurement at the median treatment duration of 6 months showed complete virological response in 24 (31%) subjects and partial virological response in 26 (34%) subjects. Interestingly, 2 subjects who showed partial virological response were detected with lamivudine resistance mutations after 6 and 9 months of treatment. Thus we show that reduction in viral load does not exclude the presence of resistance mutations. In our findings, the cumulative proportion of lamivudine resistance at the median treatment duration of 6 months was 9%. This estimated incidence of lamivudine resistance is comparably higher to that of earlier reports, which showed 5% to 6.7% at 6 months of lamivudine treatment [251, 260]. In an earlier study from India, none of the subjects treated for less than 9 months of lamivudine showed resistance mutations [43]. Our finding thus alerts the need for early monitoring of resistance mutations and clinical decision making.

Our analysis of EVR measurement for the prediction of subsequent response showed 50% of subjects with complete virological response at 6 months of lamivudine treatment to have subsequent response (undetectable HBV DNA) at 17 months versus 18% and 0% of subjects showing partial virological response and non-response respectively. Though there is no statistical significance (p=0.074), this statistical trend may suggest that EVR measurement can be a good predictor of subsequent virological response to lamivudine. This finding confirms the previously described association of greater reduction of HBV DNA levels after

6 months of lamivudine treatment and a better subsequent response to lamivudine including lower number of resistance mutations [31, 154, 262].

During ETR measurement with the median treatment duration of 12 months, 24 (38%) subjects responded to lamivudine and 44 (62%) were non-responders. Univariate analysis showed younger age, male gender, high ALT and AST levels, HBeAg-positivity and anti-HBe negative status to be associated with lamivudine failure **(Table 6)**. As the sample size was small, multivariate analysis could not be performed. Previous studies have suggested that younger subjects have higher sustained anti-HBe response and sustained virological response to lamivudine therapy [200, 263, 264]. This data contradicts our findings. However, considering that it relates to the age of sampling and not the age of infection, may be these subjects had an early age of infection and in which case the disease severity is higher [79]. Moreover, such significance was not observed in the subsequent analysis of lamivudine-experienced subjects.

In our study, the cumulative proportion of lamivudine resistance after median treatment duration of 12 months was 22%. A previous report from India has documented 6% prevalence of lamivudine resistance in 6 months; one subject showed rtM204V/I mutation among a total of 17 subjects [43]. The limited numbers in the published study might have contributed to the differences across both studies. Moreover, our findings largely agree with the results of other studies which have documented a prevalence of 22% and 23% of resistance mutations at the end of 12 months treatment [23, 24].

In our analysis, extended lamivudine treatment for a median treatment duration of 24 months showed higher number of subjects with normal ALT (67%) and AST (61%) levels when compared to EVR (58% each) and ETR (51% and 48%) measurements. However, only 22% maintained virological response with undetectable HBV DNA. Subsequent analysis of

maintained response measurement with the median treatment duration of 41 months was limited by small number of subjects. In our analysis, the cumulative proportion of lamivudine resistance after the median treatment duration of 24 and 41 months was found to be 44% and 71% respectively. This reported prevalence of lamivudine resistance is in accordance with earlier reports [22, 24].

All of the four subjects studied at the sustained virological response (SVR) measurement showed non-response and one of these subjects was identified with lamivudine resistance mutations.

In the total 147 lamivudine-experienced subjects, 50 (34%) responded to lamivudine after the median treatment duration of 13 (IQR 8-24) months and 84 (57%) were non-responders. The remaining 13 (9%) subjects continued to be positive for HBV DNA in ≤9 months of treatment and the virological response could not be categorised as baseline HBV DNA was not available. We identified typical lamivudine resistance mutations in 40 (27%) subjects. The primary rtM204V/I mutation was exclusively detected in 9 (22.5%) subjects. The rtL180M and rtM204V combination were the predominantly identified mutations (30%) followed by rtL80I and rtM204I combination (22.5%). The rtM204V/I mutation was also detected with rtL80V, rtV173L, rtL180M, rtA181V or rtM250L compensatory mutations. Especially, the rtV173L mutation was identified in 2 subjects with longer treatment duration of 72 months. The mutation patterns identified in this study largely agree with previous studies, reviewed by Bartholomeusz and Locarnini [19]. We also identified some additional HBVrt mutations, but none of these mutations showed specific association with lamivudine failure.

We analysed variables that was associated with virological response in the last follow-up of these 147 lamivudine-experienced subjects with the median treatment duration of 13 (IQR 8-

24) months. Initially, univariate analysis showed male gender, high ALT levels, high AST levels, HBeAg-positive and anti-HBe-negative status to be significantly associated with non-response **(Table 8)**. When included into a multivariate model, only HBeAg showed significant association with lamivudine response. In our study 20% of HBeAg-positive subjects responded to lamivudine when compared to 66% in HBeAg-negative subjects. This reiterates the findings of previous reports, where loss of HBV DNA is documented in 36-44% of HBeAg-positive subjects [21, 94, 124-126] when compared to higher rates of about 60-73% in HBeAg-negative subjects at the end of 24 months treatment [127-129].

Likewise, on analysing the factors associated with lamivudine resistance, the frequency of antiviral resistance mutations did not differ significantly with age, gender, ALT and AST levels, but was significantly lower in subjects with low HBV DNA levels compared to high HBV DNA levels, those with shorter treatment duration when compared to longer treatment duration, HBeAg-negative and/or anti-HBe-positive subjects than HBeAg-positive and anti-HBe negative subjects **(Table 10)**. Further, multivariate analysis showed the risk of antiviral resistance increased with HBV DNA levels and treatment duration **(Table 11)**. Subjects with HBV DNA levels >4 $\log_{10}$ IU/mL after the median treatment duration of 13 months had 5.9 fold increased chance to develop lamivudine resistance than subjects with HBV DNA levels $\leq 4$ $\log_{10}$ IU/mL. Therefore, we show that high HBV DNA levels and increased treatment duration is strongly associated with lamivudine resistance.

This is a very first report in the Indian subcontinent that have characterised the antiviral efficacy and showed the wide patterns of lamivudine resistance mutations over varying treatment duration.

### 6.3 Adefovir-experienced group

Adefovir is another oral drug shown to be effective against both wild and lamivudine resistant HBV. Since adefovir is sparingly used in our center, we could study its efficacy only in 30 subjects. Among these subjects, 18 had EVR measurement and 3 (17%) subjects showed complete virological response. In a pilot study in India, 4 (26.7%) out of 15 subjects showed undetectable HBV DNA (<12 IU/mL) after 6 months of adefovir. However, the numbers were too small for reliable comparison [213].

On sequence analysis, the well described rtN236T and rtA181T/V mutation was not identified in any of the subjects. Instead, rtI169L mutation was identified in one subject. This HBVrt amino acid position is well described to confer resistance to entecavir with replacement of threonine for isoleucine (rtI169T) [28, 265]. The subject with rtI169L mutation had no history of entecavir treatment. Recently, Li *et al*. [245] identified this rtI169L mutation in one subject with adefovir monotherapy. This report supports our finding and illustrates rtI169L mutation to be associated with adefovir resistance. In our analysis, the cumulative proportion of adefovir resistance was 6% after the median treatment duration of 5 months.

During ETR measurement, only 2 (12.5%) subjects showed virological response and the remaining 14 (87.5%) were non-responders. On sequence analysis, one subject was identified with typical rtA181V adefovir resistance mutation. The cumulative proportion of adefovir resistance was 7% after the median treatment duration of 12 months. Two reports from Taiwan and International multicenter study have documented no incidence of adefovir resistance after 12 months of adefovir treatment [266, 267].

On analysis of eight subjects with extended adefovir therapy for up to 24 months, only one subject maintained response. Also one subject who failed to show ETR initially showed

delayed response. One subject who showed complete virological response was subsequently identified with an episode of virological breakthrough. However, no adefovir resistance mutations were identified among all 6 non-responders including the subject who had virological breakthrough.

High HBV DNA levels during 6 and 12 months of therapy was shown to predict adefovir resistance [267]. A landmark study showed that 49% of subjects with serum HBV DNA >200 IU/mL (>2.3 $\log_{10}$ IU/mL) at 12 months will develop resistance when compared to 6% of subjects with <200 IU/mL (<2.3 $\log_{10}$ IU/mL) after 4 years of adefovir [27]. The median HBV DNA levels in our study subjects during 6 and 12 months was 3.82 (IQR 2.84-4.73) and 4.24 (IQR 2.84-6.2) $\log_{10}$ IU/mL respectively. This indicates that most of our study subjects had an increased risk to develop resistance. Due to loss of follow-up the subsequent response and resistance rates could not be analysed.

Among 30 subjects studied, only 2 (7%) responded to adefovir after the median treatment duration of 12 (IQR 6-18) months and 19 (63%) were non-responders. The remaining 9 (30%) subjects continued to be positive for HBV DNA in ≤9 months of treatment and the virological response could not be categorised as baseline HBV DNA was not available. Two (7%) subjects developed adefovir resistance at the median treatment duration of 12 months. This finding reiterates the fact that, adefovir is less potent and the frequency of resistance mutations is comparatively lower than lamivudine [268-270].

We attempted to study the sustained virological response for adefovir and only one subject could be followed-up. Initially this subject showed SVR after 6 (SVR-6) months cessation of therapy. Subsequently, this subject was detected with HBV DNA after 12 (SVR-12) and 18 months (SVR-18) cessation of therapy. However, no conclusions could be drawn from this one subject studied.

## 6.4 Entecavir-experienced group

In our analysis of baseline factors that would predict the virological response to entecavir, HBeAg and anti-HBe antibody showed significant association for virological response (p=0.01 in both). All 7 (16%) non-responders were HBeAg-positive and anti-HBe negative (Table 12). This finding shows that HBeAg-positive subjects have low response rate when compared to HBeAg-negative subjects. This finding is in agreement with the recent study which showed the virological response for HBeAg-negative subjects being comparably higher (98.3%) than HBeAg-positive subjects (82.9%) after 3 years of entecavir treatment [30].

During EVR measurement 40% and 49% of the subjects showed complete and partial virological response respectively. One subject with partial virological response presented exclusively with rtV173L mutation. It has been shown that entecavir requires a combination of at least 3 mutations to confer resistance [28, 113]. As the subject lost to follow-up, the clinical impact of the exclusive rtV173L mutation could not be studied.

Our analysis of EVR measurement for prediction of subsequent virological response revealed an interesting finding. Subjects who had complete virological response with undetectable HBV DNA showed higher response rate to subsequent entecavir treatment as compared to subjects who had partial virological response. Eighty percent of subjects with complete virological response at 6 months of entecavir showed subsequent response (undetectable HBV DNA) at 17 months versus 20% of subjects having partial virological response. Though there is no significant difference (p=0.058), EVR measurement can be a good predictor of subsequent response.

We could analyse the efficacy of extended entecavir treatment in 3 subjects and all were non-responders.

In the total 50 subjects analysed, 76% responded to entecavir at the median treatment duration of 6 months. The results of our finding agree with a recent study, where 81.1% showed response after 12 months of entecavir treatment [30]. On analysis of factors associated with response, subjects with anti-HBe seroconversion showed significant association with entecavir response.

Altogether, the results of our study subjects treated with lamivudine, adefovir and entecavir is shown in **Table 38**. The data is not head-to-head comparison and is limited by numbers at certain end-points of treatment. Therefore, interpretation of this data should be done cautiously.

Among the drugs analysed, maximum number of subjects were treated with lamivudine as it is widely used in this country. Adefovir is sparingly used in the treatment of chronic hepatitis B and we were successful in recruiting only 30 subjects. The use of entecavir is slowly evolving and we had sufficiently good numbers to analyse its efficacy.

During EVR measurement 62% and 68% of entecavir treated group showed normal ALT and AST levels respectively. These normal serum aminotransferases level was comparatively higher than lamivudine (50% and 48%) and adefovir (28% and 33%). The higher rate of serum ALT and AST normalization was also observed during ETR measurement in the entecavir treated group.

On analysing the virological factors at the EVR and ETR end-points, HBeAg continued to be positive in 61% and 55% in the lamivudine group; 61% and 56% in adefovir group and 53% and 47% in the entecavir group. This indicates that the loss of HBeAg is comparatively high in entecavir-experienced subjects. This substantially correlated with high anti-HBe seroconversion in entecavir-experienced subjects.

**Table 38. Comparison of antiviral efficacy of lamivudine, adefovir and entecavir in chronic hepatitis B subjects**

| Baseline and End-points of therapy | Lamivudine[$] | | | | | | Adefovir[†] | | | | | | Entecavir[ψ] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Biochemical | | Virological | | | | Biochemical | | Virological | | | | Biochemical | | Virological | | | |
| | Normal ALT | Normal AST | HBeAg positive | Anti-HBe positive | Undetectable HBV DNA | Resistance mutations | Normal ALT | Normal AST | HBeAg positive | Anti-HBe positive | Undetectable HBV DNA | Resistance mutations | Normal ALT | Normal AST | HBeAg positive | Anti-HBe positive | Undetectable HBV DNA | Resistance mutations |
| **Baseline** n=90[$]; 6[†]; 45[ψ] | 30 (33) | 27 (30) | 55 (61) | 30 (33) | 0 | 0 | 3 (50) | 3 (50) | 2 (33) | 4 (67) | 0 | 0 | 16 (36) | 24 (53) | 25 (56) | 20 (44) | 0 | 0 |
| **EVR** n=77[$]; 18[†]; 47[ψ] | 39 (50.6) | 37 (48) | 47 (61) | 32 (42) | 24 (31) | 6/64 (9) | 5 (28) | 6 (33) | 11 (61) | 7 (39) | 3 (17) | 1/17 (6) | 29 (62) | 32 (68) | 25 (53) | 24 (51) | 19 (40) | 0 |
| **ETR** n=71[$]; 16[†]; 15[ψ] | 41 (58) | 41 (58) | 39 (55) | 29 (41) | 27 (35) | 14/63 (22) | 7 (44) | 8 (50) | 9 (56) | 7 (44) | 2 (13) | 1/15 (7) | 7 (47) | 11 (73) | 7 (47) | 8 (53) | 10 (67) | 0 |
| **Maintained Response** n=36[$]; 8[†]; 3[ψ] | 24 (67) | 22 (61) | 22 (61) | 16 (44) | 8 (22) | 15/34 (44) | 4 (50) | 4 (50) | 4 (50) | 4 (50) | 2 (25) | 0 | 2 (67) | 2 (67) | 2 (67) | 1 (33) | 0 | 0 |
| **SVR** n=4[$]; 1[†]; 4[ψ] | 2 (50) | 2 (50) | 3 (75) | 1 (25) | 0 | 1/3 (33) | 1 (100) | 1 (100) | 0 | 1 (100) | 0 | 0 | 3 (75) | 3 (75) | 2 (50) | 2 (50) | 0 | 0 |

**Baseline**-treatment-naive

**EVR**- Early Virological Response, median treatment duration of 5-6 months

**ETR**- End-of-treatment-Response, median treatment duration of 12 months

**Maintained virological response**, median treatment duration of 24 months

**SVR**- sustained virological response, measured after 6 or 12 or 18 months off-therapy

Normal ALT levels (5-35 U/L)

Normal AST levels (8-40 U/L)

Undetectable HBV DNA (<82 IU/mL)

n= number of subjects

Values in parenthesis represent percentages

All subjects continued to be positive for HBsAg till the end of the study

Resistance mutations calculated after excluding the low viral load samples that failed to amplify in HBVrt PCR

Data are not head-to-head comparison and are limited by numbers at certain end-points, hence should be interpreted cautiously

Likewise, undetectable HBV DNA at EVR and ETR end-points was 31% and 35% in lamivudine group; 17% and 13% in adefovir group and 40% and 67% in entecavir group. Thus entecavir seems to be a high potent drug and adefovir being the least in terms of HBV DNA suppression.

As the numbers were limited in the subsequent end-points, comparisons could not be made.

Overall, on comparison of three drugs, entecavir showed a potent suppression of viral DNA at EVR and ETR measurements. Though the virological response was lower in adefovir group, resistance substitutions were comparatively lower than lamivudine group. Interestingly, none of the entecavir-experienced subjects showed incidence of antiviral resistance over the course of treatment. All subjects continued to be positive for HBsAg till the end of this study.

Locarnini and Mason have described antiviral resistance as the single most significant factor in treatment failure for HBV antiviral drugs [271]. Considering this, the absence of resistance mutations in most of the non-responders for all three drugs in our study subjects is contravening. The sensitivity of method applied (population sequencing) for the detection of mutations cannot be a reason, as most of the treatment failure subjects were identified with high viral load. Therefore the existence of antiviral resistant mutants is expected to be in predominant viral population and not as minority variants. It has been proposed that around 30% of virological breakthrough is associated with poor treatment compliance [272]. Therefore, the absence of resistance mutations in non-responders could probably be due to non-adherence to therapy which could not be elicited in the treatment history. In our findings, we hypothesize that high immune response as indicated by elevated baseline serum aminotransferases and anti-HBe seroconversion together with the antiviral action to play a role in treatment response. Therefore, studies aiming to identify specific immunological

markers of response and non-response can shed light on the underlying basis of such differences observed in our study.

## 6.5 Molecular modeling

All available oral drugs for HBV are nucleos(t)ide analogues that inhibit the enzymatic action of HBVrt and act as a chain terminator. A drug resistance mutation is an amino acid change that reduces the preferences of these analogues over the correct nucleotide during DNA polymerization. Using the molecular modeling approach, investigators have studied the principle of the drug resistance mechanism extensively. This study also aimed to identify novel mutations related to any one or more of these drugs used for HBV.

Initially for model building, protein BLAST of the query (target) sequence showed close identity to MULVrt (PDB: 1MU2 chain B) and HIV-2 rt (PDB: 1NND chain A). The query coverage and identity score for these templates were good when compared to previously described template of HIV-1rt (PDB: 1RTD chain A). The three aspartate amino acids that form the catalytic sites in HIV-1 rt is well conserved in HBVrt amino acid positions 85 (A domain), 203 and 204 (C Domain). Likewise, most of the amino acids interacting with the template primer and the incoming dNTP substrates are conserved in both HIV-1rt and HBVrt [40]. Moreover, the nucleos(t)ide analogues lamivudine, adefovir and tenofovir used for chronic HBV treatment were initially developed for HIV infection and their drug interactory mechanisms are very well documented [164, 165]. Therefore, modeling and docking studies of HBV using HIV-1rt template would be a suitable model for the prediction of drug resistance.

We built a homology model using HIV-1rt template. The model was evaluated by PROCHECK and the stereochemical quality of the structure was good with the overall G

factor of -0.24. Overall 98.8% of the residues were within the allowed region. We also numbered the three domains according to the nomenclature described by Stuyver *et al.*[112]. Allen *et al.* [18] and Das *et al.* [40] have modeled the interaction between lamivudine and HBV polymerase and showed that substitution of valine or isoleucine for methionine at residue 204 results in steric hindrance between the sulphur atom in lamivudine and the substituted amino acid side chains. Langley *et al*. [166] also showed this observation when examining the effect of entecavir against the lamivudine resistant HBVrt model. In order to validate our model, this well described lamivudine associated rtM204V primary resistance mutation was modelled using one of the study samples and docking analysis was performed. The rtM204V mutation showed decreased space for lamivudine binding, when compared to the wild type model **(Figure 25)**. Therefore, the spatial constraint for lamivudine resistance is further evidenced in our study.

The role of rtI233V mutation and adefovir response remains contradictory. Some studies have shown rtI233V mutation to be associated with adefovir resistance [247, 248]. In another study, it was not shown to affect adefovir response [249]. This mutation pre-existed in five (2.5%) of our treatment-naive subjects and we constructed a homology model with one representative sequence. The modelled structure showed the amino acid position rt233 to be located away from the drug interactory site. The substitution of isoleucine to valine did not affect the catalytic sites of aspartate residues at HBVrt positions 83, 205 and 206.

Warner *et al.* [167] has proposed that amino acid residues 235 to 240 form a bent structure and stabilizes the binding of incoming dNTPs. The wild type isoleucine at position 233 (I233) is just located three amino acids away from the crucial adefovir resistance amino acid position asparagines (N236), which in-part forms the bent structure. We attempted to study whether rtI233V substitution would alter the relative positions of neighbouring residues and

conformation. In wild type model the relative distance of the bent structure formed by the HBVrt amino acids L235, N236, P237, N238, K239 and T240 is 7.8 angstrom (Å). Substitution of valine reduced its relative distance to 7.7 Å. The overall conformation of the bent structure was maintained and the 0.1 Å difference in relative distance may not impose a spatial constraint to dNTP binding and adefovir efficacy **(Figure 26)**. Adding evidence to the findings of Curtis *et al.* [249]  we show that rtI233V mutation cannot affect the antiviral efficacy of adefovir.

Previous reports have shown that entecavir resistance occurs by the combination of three or more amino acid substitutions in the HBVrt region. One of the entecavir-experienced subjects in our study was exclusively detected with rtV173L mutation after 7 months treatment. However, this subject showed partial EVR with the reduction in HBV DNA levels of 1.8 $\log_{10}$ IU/mL from baseline. Delaney *et al.* [168] in their molecular modeling, showed that rtV173L together with rtL180M and rtM204V mutations alters the rtF88 residue that is crucial for DNA polymerization. We constructed HBVrt model of the sequence with rtV173L mutation and docked with entecavir. In our analysis the rtV173L mutation neither altered the entecavir binding nor the relative position of rtF88 that interacts with dNTP substrate **(Figure 27)**. In addition to the clinical evidence, our modeling results revealed that rtV173L mutation cannot confer resistance to entecavir or alter the relative amino acid residues exclusively.

**6.6 Distribution of HBV genotypes**

Our study enrolled a total of 296 subjects with chronic hepatitis B infection from 18 locations within India and adjacent countries i.e., Bangladesh, Bhutan and Maldives. Most of the subjects represented three major regions: southern India, eastern India and northeastern region. Additionally, few subjects were from southwest India and western India.

The most common genotypes identified in India are A, C and D. HBV genotypes A and D were shown to be predominant in northern and southern India, and genotype C in eastern India. Previously, our laboratory has reported prevailing circulation of HBV genotype D followed by genotypes A and C in chronic hepatitis B subjects [216, 273]. The present study also showed a preponderance of genotype D (59.1%), but the frequency of genotype C (22.6%) exceeded that of genotype A (17.9%). This may be due to the inclusion of more subjects from eastern India. Additionally, we identified 1 (0.3%) subject with newly identified genotype I.

Further, we also showed a distinct pattern of HBV genotype circulation between the three main regions of study population. HBV genotype D was the predominant genotype in south (89.1%) and east India (50.4%). These regions had a relatively lower frequency of genotype A (6.5% and 32.2%) and genotype C (4.4% and 17.4%) respectively. Genotype C (49.4%) was most common in northeast India and its surrounding regions followed by genotype D (37.9%) and A (11.5%). The newly described genotype I (1.2%) was also identified in this region. These findings were in agreement with the earlier reports [185, 215, 216, 221, 273].

On phylogenetic analysis with genotypes A to G as reference sequences, the new genotype I initially clustered with genotype G in a distinct branch. The Genafor/Arevir-geno2pheno prediction tool also determined the sequence as genotype G. As mixed HBV genotype infection has been commonly reported with genotype G, we performed clonal analysis for the sequence. All clones clustered as a separate branch with genotype G and no other co-infecting genotypes were identified.

A recent study from eastern India reported novel recombinants between HBV genotype A (nucleotide 2943-397), genotype G (nucleotides 397-1397) and genotype C (nucleotides 1397-2943) with sequence identity to Vietnam and Laos strains [185]. The HBVrt

nucleotides 130 to 1162 used in our phylogenetic analysis mostly covered the genotype G recombinant fragment of the novel genotype I. This made us to reanalyse the sequence by including the newly identified HBV genotype I. Phylogenetic analysis showed this sequence to cluster with genotype I of Vietnam/Laos and the eastern Indian strain. This was further confirmed using the newly constructed HBVSeq program in Stanford database. The overlapping surface gene analysis showed this sample to carry the classical HBV vaccine escape mutant sG145R which was also evidenced in the previous study [185]. Moreover, this subject is from Arunachal Pradesh, India, where the previous cases have also been reported.

### 6.6.1 Distribution of HBV subgenotypes

In our study, all of HBV genotype A was identified as subgenotype A1. Most of the genotype C was identified as subgenotype C1 (86.6%) with few subgenotype C2 (8.9%). Among the six subgenotypes C2, 5 (83.3%) were found to be circulating in the Arunachal Pradesh of northeast India which is bordered by china in the north where subgenotype C2 is more prevalent. This clearly suggests the transmission route of subgenotypes C2 to India. The genotype D sequences were identified as subgenotypes D1, D2, D3 and D5 in 11.4%, 64%, 18.6% and 15.4% respectively. Subgenotypes D1 (65%) and D3 (86.6%) were predominant in eastern India; subgenotype D2 (68.7%) in south India and subgenotype D5 in eastern India (51.9%) and northeast Indian subcontinent (40.7%).

The subgenotypes for 3 (4.5%) genotype C sequences and 1 (0.6%) genotype D sequence could not be assigned with the surface gene sequences. Though these sequences showed good bootstrap support, further analysis as suggested in the recent guidelines are required to confirm its circulation in Indian subcontinent [7, 180].

### 6.6.2 HBV genotypes and subgenotypes in treatment-naive subjects

In treatment-naive subjects, genotype D had significantly lower serum aminotransferases when compared to genotypes A and C (ALT, p=0.001 and AST, p=0.002). The difference between serum aminotransferases and HBV genotypes in the reported literature is contradictory. In an earlier study evaluating the clinical significance of HBV genotypes in children with chronic hepatitis B, there was no significant difference in ALT levels between genotypes A and D [274]. Similarly, a study from northern India showed no difference in serum ALT levels between HBV genotypes A and D [10]. In contrast, another study from northern India showed genotype A infected patients to have higher ALT levels as compared to patients infected with genotype D [215]. A previous study from Thailand showed genotype C to be significantly associated with high serum ALT and AST levels as compared to genotype B [275]. Another study from Taiwan did not show this association [276]. In previous finding, Kato *et al.* [277] showed higher serum ALT levels in genotype A compared to genotype C. In contrast, higher ALT and AST levels were seen in genotype C compared to genotype A in our study subjects. The clinical significance of such differences between genotypes and serum aminotransferases is unknown and requires further understanding.

The number of HBVrt amino acid substitutions was significantly higher in genotype D than genotypes A and C (p=0.0001). On subgenotype analysis of genotype D, the number of HBVrt amino acid substitutions was significantly higher in subgenotype D5 as compared to other subgenotypes of genotype D (p=0.0001). Recently, Ghosh *et al.* [222] showed the exclusive presence of subgenotype D5 in a primitive tribal community in eastern India. In their likelihood estimation of genotype D evolution, they showed subgenotype D5 to diverge much earlier than the other subgenotypes and suggested that subgenotype D5 is the most ancient of genotype D. This finding explains the reason for higher number of HBVrt substitutions seen in our subgenotype D5 sequences.

**6.6.3 Association of HBV genotypes and treatment response**

In our analysis, there was no significant difference between lamivudine response and HBV genotypes. There was also no association between number of subjects who developed lamivudine resistance and the genotypes tested. The genotype D subjects had significantly lower ALT and AST levels in comparison to other genotypes tested (p=0.003 and p=0.008 respectively). However, it should be noted that the ALT and AST levels of genotype D subjects was significantly lower in baseline illustrating that low ALT and AST levels are maintained over the course of therapy. Subgenotypes of genotype D also did not appear to influence lamivudine response and resistance **(Table 21)**.

Suzuki *et al.* [278] showed poor response to lamivudine in subjects infected with HBV genotype C compared to subjects infected with genotype B. To our knowledge, this is the only earlier published report that showed significant association between genotypes and lamivudine response. However, the numbers in this reported study were small in genotype B group (n=21) than genotype C (n=203) and therefore the reproducibility in larger studies is warranted.

In the adefovir-experienced group, 19 out of 21 subjects categorised for virological response were non-responders. This shows that the response to adefovir is not effective in all the genotypes tested. This finding is in agreement with the previous study by Westland *et al*. [92] which showed no significant difference between HBV genotypes and adefovir response.

In the entecavir group, there was no significant difference in proportion of subjects who showed response and non-response. This observation was also noticed when subgenotypes of genotype D were separately analysed. This shows that HBV genotypes have no role in predicting entecavir response. Previously, Lurie *et al.* [279] has showed entecavir response to be consistent across HBV genotypes A, B, C and D respectively.

In addition to these findings, a meta-analysis concluded lack of association between genotypes A, B, C or D and treatment response for the currently available nucleos(t)ide analogues [199]. With these earlier reported studies [92, 199, 279] together with our data it is very evident that HBV genotypes do not influence treatment response to the available nucleo(s)tide analogues. There was no significant difference in treatment duration between the genotypes or subgenotypes analysed and this excludes the chance of bias in the analysis performed.

### 6.6.4 Genetic diversity of hepatitis B virus genotypes

Hepatitis B virus genotypes have shown to be associated with disease progression and therapeutic response to immunomodulatory drugs, indicating that the genetic heterogeneity of viral genotypes may play a role in viral-host relationship. In order to identify the genetic diversity between the HBV genotypes, we measured the number of base substitutions between sequences (genetic distance, $d$); the number of synonymous substitutions per synonymous site ($dS$) that are silent nucleotide changes and do not alter the amino acid codons; and the number of non-synonymous substitutions per non-synonymous site ($dN$) where the nucleotide changes alter the amino acid codons.

Interestingly, we found $d$, $dS$ and $dN$ to be significantly different between genotypes of treatment-naïve sequences (p=0.0001 for all; **Table 27**). The $d$, $dS$ and $dN$ were higher in genotype D sequences when compared to genotypes A and C. This indicates genotype D to be highly divergent than other two genotypes identified in this study. This finding also contributes to our earlier results, where we have showed higher number of HBVrt amino acid substitutions in genotype D subjects than genotypes A and C (section 5.6.3.1). This is also evidenced in two other studies, where De-Maddalena *et al.* [280] showed high genetic heterogeneity in genotype D as measured by higher $dN$ values as compared to other

genotypes and Solmone *et al*. [253] showed higher number of amino acid substitutions in genotype D than genotype A. In our analysis, the mean genetic distance ($d$) and synonymous substitutions ($d$S) were high in genotype C than genotype A. However, the number of non-synonymous substitutions ($d$N) were high in genotypes A than genotype C **(Table 27)**. This indicates that higher proportion of nucleotide change in genotype C sequences is accompanied by amino acid changes.

Intriguingly, on analysis of baseline sequence and subsequent response to nucleos(t)ide analogues, the $d$, $d$S and $d$N was always higher in responders as compared to non-responders irrespective of the genotypes tested **(Table 28)**. Subsequently, on analysis of lamivudine-experienced subjects with partial virological response and non-response, the genetic diversity of responders was found to be significantly higher than those of non-responders **(Table 29)**.

These results contradicts the findings of Chen *et al*. [281]. In their analysis, the $d$, $d$S and $d$N of lamivudine responders and non-responders were not different at baseline. Moreover, the $d$, $d$S and $d$N were significantly lower in non-responders after four weeks of lamivudine treatment. They suggested that the antiviral pressure would have led to the selection of mutants and hence the viral genetic diversity in non-responders was higher than lamivudine responders.

Another study comparing the viral quasi-species evolution between HBeAg seroconversion and non-seroconversion showed high viral genetic diversity among responders than non-responders [282]. Fukai *et al*. [161] showed higher number of HBVrt substitutions in the lamivudine responders than in non-responders. These earlier published findings corroborate the findings of the present study.

The concept of intermediate antiviral pressure and selection of mutants (reviewed in section 3.7.5) can be extended in terms of host immune pressure and adaptive mutations to explain this finding. At low immune pressure, the virus replication is active and undergoes only few adaptive mutations. When immune pressure is high there is a complete suppression or very low levels of viral replication and therefore no chance of mutant selection. However, at intermediate levels of immune response, the virus evolves strategies to counteract the selection pressure and therefore higher numbers of mutations are observed. This hypothesis has also been proposed by Lim and others [282, 283]. Therefore, high immune response and subsequent action of nucleos(t)ide analogues showed high viral genetic diversity in responders over the course of therapy. This finding is again supported by our results where responders had elevated serum aminotransferases and anti-HBe response at baseline (section 5.2.1.3 and 5.4.1.2.1).

Therefore, high viral genetic diversity, elevated baseline serum aminotransferases and spontaneous anti-HBe seroconversion (suggesting a high immune response) combined with the action of nucleos(t)ide analogues showed better response to lamivudine.

## 6.7 HBV subtypes

We have developed a new programme to determine the HBV subtypes. This programme uses the algorithm determined by Purdy *et al.* [9] to identify the currently known nine major subtypes. This programme will enable the determination of subtypes automatically and reduces time and error rates caused by manual procedures. Hence it should be a useful tool especially in clinical settings and for epidemiological studies. The HBV subtyping programme was validated by comparing the results generated by this tool with our earlier published HBV subtypes [217]. In the previous study, the subtypes for 97 subjects were determined manually by positioning the HBV surface gene amino acids using BioEdit. In the

present study, the deduced subtypes were re-analysed using the new subtyping programme and all 97 subtypes showed concordance to those of the previously determined subtypes.

Previously, in a report from western India, subtype *ayw3* (50%) and *ayw2* (37%) were identified to be the major circulating subtypes [225]. Another study from eastern India documented the prevailing subtypes in 11 subjects: 3 (27.3%) were detected with subtype *adr*, 3 (27.3%) with subtype *adw2*, 3 (27.3%) with *adw3* and 2 (18.1%) with subtype *ayw2* [178]. Though there are other earlier reports of prevailing subtypes in India, the specific subdeterminants were not identified. In south India, subtype *ad* was documented in about 56.5% to 68.7% individuals [284]. In north India, subtype *ayw* was identified as a predominant subtype in 59% to 100% individuals [284].

In our study we have identified six of nine subtypes circulating in the Indian subcontinent. Subtypes *ayw3*, *adr*, *adw2* and *ayw2* were the most common subtypes identified in 54%, 22%, 18% and 12% of study subjects respectively. The remaining two subtypes *ayw1* and *adw3* were identified only in 0.7% and 0.3% subjects respectively. So far there are no studies which reported the prevalence of circulating subtypes across three regions in this subcontinent.

The subtypes could not be determined in 5 treatment-experienced (1.7%) subjects, as they presented with unusual amino acid substitutions at surface gene positions that are crucial for subtype determination. All these uncategorised subtypes were identified in treatment-experienced subjects and antiviral pressure would have selected these variants to occur.

As showed by Purdy *et al.* [9] we also noticed a significant association between genotypes and subtypes (p<0.0001). Therefore, distribution patterns of HBV subtypes were mostly similar to that of the closely related genotypes. However, few subtypes were predominantly

identified in certain geographical regions. Subtype *adw2* was predominantly identified in eastern India (72.2%). Likewise, all the subjects with subtype *adw3* in the southern region had subgenotype D2.

In our analysis of treatment-naïve subjects, serum ALT and AST levels were lower in *ayw2* and *ayw3* subtypes subjects when compared to *adr* and *adw2* subtypes, Subtype *ayw3* had higher number of HBVrt amino acid substitution as compared to other 3 major subtypes. These associations were similar to those observed for the closely related genotype D.

**6.7.1 Association of HBV subtypes and treatment response**

Zollner *et al.* [12] showed subtype *adw* to have a 20-fold increased risk of lamivudine resistance compared to subtype *ayw*. To address whether HBV subtypes influence lamivudine response, we analysed all 147 lamivudine-experienced subjects. Our results indicated that there was no significant difference in lamivudine response or resistance for the subtypes tested.

Among the 30 subjects analysed, only 2 subjects showed virological response. This show that antiviral efficacy of adefovir is poor for all subtypes tested. Among these subjects, one infected with subtype *adr* developed adefovir resistance mutations. Since the numbers are limited no conclusions could be drawn for the association of HBV subtypes and adefovir response/antiviral resistance.

There was also no significant association between HBV subtypes and entecavir response. To our knowledge, this is the first study that had analysed subtype-dependent response rate in adefovir and entecavir-experienced subjects.

Our region-wise analysis of HBV subgenotypes/subtypes showed, D2/*ayw3* (79%), A1/*adw2* (32%) and C1/*adr* (41%) were the predominant HBV subgenotype/subtype circulating in southern India, eastern India and north eastern region respectively (Figure 37).

We have also identified some genotype, subgenotype and subtype specific HBVrt amino acid substitutions in our study subjects. Further investigations of these amino acid substitutions will aid in determining HBV at least to the level of subgenotype by framing algorithms similar to that of HBV subtype classification used in this study.

To briefly summarize, this study has investigated in detail the sequences of almost 200 treatment-naive subjects and almost 250 treatment-experienced subjects from 3 different regions in the Indian subcontinent. An investigation into the spatial configuration of wild and resistant HBVrt sequences in the context of drug binding was also attempted. Further, an attempt was also made to investigate the possible association of various HBV genotypes, subgenotypes and subtypes with treatment response in subjects hailing from three regions of the Indian subcontinent.

# 7. SUMMARY & CONCLUSION

## 7. SUMMARY AND CONCLUSIONS

The study aimed to characterize the antiviral resistance mutations in Indian subcontinent subjects with chronic hepatitis B. It was also aimed to identify the association between HBV genotypes, subgenotypes, subtypes and treatment outcome in these subjects. The antiviral efficacies of three widely used drugs in this country i.e., lamivudine, adefovir and entecavir were studied.

### 7.1 Treatment-naive group

In the treatment-naive subjects analysed, no known signature mutations that could independently affect the antiviral efficacy to any of the currently available drugs were identified. However, other additional antiviral resistance-related putative, atypical, compensatory, novel amino acid substitutions and naturally occurring polymorphisms were seen. We proceeded to see if these pre-existing substitutions would impact on the efficacy of antiviral drugs over the course of treatment. On follow-up analysis, 75% subjects with these pre-existing substitutions responded to treatment illustrating that the observed amino acid substitutions were merely random mutations and did not have any impact on subsequent therapy. Thus, baseline monitoring of HBV antiviral resistance mutations as a prerequisite to treatment among these patients is not suggested.

### 7.2 Lamivudine-experienced group

We have identified certain baseline and on-treatment predictors of lamivudine response. In the lamivudine-experienced subjects, high baseline AST levels were significantly associated with subsequent response. We speculate that high immune response indicated by elevated serum AST or ALT levels together with the antiviral action of lamivudine might lead to better clinical outcome. According to our study, the baseline HBV DNA levels did not appear to affect subsequent response. However, subjects who showed complete virological response

with undetectable HBV DNA after 6 months of treatment [Early Virological Response (EVR) measurement] had better response to subsequent lamivudine treatment. This observation suggests that complete loss of HBV DNA is a better predictor of subsequent response than baseline HBV DNA levels. In our analysis, few subjects who showed partial virological response with reduction in HBV DNA levels of $\geq 1$ $\log_{10}$ IU/mL after 6 months of lamivudine treatment were detected with antiviral resistance mutations. This illustrates that mere reduction in HBV DNA levels does not exclude the presence of resistance mutations.

The lamivudine resistance mutations identified in our study population were rtL80V, rtV173L, rtL180M, rtA181V, rtM204V/I and rtM250L. None of the additional mutations identified showed specific association with lamivudine failure. Overall, the cumulative proportion of lamivudine resistance mutations were 9%, 22%, 44% and 71% for median treatment duration of 6, 12, 24 and 41 months respectively. On the last follow-up analysis, multivariate analysis showed that subjects who continued to be positive for HBeAg have increased risk for lamivudine failure. Further, high HBV DNA levels of $>4$ $\log_{10}$ IU/mL and increased treatment duration were strongly associated with lamivudine resistance. These findings are largely in agreement with earlier published studies.

To our knowledge, this is the first report from the Indian subcontinent that brings collective information in sufficiently good number of lamivudine-experienced subjects. Considering limited potency and high resistance rates, our study emphasises the use of more potent drugs in the management of HBV to ensure an optimum virological response and prevent the progression of disease.

## 7.3 Adefovir-experienced group

In the 30 adefovir-experienced subjects, only 7% showed virological response with the median treatment duration of 12 months. Our identification of rtI169L mutation further adds

evidence to a very recent finding that shows the association between rtI169L mutation and adefovir resistance. Additionally, we identified the typical adefovir resistance rtA181V mutation in one subject. The cumulative proportion of adefovir resistance mutations were 6% and 7% with median treatment duration of 6 and 12 months respectively. Though the sample size is small, this finding reiterates the fact that, adefovir is less effective though the frequency of resistance mutations is comparatively lower than lamivudine. Therefore, adefovir in the management of HBV should be used judiciously.

## 7.4 Entecavir-experienced group

In the entecavir group, subjects who had spontaneous anti-HBe seroconversion showed better response to entecavir. At the time of EVR measurement, subjects who had complete virological response with undetectable HBV DNA showed higher response to subsequent entecavir treatment than subjects who showed partial virological response, illustrating that complete loss of HBV DNA after 6 months of treatment is a better predictor of subsequent response than mere reduction in HBV DNA levels. Overall 76% of entecavir-experienced subjects responded to entecavir with the median treatment duration of 6 months and none showed entecavir resistance mutations. These findings largely agree with previous reports. To our knowledge, this is the very first report from the Indian subcontinent which analysed the antiviral efficacy of entecavir. This would expand the choice of antivirals for the treatment of chronic HBV subjects in our population.

On comparing the three drugs, entecavir was found to be a potent drug in terms of HBV DNA suppression, normalization of serum aminotransferases, loss of HBeAg and anti-HBe seroconversion. Moreover, none of the subjects developed resistance to entecavir for at least 24 months. Thus entecavir seems to be a suitable drug of choice in the management of HBV.

However, due to cost constraints, long-term use of entecavir is currently not feasible in resource limited countries like India.

All subjects continued to be positive for HBsAg till the end of study, demonstrating that nucleos(t)ide analogues had reduced efficacy in elimination of HBsAg when compared to the previous reports of results with interferon treatment.

This study still holds some limitations in attempting to study the antiviral efficacies of drugs. Many subjects in our study were lost to follow-up and we could not analyse the efficacy of antiviral drugs at each of the time-points of therapy. Therefore, most of our analysis is cross-sectional and a larger sample size with longitudinal analysis is of utmost importance. Moreover, few low viral load samples failed to amplify in HBVrt PCR and these samples were not included for the analysis. The presence of resistance mutations in these low viral load samples is less likely. However, our estimated prevalence rates concur with those of previous findings.

**7.5 Molecular modeling studies**

This study enabled us to identify the impact of rtI233V mutation which has been debated in the recent years. In molecular modeling studies, we have shown that the rtI233V mutation does not affect the antiviral action of adefovir. Furthermore, two subjects with pre-existing rtI233V mutation at baseline responded to lamivudine and entecavir subsequently. This again shows that rtI233V mutation does not alter the antiviral efficacy to any of these drugs.

We also attempted to study the impact of rtV173L mutation which was exclusively detected in one subject who showed partial virological response to entecavir. In addition to clinical evidence, our modeling results revealed that the rtV173L mutation cannot independently confer resistance to entecavir or alter the relative amino acid residues. Therefore, extending

the molecular modeling approach will aid in the identification of the true impact that mutations have on antiviral action.

**7.6 Determination of HBV genotypes, subgenotypes and treatment outcome**

The most common genotypes identified in our study are genotypes A, C and D. We show a distinct pattern of genotypes distribution across three regions in this subcontinent. Genotype D was the predominant genotype in southern and eastern India. These regions had a relatively lower frequency of genotypes A and C. In northeast India and its surrounding regions, genotype C was predominantly identified followed by genotypes D and A. The newly described genotype I was also identified in this region.

Likewise, all of genotype A was identified as subgenotype A1. Most of genotype C belonged to subgenotype C1 with few subjects harbouring subgenotype C2. The genotype D sequences were identified as subgenotypes D1, D2, D3 and D5. Subgenotypes D1 and D3 were predominantly distributed in eastern India; subgenotype D2 in south India; subgenotype D5 in eastern India and the northeast region respectively.

The increasing recognition of genotype I and other subgenotypes in our population suggests that further studies may reveal genotypes from other geographically distant regions. We also stress careful analysis of widely used surface gene sequences for determining HBV genotypes. The recombinant fragment of genotype G largely occupies the surface gene sequence of the newly identified genotype I and could hence lead to misclassification of genotype I.

It is very evident from our finding that HBV genotypes do not influence treatment outcome to all the three nucleos(t)ide analogues studied. Therefore, HBV genotypes have limited scope in the clinical decision making but has significant role in epidemiology. Our attempt to

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identify the genetic diversity between the HBV genotypes showed genotype D to be highly divergent as compared to genotypes A and C.

In our analysis, HBV genetic diversity was always higher in responders than non-responders irrespective of the genotypes tested. Hence, we hypothesize that subjects with heightened immune response inherently had high viral genetic diversity due to the immune pressure. Therefore, high immune response together with the antiviral action of nucleos(t)ide analogues resulted in better clinical outcome. This is also supported by our earlier finding where responders had elevated serum aminotransferases and spontaneous anti-HBe seroconversion. This study is unique in its analysis showing the predominant HBV genotypes and subgenotypes circulating in three major regions of India and the surrounding subcontinent.

**7.7 Determination of HBV subtypes and treatment outcome**

The newly developed programme for HBV subtype determination should be a useful tool in clinical settings and epidemiological studies. In our analysis, HBV subtypes *ayw3*, *adr*, *adw2* and *ayw2* were the most common subtypes with low frequency of *ayw1* and *adw3*. We noticed a significant association between genotypes and subtypes. Therefore distribution patterns were mostly similar to that of the closely related genotypes. In addition to HBV genotypes, subtypes also did not influence treatment outcome nor development of antiviral resistance. This finding suggests that there is no association between HBV subtypes and treatment response to all three antiviral drugs studied but knowledge of subtypes has importance in epidemiological investigations.

In addition we have identified some novel HBVrt amino acid substitutions specific to certain HBV genotypes, subgenotypes and subtypes. Further investigations of these amino acid

substitutions will aid in studying HBV at least to the level of subgenotype by framing algorithms similar to that of the HBV subtype classification used in this study.

D2/*ayw3*, A1/*adw2* and C1/*adr* were the predominant HBV subgenotype/subtype circulating in southern India, eastern India and north eastern India respectively. This region specific classification of HBV subgenotypes and subtypes is very unique and has not been reported in Indian literature. Thus the study would provide new insights about the evolution and transmission routes of HBV within the Indian subcontinent.

Three hundred and seventy-two sequences generated from this study have been deposited in GenBank. The purpose is to make it publicly accessible and serve as a valuable resource for epidemiological studies and to facilitate reliable comparison of drug resistance mutations for future studies.

**Conclusions**

In view of the limited potency and high resistance rates to lamivudine, our study emphasises the use of more potent drugs in the treatment of chronic hepatitis B to ensure an optimum virological response and prevent the progression of disease. Though the frequencies of adefovir resistance mutations are low, there is paucity in the proportion of subjects who showed virological response. Therefore, adefovir in the management of HBV should be used judiciously. Among the three drugs studied, entecavir seems to be a suitable drug of choice in the management of HBV. However, due to cost constraints long-term use of entecavir is implausible in resource limited countries like India. Our study has identified some baseline and on-treatment predictive factors of response and non-response. Our study has also revealed valuable information that would widen the scope of testing antiviral resistance mutations for appropriate tailoring of therapy. The absence of resistance mutations in most of the non-responders for all three drugs in our study subjects is contradictory to the

expectation. We also show that HBV genotypes and subtypes do not influence treatment outcome to all three antiviral drugs studied. Further, based on the findings of this study we hypothesize that high viral genetic diversity, elevated baseline serum aminotransferases and spontaneous anti-HBe seroconversion (suggesting a high immune response) coupled with antiviral action play an effective role in clearing the viral infection. We also propose that future studies be directed towards extending the combination therapy approach to HBV, as currently practiced in the management of HIV infection.

# 8. IMPACT OF THE STUDY

## 8. IMPACT OF THE STUDY

- This study shows that pre-existing hepatitis B virus reverse transcriptase (HBVrt) amino acid substitutions in treatment-naïve subjects are mainly random mutations and do not have any impact on subsequent therapy. Therefore, baseline HBVrt sequence analysis is not a requisite before initiation of therapy and has a limited scope in Indian subcontinent.

- We have identified elevated serum aminotransferases as a baseline predictive factor of virological response with lamivudine therapy. Anti-HBe response was a baseline predictor factor of virological response for entecavir therapy. Loss of HBV DNA after 6 months of treatment was identified as an on-treatment predictor of virological response both with lamivudine and entecavir therapy. This would be helpful in future management and clinical decision making.

- Our study has revealed typical HBVrt mutations that confer resistance to widely used antiviral drugs. These points to the important role of antiviral resistance testing for appropriate tailoring of therapy.

- In view of the limited potency and high resistance rates in lamivudine, our study emphasizes the use of more potent antiviral drugs or combination therapy in the management of HBV to ensure an optimum virological response and prevent the progression of disease.

- Our study reiterates the fact that the virological response for adefovir is slow though the frequency of resistance mutations is comparatively lower than lamivudine. Therefore, adefovir in the management of HBV should be used judiciously.

- Our study is the first report from the Indian subcontinent that analysed the antiviral efficacy of entecavir. This would widen the scope of antiviral options in the treatment of chronic HBV subjects in our population.

- We compared three drugs and have found entecavir to be a more potent drug in terms HBV DNA suppression, normalization of serum aminotransferases, loss of HBeAg and anti-HBe seroconversion. In addition, there were no antiviral resistance mutations for up to 24 months of entecavir treatment.

- This is the first study in the Indian subcontinent to give such collective information in sufficiently good numbers at all end-points recommended for therapeutic monitoring.

- Molecular modeling analysis enabled us to identify the impact of rtI233V mutation and antiviral efficacy of adefovir which has been debated up on in the recent years. Additionally, we have also showed the impact of the exclusive rtV173L mutation on entecavir action not yet reported in the literature.

- This study is very unique in showing the prevailing HBV genotypes, subgenotypes and subtypes in three geographical regions i.e., southern India, eastern India and north eastern India and its surrounding regions. This would provide new insight into the virus evolution and disease transmission routes in the Indian subcontinent.

- This study reveals the presence of the newly identified genotype I in our population and illustrates the need for further studies which might reveal new genotypes from geographically distant regions.

- This study also illustrates the importance of a careful analysis of widely used surface gene sequences for determination of HBV genotypes to avoid its misclassification.

- The newly developed tool for HBV subtype determination in our study shows a wider applicability potential in clinical settings and epidemiological studies.

- It is very evident from our findings that HBV genotypes and subtypes do not influence treatment but have epidemiological importance.

- This is the first report in the country that has characterized the genetic diversity of three major HBV genotypes in the Indian subcontinent and correlated its finding with treatment response and non-response.

- This study suggests that immune mediated response plays a vital role in controlling viral replication and emphasizes the need for studies aiming to identify immunological markers of response and non-response.

Overall, these findings expand our understanding of the role of three major antiviral agents used for the treatment of chronic hepatitis B and has shed light on the role of antiviral resistance testing in the management of HBV in the Indian subcontinent. Molecular modeling and docking analysis provided a better understanding of the prediction of antiviral resistance mutations to the commonly used oral nucleos(t)ide analogues. Though there is no evidence of HBV genotype, subgenotype or subtype-dependent antiviral response, understanding their distribution pattern provides valuable epidemiological information.

# 9. APPENDIX

## 9. APPENDIX

### 9.1 Tris-EDTA buffer (pH 8.0)

- Trizma.HCl (MW-157.6)    -0.157 g
- EDTA (MW-372.2)          -0.037 g

Dissolved in 100 mL of sterile milliQ water to make a final concentration of 10mM Tris.HCl and 1mM EDTA. Adjusted the pH to 8.0 and sterilised by autoclaving.

### 9.2 TAE buffer (50X)

- Tris base                -242 g
- Glacial acetic acid      -57.1 mL
- EDTA (0.5M)              -100 mL

Prepared 0.5M EDTA by dissolving 18.61 g of EDTA (pH 8.0) in 100mL of distilled water. The specified amount of Tris base and Glacial acetic acid was then added and the solution was made up to 1000 mL.

The final 1X working solution had 40mM Tris-acetate and 1mM EDTA.

### 9.3 Ethidium bromide stock (0.1%)

10 mg of Ethidium bromide was dissolved in 10 mL of milliQ water. The stock solution was stored at room temperature in a dark brown bottle.

The final concentration of ethidium bromide was 0.5µg per mL

**9.4 Gel loading buffer (6x )**

- Sucrose                    - 4 g

- Bromophenol blue        - 25 mg

Dissolved in 10 mL of distilled water and stored at 4°C

**9.5 Luria bertani broth**

Luria bertani broth             - 2.5 g

Dissolved in 100ml of distilled water and sterilized by autoclaving

**9.6 Luria bertani agar**

Luria bertani agar               - 4.0 g

Dissolved in 100ml of distilled water and sterilized by autoclaving

**9.7 Ampicillin antibiotic**

200 mg of Ampicillin was rehydrated in 20 mL of sterile water. From this stock, 0.5 mL was added to 100ml of Luria bertani broth to obtain a final concentration of 25 µg/ml.

**9.8 X-gal**

40 mg of X-gal was dissolved in 1 mL of Dimethyl formamide and 40 µl was spread over Luria bertani agar medium

<div align="center">**Appendix-II**</div>

<div align="center">**PATIENT INFORMATION SHEET**</div>

**Introduction**

Informed consent refers to participation in this study after gaining an understanding about the purpose, risks and benefits of the study. This form provides information about the study which has been already explained to you. Your decision to participate in this study is voluntary.

**Purpose and Background**

Hepatitis B virus (HBV) is a virus that infects the liver and causes jaundice which is an inflammatory condition of the liver. It causes mild to serious liver disease in the infected individuals. It is transmitted through blood and blood products, from mother to child and sexually. Approximately 5-10% of the infected individuals develop chronic infection. Chronic infection can lead to fibrotic changes in liver, end stage liver disease and liver cancer. In this study we have planned to study the predominant viral mutations that confer drug resistance to HBV and its possible influence on diagnosis, antiviral treatment and response. Other viruses like Hepatitis C virus and Human Immunodeficiency virus which would influence the outcome of Hepatitis B infection will be screened for. In the event of you being positive for either of these viruses, you will be referred to an appropriate clinic for further counseling and management and will be excluded from the study.

**Procedures**

Participation in this study requires information about your age, history of past/present liver disease, treatment and vaccination details. A blood sample will be collected for serological and

molecular tests related to this study. If there is an archived (earlier collected) sample of yours in the laboratory, it may be also tested.

These tests are currently only for research. Data generated from this study could be put to clinical use in the future. Hence results will not be informed to you by lab staff. However, any finding contributing your treatment outcome will be informed to the concerned physician.

**Possible risks/discomforts**

1. In many individuals, collection of blood from vein may cause discomfort.
2. You might or might not feel uncomfortable to give information like history of liver related diseases that would be collected from you for research purpose.

**Possible benefits**

From the findings of this study, there may be possible benefit to medical knowledge. Any valuable insights into the genetic basis of viral resistance could contribute to the diagnosis, and therapeutic management of HBV infection.

**Alternatives**

Your alternative is not to participate in this study

**Costs**

No additional cost to the participant in this study

**Privacy**

Clinical medical records will be reviewed by the research investigators confidentially to obtain clinical information to benefit the study. The patient samples will be coded and will not be easily identified. Individual identities of the participants of the study will not be revealed in any reports or in any scientific publication.

# INFORMED CONSENT FORM

Study Title: **Characterization of hepatitis B viral drug resistance in Indian subcontinent patients with chronic liver disease**

Study Number:

Subject's Initials: _____ Subject's Name: _____

Date of Birth / Age: _____

(Subject)

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s) [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative:_____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____    Signature of the Witness: _____

Date: _____/_____/_____                          Date: _____/_____/_____

Study Investigator's Name: **A. Mohamed Ismail**   Name of the Witness: _____

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# Antiviral Resistance Mutations and Genotype-Associated Amino Acid Substitutions in Treatment-Naïve Hepatitis B Virus-Infected Individuals from the Indian Subcontinent

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**Abstract**

***Background/Aims:*** Antiviral resistance is a major challenge to the treatment currently available for hepatitis B virus (HBV). In this study, mutations that may affect the antiviral efficacy in treatment-naïve HBV-infected individuals were analyzed. ***Methods:*** Ninety-seven treatment-naïve HBV-infected individuals were included in this study. HBV reverse transcriptase (rt) domains were sequenced and nucleotide differences were compared to GenBank wild-type sequences. Furthermore, HBV genotypes, subgenotypes and subtypes were determined by analyzing surface gene sequences. ***Results:*** An adefovir-related rtI233V mutation was identified in 4 subjects. The rtS213T lamivudine and entecavir refractory mutant was presented in 3 individuals. Altogether, drug-related, atypical and novel HBVrt amino acid substitutions were seen in 73 positions. The HBV genotypes A, C, D and G were depicted in 15, 21, 60 and 1 individuals, respectively. There were 17 HBVrt amino acid substitutions that are associated with certain genotypes of HBV. Mutations in HBVrt corresponded to established surface gene mutations in 9 patients. ***Conclusion:*** This data shows that antiviral-resistant HBV strains do exist in treatment-naïve individuals in this region. Further studies are essential to characterize the role of HBVrt amino acid substitutions in response to anti-HBV therapy.

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## Introduction

The goal of antiviral treatment is to achieve viral suppression. However, the virus evolves strategies to overcome the drug selection pressure, thereby escaping the antiviral action. Antiviral resistance is a major concern for the therapeutic management of hepatitis B virus (HBV) infection. The incidence of lamivudine resistance at the end of 24 months in hepatitis B e antigen (HBeAg)-positive and -negative patients was 39.5 and 25.9%. Likewise, for telbivudine it is 25.1 and 10.8%, respectively [1]. The cumulative probability of adefovir and entecavir resistance for the same duration was found to be 3 and 0.5% [2, 3].

With 50 million carriers, India has the second largest population of individuals with chronic HBV infection worldwide [4]. Locarnini and Mason [5] have described HBV drug resistance as the single most significant factor in treatment failure of HBV. All the available nucleoside/nucleotide analogues for HBV target the reverse transcriptase (rt) domain of polymerase gene, and mutations

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occurring in the conserved domains of HBVrt have been shown to confer resistance to antiviral drugs [6, 7]. Furthermore, HBVrt lacks the proofreading activity and constantly evolves with substitution rates of $4.2 \times 10^{-5}$ nucleotide substitutions/site/year [8, 9]. Consequently, viral mutants are generated during the course of disease, leading to the development of viral quasispecies [5]. The presence of pre-existing antiviral-resistant mutants and the mutation patterns that accumulate over time may affect the efficacy of subsequent treatments.

The typical mutations considered as primary mutations to lamivudine involve rtM204V/I, and for adefovir it is rtA181V/T and rtN236T [10, 11]. Other amino acid substitutions at sites rtL80I, rtI169T, rtV173L, rtL180M, rtA181T/S and rtQ215S occur during lamivudine therapy to restore the replication capability, and are called secondary/compensatory mutations [12]. Likewise, entecavir-related mutations require combinations of substitutions at positions rtI169T, rtL180M, rtT184G, rtS202I, rtM204V and rtM250V [13, 14]. The frequency of rtM204I/V mutations in lamivudine-experienced Indian patients was found to be 29% at 18 months [15].

Eight HBV genotypes (A–H) and subgenotypes within certain HBV genotypes have been identified [16]. The HBV strains are also distinguished into hepatitis B surface antigen (HBsAg) subtypes and there is a correlation between subtypes and genotypes [17]. Recently, genotype-dependent polymorphic amino acid positions in HBVrt region were identified, illustrating different genomic variability among HBV genotypes that may influence the development of drug resistance mutants [18].

Recent reports showing the existence of antiviral resistance strains in treatment-naïve hepatitis B individuals alert the need for baseline monitoring of antiviral resistance mutants [19–21]. Hence in the present study we attempted to identify and analyze the HBVrt amino acid substitutions capable of conferring resistance to antivirals in treatment-naïve HBV-infected individuals from the Indian subcontinent. In addition, the effect of HBVrt amino acid substitutions on the overlapping surface (S) gene was also studied.

## Materials and Methods

### Subjects
Blood samples obtained from 97 HBV-infected individuals attending the liver clinic of a tertiary care teaching hospital in South India who were HBV DNA-positive were included in this study by convenient sampling. Subjects were recruited between April 2007 and August 2009 and were referred to the Department of Clinical Virology for HBV quantification. Plasma was separated on the same day of blood collection and was stored in aliquots at –60° until further analysis. All subjects were HBsAg-positive, hepatitis C virus (HCV) antibody (Ab)-negative, human immunodeficiency virus (HIV)-negative and treatment-naïve. The study was approved by the institutional review board and informed written consent was obtained from all the subjects. Clinical details and serum alanine transaminase levels (ALT) were obtained from the patient's hospital records.

### Serology Markers
HBsAg and HBeAg testing were performed in EIA (Diasorin S.P.A., Saluggia, Italy). HCV Ab and HIV were screened in Ortho HCV 3.0 (Ortho Clinical Diagnostics, Raritan, N.J., USA) and Axsym HIV Ag/Ab combo (Abbott, Wiesbaden, Germany).

### DNA Isolation
DNA was extracted from 200 μl of blood plasma using the QIAamp DNA Blood MiniKit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's instructions. Finally, the extract was resuspended in 50 μl of elution buffer.

### HBV DNA Quantification
HBV DNA was quantified using artus® HBV RG PCR (Qiagen GmbH, Hilden, Germany) in the Rotor-Gene 3000 or 6000 platform (Corbett Research, Mortlake, Vic., Australia).

### HBV Polymerase/rt Gene PCR
HBV polymerase gene covering the entire rt region was amplified (1,323 bp) using high-fidelity platinum Taq DNA polymerase (Invitrogen, Carlsbad, Calif., USA) with a HBV DNA template of 5 in 50 μl reaction volume. The primer sequences and PCR cycling conditions used have been described previously [13].

### DNA Purification and Sequencing Analysis
The amplified PCR products were purified by Multiscreen$_{HTS}$ PCR plate (Millipore, Billerica, Mass., USA). Sequencing reaction was carried out with two sets of primer sequences, SP1- CTC CAG TTC AGG AAC AGT AAA CCC, ISP2- CGA ACC ACT GAA CAA ATG GC, HBVFS4- TGT ATT CCC ATC CCA TC, HBV4- GCT AGG AGT TCC GCA GTA TGG A [13, 22], and the ABI Prism Big Dye terminator cycle sequencing ready reaction kit. Briefly, 1 μl of the purified PCR product is mixed with 1.6 pmol of the primer, and 1 μl of the ready reaction mix with 2 μl of the sequencing buffer, making the volume to 10 μl with nuclease-free water. The cycling conditions consisted of 25 cycles of 96° for 15 s, 50° for 20 s and 60° for 4 min. Excess salts and dye terminators were removed from the sequencing mixture using Montage SEQ$_{96}$ filtration (Millipore). The sequencing reactions were run on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster City, Calif., USA). Obtained bidirectional sequences were analyzed using BioEdit v7.0.9 and multiple sequence alignment was performed using the built-in CLUSTALW integrated in MEGA4 [23]. Nucleotide substitutions were revealed by comparing the study sequences with the consensus sequence of the 600 HBV GenBank sequence (accession numbers in suppl. material www.karger.com/doi/10.1159/000323521). The consensus was generated aligning 3 different datasets. An amino acid differences table was generated and analyzed by CUBIT GUI

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**Table 1.** HBVrt amino acid substitutions observed in this study

| | | | | |
|---|---|---|---|---|
| rtA/P/S/T7D | rtR110G | rtK149R/Q | rtY245C/H | rtM309K/I |
| rtH9Y | rtL115V | rtF151Y | rtS246H | rtA313S |
| rtI14L | rtI/N121S | rtR153Q/W | rtG255D | rtC314S |
| rtI16T | rtF/I/L/V122H/N | rtI187L | rtW257Y | rtQ316H |
| rtA21S | rtN123D | rtS213T*/N | rtT259S | rtQ319R |
| rtV23I | rtQ125K | rtV214A*/E | rtD/E263S | rtT322S |
| rtA/S/T38K/E | rtH126Y/R | rtQ215S*/H/P | rtI/L/V266R/K | rtP325L |
| rtI/N/S/T53Y/D | rtT128N*/L | rtL217R | rtH/Q267L | rtT326A |
| rtA/P/S/T54Y/H | rtM129V/L | rtE218N | rtH/Q271K | rtY327F |
| rtN76S/D | rtQ130P | rtS219T*/A | rtV278I/T/L | rtA329T/V |
| rtS78T/Y | rtL132M | rtT225S | rtN279D | rtC332S/R/Y |
| rtV84I | rtD/E134N | rtI233V* | rtI290L | rtK333A/N/Q |
| rtI91L | rtS135N/Y | rtP237T | rtV291T | rtN337H |
| rtV103I | rtR138K | rtN238S*/Q/T/H/D | rtL293I | |
| rtS106C | rtV142E | rtK241Q | rtY305F | |

On comparison with HBV GenBank consensus sequence, amino acid substitutions in 73 positions were seen. Shaded positions include rt-conserved domain substitutions and * indicates amino acid substitutions that are crucial for antiviral resistance to HBV.

(Free Software Foundation Inc., Boston, Mass., USA). These sequences were also analyzed for in vitro phenotypic prediction of HBV drug resistance mutations using the Genafor/Arevir-geno-2pheno (hbv) drug resistance tool (Genafor, Bonn, Germany; http://hbv.bioinf.mpi-inf.mpg.de/index.php).

Nucleotide sequences generated from this study are deposited in GenBank database under accession numbers GU798963 to GU799059.

*HBV Surface Gene Analysis*
HBV S gene sequences were similarly analyzed to identify the overlapping mutations. HBV genotypes and subgenotypes were determined by aligning study sequences with published sequences representing all HBV genotypes and subgenotypes [16, 24, 25]. Phylogenetic analysis was performed in MEGA4 using the neighbor-joining method with a bootstrap test of 1,000 replicates and maximum composite likelihood algorithm. HBsAg subtypes were predicted by S gene amino acid codons 122, 160, 127, 159 and 140 as described elsewhere [17].

*Statistical Analysis*
The number of HBVrt amino acid substitutions and HBV DNA level between the genotypes were compared using a Kruskal-Wallis test. A p value of <0.05 was considered statistically significant. Correlations between the HBV DNA level and ALT, rt amino acid substitution and age were analyzed using Spearman's correlation coefficient. The median HBV DNA and median number of rt amino acid substitution was compared between HBeAg status using a Mann-Whitney U test. All analysis was done using STATA 11 (StataCorp, College Station, Tex., USA).

## Results

In 97 subjects, 80 (83%) were male and the median age was 35 years (4–75 years). Sixty-three (65%) were HBeAg-positive and there was a significant association between HBeAg status and median HBV DNA level ($4 \times 10^5$ IU/ml; $p < 0.001$), range $219–9 \times 10^8$ IU/ml. The median ALT was 40 U/l (12–624 U/l) and there was a correlation between ALT and viral load ($r = 0.26$, $p = 0.008$). Twenty-six (27%) individuals had a family history of HBV infection and 13 (13%) had undergone surgical or dental procedures.

Comparison with HBV GenBank sequences revealed rt amino acid substitutions in 73 amino acid sites (table 1). An adefovir-related rtI233V mutation was seen in 4 patients. An antiviral-resistant mutant (rtS213T) that restores the replication fitness in lamivudine- and entecavir-experienced patients was seen in 3 patients. Likewise, rtT128N, rtV214A, rtQ215S, rtS219T and rtN238S compensatory mutations were identified individually. Atypical mutations with new amino acid substitutions in positions rtV84, rtT128, rtS213, rtV214, rtQ215, rtS219, rtP237, rtN238 and rtY245 were seen in 19 individuals. Altogether, functional domain mutations in HBVrt domains F, A, D and E were seen in 2, 5, 9 and 2 individuals, respectively. One subject had a single mutation in both the A and D domains. Additionally, novel amino acid substitutions were seen in 62 amino acid positions of the HBVrt region. rtI91L and rtL217R naturally occurring polymorphisms
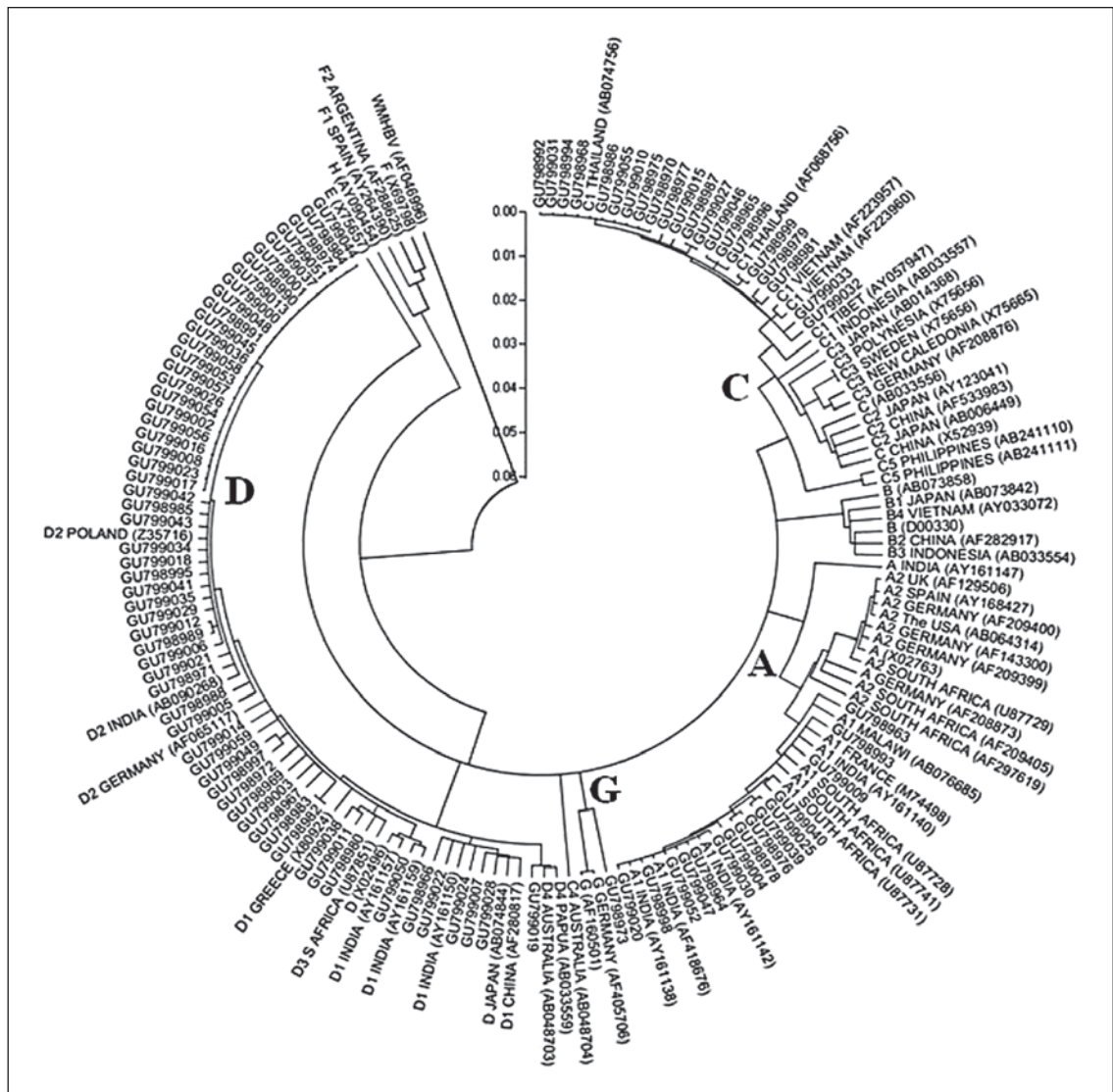
**Fig. 1.** Phylogenetic analysis of HBV 'S' gene sequences (649 positions) conducted in MEGA4 using the neighbor-joining method and maximum composite likelihood model. GenBank reference strains are shown by subgenotype, accession number and country of origin. Study sequences are designated by accession number prefixed by GU. Woolly monkey HBV (WMHBV) was used as an out-group.

that respond poorly to lamivudine and adefovir were seen in 75 and 3 individuals, respectively [26, 27]. On statistical analysis, there was a significant association between age and number of rt amino acid substitutions (r = 0.39, p < 0.0001) and a significant difference between median number of rt amino acid substitution and HBeAg status (p = 0.01). All the study sequences when analyzed for the phenotypic prediction of drug resistance in geno2pheno (hbv) database showed adefovir resistance in 4 individuals carrying the rtI233V mutation.

On analyzing the S gene, HBV genotype D was seen most commonly (n = 60) with the subgenotype/subtype split of D1/ayw2 (n = 6), D2/ayw2 (n = 3), D2/ayw3 (n = 45), D3/ayw2 (n = 3), D4/ayw2 (n = 1) and D5/ayw3 (n = 2). The HBV subgenotype/subtype A1/adw2 and C1/adr was seen in 15 and 21 individuals. One subject was infected with HBV genotype G/ayw1 (fig. 1 and 2). Sequence analyses also showed some HBVrt amino acid substitutions that are common and specific to certain genotypes. In the present study, rtA/P/S/T54H/Y (100%),

Ismail/Samuel/Eapen/Kannangai/
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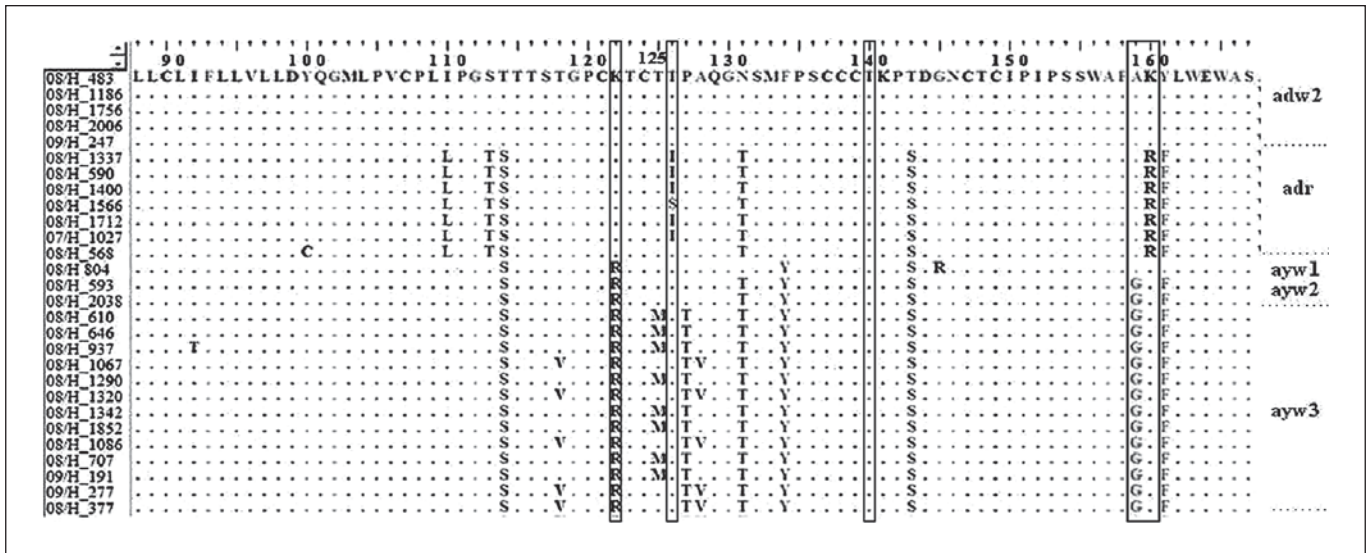
**Fig. 2.** HBV S gene amino acid codons showing representative samples of HBV subtype class identified in this study. Amino acid positions 122, 160, 127, 159 and 140 are used for subtype determination. Amino acid substitution sT125M is only seen in subtype ayw3 of genotype D subjects (n = 8).

rtS135Y/N (78%), rtK149Q (83%), rtW257Y (88%), rtT259S (98%) and rtI/L/V266R/K (18%) were only seen in HBV genotype D. Specifically, rtI/N/S/T53D and rtH126R was observed in subgenotypes D2 with a frequency of 83 and 87% respectively. Likewise, rtF151Y and rtR153W/Q was specific to all study samples of HBV genotype A, while rtF/I/L/V122N and rtC332S were seen in 53 and 87% of this genotype, respectively. In HBV genotype C populations, rtH9Y (100%) is the only substitution that was specific to this genotype. In addition, some of the rt amino acid substitutions are specific to certain genotypes and are shown in table 2.

There was a significant difference between HBV genotypes and the mean number of HBVrt amino acid substitutions between the A and C genotypes, and between the C and D genotypes (p < 0.0001, respectively), but no significant difference was found between genotypes A and D. However, 5 (12%) of the genotype D subjects had >10 amino acid substitution (table 3). The difference in HBV DNA load and genotype was not statistically significant.

On comparison of overlapping S gene sequences with reference sequences, amino acid substitutions were seen in 57 amino acid positions. The rtR153Q substitution corresponded to the sG145R vaccine escape mutant in 1 patient infected with HBV genotype G, and amino acid substitution sP120T corresponding to rtT128N was seen in another patient. Likewise, neutralizing antibody es-

**Table 2.** HBV genotype-associated rt amino acid substitutions identified in this study

| Genotype | rtV103I | rtF/I/L/V122H | rtM129L | rtV278I/T/L |
|---|---|---|---|---|
| A (n = 15) | 15 (100) | 7 (47) | 15 (100) | 0 |
| C (n = 21) | 0 | 0 | 2 (9) | 14 (67) |
| D (n = 60) | 1 (1.67) | 4 (6.66) | 2 (3.3) | 1 (1.67) |
| p | <0.0001 | 0.0004 | <0.0001 | <0.0001 |

Values in parentheses represent percentages.

**Table 3.** Number of HBVrt amino acid substitutions with respect to HBV genotype

| Number of HBVrt amino acid substitution | HBV genotype | | | |
|---|---|---|---|---|
| | A | C | D | G |
| 1–5 | 0 | 20 | 7 | 0 |
| 5–10 | 15 | 1 | 48 | 1 |
| >10 | 0 | 0 | 5 | 0 |
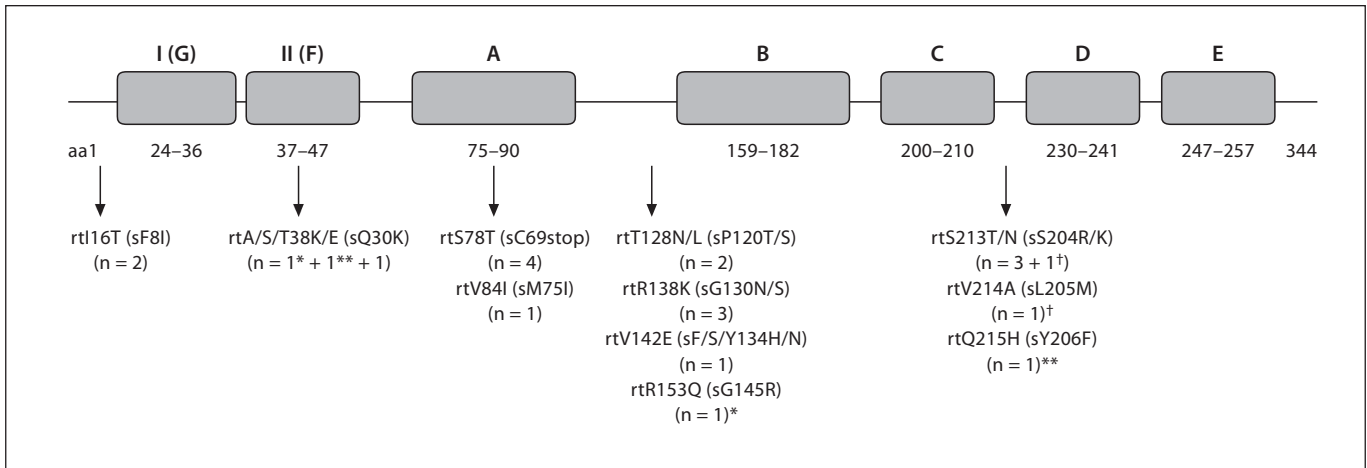
**Fig. 3.** HBVrt amino acid substitutions conferring overlap surface gene mutations. HBVrt domains (A–G) and respective amino acid (aa) positions are shown [45]. 'n' indicates number of patients. One patient (*) presented with substitution in positions rt38 (s30) and rt153 (s145), another patient (**) had rt38 (s30) and rt215 (s206) substitutions and one patient (†) carried rt213 (s204) and rt214 (s205) amino acid substitutions.

cape mutants sI/N/T126S and sK141E were detected in 2 and 1 patients, respectively. The rtS78T substitution resulted in a sC69 stop codon mutation in 3 patients. Overall, HBVrt amino acid substitutions resulted in 'S' gene amino acid changes in 11 positions (fig. 3).

## Discussion

In this study, we have described HBVrt amino acid substitutions from 97 HBV-infected subjects who have not experienced antiviral drugs to HBV. As is widely reported, the HBeAg positivity and raised ALT levels correlated with higher HBV DNA loads in these individuals. We attempted to identify novel amino acid substitutions by comparing the study sequences with maximum wild-type sequences from GenBank. This allowed us to identify some unique mutations that are drug-related, atypical amino acid substitutions in positions of known anti-viral resistant target sites [20] and some novel amino acid substitutions not yet reported in the literature. There was a significant positive relationship between the age of HBV-infected individuals and the number of rt amino acid substitutions. As discussed by Solmone et al. [28], this may be due to the accumulation of substitutions in older age groups.

Previously, this laboratory has reported prevailing circulation of HBV genotype D followed by A and C from chronic hepatitis B groups [29, 30]. The present study also shows a preponderance of genotype D, but the frequency of genotype C (22%) exceeded that of genotype A (15%). This may be due to the inclusion of more subjects from eastern India (42/97, 43%) where genotype C is common [31, 32]. In our study, subgenotype D1, D2, D3 and D5 were seen with a frequency of 10, 80, 5 and 3.3%, respectively, which is comparable to an earlier report [33]. One patient from Bhutan was infected with HBV subgenotype D4 and had a unique rtE/D263S and rtA329V substitution. Banerjee et al. [34] and Kumar et al. [35] reported a sT125M mutation in the 'a' determinant region of the S gene to be associated with the ayw3 subtype of genotype D. A similar finding was obtained in the ayw3 subtype of D2 (16%) and D5 (100%) subgenotype in our study subjects. It has been reported by Norder et al. [16] that this mutant is associated with intravenous drug use. However, family history of jaundice and surgical procedures were the only risk factors of HBV infection in our study subjects. One patient carried HBV genotype G, which has been previously reported from countries like France, Germany, USA, Mexico and more recently from an Indian patient with occult HBV [35]. Intriguingly, there was no history of travel or receipt of imported blood products in this individual. The presence of HBV genotype G in our study population alerts us that larger studies may reveal genotypes from geographically distant regions.

In samples analyzed, no known 'hot-spot' mutations that can independently affect the antiviral susceptibility were seen. The rtI233V mutation common in HBV genotype D, which can affect the antiviral efficacy to adefovir, was seen in 4 subjects: 3 (5%) HBV genotype D and 1 (4.76%) genotype C, respectively [36]. In analyzing our study sequences, this is the only HBVrt mutation that showed a resistance profile to adefovir in the geno2pheno (hbv) drug resistance tool. Recently, Schildgen et al. [37] showed adefovir failure due to the pre-existing rtI233V mutation. However, the role of the rtI233V mutation and adefovir response remains contradictory [10, 38, 39].

Structural biology studies have shown that HBVrt C–D interdomain mutations at rt215 and rt219 can alter the nucleotide triphosphate binding active site [7, 12]. Amino acid substitutions in each of these positions were identified in 5 individuals. Likewise, the rtS213T mutation that re-establishes the replication fitness to lamivudine and entecavir therapy was seen in 3 individuals. Amino acid substitutions at rt214, rt237 and rt238 that lie in close proximity to lamivudine-, adefovir- and entecavir-related key signature mutations were also seen. In addition to these HBVrt interdomain substitutions, functional mutations in the conserved domains A–E were seen in 17 subjects. HBVrt shares a good sequence homology in the catalytic regions of HIV domains A–G [7]. Unlike HIV, there have been no reports of domain F antiviral-related mutations for HBV [40]. In our study, rtA/S/T38K in the F domain was seen in 2 subjects and the role of this substitution needs to be established. Likewise, predominant substitution of rtW257Y and rtN248H in E domain is noteworthy.

Comparison to the GenBank consensus sequence allowed us to identify some unique amino acid substitutions that are genotype-specific and some substitutions that are common across certain subgenotypes/subtypes. Identification of more specific genotypic markers will aid in framing an algorithm for genotype/subgenotype classifications just like specifying HBsAg subtypes. This will also pave the way to identify markers that are predictors of response and nonresponse to HBV drugs. A2 subgenotypes containing L217R polymorphisms are shown to respond poorly in adefovir-treated individuals. This polymorphism was seen in 2 of our D2 subgenotypes and 1 C1 subgenotypes. The rtI91L naturally occurring polymorphism is predominantly seen in lamivudine-failure patients. This polymorphism was found in HBV genotype C, D and G in our study subjects and all genotype A patients in this study had rt91I. Better characterization of naturally occurring polymorphisms and drug-related re-

sistance mutations are required for pointing out genotypic markers of response and nonresponse. All individuals carried at least 1 amino acid substitution when compared to the previously reported GenBank sequences. Statistical analysis of the number of HBVrt mutations in relation to HBV genotypes showed a significant difference between genotypes A and C and between C and D. HBV genotype D showed a larger number of substitutions (10–14 substitutions) in 5 individuals. This can potentially have clinical significance in India due to the high burden of chronically infected individuals, extensive usage of antiviral drugs and predominance of genotype D.

The surface gene of HBV is entirely overlapped by the HBVrt region, so mutations occurring in one region may affect the protein coding sequences of the other. HBVrt substitutions resulted in HBV S codon changes in 12 positions. The sG145R vaccine escape mutant and sP120T mutant seen in our study subjects have been shown to partially restore the replication of lamivudine resistant strains in vitro [41]. As reported, these vaccine escape mutants and truncated proteins may have an important effect on immune recognition and diagnostic testing [42]. Some naturally occurring baseline S gene mutations previously reported in Indian patients with virological breakthrough was not seen in our patients, except the sA128V mutation that was seen in 40% of the individuals [43].

This is the first report from the Indian subcontinent to characterize the HBVrt sequence from treatment-naïve individuals. Our findings expand the results of earlier published studies on antiviral-resistant mutants to HBV and overlap S gene mutants. According to present knowledge, the incidence of antiviral resistance is comparatively higher in HBeAg-positive individuals. In our study, HBeAg-negative variants had a higher number of rt amino acid substitutions compared to the HBeAg-positive group. It is reasoned that higher number of rt amino acid changes in the active domain may affect the enzyme activity reducing the replication efficacy of the virus and contribute to treatment response [44]. Follow-up of these individuals is essential to identify if a greater number of rt amino acid substitutions in HBeAg-negative patients can reduce the risk of treatment failure.

Lamivudine is the first orally available drug for HBV and adefovir remains an alternative drug of choice from 2002. As observed in this study, the chance for the presence of pre-existing HBV variants resistant to these drugs is higher when compared to the more newly introduced drugs like entecavir, telbivudine and tenofovir. Hence, to

better understand the significance of the observed amino acid substitutions, structural biology studies or phenotypic testing and follow-up of these patients is required.

In summary, we have identified HBVrt amino acid substitutions in treatment-naïve individuals that could reduce the efficacy of currently used antivirals like lamivudine, adefovir and entecavir. Though no established mutations against any of these drugs was seen in these 97 subjects, the presence of compensatory/secondary mutations that usually occur in treatment-experienced individuals alerts that the chance of development of 'hot-spot' primary mutations in these treatment-naïve individuals is higher. There were 17 HBVrt positions that showed genotype-specific amino acid substitutions. Future studies are warranted to identify mutations that would definitely impact on antiviral efficacy, to explore the role of atypical and novel amino acid substitutions, and to determine baseline genotype-specific markers of HBV drug resistance. Baseline characterization of HBVrt sequences will have a promising role in therapeutic management of HBV as all of the currently available oral drugs target this region.

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